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Full Length Research Paper

Cytotoxic potential of ethanol extract of *Parquetina nigrescens* on MCF-7, C4-2WT, HT 29 and HTC 116 cell lines

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The cytotoxic activity of ethanol extract of *Parquetina nigrescen* was investigated using a (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, methylene blue and Trypan Blue exclusion assay on four human cancer cell lines, MCF-7, HT 29, HTC 116 and C4-2WT. A mitochondrial enzyme in living cells, succinate-dehydrogenase cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan whose intensity is directly proportional to the presence of viable cells in the microwell plate. Results showed a significant ($p<0.05$) cytotoxic effect of the extract in a dose dependent manner. Cytotoxicity increased with increase in the concentration of the extract used. GI50 results calculated after MTT test showed the concentration of ethanol extract of *P. nigrescens* required for 50% inhibition of the different cell lines as follows: MCF-7 = 2.61 µg/ml, C4-2WT = 8.33 µg/ml, HCT 29 = 3.47 µg/ml and HCT 116 =1.75 µg/ml. For the methylene blue assay, the number of viable cells present was significantly reduced ($p<0.05$) with an increase in the concentration of the extract and duration of exposure of the cells to the extract. The result of trypan blue assay showed a significant reduction ($p<0.05$) in the total count of viable cells and a significant increase ($p<0.05$) in the total count of non-viable cells over 72 h post-treatment with an extract of *P. nigrescens*. Comparatively, results obtained indicate that there is a correlation between the various methods adopted in establishing the antiproliferative and cytotoxic activity of ethanol extract of *P. nigrescens* obtained in this study.

Key word: *Parquetina nigrescens*, cytotoxicity, human cell lines, MTT, trypan blue, methylene blue.

INTRODUCTION

Cancer is a generic term for a large group of diseases that can affect any part of the body and is characterized by the rapid creation of abnormal cells that grow beyond their usual boundaries. Abnormal proliferating cells can invade adjoining parts of the body and spread to other organs. This latter process is referred to as metastasizing

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and metastases are a major cause of death from cancer. The World Health Assembly in 2017, passed the resolution Cancer Prevention and Control through an Integrated Approach (WHA70.12) urges governments and WHO to accelerate action to achieve the targets specified in the Global Action Plan *and* 2030 UN Agenda for Sustainable Development to reduce premature mortality from cancer (WHO, 2013). Despite the promotion of synthetic chemistry as a method of drug discoveries and drug productions, the contribution of new and novel products from potential bioactive plants or their extracts for disease treatment and prevention is still vast (Kviecinski et al., 2008). Numerous phytochemical compounds found in plants with anticancer properties include: alkaloids, phenylpropanoids, and terpenoids (Kintzios, 2006; Park et al., 2008). Some plant-derived drugs like vinblastine, vincristine, taxol, and camptothecin, with antitumor potentials, have been reported to be efficacious (Yousefzadi et al., 2010). Plants contain the unlimited capacity to generate compounds that fascinates researchers in the quest for new and novel chemotherapeutics (Reed and Pellecchia, 2005).

Phytochemicals in plants over the past century have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest in the biological activities of these substances (Moghadamtousi et al., 2013). Despite these studies, a limited range of plant species has experienced detailed scientific inspection. The attainment of an understanding of natural products necessitates comprehensive investigations on the biological activities of these plants (Moghadamtousi et al., 2014). Certain African plants have a long history of use in ethnomedicine and are a rich source of active phytoconstituents that provide medicinal or health benefits against various diseases. One such plant with extensive traditional uses is *Parquetina nigrescens*.

Also known as bullock, *P. nigrescens* is a shrub found in equatorial West Africa and its leaves, roots and latex have been in traditional medicine practice for centuries (Owoyele et al., 2011). It occurs in secondary forest, savanna, vegetation bordering roads and gallery forest, also commonly growing on ant-hills. It grows on various types of soil, including marshy areas (Alvarez Cruz, 2012). *P. nigrescens* is used in traditional medicine in small amounts, as the plant is toxic, especially the latex. Many fatal accidents have been recorded. The plant or leaf decoction is taken as an enema to treat serious kidney problems, severe constipation and to induce abortion.

