Anti-proliferative Activity of Ethyl Acetate Leaf Extract of *Annona Muricata* L. on Selected Carcinoma Human Cell Lines

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ABSTRACT

Medicinal plants are a rich source of active phytochemicals and have been in use in ethnomedicine. This study investigates the anti-proliferative activity of ethyl acetate leaf extract of Annona muricata L. on selected carcinoma human cell lines of MCF 7, HT 29, HCT 116 and C4-2WT. MTT (3- (4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide) assay, methylene blue, Trypan Blue exclusion assay and wound or scratch assays were carried out to evaluate, in vitro, the cytotoxicity potential of the ethyl acetate leaf extract of Annona muricata L. Results showed that anti-proliferation activity increased with increase in the concentration of the extract used. The minimum concentration of the extract required for 50% inhibition (GI_{50}) of the different cell lines calculated after MTT test were as follows: MCF-7 = 2.61 µg/ml, C4-2WT = 9.33 µg/ml, HT 29 = 4.97 µg/ml and HCT 116 =2.13 µg/ml. There was a significant reduction ($p<0.05$), using the methylene blue assay, in the total number of viable cells when their optical densities were measured at 24 hrs, 48 hrs and 72 hrs. Trypan Blue exclusion 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 the extract. Photomicrographs of scratch assay obtained after 48 h using MCF 7 as the experimental model showed an increase in cell proliferation and wound healing up to approximately 100% confluence in control cultures not treated with the extract. However, cells treated with GI_{50} of the extract showed non-proliferation, expansion and deterioration of the wound or gap created by the scratch. Conclusively, there was linearity in the relationship between different concentrations of the extract used and the anti-proliferative activity of the extract.

Keywords: annona muricata l., human carcinoma cells, anti-proliferative, scratch assay.

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I. ABSTRACT

Medicinal plants are a rich source of active phytochemicals and have been in use in ethnomedicine. This study investigates the anti-proliferative activity of ethyl acetate leaf extract of Annona muricata L. on selected carcinoma human cell lines of MCF 7, HT 29, HCT 116 and C4-2WT. MTT (3- (4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide) assay, methylene blue, Trypan Blue exclusion assay and wound or scratch assays were carried out to evaluate, in vitro, the cytotoxicity potential of the ethyl acetate leaf extract of Annona muricata L. Results showed that anti-proliferation activity increased with increase in the concentration of the extract used. The minimum concentration of the extract required for 50% inhibition (GI_{50}) of the different cell lines calculated after MTT test were as follows: MCF-7 = 2.61 µg/ml, C4-2WT = 9.33 µg/ml, HT 29 = 4.97 µg/ml and HCT 116 =2.13 µg/ml. There was a significant reduction ($p<0.05$), using the methylene blue assay, in the total number of viable cells when their optical densities were measured at 24 hrs, 48 hrs and 72 hrs. Trypan Blue exclusion 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 the extract. Photomicrographs of scratch assay obtained after 48 h using MCF 7 as the experimental model showed an increase in cell proliferation and wound healing up to approximately 100% confluence in control cultures not treated with the extract. However, cells treated with GI_{50} of the extract showed non-proliferation, expansion and deterioration of the wound or gap created by the scratch. Conclusively, there was linearity in the relationship between different concentrations of the extract used and the anti-proliferative activity of the extract.

Keywords: annona muricata l., human carcinoma cells, anti-proliferative, scratch assay.

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II. INTRODUCTION

Cancer is the second leading cause of death globally and was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer. Approximately 70% of deaths from cancer occur in low- and middle-income countries. Around one-third of deaths from cancer are due to the 5 leading behavioural and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use (Ferlay *et al.,* 2013; WHO, 2018). In a pharmaceutical landscape, plants with a long history of use in ethnomedicine are a rich source of active phytoconstituents that provide medicinal or health benefits against various ailments and diseases. The goal of screening medicinal plant is to search for excellent anticancer agent avertable to human malignancies. In defiance of astonishing advances in modern medicine, such as surgery, radiotherapy, chemotherapy, and hormone therapy, cancer disease remains a worldwide

