Phytochemical Screening and Cytotoxic Activities of Methanolic Extracts of *Physalis peruviana* Studied on Normal and Cancerous Mammalian Cell lines

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Abstract:- **In modern era plants are widely used in pharmaceutical industry as sources of raw materials and essential ingredients for medicine. Cancer is the second leading cause of death worldwide with high percentage of deaths occurring in developing countries. In Kenya, new cancer cases have been witnessed all over the country. The attention on diseases like malaria, HIV and tuberculosis has resulted in the neglect of diseases like cancer. This is caused by lack of awareness, non-qualified personnel, inadequate facilities and financial shortages. In Kenya, majority of the population relies on traditional medicine as an alternative treatment since the conventional health system provides for only 30% of the population. Although medicinal plants in Kenya have been used for treatment of cancer by the traditional healers, no studies have been carried out to verify their healing claims. This study focused on the analytical methodologies, which included the extraction of crude extract from *Physalis peruviana* using methanol, dichloromethane petroleum ether, water and hexane. It also included the phytochemical screening assay through the TLC method and was observed under UV lamb and the in vitro cytotoxicity activity of *Physalis peruviana* on normal and cancerous mammalian cell lines. The *in vitro* assays involved determination of the cytotoxic concentration levels (CC_{50}) of the plant extracts on cancer cell lines as well as calculating the inhibitory concentration (IC_{50}) of the plant extracts on cancer cell lines. The biochemical responses of cells after exposure with organic plant extract was observed using the MTT dye {3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide}. *Physalis peruviana* extract was expected to have a cytotoxicity effect on selected cell lines.**

Key words: **Cancer; medicinal plants; phytochemical; cytotoxic**

I. INTRODUCTION

Over the years, people in all corners of the world have been using plants for different purposes. more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (WHO, 2002). Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al*., 2006). Consequently, nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, characterization of bioactive compound then isolation. Medicinal plants and their extracts have been applied as herbal remedies for diverse human ailments. Presently, medicinal plants are still being used in various developing countries as source of therapeutic agents due to believe that they are readily available, accessible, affordable, potent and with lower adverse side effects incidences as compared to the modern drugs (Gurib-Fakim, 2006). They contain active compounds or ingredients which are substances of therapeutic activity, that is, the successfulness of prevention, diagnosis and treatment of physical and mental illness as well as beneficial alteration or regulation of the physical and mental status of the body (Shirota ,1996). In Kenya, these plants are still popularly used to cure various kinds of ailments. This research is therefore aiming on extraction and test the cytotoxicity activity on cancer cells lines of the extract.

Nowadays, certain methods have been developed to monitor the quality of medicinal plants used by many people. One such method is by the use of chromatographic technique. Chromatography, though simple and use basic instruments, is a key analytical tool in the discovery of new active compounds. Research on active components in plants is very important because it would pave way to the verification of the common and popular notion that plants, when used in treating diseases, is totally safe (Samuelsson G. 1999). Many believe that because it is "natural" then there would be no overdose and toxification from other compounds used to generate artificial medicines. Meaning, it can be taken anytime of the day without concern on how much is supposed to be taken in, thus the more you take it in higher doses, the better. On the other hand, Kenya is a third world country and many of its citizens don't have the access to effective and costly medicines. This has propelled people resort to what is available in nature such as plants extracts. . Plants continue to provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cosa, P. et al., 2)006. Worldwide, cancer ranks second as the leading cause of death (Joyce et al, 2011).

Cancer is a disease caused by uncontrolled division of abnormal cells in the body. The cell proliferation in cancer is mainly activated by extracellular growth factors which are bound to the surface of the cell tyrosine kinase receptor (TRKs). The activation of intercellular signal transduction pathway usually results in altered gene expression resulting in proliferation. Cells become cancerous once there is accumulation of mutations in the genes that controls cell proliferation. Normally mutation in genes usually cause cancer by accelerating cell division rate or by inhibiting normal control of things like cell cycle arrest or programmed cell death in the system. Ways of blocking this process has been the important target in current treatment of several tumor types. Cancer being a major scourge globally (Greg and Jener, 2006), the significance of medicinal plants have been prompted by the discovery of natural anti-cancer compounds which have promising biological activities (Roja and Rao , 2000). So far, about 30 compounds derived from plants have been proven to be clinically active against various types of cancer cells, therefore further research in this area may lead to better cancer treatment (Joyce et al 2011).