Sometimes freshly crushed leaves are taken as an emetic to treat severe constipation (Imaga et al., 2009). A leaf decoction or infusion of *P. nigrescens*, sometimes with parts of other plant species, is drunk to treat measles, intestinal worms, diarrhoea, dysentery, diabetes, menstrual disorders and venereal diseases. It is given to children in very small quantities, to treat respiratory diseases (Agbor and Odetola, 2005). A leaf decoction with honey added is drunk to treat fatigue, jaundice, stomach ulcers and anaemia, as a tonic. It is also taken to treat hypotension and to ease child birth. The body is washed with a leaf decoction to treat general fatigue. The leaves are a common ingredient in medications to treat insanity (Kayode et al., 2009). In Nigeria *P. nigrescens*, *Sorghum bicolor* (L.) Moench and *Harungana madagascariensis* Lam. ex Poir, (Jubi formular), is marketed as a constituent of a commercial herbal preparation to treat anaemia (Alvarez Cruz, 2012). Extract of *P. nigrescens* has been shown to have a high content of flavonoids, saponins, glycosides, cardiac glycosides, tannins, anthraquinones, phlo- batannins and oils and its antioxidative properties have been reported (Ayoola et al., 2011).

Chemoprevention by natural products may be considered a promising approach to cancer control and management (Karikas et al., 2010). Many studies have demonstrated antiproliferative, cytostatic and cytotoxic activities of phytochemicals against cancer cells (Cordaliza et al., 2007). In this study, the ethanol extract of *P. nigrescens* was tested as potential anticancer agent. The antitumoral activity of this plant extract was tested on four human cancer cell lines: MCF-7 (breast carcinoma cells), C4-2WT (prostate carcinoma cells, HCT 29 and HCT 116 (Colorectal carcinoma cells). Cytotoxicity tests implored include MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), methylene blue proliferative assay and trypan blue assay for cell count.

MATERIAL AND METHODS

Plant material and extraction

The dried leaves of *P.* nigrescens were collected from Ghana. Plant materials were ground to a 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. nigrescens* was macerated in 250 mL of aqueous ethanol (70:30) for 72 h. The vacuum pump was used for filtering 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

Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) and all of other chemicals and reagents used were obtained from Sigma Aldrich and are of analytical grade.

Cell lines

All cell lines used during the present study were obtained from

Tissue Culture Unit of Gene Regulation and RNA Biology Laboratory of the School of Pharmacy, University of Nottingham, United Kingdom. These cell lines were: 1) MCF-7 (breast carcinoma cells), 2) C4-2WT (prostate carcinoma cells, 3) HCT 29 and 4) HCT 116 (Colorectal carcinoma cells). The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

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.

Ethanol extracts of *P. nigrescens* 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.

Preparation of extract stock and working solution

Fifty milligrams of the extract was 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. The 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 phenyltetrazolium bromide (MTT): Cells were seeded into 96-well microtitre plates at a density of 3.0 - 4 x 10^3 per well and allowed 24 h to adhere. Before drugs were introduced (final concentration 0.1 µg to 100 µg/ml, n=6), extract dilutions as well as DMSO control were prepared using DMEM as diluents immediately prior to each treatment. Ethanol fractions of *P. nigrescens* were dissolved in DMSO and diluted with complete DMEM medium to get a range of test concentration (0.1 µg to 100 µg/ml). 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 h. Control groups received the same amount of DMSO. Viable cells at the time of extract addition were time zero; (T0), and following 72 h, the effect of exposure to extract were determined by cell-mediated 3-(4,5-dimethylthiazol-2-yl)-2,5 phenyltetrazolium bromide (MTT) reduction. MTT was added to each well (final concentration 400 µg/ml) and plates were incubated at 37ºC for 4 h to allow reduction of MTT by viable cell dehydrogenases to an insoluble formazan product. Well supernatants were aspirated and cellular formazan solubilised 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 extract of *P. nigrescens* were calculated for the four different cell lines - MCF7-, HT 29, HTC 116 and C4-2WT and compared statistically with the control. The American National Cancer Institute renamed the IC_{50} value, the concentration that causes 50% growth inhibition as GI_{50} value to emphasize the correction for the cell count at time zero; therefore, the GI_{50} measures the growth inhibitory power of the test agent and is calculated thus:

