Journal of Advanced Research in BioChemistry and Pharmacology
Volume 2, Issue 1 - 2019, Pg. No. 5-10
Peer Reviewed & Open Access Journal

Research Article

In vitro Cytotoxic Evaluation of Ethanol Leaf Extract of *Physalis angulata* Linn on Some Human Carcinoma Cell Lines

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How to cite this article:
Onyegeme-Okerenta BM, Bradshaw TD, Agyare C et al. *In vitro* Cytotoxic Evaluation of Ethanol Leaf Extract of *Physalis angulata* Linn on Some Human Carcinoma Cell Lines. *J Adv Res BioChem Pharma* 2019; 2(1): 5-10.

Date of Submission: 2019-01-16
Date of Acceptance: 2019-03-14

A B S T R A C T

The search for the use of herbal remedies as an alternative medicine for the treatment and management of cancer is on-going. This study evaluates the *in vitro* cytotoxic potential of ethanol leaf extract of *P. angulata* L. on some human carcinoma cell lines. Four different human cell lines: MCF 7 (human breast), C4-2WT (prostate), HT 29 and HCT 116 (colorectal) were used for this study while cytotoxicity screening methods used are - MTT (3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Methylene blue proliferation and Trypan Blue exclusion assays. The extract significantly ($p<0.05$) induced cytotoxicity at 50% inhibition (GI_{50}) as follows: MCF-7 = 3.86 µg/ml, HT 29 = 14.99 µg/ml, HCT 116 = 15.71 µg/ml and C4-2WT = 20.16 µg/ml. Methylene Blue proliferation assay response of the carcinoma cell lines to ethanol leaf extract of *P. angulata* L. indicated a significant decrease ($p<0.05$) in the total number of proliferating cells in the different concentrations of the extract used when compared to the optical densities of the controls at 24 hours, 48 hours and 72 hours. Similarly, results for Trypan Blue exclusion assay showed there was a significant decrease ($p<0.05$) in the total number of viable cells (VC) and a significant increase ($p<0.05$) in the total number of non-viable cells (NVC) over a period of 72 hours. There is a correlation between the results of the cytotoxic assays which indicated that the extract induced cytotoxicity on the human carcinoma cell lines in a time and concentration-dependent manner.

Keywords: Cytotoxicity, Human Carcinoma Cell Lines, *In vitro*, *Physalis angulata* Linn

Introduction

Cancer refers to a group of diseases characterized by abnormal cell growth that proliferates uncontrollably and have the potential to invade or infiltrate to other parts of the body and destroy normal body tissue. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes.[1,2] The World Health

Organization (WHO) reported in its fact sheet of February 2017, that cancer is one of the leading causes of morbidity and mortality worldwide, with nearly 14 million new cases in 2012, which is expected to rise by about 70% over the next two decades. With 8.8 million deaths recorded in 2015 as a result of cancer, it is a public health problem worldwide affecting all categories of persons and has been rated by WHO as the second leading cause of death globally. Seventy percent of these deaths were from low and middle-income countries.[3,4] In Nigeria, some 100 000 new cases of cancer occur every year, with high case fatality ratio.[5] With approximately 20% of the population of Africa and slightly more than half the population of West Africa, Nigeria contributed 15% to the estimated 681,000 new cases of cancer that occurred in Africa in 2008.[6] Records obtained showed a worrisome increase in the trend of new cancer cases and deaths worldwide. In 2004, cancer was ranked as the 7th leading cause of death in Africa, with an expected annual incidence of 1.28 million cases and 970,000 deaths by 2030.[5] The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in 2010 was estimated at approximately US$ 1.16 trillion.[4] The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in 2010 was estimated at approximately US$ 1.16 trillion.[4] This high cost of cancer treatment has led to the search for the use of herbal remedies as alternative medicine for the treatment and management of cancers. According to the WHO, 70-80% of the world population uses the plant-derived traditional methods for the treatment of various health problems.[7,8] Medicinal plants are available and cost-effective when compared to modern therapeutic agents. This makes them more attractive as therapeutic agents.[9] Several plants are understood to have anti-cancer effect.[10] One of such plant is *Physalis angulata* Linn (*P. angulata* L.). *P. angulata* L. is an annual herb belonging to the family Solanaceae and is widely distributed in tropical and temperate regions. It is known by different names, including camapu; cut leaf groundcherry; wild tomato, mullaca, winter cherry etc. In Southwest Nigeria, it is known as Koropo. Its biological properties include antimycobacterial, anticancerous, antitumorous, anticoagulant, hypotensive, immunostimulant etc.[11-14] The Plant prefers moist drained sandy loamy soil with full sun or partial shade; it is renowned as an effective stimulant for the immune system. The juice is used in the treatment of an earache, jaundice, fever, bladder diseases etc. The fruit and other aerial parts are used in the treatment of boils, sores, cuts, constipation, intestinal and digestive problems[15], and used as an antimutagenic, anticoagulant, antispasmodic, antileukemia agents.[16]