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health problem, thus endeavouring the search for a new alternate approach. The nature as a huge valuable contributor to a potential source of chemotherapeutic agents has recently been reviewed (Newman and Cragg, 2007). Newman and Cragg (2007) reported in their analysis that the sources of new drugs over the period 01/1981-06/2006 possess 974 small molecules, out of which 66% were new chemical entities which are classified synthetic, 17% correspond to synthetic molecules containing pharmacophores derived directly from natural products, and 12% are actually modelled on a natural product inhibitor of the molecular target of interest, or mimic (i.e., competitively inhibit) the endogenous substrate of the active site, such as ATP. These facts are in favour of the new call for medicinal plant identification namely local plants, in conjunction with anticancer properties (Newman and Cragg, 2007; Soundararajan and Sreenivasan, 2012). Though the number of cancer survivors continues to increase due to the improvements in early detection, cancer incidence and deaths still escalating each year. Even though there are major advancement in medical techniques such as chemotherapy, radiotherapy and nuclear medicine, people in developing countries, especially in Asian countries, are looking towards natural product as an alternative medicine especially in cancer treatment and prevention; primarily because of the general belief that herbal drugs are without any side effects besides being cheap and locally available (Fakhrurazi *et al.*, 2012). One of such medicinal plant that has been reportedly used in the treatment of different types of cancer disease is *Annona muricata* L.

Annona muricata L. commonly known as soursop, graviola, guanabana, paw-paw and sirsak, is a member of the Annonaceae family comprising approximately 130 genera and 2300 species. *Annona muricata* is a small, upright, evergreen tree that can grow to about 4 metres (13 ft) tall (Royal Botanic Gardens, 2005; *Annona muricata*, 2008). It is a broadleaf, flowering, tree that is native to Mexico, Cuba, Central America, the Caribbean islands of Hispaniola and Puerto Rico, and northern South America, primarily Colombia, Brazil, Peru, Ecuador, Venezuela and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia and Nigeria (Leboeuf *et al.,* 1980; Adewole *et al.,* 2006; Mishra *et al.,* 2013).

Annona muricata L. plant

All portions of the *A. muricata* tree are extensively used as traditional medicines against an array of human ailments and diseases, especially cancer and parasitic infections. The fruit is used as natural medicine for arthritic pain, neuralgia, arthritis, diarrhoea, dysentery, fever, malaria, parasites, rheumatism, skin rashes and worms, and it is also eaten to elevate a mother's milk after childbirth. The leaves are employed to treat cystitis, diabetes, headaches and insomnia. Moreover, internal administration of the leaf's decoction is believed to exhibit anti-rheumatic and neuralgic effects, whereas the cooked leaves are topically used to treat abscesses and rheumatism (Mishra, 2013). In addition to ethnomedicinal uses, the fruits are widely employed for the preparation of beverages, candy, ice creams, shakes and syrups (Jaramillo-Flores et al., 2000). Studies have reported the significant antiproliferative effects of different extracts of the plant against colon cancer cells (HT-29 and HCT-116) and lung cancer cells (A549). The leaf extract was able to induce apoptosis in colon and lung cancer cells through the mitochondrial-mediated pathway (Ezirim *et al.,* 2013). Similarly, Moghadamtousi and

colleagues in (2014) also examined the *in vivo* chemopreventive potential of the ethyl acetate extract of the *A. muricata* leaves against azoxymethane-induced colonic aberrant crypt foci (ACF) in rats. The oral administration of the extract at two doses (250 and 500 mg/kg) for 60 days significantly reduced ACF formation in rats, as assessed by methylene blue staining of colorectal specimens. These substantial anticancer and antitumor activities mentioned for *A. muricata* leaves led to tablet formulations of the ethyl acetate-soluble fraction of the leaves, which contains AGEs that can be used as a cancer adjuvant therapy (Elisya *et al.,* 2014). In view of the above reports and findings, this study set out to ascertain the anti-proliferative activity of ethyl acetate leaf extract of *Annona muricata* L. on selected carcinoma human cell lines of MCF 7, HT 29, HCT 116 and C4-2WT.

III. MATERIAL AND METHODS

3.1 Plant material and extraction

Fresh leaves of *A. muricata* were collected from farmland in Obohia-Ndoki, Ukwa-East Local Government Area of Abia State, Nigeria in March 2017. The plant materials were identified and authenticated by Mr. Alfred Ozioko of International Centre for Ethnomedicines and Drug Development Nsukka, Nigeria and deposited in herbarium with Voucher Number: Intercedd/16091. Plant materials were were air dried in a shade for 21 days and 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 *A. muricata* was macerated in 250 mL of ethyl acetate for 72 h. The vacuum pump was used to filter and the ethyl acetate plant material was air dried in a fume chamber and the resulting extract was kept in the refrigerator at -4^{0}C.