$$OD\ GI_{50} = (Cont - To)/2+To \quad (1)$$

Insert computed OD GI_{50} value into Equation 2

$$GI50 = (HOD - OD\ GI_{50})/(HOD - LOD) * (HC - LC) + LC \quad (2)$$

Where, OD GI_{50} = Optical Density of GI_{50}; Cont = optical density of non- treated; To = optical density at time zero; HOD = high optical density within which GI_{50} falls; LOD = low optical density within which GI_{50} falls; HC = High Conc. within which GI_{50} falls; LC = Low Conc. within which GI_{50} falls. Viable cells were determined by the absorbance at 580 nm after MTT. Measurements were performed and the concentration required for a 50% inhibition of viability (GI50) was determined graphically.

Methylene blue proliferation assay

A modified method of Oliver et al. (1989) was adopted. The cells were counted in a haemocytometer and the cell suspension was diluted with DMEM to give a density of 5.0 x 10^3 per well. Cell suspensions were introduced into 96- microtitre plates using a repeating pipette with sterile tip. Cells were seeded for 24 , 48 and 72 h for each cell line and allowed 24 h to adhere before extract was introduced (final concentration 10 µg -100 µg/ml, n=6). Cells for day 0 were counted 3-4hrs giving time for cells to adhere and then methylene blue assay was carried out. Assays were carried out 24, 48 and 72 h respectively.

Fixation of cells: The culture medium in each well was removed by gentle vacuum aspiration using a Pasteur pipette with a fine angled tip. The cell layer was then fixed by adding 100 µl of 100% methanol to each well and let stand for 30 min.

Cell staining: The fixative was removed by gentle vacuum aspiration using a Pasteur pipette and 100 µl of filtered 1 % (w/v). Methylene blue was added to each well. Methylene blue stains only the viable (live) cells. After 30 min, excess dye was removed by another gentle vacuum aspiration using a Pasteur pipette. The remaining dye was then washed off by serially dipping the plate into each of four tanks of distil water, shaking the water off between each immersion. This was done in a uniform manner to minimize between-plate variation. After the last rinse and shake, the cell layer, still stained with methylene blue, was examined microscopically. To elute the dye, 100 µl of 1:1 (v/v) ethanol and 0.I M-HCl was added to each well. The plates were then agitated on a plate shaker for 30 min to release the fixed stain and 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 h assay.

Trypan blue exclusion assay

A modified method stated by Karthik Raman (2016) was adopted for this study. 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. Cell suspensions were introduced in triplicates into 6- well plates using a sterile disposable pipette. Cells were seeded for 24, 48 and 72 h for each cell line and allowed 24 h to adhere before extract was introduced (final concentration; 10 µg - 40 µg/ml, n=6). Assays were carried out 24, 48 and 72 hrespectively for each cell line.

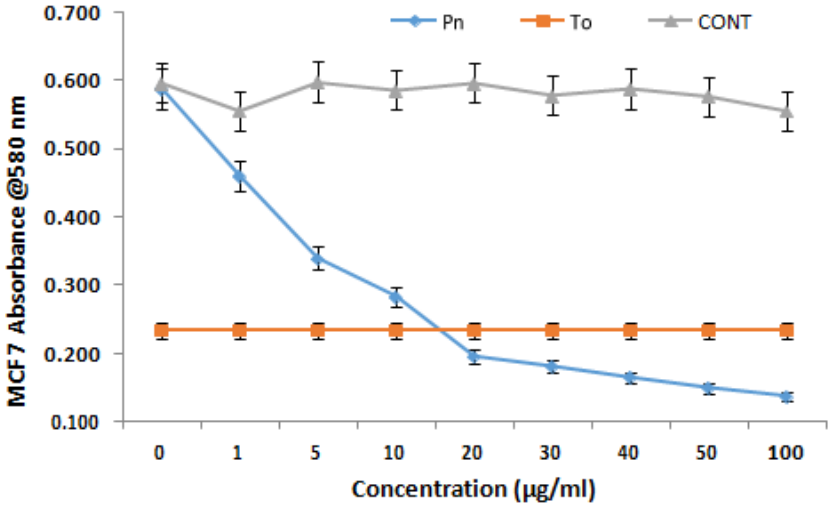

Figure 1. Cytotoxic effect ethanol extract of *P. nigrescens* on MCF 7 after 72 h treatment.