Physalisperuviana belongs to solanace family pysalis genus from Amazon and Andes .It is exported from several countries, Kenya Egypt with Colombia are the largest producer. It is a fuzzy tropical hairy plant which is slender pointed, heart-shaped leaves and orange edible fruits and it bares yellowish flowers. Fruits are protected by papery husks from insects' birds and adverse conditions. *Physalisperuviana* is used in many ways such as cooked dishes food salads jam and dessert. It is also used as a traditional medicine for anticancer, immunomodulatory, antileukemic, ant mycobacterial, hepatitis, dermatitis, diuretic, malaria and rheumatism (Chiang et al.1992a & b; Ismail and Alam, 2001). This plant is believed to be diuretic, juice from it fruits is administered for bowel and worms complaints and also has antibiotic activity against staphylococcus bacteria (Metzger and Perry , 1980).Oleaginous from the plant extract is an important source of essential oil which is rich in Fatty acids Phytosterols and natural antioxidants (Ramadan, 2003*). P. peruviana* pulp of the fruits is usually nutritious and contains high levels of carotenoids, minerals and vitamin C. There are chemical compounds which are present in plant such as viz-28 hydroxywithanolide, withanolides, phygrine, quercoletin di- and tri- glycosides and kaempferol (Keith et al., 1992). The fruit of this plant is of commercial interest due to it nutritional properties such as the presence of high vitamin content, antioxidant, anti-inflammatory, anti-cancer and other medicinal properties. A lot of study has been done on this plant and this research is targeting on leaves extract and test of cytotoxicity activity on cancer cells.

II. MATERIALS AND METHODS

2.1 Study site

This study was conducted at the Kenya Medical Research Institute (KEMRI) Centre for Traditional Medicine and Drug Research (CTMDR) and Center of Biotechnology and Research development.

2.2 Plant Materials

The plant was selected for screening using a focused method based on ethnobotanical information derived from CTMDR database of medicinal plants containing the traditional and folk medicine uses Plant materials were obtained from the previously collected samples stored at CTMDR herberium.

2.3 Plant Extracts (Methanol Extraction)

Ground powdered part was placed in glass percolator. Sufficient quantity of solvent was added to submerge the plant material. After standing for about 16 hours percolate was collected and filtered. The process was repeated four times for exhaustive extraction of the plant material. The methanolic extract was evaporated under reduced pressure at 50^{o}C using rotavapor and round bottom flask. Then finally it was concentrated in a vacuum desiccators. The extract was transferred to glass container of appropriate size. This formed the stock extract.

2.4 Cytotoxicity activity procedure

For testing, cells were washed by phosphate buffer saline (PBS) and harvested by tripsinization and were plated in 96 well plates (20,000 cells/well) and incubated under 5% CO_2 and 95% air at 37^{o}C for 24 hours. The cells were treated with different concentrations of plants extracts including 0, 1.371, 4.115, 12.345, 37.037, 111.111, 333.333, and 1000µg/ml. These treatment concentrations were made in triplicate dilution using culture medium yielding final extracts concentrations with a final DMSO concentration of 0.1%. This concentration of DMSO did not affect cell viability. Control cells were incubated in culture medium only. All concentrations of plants extracts were in triplicates on the each cell batch.

2.4.1 Study design: Cell based assay

2.4.2 Cell Line Culture Procedure

This test was carried out alongside the promega protocols and application guide. Vero cell line passage 48, breast cancer cell line (4T1 ATCC®CRL-2539™), Colon cancer cell line (CT26.WT-ATCC® CRL-2638™), DU-145 and cervical cancer cell lines. The cells were cultured on ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003 supplemented with 10% foetal bovine serum,L-glutamine (2mM) and 1% penicillin-streptomycin in static 75 cm2 T-Flask (GIBCO, USA). The cells were incubated in a humidified enclosure with 5 % CO_2 at 37^{0}C.