3.2 Reagents

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

3.3 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 are: 1) MCF-7 (breast carcinoma cells), 2) C4-2WT (prostate carcinoma cells, 3) HT 29 and 4) HCT 116 (Colorectal carcinoma cells). The cell lines were cultured at 37 ^{0}C 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.

Ethyl acetate extracts of *A. muricata* were prepared as 50 mg stock solutions dissolved in dimethyl sulfoxide (DMSO) and stored at -4^{0}C, for a maximum period of 4 weeks. Extract dilutions were made in culture medium immediately prior to use.

3.4 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. Remaining working solutions were discarded. DMSO of corresponding concentrations was used as a control.

3.5 Cytotoxicity Screening

3.5.1 Growth inhibitory assays.

1. 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-100 µg/ml, n=6). Extract dilutions as well as DMSO control were prepared using DMEM as diluents immediately prior to each treatment. Ethyl acetate fractions of *A. muricata* 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; (To), 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 37 ºC 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 ethyl acetate extracts of *A. muricata* were calculated for the four different cell lines - MCF7-, HT 29, HCT 116 and C4-2WT and compared statistically with the control. The GI_{50} measures the growth inhibitory power of the test agent and is calculated thus:

Eqn. I: $ODGI_{50}$ = (Cont - To)/2+To
Insert computed $ODGI_{50}$ value into equation II
Eqn. II: GI_{50} = (HOD - ODGI50)/(HOD - LOD) * (HC - LC) + LC

Where:

$ODGI_{50}$ = 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 580nm after MTT. Measurements were performed and the concentration required for a 50% inhibition of viability (GI_{50}) was determined graphically

3.6 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 h, 48 h 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 hours 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 30min.

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 30min, 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 distill 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.IM-HCIwere added to each well. The plates were then agitated on a plate shaker for 30 minutes 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 hrs assay.

3.7 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 hr, 48 hr and 72hr 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 hours respectively for each cell line.

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 minutes 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 ImL. To check the concentration of dead cells, 95µl 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.

3.8 Wound Healing or Scratch Assay

This method is based on the observation that, upon creation of a new artificial gap, so-called "scratch", on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the "scratch" until new cell-cell contacts are established again. Wound or "scratch" assay was done using the modified method by Liang *et al.*, 2007 and Straatman, (2008). Cell suspensions were introduced in triplicates into 6- well plates using a sterile disposable pipette. A concentration of 5.0 × 10^5 of the different cells was seeded in a 6-well plate and incubated overnight and observed for confluence. The next day, a straight wound line was drawn across the 100 % confluent attached cell layer with p200 pipette tips. The floating cells were removed with PBS and replaced with new fresh DMEM medium. Images were taken and recorded as time 0. The concentration of *A. muricata* crude extract that caused 50% growth inhibition (GI_{50}) for MCF 7 cell line tested and confirmed by the MTT assay as well as the DMSO controls were added to their respective labelled wells and images of the closure of the wound due to cell migration were recorded at intervals of 24, 36 and 48 h using the inverted microscope equipped with a camera (Nikon, Japan) and the images were compared to determine the rate of cell migration.

IV. RESULTS

4.1 MTT

Concentrations of 0.1 to 100 g/mL of *A. muricata* extracts showed an increase ($p<0.05$) in cytotoxicity activity on MCF-7 C4-2WT, HT 29 and HCT 116 as compared to the untreated control cells (Figs. 1–4). The concentration required for a 50% inhibition of viability (GI_{50}) was determined by substituting the values in the equation II for calculation of GI_{50}. GI_{50} results calculated after MTT test showed the concentration of ethyl acetate extracts of *A. muricata* required for 50% inhibition of the different cell lines as follows: MCF-7 = 2.61 µg/ml, C4-2WT = 9.33 µg/ml, HT 29 = 4.97 µg/ml and HCT 116 =2.13 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.

