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Cytotoxic Activity of *Amorphophallus paeoniifolius* Tuber on Cervical CancerCell Line

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Abstract: The present study explores the cytotoxic activity of *Amorphophallus paeoniifolius* (Elephant foot yam) tuber on cervical cancer cell line (HeLa cell line). The extracts of the tuber in Petroleum Ether, Chloroform, Ethyl Acetate, Methanol and Distilled Water were evaluated by direct observation of cells using inverted phase contrast microscope followed by MTT assay using tetrazolium dye. All the five extracts of the tuber showed a dose-dependent MTT reduction in HeLa cell line. Petroleum ether extract with half-maximal inhibitory concentration, IC_{50} 26.9478 µg/mL in MTT assay have shown greater cytotoxic activity. The toxicity of the petroleum ether extract to normal cells were studied using L6 - Rat myoblast cell lines. In the case of normal cell lines, IC_{50} value obtained is 95.37 µg/mLwhich supports the non-toxic effect of the extract at therapeutic concentration on normal cells. Qualitative analysis of the petroleum ether extract shows the presence of phenols, tannins and saponins. The quantitative study is carried out using colorimetric method. The quantitative study showed that the petroleum ether extract contains higher number of phenolic compounds, 84.51 µg per mg equivalent of standard Gallic acid**.** Petroleum Ether extract with the presence of these phytochemicals hence serves as a potential source of anti-cervical cancer agents. The isolation and characterization of the phytochemicals may open up opportunities for further study and application of *Amorphophallus paeoniifolius* tuber in anti-cancer research.

Keywords: *Amorphophallus paeoniifolius*, Cytotoxicity,HeLa Cell line, MTT assay, Petroleum ether extract

1.Introduction

Cervical cancer,a life-threatening disease is the fourth most common cancer seen in women above thirtyyears of age [1].This diseasemainly arises due to continuous infection produced by human papillomavirus (HPV) in the cells of cervix connecting the vagina (birth canal) to the upper part of the uterus [2].

Though vaccination and early detection methods are available [3,4],this type of cancer is common among women over

age 30.The cancer become worse when it spread to other internal organs. The methods like surgery, radiation therapy [5],chemotherapy, targeted therapy[6] commonly used for the treatment possess serious side effects. The present work aims to find a substitute from dietary plant kingdom with minimal side effect namely,*Amorphophallus paeoniifolius*. Furthermore, the present study provides a platform towards the isolation and characterization of the pharmacologically active phytochemical constituents from *Amorphophallus paeoniifolius.*The extraction of phytochemicals from the tuber may lead to their wider analysis and applications.

*Amorphophallus paeoniifolius*is a highly potential, nutritious tropical tuber crop of Araceae family,known as the king of tuber crops[7].

It is basically a crop of south East Asian origin. It grows in wild form in Philippines, Malaysia, Indonesia and South East Asian countries. The tuber is consumed by many people as a food and widely used in many ayurvedic medicine preparations.

The tuberous roots of the plant have been used traditionally for the treatment of abdominal disorders, tumours, asthma, rheumatism, arthralgia, elephantiasis, inflammations, haemorrhoids, haemorrhages, vomiting, cough, bronchitis, asthma, anorexia, dyspepsia, flatulence,constipation,weakness,fatigue and anaemia. The multiple potentiality of the tuber arises due to the presence of various phytoconstituentslike alkaloids, triterpenoids, flavonoids, fatty acids, tannins and steroids.The tuber has been reported to possess hepatoprotective[8] antioxidant[9], cytotoxic[10], antibacterial and antifungal activities [11].

2.Materials and Methods

2.1Invitro Determination of Antiproliferative Effect by MTT Assay

The in-vitro anticancer study of the five different extracts of the tuber of *A. paeoniifolius* was carried out using HeLa cell lines [12]. HeLa cell lines was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in 25 sq.cm tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100Um/L), Streptomycin (100µg/mL), and Amphoteracin B (2.5µg/mL). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (Galaxy® 170 Eppendorf, Germany).