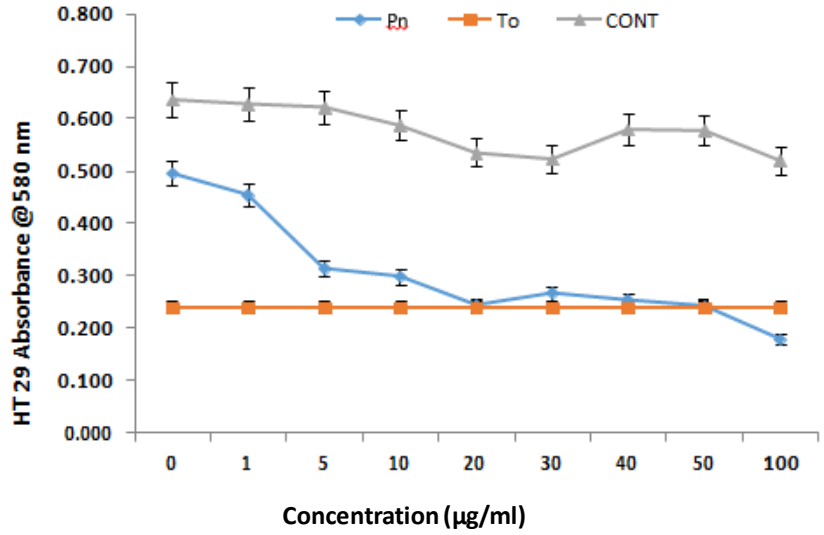

Figure 2. Cytotoxic effect ethanol extract of *P. nigrescens* on HT 29 after 72 h treatment.

The culture medium in each 6-well plate was removed by gentle vacuum aspiration using a Pasteur pipette with a fine angled tip. The wells were washed with warm sterile phosphate buffered saline (PBS) and aspirated off into the waste pot and 500 µl of 0.05% trypsin in 0.53 mM EDTA (enough to cover the cell surface) was added. This was incubated at 37°C for 5 min until the cells have dissociated. A tap to the side of the flask can encourage recalcitrant cells to let go. Cells were resuspended in 500 µl of fresh medium bringing the total volume to 1 mL. To check the concentration of dead cells, 95 µl cell suspension from each well was transferred into well labelled 0.5 ml Eppendorf tubes and 5 µl trypan blue added and count using the haemocytometer. Dead cells stained blue.

RESULTS

MTT

Concentrations of 0.1 to 100 g/mL of *P. nigrescens* extracts showed an increase ($p<0.05$) in cytotoxicity activity on MCF-7 C4-2WT, HCT 29 and HCT 116 as compared to the untreated control cells (Figures 1 to 4). The concentration required for a 50% inhibition of viability (GI_{50}) was determined by substituting the values in Equation 2 for calculation of GI_{50}. GI_{50} results calculated