This study's aim was to evaluate, *in vitro*, the cytotoxic potential of ethanol leaf extract of *P. angulata* L. on some human carcinoma cell lines.

Material and Methods

Plant Material and Extraction

The dried leaves of *P. angulate* L. were collected by Prof. Christian Agyare from Ghana around November 2017. Plant materials were ground to powder form using an electric mill. The powdered sample was kept in an airtight container until required. About 50 g of the powdered leaves of *P. angulata* L. was macerated in 250 ml of aqueous ethanol (70:30) for 72 h. The vacuum pump was used to filter and the ethanol plant material was dried over a water bath at 40°C and the resulting extract was kept in the refrigerator at -4°C.

Reagents

Dimethyl sulfoxide (DMSO), Trypsin-EDTA, Phosphate buffered saline (PBS), methylthiazolyl diphenyl-tetrazolium bromide (MTT), Methylene blue and all other chemicals and reagents used were obtained from Sigma Aldrich and are of analytical grade.

Cell Lines

The following human cell lines 1) MCF-7 (breast carcinoma cells), 2) C4-2WT (prostate carcinoma cells, 3) HT 29 and 4) HCT 116 (Colorectal carcinoma cells) were used for this present study and they were all obtained from the Tissue Culture Unit of Gene Regulation and RNA Biology Laboratory of the School of Pharmacy, University of Nottingham, United Kingdom. The cell lines were cultured at 37ºC in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine and 10% foetal calf serum (FCS), and routinely sub-cultured twice weekly to maintain continuous logarithmic growth.

Preparation of Extract Stock and Working Solution

Ethanol leaf extract of *P. angulate* L. were prepared as 50 mg stock solutions dissolved in dimethyl sulfoxide (DMSO) and stored at -4ºC, for a maximum period of 4 weeks. Extract dilutions were made in culture medium immediately prior to use.

Fifty milligrams of the extract were dissolved in 1 ml of DMSO to give a stock solution of 50 mg/ml. A working stock of 500 µg/ml was freshly prepared from the 50 mg/ml stock solution using DMEM and various working concentrations of equal volume made by dilution with DMEM to obtain the desired concentration of the extract. The working concentration was prepared freshly and filtered through 0.45-micron filter before each assay. Remaining working solutions were discarded. DMSO of corresponding concentrations was used as a control.

Cytotoxicity Screening

Growth Inhibitory Assays: 3-(4,5-dimethylthiazol-2-yl)-2,5 phenyl tetrazolium bromide (MTT):

The confluent monolayer of four different cell lines - MCF7,

HT 29, HCT 116 and C4-2WT were trypsinized, counted and seeded into 96-well microtitre plates at a density of 3.0 - 4 x 10^3 per well and allowed 24 hours to adhere. Ethanol leaf fraction of *P. angulate* L. were dissolved in DMSO and diluted with complete DMEM medium to get a range of test concentration (0.1 µg to 100 µg/ ml), each concentration was done six times (n=6). DMSO concentration was kept less than 0.1% in all the samples. Prepared dilutions were added to different wells, and cells were incubated for 72 hours. Control groups received the same amount of DMSO. Viable cells at the time of extract addition were time zero; (T0), and following 72 hours, the effect of exposure to the leaf extract was determined by cell-mediated 3-(4, 5-dimethylthiazol-2-yl)-2, 5 phenyl tetrazolium bromide (MTT) reduction. MTT was added to each well (final concentration 400 µg/ ml) and plates were incubated at 37ºC for 4 hours to allow reduction of MTT by viable cell dehydrogenases to an insoluble formazan product. Well supernatants were aspirated and cellular formazan solubilized by addition of DMSO: glycine buffer (pH 10.5; 4:1). Cell growth and agent activity were determined by measuring absorbance at 580 nm using the BioTek Synergy HTX Multi-Mode Microplate Reader. The GI_{50} values of ethanol leaf fraction of *P. angulate* L. were calculated for the four different cell lines and compared statistically with the control.[17] Viable cells measurements were performed and the concentration required for a 50% inhibition of viability (GI_{50}) was determined graphically. The GI_{50} measures the growth inhibitory power of the test agent.