2.2Preparation of plant extracts and compound stock

Amorphophallus paeonifoliius tubers were harvested from Pathanamthitta district, Kerala, India. After removing thepeel,the tuber is cut into pieces, shade-dried andfinely powdered. Then the powdered sample is subjected to Soxhlet extraction with Petroleum Ether, Chloroform, Ethyl Acetate,Methanoland Distilled water and concentrated under reduced pressure using a rotary evaporator.

1mg of plant extract was added to 1ml of DMEM and dissolved completely by cyclomixer. The solution was then filtered

through 0.22 µm millipore syringe filter to ensure sterility.

2.3Anti-proliferative effect Evaluation

The viability of cancer cell lines and normal cell lines(L6 - Rat myoblast cell lines) was evaluated by direct observation of cells by Inverted phase contrast microscope followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay[13,14].

After 24 hours ,the growth medium was removed and different concentrations (6.25µg, 12.5µg, 25µg, 50µg, 100µg in 100µl of 5% DMEM) of freshly prepared samples were added in triplicates to the wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

2.3.1Anti-proliferative effect by Direct Microscopic observation

Entire plate was observed at an interval of 24 hours up to 72 hours in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPSTM HD camera) and microscopic observation were recorded as images. Any detectable changes inside the morphology of the cells, together with rounding or shrinking of cells, granulation and vacuolization inside the cytoplasm of the cells have been considered as signs of cytotoxicity.

2.3.2Anti-proliferative effect by MTT Method

Inthe MTT assay, the tetrazolium dye MTT is transformed into formazan by means of some unique enzymes within theliving cells [14]. The quantity of formazan product formed can be correlated with the viability of cells. It isquantified via measuring the absorbance with the help of a spectrophotometer. Fifteen mg of MTT (Himedia, M-5655) become reconstituted in three ml PBS until absolutely dissolved and sterilized viafilter out sterilization.After 24 hours of incubation duration, the sample content material in wells were eliminated and 30µl of reconstituted MTT solution became introduced to all including control wells,then the plate was gently shaken and incubated at 37ºC in a humidified 5% CO2 incubator for 4 hours. After the incubation, the supernatant was removed and 100µl of MTT solubilisation solvent (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. [15].The absorbance values were measured by using micro plate reader at a wavelength of 570 nm.The percentage of growth inhibition was calculated using the formula. Percentage of viability= (Mean Optical Density of Samples /Mean Optical Density of control group) x 100

2.4Qualitative and Quantitative analysis of phytochemicals

Qualitative analysis of the extract is done by the following standard methods using chemicals and reagents of analytical gradeand quantitative analysis is done by colorimetric method.

2.4.1Qualitative analysis of Phytochemicals

Phenols

2 ml of sample was taken in a test tube and add 1% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compound[16].

Flavonoids

2ml of sample was treated with 2ml of 10% lead acetate solution. Appearance of yellowish green colour indicated the presence of flavonoid [17].

Alkaloids

2ml Wagner's reagent is added to 2 ml of sample. The appearance of reddish-brown precipitate shows the presence of alkaloids [17,18].

Tannins

5 % $FeCl_3$ (1 ml) was added to 1ml of the sample. Iftannin is present, brownish green colour appears [17].

Glycosides

The presence of glycosides is indicated by the formation of brown ring when2ml glacial acetic acid, one drop of 5% $FeCl_3$ and conc. $H_2SO_4$is added to 5ml sample[17,18].

Saponins

1ml of sample was added to 2ml of distilled water in a test tube and shaken vigorously with few drops of olive oil. Foam which persisted was taken as evidence for the presence of saponins [18].

Steroids

1ml of sample was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added by the sides of the test tube. The formation of two layers, red upper layer and yellow lower layer with green fluorescence shows the presence of steroids[18].

Terpenoids

2ml of each sample was mixed with 2ml of chloroform. Then allow to evaporate and add 2ml of concentrated sulfuric acid, then heat for 2 minutes.Greyish colour indicates the presence of terpenoids [17,18].

Quinones

2ml of each sample was mixed with 3 or 4 drops of concentrated HCl. A yellow colour precipitate indicates the presence of quinones[19].