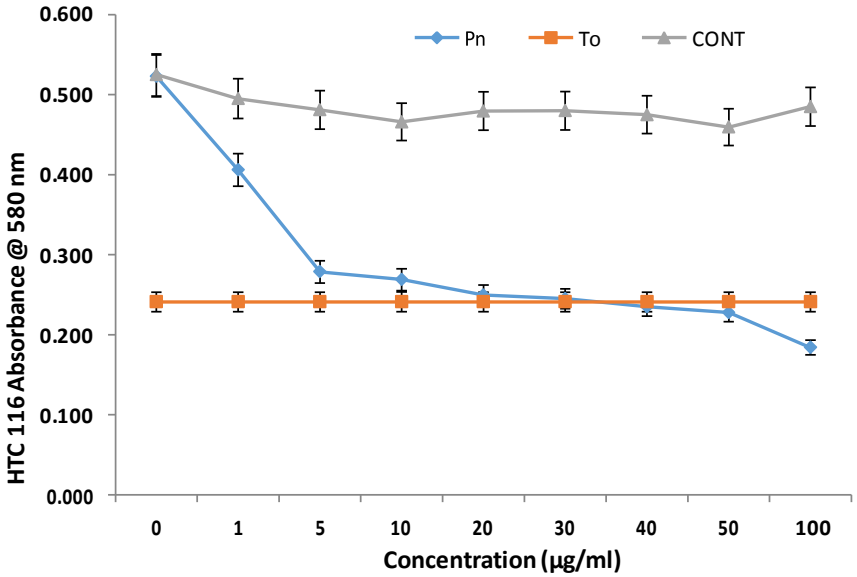

Figure 3. Cytotoxic effect ethanol extract of *P. nigrescens* on HTC 116 after 72 h treatment.

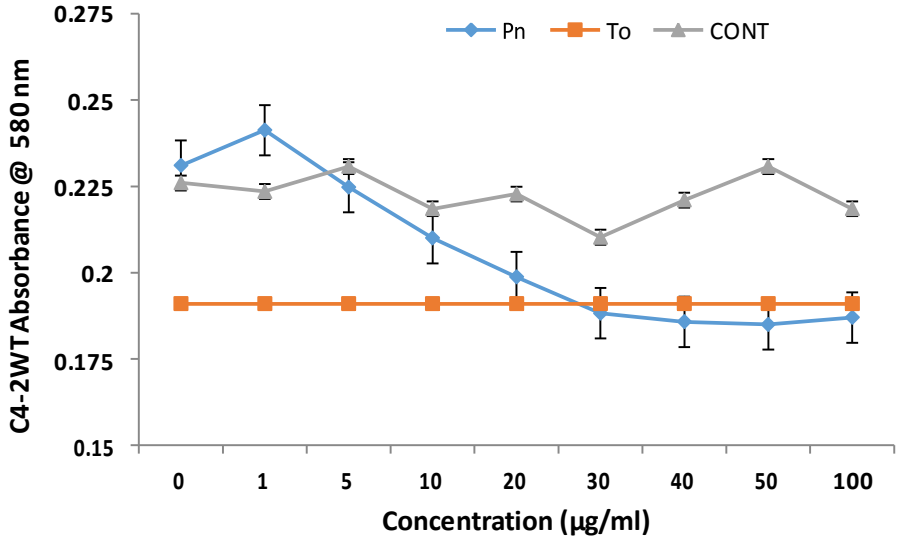

Figure 4. Cytotoxic effect ethanol extract of *P. nigrescens* on C4-2WT after 72 h treatment.

after MTT test showed the concentration of ethanol extract of *P. nigrescens* required for 50% inhibition of the different cell lines as follows: MCF-7 = 2.61 µg/ml, C4-2WT = 8.33 µg/ml, HCT 29 = 3.47 µg/ml and HCT 116 =1.75 µg/ml. However, according to the criteria of the American National Cancer Institute, the GI_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/ml.

Methylene blue assay

The result of the methylene blue colorimetric micro titre plate assay for determining the response of monolayers of the different cell lines to ethanol extract of *P. nigrescens* showed linearity in the relationship between different concentrations of the extract used and their optical densities at 24, 48 and 72 h (Figures 5 to 7). This