Methylene Blue Proliferation Assay:

A modified method[18] was adopted for Methylene Blue Proliferation assay and has been reported previously.[19] The optical density was measured at 650 nm for each well by BioTek Synergy HTX Multi-Mode Microplate Reader. The photometer was blanked on the last two rows of control wells containing elution solvent alone. Results were reported based on the 72 hours assay.

Trypan Blue Exclusion Assay:

A modified method[20] was adopted for this study and has been reported previously.[19] The cells were counted in a haemocytometer and the cell suspension was diluted with DMEM to give a density of 10.0 X 10^3 per mL per well.

Results

GI_{50} results calculated after MTT test showed the concentration of ethanol leaf extract of *P. angulate* L. required for 50% inhibition of the different cell lines as follows: MCF-7 = 3.86 µg/ml, HT 29 = 14.99 µg/ ml, HCT 116 = 15.71 µg/ ml and C4-2WT = 20.16 µg/ ml. Ethanol leaf extract of *P. angulate* L. strongly decreazed the proliferation of MCF-7, HT 29, HCT 116 and C4-2WT when compared to the untreated control cells (Figure 1-4).

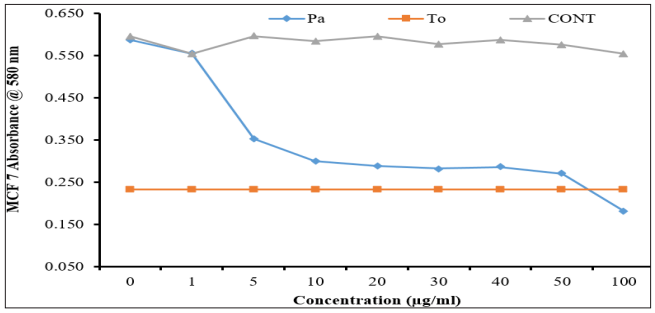

Figure 1.*In vitro* cytotoxic activity of ethanol leaf extract of *P. angulata* L. on MCF 7 after 72 hours treatment

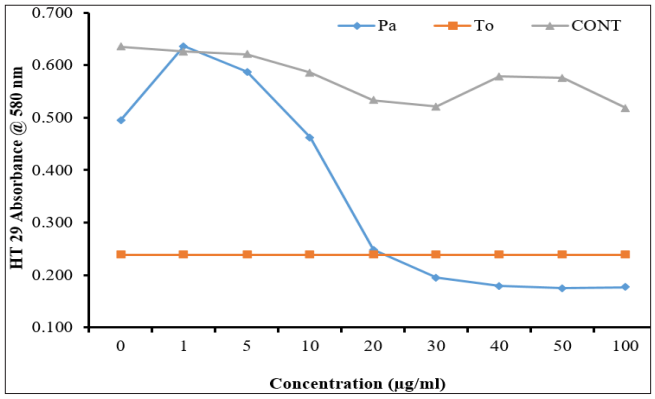

Figure 2.*In vitro* cytotoxic activity of ethanol leaf extract of *P. angulata* L. on HT 29 after 72 hours treatment

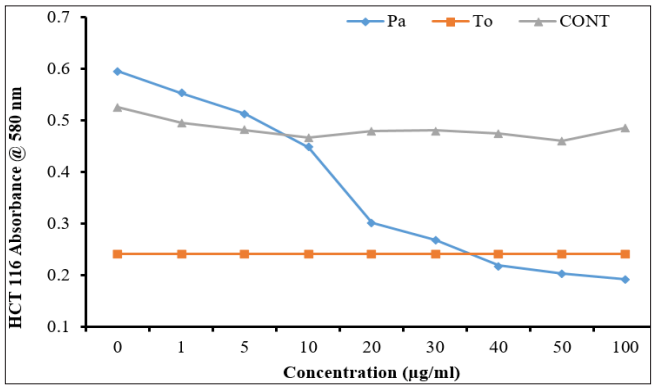

Figure 3.*In vitro* cytotoxic activity of ethanol leaf extract of *P. angulata* L. on HCT 116 after 72 hours treatment