2.4.3 In Vitro Assay for Cytotoxic Activity

2.4.3.1 Cell seeding procedure

Cells were plated in a 96- well plate 2×10^4 cells/well in a suspension of 100 µl. The cells were left overnight (24 hours) to adhere before exposed to the drug. Starting drug

Concentration was 1000 µg/ml and three fold serial dilutions were done from row H up to Row B. The overlying growth media was discarded and the wells filled with 100 µl of fresh growth media leaving out wells in row H across which was added growth media measuring 135 µl. The testing drug measuring 15 µl was added across row H. From row H 50 µl of the testing drug was aspirated up to row B by mixing well and in row H 50 µl was aspirated and discarded to make sure each well contains 100 µl consisting of the drug and growth media. Incubation was done for 48 hours. 10µl of 5mg/ml MTT reagent in sterile PBS was added directly to the wells. The cells were further incubated for 4 hours for formation of insoluble purple formazan from yellowish MTT by enzymatic. The insoluble formazan was solubilized by adding 100 µl of DMSO after removal of supernatant. The optical density of solution was measured at 562 using microplate reader (Multiskan EX, Labsystems).

2.4.3.2 MTT Assay

Growth of tumoral cells quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product (12). At the end of 72 hours incubation, the medium in each well was replaced by MTT solution (20,000 cell/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for 4 hours under 5% CO_2 and 95% air at 37ºC. MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in 100ml DMSO and gently shaken. The absorbance was then determined photometrically at 562nm.

The percentage growth inhibition was calculated using following formula,

$$\% \text{ cell inhibition} = 100 - [(A_t - A_b)/(A_c - A_b)] \times 100$$

Where, A_t = absorbance value of test compound,
A_b = Absorbance value of blank and
A_c = Absorbance value of control.

The effects of extracts were expressed by IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

2.5 Preparation of the plant extract

The leaves material were artificially dried in cold room, powdered through electric miller and extracts obtained through organic extraction. The desired extract of a *physalis peruviana* leaves material was obtained through soaking of powdered leaves in methanol and dichloromethane (DCM) solvent in the ratio of 1:1 for 72 hours. After, filtration was done and the filtrate put in a rotavopor for evaporation of the solvent. The extract was left for some time to dry.

2.6 Phytochemical tests

2.6.1 Test for alkaloids

A drop of the extract solution was spotted on a TLC plate. The spot was allowed to dry at room temperature and then transferred into the deployment chamber containing petroleum ether and ethyl acetate in the ratio 7:3 respectively. The mobile phase was allowed to move to at least three quarters of the page, removed and air dried at room temperature. After drying, it was observed under long wave Uv lamb and then it was sprayed with Dragendorff's reagent. Then observation was done.

2.6.2Test for phenols

A drop of the extract solution was spotted on a TLC plate. The spot was allowed to dry at room temperature and then transferred into the deployment chamber containing petroleum ether and ethyl acetate in the ratio 7:3 respectively. The mobile phase was allowed to move to at least three quarters of the page, removed and air dried at room temperature. After drying, it was observed under long wave Uv lamb and then it was sprayed with a reagent prepared through mixing of 0.1g of ferrichloride in 10 ml water with o.1g ferricyanide in 10 ml water. Then observations were done.

2.6.3 Test for Anthraquinones

A drop of the extract solution was spotted on a TLC plate. The spot was allowed to dry at room temperature and then transferred into the deployment chamber containing petroleum ether and ethyl acetate in the ratio 7:3 respectively. The mobile phase was allowed to move to at least three quarters of the page, removed and air dried at room temperature. After drying, it was observed under long wave Uv lamb and then it was sprayed with a solution of 0.12g of potassium hydroxide pellets in12.5 ml methanol. Then observations were done.

2.6.4 Test for flavanoids

A drop of the extract solution was spotted on a TLC plate. The spot was allowed to dry at room temperature and then transferred into the deployment chamber containing petroleum ether and ethyl acetate in the ratio 7:3 respectively. The mobile phase was allowed to move to at least three quarters of the page, removed and air dried at room temperature. After drying, it was observed under long wave Uv lamb and then it was exposed to ammonia. Then observations were done.