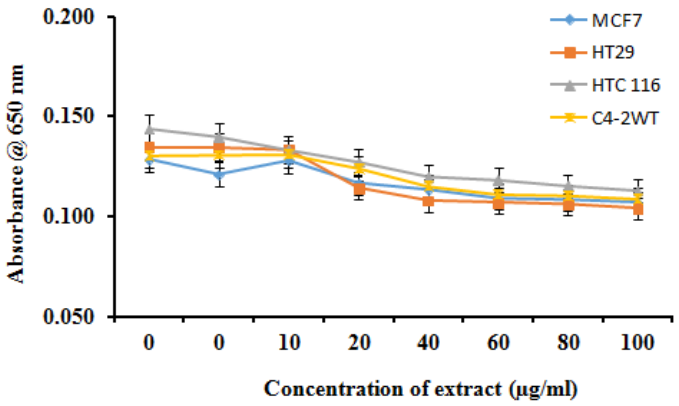

Figure 5. Optical density of viable cell lines 24 h after treatment with ethanol extract of *P. nigrescens.*

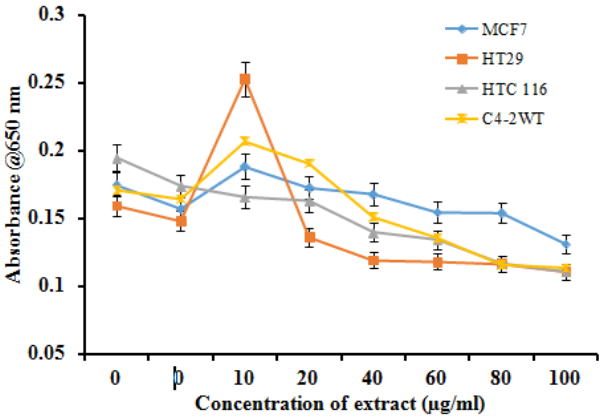

Figure 6. Optical density of viable cell lines 48 hrs after treatment with ethanol extract of *P. nigrescens.*

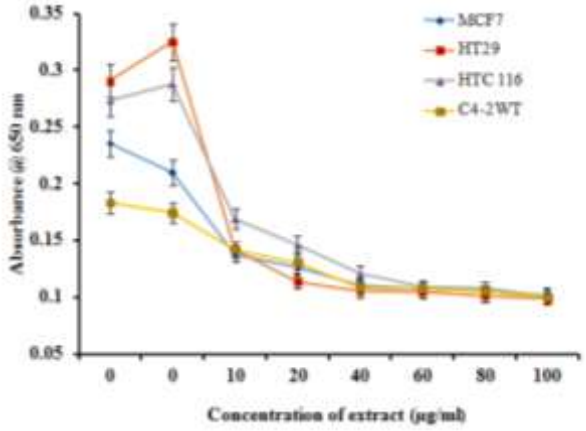

Figure 7. Optical density of viable cell lines 72 hrs after treatment with ethanol extract of ***P. nigrescens.***

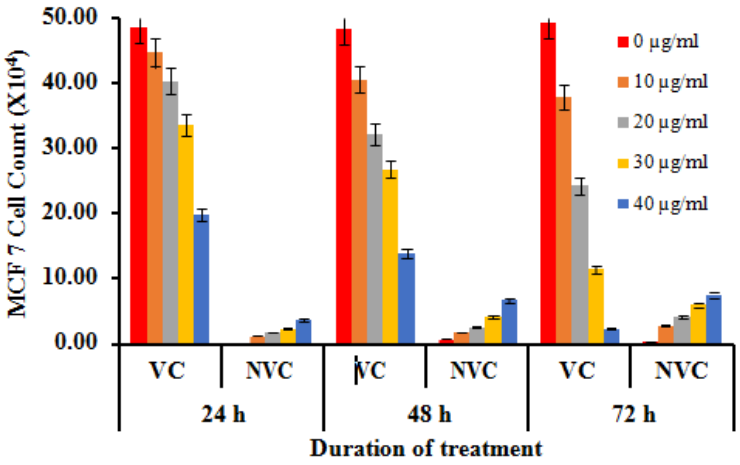

Figure 8. Cell count of viable (VC) and non-viable (NVC) MCF 7 cells after treatment with ethanol extract of *P. nigrescens.*