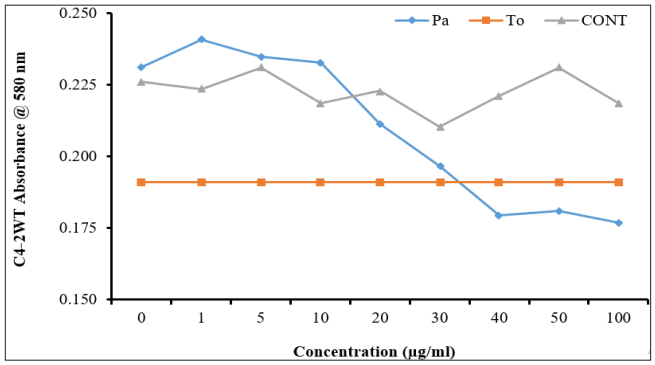

Figure 4.*In vitro* cytotoxic activity of ethanol leaf extract of *P. angulata* L. on C4-2WT after 72 hours treatment

Effect of Different Concentrations of Ethanol Leaf Extract of *P. angulata* L. on Cell Growth and Proliferation

The results obtained for Methylene Blue proliferation assay response of monolayers of the carcinoma cell lines to ethanol leaf extract of *P. angulata* L. indicated a significant decrease ($p<0.05$) in the total number of proliferating cells in the different concentrations of the extract used when compared to the optical densities of the controls at 24 hours, 48 hours and 72 hours (Figure 5-7).

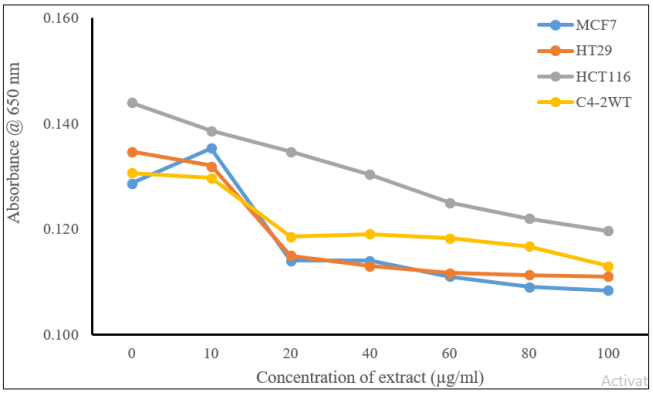

Figure 5.Optical density of viable cell lines 24 hours after treatment with ethanol leaf extract of *P. angulata* L.

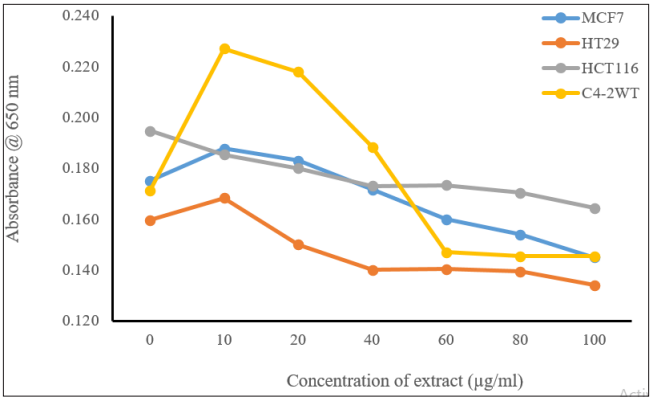

Figure 6.Optical density of viable cell lines 48 hours after treatment with ethanol leaf extract of *P. angulata* L.

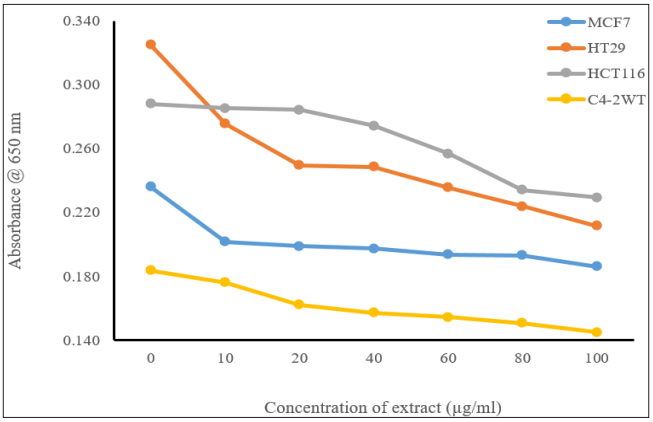

Figure 7.Optical density of viable cell lines 72 hours after treatment with ethanol leaf extract of *P. angulata* L.