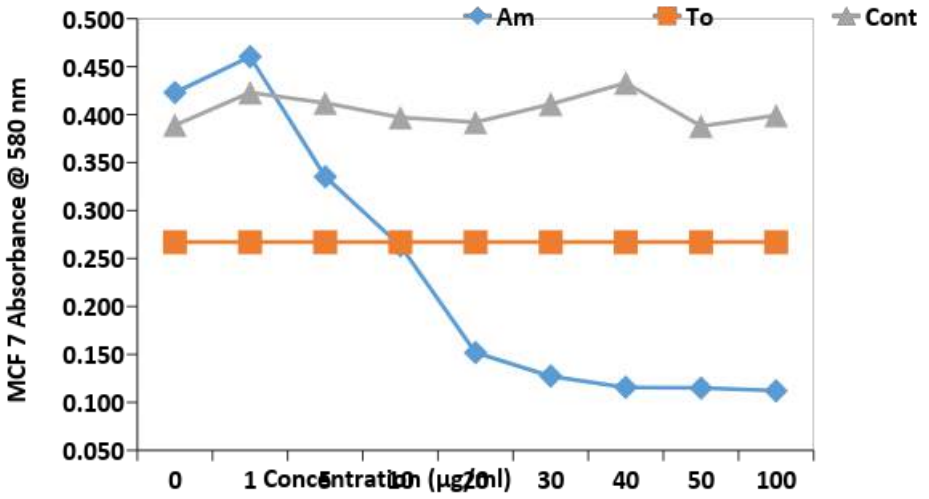

Figure 1: Anti-proliferative activity of ethyl acetate extract of *A. muricata* on MCF 7 after 72 hr treatment

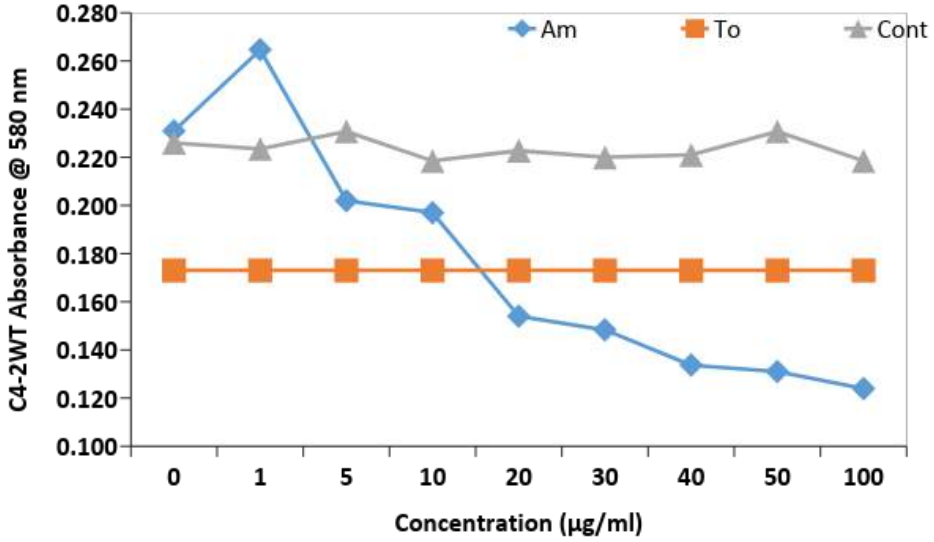

Figure 2: Anti-proliferative activity of ethyl acetate extract of *A. muricata* on C4-2WT after 72 hr treatment