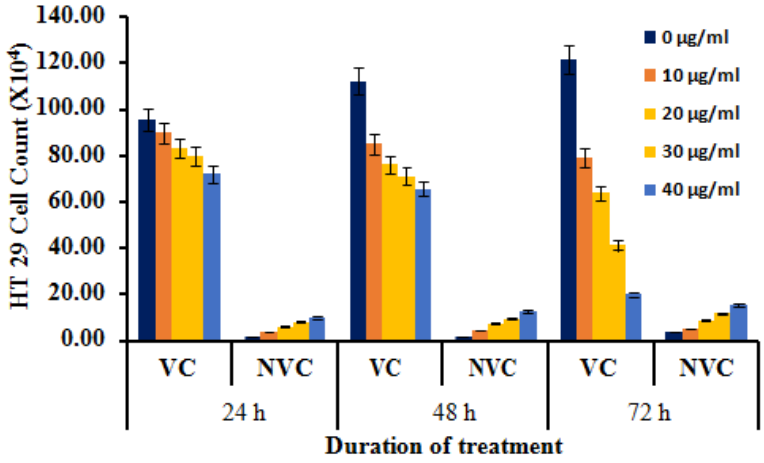

Figure 9. Cell count of viable (VC) and non-viable (NVC) HT 29cells after treatment with ethanol extract of *P. nigrescens.*

was confirmed for each cell line and when initial cell density was optimized to give exponential growth over the assay period, differences in response to different concentrations of the extract were obvious. The methylene blue colorimetric microtitre plates assay was found to be a simple, reliable, sensitive method with low variability, for determining the response of cultured cells lines to the inhibitory agent.

Trypan blue exclusion assay for cell count

Trypan blue exclusion assay counts the number of dead cells in a given cell population. Because trypan blue is a charged dye, it cannot permeate through living cells. So, simply incubating cells with trypan blue and looking under a microscope allows you to visually determine the number of viable cells (unstained), a number of non-viable cells (dark blue), and the number of damaged cells (slightly blue). Viable cells (VC) and non-viable cells (NVC) were counted for each concentration of extract and cell lines. Results obtained showed a significant decrease ($p<0.05$) in the total number of viable cells and a significant increase ($p<0.05$) in the total number of non-viable cells with an increase in extract concentration (Figures 8 to 11). Inhibition of cell growth and proliferation of cells by *P. nigrescens* occurred in a dose dependent manner.

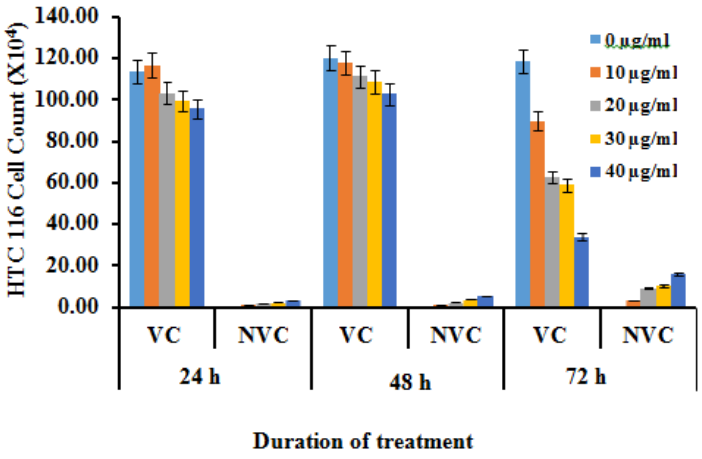

Figure 10. Cell count of viable (VC) and non-viable (NVC) HT 116cells after treatment with ethanol extract of *P. nigrescens.*