Effect of Different Concentrations of Ethanol Leaf Extract of *P. angulata* L. on Cell Growth

The effect of the different concentrations of ethanol leaf extract of *P. angulata* L. on cell growth using Trypan blue exclusion assay showed that inhibition of cell growth and proliferation by extract occurred in a concentration-dependent manner (Figure 8-11). There was a significant decrease ($p<0.05$) in the total number of viable cells (VC) and a significant increase ($p<0.05$) in the total number of non-viable cells (NVC) over a period of 72 hours.

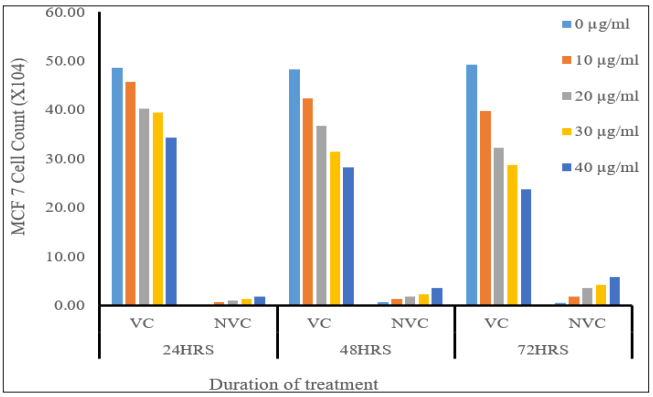

Figure 8.Cell count of viable and non-viable MCF 7 cells after treatment with ethanol leaf extract of *P. angulata* L.

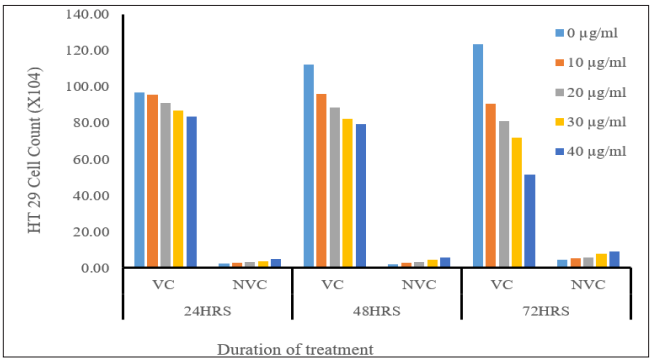

Figure 9.Cell count of viable and non-viable HT 29 cells after treatment with ethanol leaf extract of *P. angulata* L.

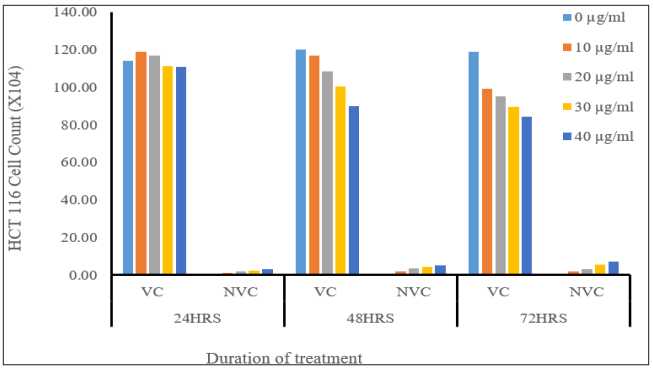

Figure 10.Cell count of viable and non-viable HCT 116 cells after treatment with ethanol leaf extract of *P. angulata* L.

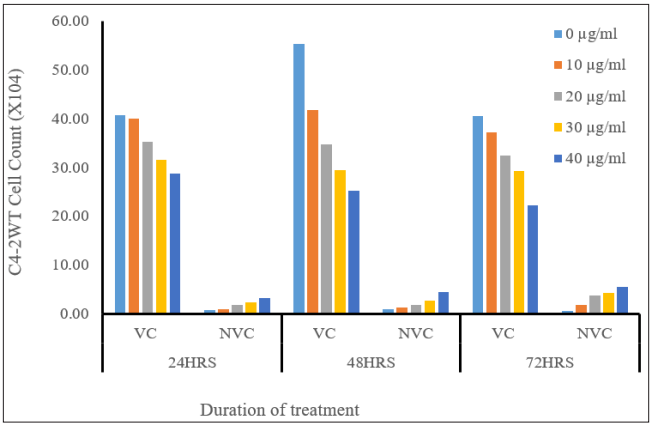

Figure 11.Cell count of viable and non-viable C4-2WT cells after treatment with ethanol leaf extract of *P. angulata* L.