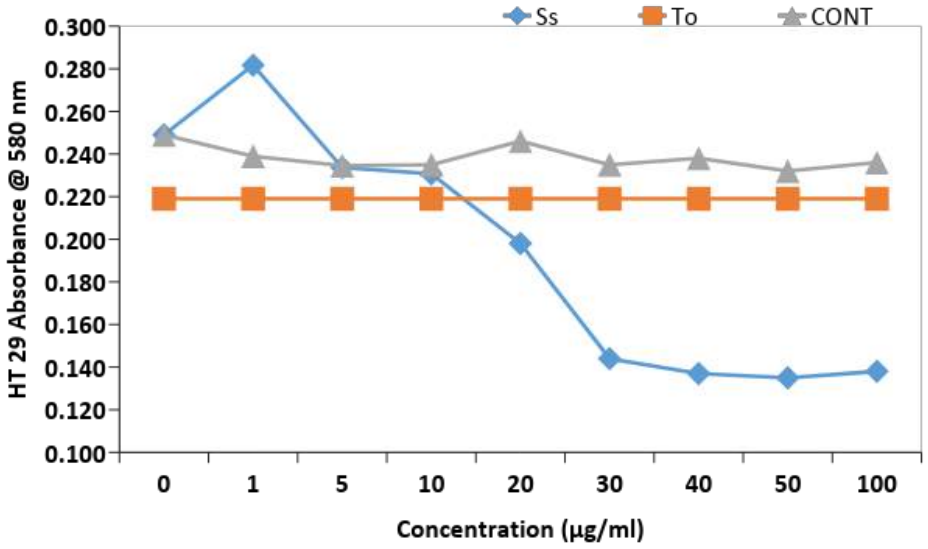

Figure 3: Anti-proliferative activity of ethyl acetate extract of *A. muricata* on HT 29 after 72 hr treatmnt

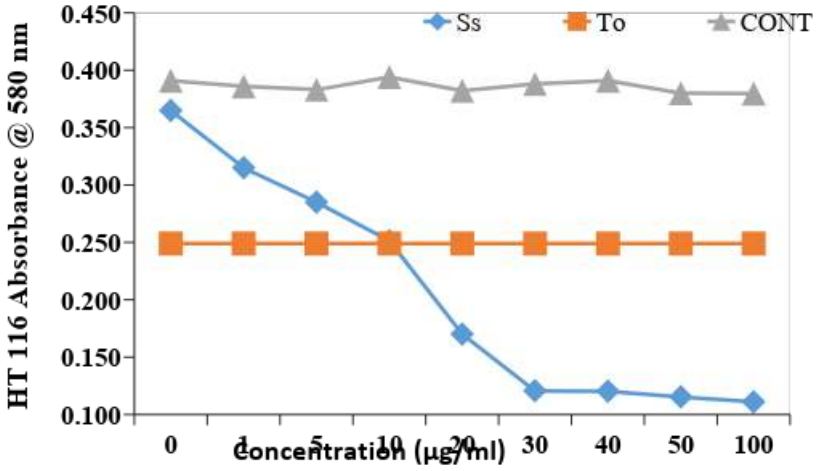

Figure 4: Anti-proliferative activity of ethyl acetate extract of *A. muricata* on HCT 116 after 72 hr treatment

4.2 Methylene Blue Proliferation assay

The response of monolayers of MCF-7 C4-2WT, HT 29 and HCT 116 cell lines to ethyl acetate extract of A. muricata for methylene blue colorimetric microtitre plate assay showed a direct relationship between different concentrations of the extract used and their optical densities at 24 hrs, 48 hrs and 72 hrs (Figures 5-7). When initial cell density for each cell line was optimized to give exponential growth over the assay period, differences in response to different concentrations of the extract were obviou.

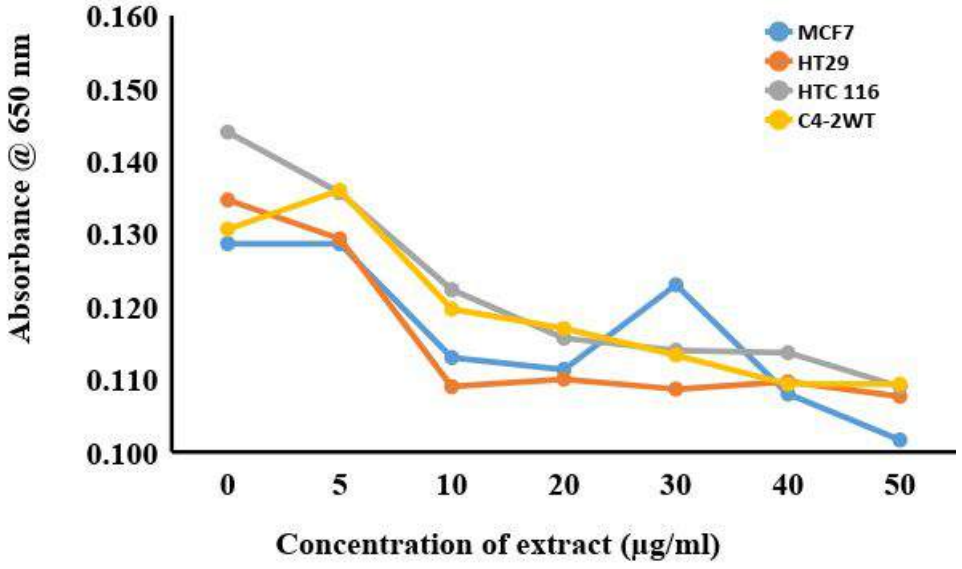

Figure 5: Optical density of viable cell lines 24 hrs after treatment with ethyl acetate extract of *A. muricata*