2.6.5 Test for terpenoids

A drop of the extract solution was spotted on a TLC plate. The spot was allowed to dry at room temperature and then transferred into the deployment chamber containing petroleum ether and ethyl acetate in the ratio 7:3 respectively. The mobile phase was allowed to move to at least three quarters of the page, removed and air dried at room temperature. After drying, it was observed under long wave Uv lamb and then it was sprayed with a solution of 5g vanillin in 50ml concentrated sulphuric acid. After that observations were done.

2.6.6 Test for saponins

Small amount of the extract bwas put in a test tube, shaking was done and observations were recorded.

III. RESULTS

3.1 Extraction

Table 3.1: Plant extract and percentage yields of water and methanol extracts

Plant	Part	Extraction method	Weight after extraction (g)	% yield
Physalis peruviana	Leaf	Water	50	50
Physalis peruviana	Leaf	Methanol	49	49

3.2 Test for petrochemicals results in table form.

Test	Observation	Result	Intensity
Alkaloids	Yellow, Blue and green color observed	Present	++
Phenols	Blue color observed	Present	++
Anthraqunons	No color change	Absent	-
Flavonoids	No color change	Absent	-
Terpenoids	Dark blue, purple and navy blue color observed	Present	++
Saponins	Little foam was foamed	Present	+

Key:

- indicates absence of phytochemicals.
++ shows high concentration.
+ shows moderate concentration.

3.3 Phytochemicals on TLC plates and test tubes.

3.4 Cancer cell lines passage result

Table 3.4 Optical density reading at 562 nm for du 145crl-18 passage 22

	1	2	3	4	5	6	7	8	9	10	11	12
	IC_{50} 47.69µg/Ml			IC_{50} 51.7µg/mL			IC_{50} 62.31µg/mL			IC_{50} 50.37µg/mL		
A	0.499	0.513	0.075	0.456	0.5	0.098	0.441	0.465	0.093	0.459	0.525	0.08
B	0.503	0.498	0.079	0.447	0.47	0.101	0.469	0.454	0.097	0.457	0.495	0.077
C	0.466	0.441	0.078	0.387	0.458	0.076	0.466	0.441	0.078	0.387	0.458	0.076
D	0.423	0.474	0.097	0.443	0.446	0.094	0.423	0.474	0.097	0.451	0.388	0.109
E	0.356	0.313	0.083	0.316	0.339	0.1	0.356	0.313	0.083	0.321	0.339	0.083
F	0.186	0.168	0.082	0.169	0.187	0.074	0.194	0.187	0.09	0.194	0.187	0.09
G	0.155	0.109	0.078	0.098	0.097	0.107	0.106	0.118	0.08	0.106	0.118	0.08
H	0.093	0.125	0.118	0.09	0.09	0.096	0.09	0.09	0.096	0.09	0.09	0.096

3.5 Vero passage 48 results

Table 3.5 Vero passage 48												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.503	0.508	0.09	0.532	0.571	0.084	0.503	0.508	0.09	0.5	0.564	0.088
B	0.545	0.512	0.09	0.54	0.517	0.089	0.545	0.512	0.09	0.476	0.521	0.128
C	0.533	0.484	0.081	0.533	0.484	0.081	0.533	0.484	0.081	0.497	0.472	0.08
D	0.41	0.472	0.102	0.41	0.472	0.102	0.41	0.472	0.102	0.535	0.476	0.097
E	0.396	0.405	0.151	0.396	0.405	0.151	0.396	0.405	0.151	0.408	0.425	0.123
F	0.225	0.214	0.087	0.225	0.214	0.087	0.225	0.244	0.087	0.225	0.214	0.087
G	0.099	0.094	0.083	0.099	0.094	0.083	0.099	0.094	0.083	0.099	0.094	0.083
H	0.114	0.107	0.117	0.105	0.106	0.101	0.114	0.107	0.117	0.105	0.106	0.101

3.6 CC_{50} results with VERO cells

The concentration of plant extracts that killed (reduced cell viability) in 50% of the cells (cytotoxic concentration, CC_{50}) was calculated for Vero Cells which was **60.33** µg/mL and was used as standard.