Discussion

The active principles in extracts from a variety of plant sources from tropical and sub-tropical regions of the world are studied for possible applications in human health and reports indicate that approximately 30% of drugs available worldwide are based on natural products.[21,22] Many studies demonstrated significant anti-inflammatory, anti-cancer, anti-asthmatic, anti-diabetic and anti-bacterial activities, etc., which are reported in the extracts of different parts of *P. angulata* L. and also from its phytoconstituents.[23] In the present work, cytotoxic potential of ethanol leaf extract of *Physalis angulate* Linn was evaluated *in vitro* against four different human carcinoma cell lines: MCF 7, C4-2WT, HT 29 and HCT 116 using three different cytotoxic screening assays. MTT was used to explore the concentration required for a 50% inhibition of viability (GI_{50}) of the different cell lines and the result is as follows: MCF-7 = 3.86 µg/ml, HT 29 = 14.99 µg/ml, HCT 116 = 15.71 µg/ml and C4-2WT = 20.16 µg/ml. According to the criteria of the American National Cancer Institute, before a crude extract will be considered promising for further purification, the GI_{50} limit of the crude extract must be lower than 30 µg/ml while a crude extract with IC_{50} less than 20µg/ml is considered highly cytotoxic.[24,25] A group of researcher,[26] reported that extracts of Withangulatin A (1) and Withangulatin I (2) from the whole plant of *Physalis angulata* exhibited cytotoxic activities *in vitro* against two human cancer cell lines, colorectal carcinoma COLO 205 and gastric carcinoma cells with IC_{50} values of 16.6 and 1.8 and 53.6 and 65.4mM, respectively. Also, a single treatment of ethanol extract of *P. angulata* L. assayed by MTT method on T47D cells produced cytotoxicity effect with IC_{50} value of 160 µg/ml[27], and inhibits COX-2 activity in MCF-7 cells with IC_{50} of 37.57±3.11 µg/ml.[28] Similarly, the chloroform extract of *Physalis minima* produced a significant growth inhibition against human T-47D breast carcinoma cells as compared with other extracts with an EC_{50} value of 3.8 mg/ml.[29] A research on cytotoxic activity of extracts of *P. angulata* identified physalin-F as the active cytotoxic molecule present in the plant extract and concluded that Physalin-F induced cell apoptosis through the ROS-mediated mitochondrial pathway and suppressed NF-κB activation in human renal cancer A498 cells. Thus, physalin F appears to be a promising anti-cancer agent worthy of further clinical development. Therefore, ethanol leaf extract of *P. angulata* L., from this study, has a strong cytotoxic potential against the different carcinomas investigated.[30]

Results obtained for the methylene blue proliferation assay showed that the significant decrease ($p<0.05$) in the number of proliferating cells when treated with ethanol leaf extract of *P. angulata* L. occurred in a time and concentration-dependent manner. This indicates a direct proportionality to the concentration of the dye eluted from the viable cells and this is a function of the total number of viable cells present in the microplate wells. Similarly, a significant reduction ($p<0.05$) in the total number of viable cells and a significant increase ($p<0.05$) in the total number of non-viable cells over 72 hours post-treatment with the leaf extract was observed using the Trypan blue exclusion assay to evaluate cytotoxicity. According to a report,[19] the decrease in the number of proliferating cells is as a result of cytotoxicity induced by varying concentrations of the extract used.

There is a correlation between the three cytotoxicity methods implored in evaluating *in vitro*, the cytotoxic potential of ethanol leaf extract of *P. angulata* L. on some human carcinoma cell lines. Results indicates that the ethanol leaf extract of *P. angulata* L. induces cytotoxicity and inhibits proliferation of human carcinoma cells with GI_{50} MCF-7 = 3.86 µg/ml, HT 29 = 14.99 µg/ml, HCT 116 =15.71 µg/ml and C4-2WT = 20.16 µg/ml in a time and concentration-dependent manner.

Acknowledgment

This research was sponsored by the Commonwealth Scholarship Commission, United Kingdom and supported by the Gene Regulation and RNA Biology Laboratories, School of Pharmacy, University of Nottingham, United Kingdom.

Conflict of Interest: None

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