2.4.2Quantitative analysis of Phytochemicals

The qualitative analysis showed the presence of phenol,tannin and saponin in the selected plant extract.The qualitativestudies are supported by quantitative analysis using colorimetric method.

Estimation of phenol

Content of phenol in sample was determined by Folin-Ciocalteumethod. One ml of sample was taken and added 3.0 ml of distilled water. Folin-Ciocalteu reagent (0.5ml) and 2mL 20% Na_2CO_3 were added and the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 750nm in a spectrophotometer against blank. Standard gallic acid solutions (2.5-100µg/ml) were also treated as above[20].

Estimation of Tannin

Content of tannins in sample was determined by Folin-Ciocalteu method. Colorimetric estimation of tannins is based on the measurement of blue colour formed by the reduction of phosphotungsto molybdic acid by tannin like compounds in alkaline medium. 1ml of sample and standard solution of tannic acid (20-100 µg) was made up to 7.5mL with distilled water. Then 0.5mL of Folin-Ciocalteu reagent and 35% 1mL sodium carbonate solution was added. The volume was made upto 10mL with distilled water and the absorbance was measured at 700nm [21].

Estimation of saponin

The Vanillin-Sulphuric acid assay for determining the total saponin content is usually done by incubating 100µl of sample, standards or reagent blank with 0.25 mL of 0.8% (w/v) vanillin in ethanol and 2.5 mL of 72% (v/v) sulphuric acid in water for 15 min at 60°C in a shaking water bath, with the standard as diosgenin and the reagent blank made up with the extraction solvent. After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extracts are measured at 544 nm using a UV–VIS spectrophotometer [22].

2.5Statistical Analysis

All statistical comparison were made by means of Two-Way ANOVA and p-values less than 0.05 were considered significant.

3.Results and Discussion

MTT assay result of Petroleum Ether, Chloroform, Distilled Water, Ethyl Acetate and Methanol extracts of the tuber on HeLa cell lines are graphically shown in Fig.1.

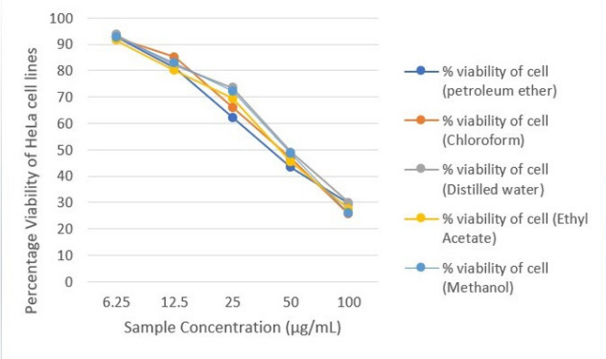

Fig. 1. Percentage viability of HeLa cell lines in five different concentrations of Petroleum Ether, Chloroform, Distilled Water, Ethyl Acetate and Methanol extracts of the tuber.

The MTT assay results showed that in the case of five extracts, the percentage viability of the HeLa cell line decreases with increase in concentration of the extracts.As p value obtainedusing two-way ANOVA calculator with concentration treatment is less than 0.05 ($p<0.05$), itstatisticallysupportsthe observation of dose-dependent MTT reduction in HeLa cell line.Thehalf maximal inhibitory concentration, IC_{50}value obtained for Petroleum Ether,Chloroform,Distilled Water,Ethyl Acetate and Methanol extracts are 26.9478µg/mL,44.4689µg/mL, 52.2723µg/mL,44.7766µg/mL,59.364µg/mL respectively. Hencepetroleum ether extract with IC_{50}26.9478 µg/mLwas found to have greater cytotoxic activity than other extracts. Thus, the present study is focussed on petroleum ether extract as it may be a potent source of anti-cervical cancer agents.The cytotoxic activity of the petroleum ether extract on normal cell line was also studied using MTT

assay. The result of the MTT study is shown in Fig.2.