3.7 IC_{50} results of the plant extracts with DU-145CRL-18

The concentration that inhibited growth in 50% of the cells (IC_{50}) was calculated for the DU145CRL-18 cells. This was 47.69 µg/mL and was compared with Vero cells used as standard.

Table 3.7 Efficacy of the plant extracts against DU145CRL-18 cell lines

	Conc. µg/mL	Absorbance at 562nm			Absorbance at 562nm		
		DU145CRL-18		% Cell viability	VERO		% Cell viability
		Test	Blank		Test	Blank	
Physalis peruviana	0.46	0.486	0.075	100%	0.525	0.09	100%
	1.371	0.477	0.079	97%	0.513	0.13	94%
	4.115	0.437	0.078	87%	0.505	0.08	91%
	12.345	0.440	0.097	81%	0.472	0.1	92%
	37.037	0.339	0.083	58%	0.403	0.12	66%
	111.111	0.189	0.082	22%	0.221	0.09	30%
	333.333	0.119	0.078	12%	0.097	0.08	3%
	1000	0.091	0.118	-2%	0.108	0.1	1%
IC_{50}	**47.69** µg/ml				**60.33** µg/Ml		

3.8 IC_{50} results of the plant extracts with Cervical Cancer cells.

The concentration that inhibited growth in 50% of the cells (IC_{50}) was calculated for the Cervical Cancer cells. This was 51.70 µg/mL and was compared with Vero cells used as standard.

Table 3.8 Efficacy of the plant extracts against cervical cancer cell lines

	Conc. µg/mL	Absorbance at 562nm			Absorbance at 562nm		
		Cervical Cancer Cell		% Cell Viability	VERO		% Cell Viability
		Test	Blank		Test	Blank	
Physalis peruviana	0.46	0.478	0.098	100%	0.525	0.09	100%
	1.371	0.459	0.101	94%	0.513	0.13	94%
	4.115	0.267	0.076	91%	0.505	0.08	91%
	12.345	0.270	0.094	92%	0.472	0.1	92%
	37.037	0.220	0.1	60%	0.403	0.12	66%
	111.111	0.178	0.074	27%	0.221	0.09	30%
	333.333	0.098	0.107	-3%	0.97	0.08	3%
	1000	0.09	0.096	-2%	0.108	0.1	1%
IC_{50}	**51.70** µg/mL				**60.33**µg/mL		

3.9 IC_{50} results of the plant extracts with 4T1 cells.

The concentration that inhibited growth in 50% of the cells (IC_{50}) was calculated for the 4T1 cells This was 62.31 µg/mL and was compared with Vero cells used as standard.

Table 3.9 Efficacy of the plant extracts against 4T1 cell lines

	Conc. µg/mL	Absorbance at 562nm			Absorbance at 562nm		
		4TI Cells		% Cell Viability	VERO		% Cell Viability
		Test	Blank		Test	Blank	
Physalis peruviana	0.46	0.453	0.093	100	0.525	0.09	100%
	1.371	0.462	0.097	101%	0.513	0.13	94%
	4.115	0.454	0.078	104%	0.505	0.08	91%
	12.345	0.449	0.097	98%	0.472	0.1	92%
	37.037	0.335	0.083	70%	0.403	0.12	66%
	111.111	0.191	0.09	28%	0.221	0.09	30%
	333.333	0.112	0.08	9%	0.97	0.08	3%
	1000	0.90	0.096	-2%	0.108	0.1	1%
IC_{50}	**62.31** µg/mL				**60.33** µg/mL		

3.10 IC_{50} results of the plant extracts with Colon Cancer cells.

The concentration that inhibited growth in 50% of the cells (IC_{50}) was calculated for the Colon cancer cells. This was 50.37 µg/mL and was compared with Vero cells used as standard.