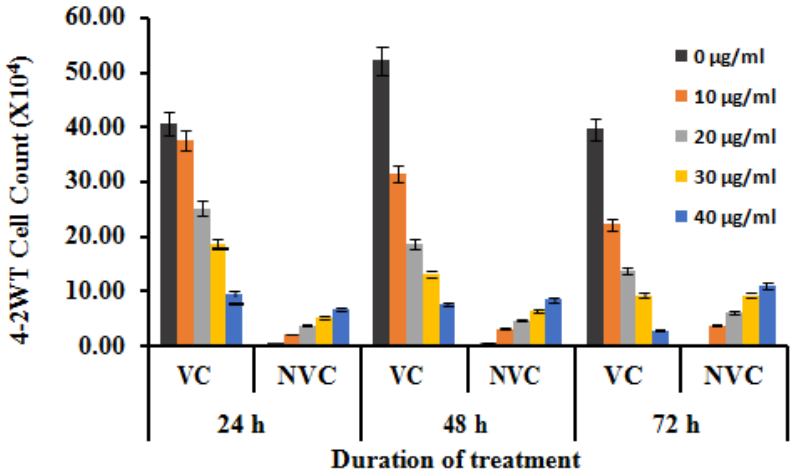

Figure 11. Cell count of viable (VC) and non-viable (NVC) C4-2WTcells after treatment with ethanol extract of *P. nigrescens.*

DISCUSSION

MTT reduction assay is carried out to evaluate the activity of mitochondrial and non-mitochondrial dehydrogenases of compounds as a potential indication of their cytotoxic effect (Döll-Boscardin et al., 2012). Also, a mitochondrial enzyme in living cells, succinate-dehydrogenase cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan. The amount of formazan produced is directly proportional to the number of viable cells. The *nigrescens* as a potential anticancer agent. Cytotoxicity may be due to loss of cellular function and viability either through necrosis or by apoptosis caused by *P. nigrescens* which is an exogenous or foreign agent to the affected cells. Mosmann (1983), showed that methanol cytotoxic activity of ethanol extract of *P. nigrescens* was investigated using an MTT assay on four human cancer cell lines, MCF-7, HT 29, HTC 116 and C4-2WT. Results showed a significant ($p<0.05$) cytotoxic effect of the extract in a dose dependent manner. Cytotoxicity increased with increase in the concentration of the extract used. Similarly, 50% inhibition of viability (GI_{50}) of the extract on the four cell lines was less than 30 µg/ml; this is below the criteria of the American National Cancer Institute and indicates the prospect of *P.* extract significantly inhibited cancer cell growth at a concentration of 100 µg/ml due to the presence of compounds in the extract. Similarly, Akiriti et al. (2014) in their *in- vitro* cytotoxicity study of methanolic fraction from *Ajuga bracteosa* wall ex. benth on MCF-7 breast

adenocarcinoma and hep-2 larynx carcinoma cell lines showed that significant cytotoxic activity was detected for the methanolic fraction of *Ajuga bracteosa* (aerial part) presenting IC_{50} values lower than 5 and 10 µg/ml against two cell lines (MCF-7 and Hep-2). Furthermore, Adu-Amoah et al. (2014), reported that significant reduction in the viability of the HaCaT keratinocytes was observed from treatment with 10, 50 and 100 81 µg/mL of leaf extract of *E. ivorense*, 0.1 to 100 µg/mL of bark extracts of *E. ivorense* ($p<0.0001$) and 100 µg/mL leaf and other aerial parts extract of *P. nigrescens* ($p<0.01$) as compared with the untreated cells.

The linearity of the methylene blue and Trypan Blue exclusion assay was carried out to demonstrate the presence of viable and non-viable cells in the media after 24, 48 and 72 h respectively. For the methylene blue assay, the number of viable cells present was significantly reduced ($p<0.05$) with an increase in the concentration of the extract and duration of exposure of the cells to the extract. The intensity of the medium is directly proportional 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. This observation suggests that cytotoxic effect of ethanol extract of *P. nigrescens* is dose and time dependent. On the other hand, the Trypan Blue exclusion cell count is a measure of the number of non-viable cells observed during cell count. The non-viable cells stained dark blue under the light microscope. A significant reduction ($p<0.05$) in the total count of viable cells and a significant increase ($p<0.05$) in the total count of non-viable cells over 72 h post treatment with an extract of *P. nigrescens* was an indication of non-proliferation of the cells due to the cytostatic or cytotoxic activity of the extract.

Conclusion

Results indicate that there is a correlation between the various methods adopted in establishing the anti-proliferative and cytotoxic activity of ethanol extract of *P. nigrescens* obtained in this study. However, this plant has shown pronounced cytotoxic activity against some human cell lines and will be evaluated further for the possible isolation of active anticancer compounds.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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