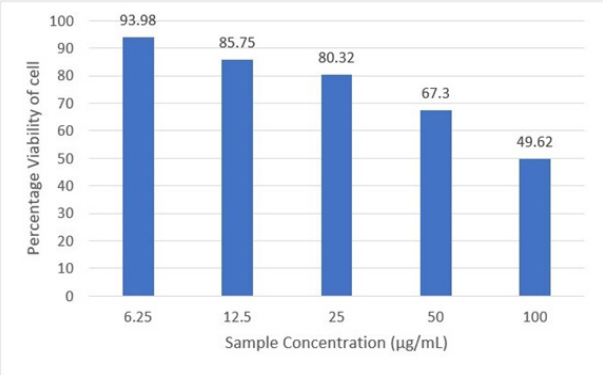

Fig. 2. Percentage viability of the normal cell lines with increase in sample concentration in petroleum ether extract.

The IC $_{50}$ value was observed with 95.37 µg/mL concentration of the test sample. The value shows that the petroleum ether extract of the sample is less toxic to normal cell line.The microscopic observation of the control and the cell lines (HeLa cell line and normal cell line) treated with different concentration of the petroleum ether extract of the sample were recorded as images. The images obtained are shown below.(Figure 3 &4)

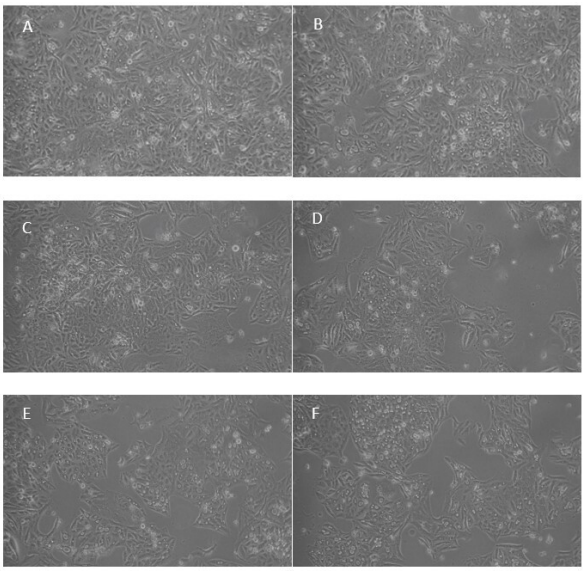

Figure.3. Microscopic observation of the control and the treated HeLa cell line. (A) HeLa cell line (Control) and HeLa cell line treated with (B) 6.25 µg/mL of sample (C) 12.5 µg/mL of sample (D) 25 µg/mL of sample (E) 50 µg/mL of sample (F) 100 µg/mL of sample.

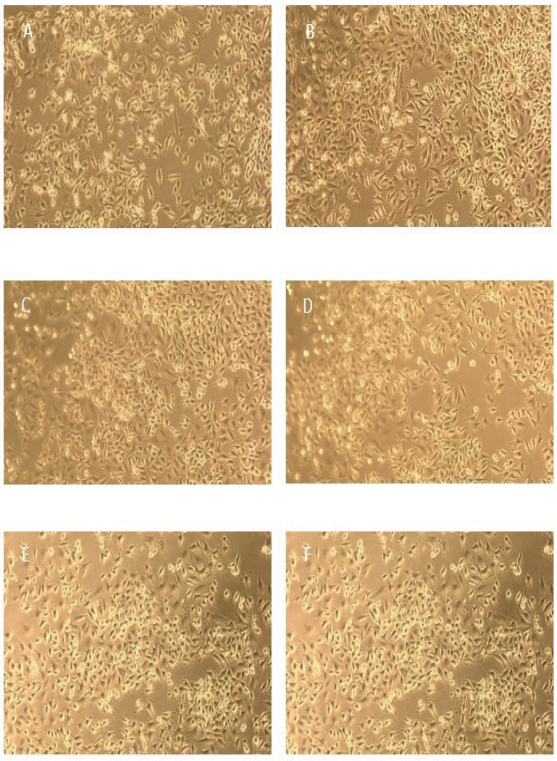

Figure. 4. Microscopic observation of the control and the treated normal cell line. (A) L6 - Rat myoblast cell (Control) and L6-Rat myoblast cell treated with (B) 6.25 µg/mL of sample. (C) 12.5 µg/mL of sample (D) 25 µg/mL of sample. (E) 50 µg/mL of sample. (F) 100 µg/mL of sample.