Table 3.10 Efficacy of the plant extracts against colon cancer cell lines

	Conc. µg/mL	Absorbance at 562nm			Absorbance at 562nm		
		Colon Cancer Cell		% Cell Viability	VERO		% Cell Viability
		Test	Blank		Test	Blank	
Physalis peruviana	0.46	0.492	0.08	100%	0.525	0.09	100%
	1.371	0.476	0.077	97%	0.513	0.13	94%
	4.115	0.423	0.076	84%	0.505	0.08	91%
	12.345	0.420	0.109	75%	0.427	0.1	92%
	37.037	0.330	0.083	60%	0.403	0.12	66%
	111.111	0.191	0.09	24%	0.221	0.09	30%
	333.333	0.112	0.08	8%	0.97	0.08	3%
	1000	0.09	0.096	-2%	0.108	0.1	1%
IC_{50}	**50.37** µg/mL				**60.33** µg/mL		

IV. DISCUSSION

Vero cells have been recommended for cytotoxicity studies and for the analysis of cell-substrate interactions in biomaterial research (ISO, 1992; Kirkpatrick, 1992). This study shows investigations of anti-cancer potential of *Physalis peruviana* plant extract which has not been studied in Kenya, by screening for cytotoxic activity against healthy cells and cancer cell lines. In the first test concentration that inhibited growth in prostate cancer cell line (DU-145) was 47.69 µg/ml and 60.33 µg/mL showed toxicity against the control cell lines (Vero cells) which was used as standard for this test. This indicated that the plant leaves extract was able to stop proliferation of the prostate cancer cell lines at a lower concentration and wasn't toxic to the normal cells since it was lower than the toxic level to the normal cells.

A concentration of 57.70 µg/mL of plant extract was required to inhibit growth of cervical cancer indicating a lower concentration compared to that of the Vero cell concentration which was toxic to the cell used as standard. Therefore, the plant extract was considered effective since it could stop proliferation of the cervical cell line growth without being toxic to the normal cell.

For the breast cancer cell lines the concentration that inhibited growth was 62.31µg/mL, this recorded a much higher concentration compared to the standard concentration of 60. 33 µg/mL which was toxic to the Vero cells. The plant extract was therefore considered ineffective to the 4TI cells since beyond 60. 33 µg/mL concentration the plant extract was considered toxic to the normal cells. A concentration of 50.37 µg/mL of plant extract was required to inhibit growth of colon cancer indicating a lower concentration compared to that of the Vero cell concentration which was toxic to the cell used as standard. Therefore, the plant extract was considered effective since it could stop proliferation of the cervical cell line growth without being toxic to the normal cell. This was the second cancer cell line which the plant extract was most effective on followed by cervical cancer cell line.

The effectiveness of this plant on prostrate, cervical and colon cancer cell line could be due to phytochemicals present in this plant especially the alkaloids which have previously been reported to inhibit growth of cancer cell lines. From the previous studies it has been reported that 4β-Hydroxywithanolide E (4βHWE) compound is a steirodal lactone which from *Physalis peruviana* has anticancer properties especially inhibition of growth of human lung cancer cell line (Wang, 2012). Other withanolis such as Withaferi A from Physalis peruviana fruit extract also has been reported to have antiproliferative activity on breast cancer cell lines (Hahm ,,2011) ,Leukemia (Mandal, 2008).From the previous studies it is seen that the effectiveness of the *Physalis peruviana* leaves crude extract could be because of the presence of this compounds in this plant.

Therefore this study provides an important basis for further investigation into the isolation, characterization and mechanism of cytotoxic compounds from the screened leaves plant extracts esnpecially the Withanolis and Withaferin, thus this plant could be used as a source of new lead structures in drug design to combat cancer.

V. CONCLUSION

Anti-proliferative activity of the plant extracts was experienced with the methanol extracts from on all cancer cell lines with prostate cancer indicating the lowest concentration required for the ant proliferative activity that is IC_{50} of 40 µg/ml. The cancer cell lines that exhibited IC_{50} values of >60.33µg/ml of Vero cells was considered non effective since beyond this concentration the plant extract was toxic to normal cells.

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