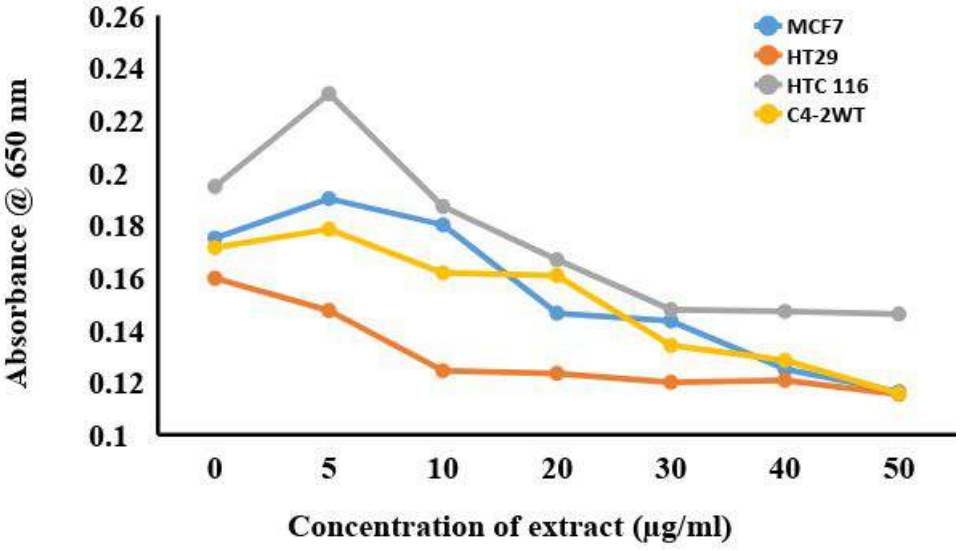

Figure 6: Optical density of viable cell lines 48 hrs after treatment with ethyl acetate extract of *A. muricata*

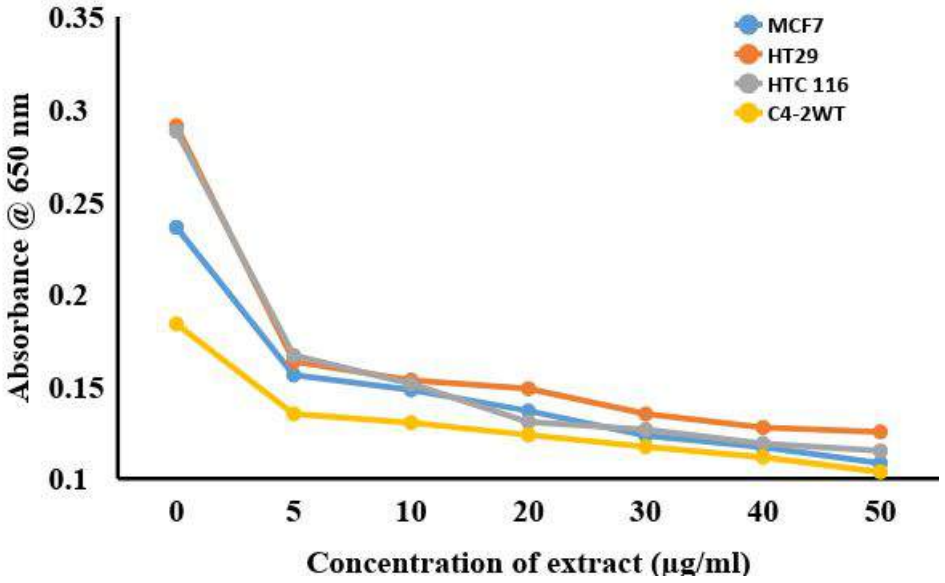

Figure 7: Optical density of viable cell lines 72 hrs after treatment with ethyl acetate extract of *A. muricata*

4.3 Trypan blue exclusion assay for cell count

Dead cells take up the blue colour of trypan blue while live cells don't take the dye. The number of stained and unstained cells were counted separately. Viable cells (VC) and non-viable cells (NVC) were counted for each concentration of extract used on MCF-7 C4-2WT, HCT 29 and HCT 116 cell lines. Results obtained (figures 8 – 11) showed a significant decrease ($p<0.05$) over a period of 72 hrs 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. Inhibition of cell growth and proliferation of cells by ethyl acetate extract of A. muricata occurred in a concentration-dependent manner.