With increase in sample concentration (petroleum ether extract) there occurs a detectable change in the morphology of the HeLa cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells, that shows the anti-cancer potential

of the sample extract. But there is not much changes in the morphology of treated L6-Rat myoblast cell, supporting the non-toxic nature of the extract to the normal cells.The pharmacologically active phytochemical constituents present in the petroleum ether extract are then evaluated. The result of the qualitative studies done are shown below in Table 1.

Table 1. **Phytochemicals present in the extract**

	Name of secondary metabolites	Name of test	Observation
1	Phenol	Lead acetate Test	White precipitate is formed
2	Tannin	Ferric chloride Test	Brownish precipitate is formed
3	Flavanoid	Lead acetate test	No yellowish green colour
4	Saponin	Foam Test	Persistant foam is observed
5	Terpenoid	Liebermann-Burchard Test	No greenish colour
6	Alkaloid	Wagner's Reagent	Absence of reddish brown precipitate
7	Glycoside	Kellar-Killani Test	Brown ring is not formed
8	Steroid	Liebermann-Burchard Test	No characteristic reaction
9	Quinones	HCl method	Absence of yellow precipitate

The qualitative study showed the presence of phenols, tannin and saponin in the extract. This study is further supported by the quantitative analysis of these phytochemicals using colorimetric method.The concentration of phytocompounds in terms of various standard as obtained by absorbance versus concentration plots are listed in following table 2.

Table 2. Concentration of phenol, tannin and saponin in their corresponding standard equivalents µg/mg of sample.

Optical Density (OD) at 750 nm	1.043	Concentration of Phenol in Gallic acid equivalent of sample	84.51 µg /mg
Optical Density at 700 nm	1.242	Concentration of Tannin in Tannic acid equivalent	58.63 µg/ mg
Optical Density at 544nm	0.358	Concentration of Saponin in Diosgenin of extract	68.85 µg/ mg

The quantitative studies showed the presence of higher number ofphenolic phytochemicals in the petroleum ether extract. Further studies can only reveal the nature of phenolic compounds present in the extract. The isolation and characterisation of that compound may help in the development of a potent and a saferanticancer drug,will be a remarkable milestone in the chemotherapeutic path. The extraction of the bioactive compound from the tuber extract needs appropriate and efficient method. Furthermore, itsfuture application demands the checking of bioavailability and the mode of its action with the receptor binding site.

4.Conclusion

The MTT studies using HeLa cell lines showed that of the five different extracts of the tuber *A.paeoniifolius*,the petroleum ether extract possesses greater cytotoxic activity,thoughthe five extracts of the tuber showed a dose-dependent MTT reduction in HeLa cell line. The p value obtained by taking concentration parameter with two-way

ANOVA calculator is less than 0.05 ($p<0.05$), it statistically supports the dose-dependent MTT reduction in HeLa cell line.Petroleum ether extract showed IC_{50} 26.9478 µg/mL in the case of cancer cell lines and95.37 µg/mL in the case of normal cell lines in MTT assay. The IC_{50} value 95.37 µg/mL supports the non-toxic effect of the extract at therapeutic concentration on normal cells.

Phytochemical analysis suggested the presence of tannins,saponins and phenols in the extract. The quantitative colorimetric study using gallic acid as the standard showed the presence of higher number of phenolic compounds in the extract.Petroleum ether extract with the presence of these phytochemicals, hence serve as a potential source of anti-cervical cancer agents. The isolation and characterization of these bioactive componentswill become a remarkable milestone in chemotherapeutic path. Hence this work opens up opportunities for further study and application of Amorphophallus paeoniifolius tuber in anti-cancer research.However, further studies are needed to realize the different mechanisms by which the bioactive component in the petroleum ether extract of *Amorphophalluspaeoniifolius*tuber exert their cytotoxic effects on the cervical cancer cell line, HeLa cell line.

5.References

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