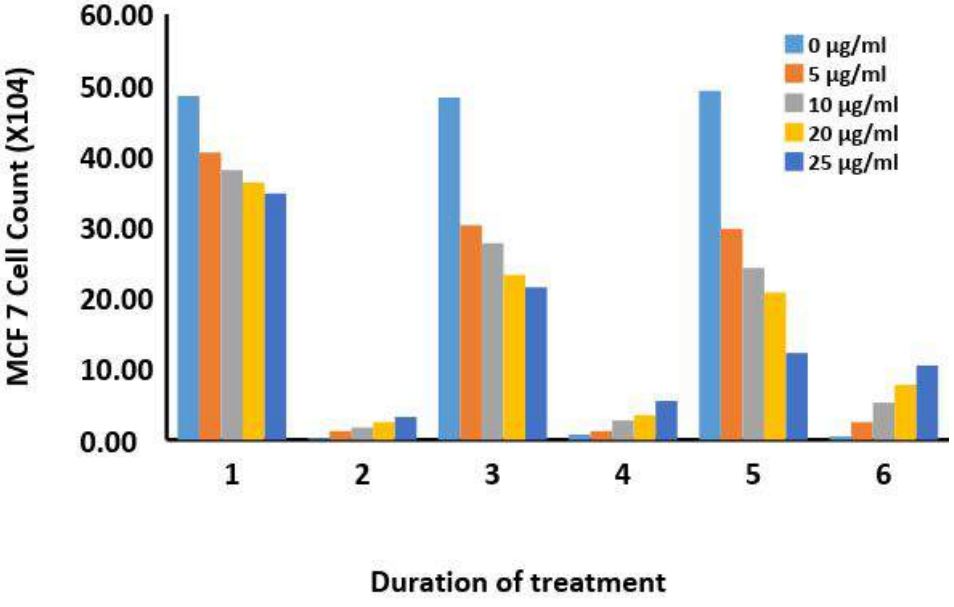

Figure 8: Cell count of viable and non-viable MCF 7 cells after treatment with ethyl acetate extract of *A. muricata*

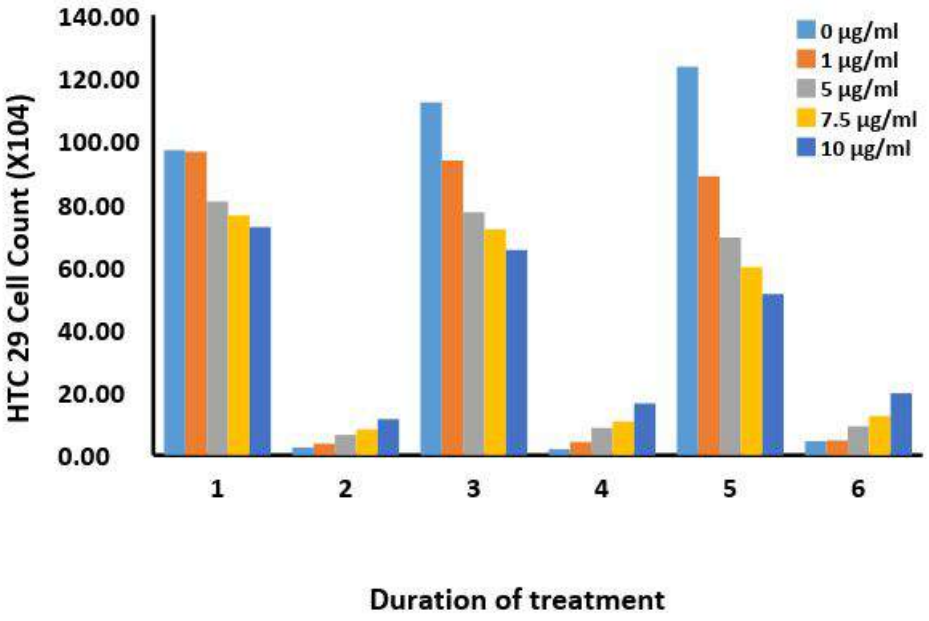

Figure 9: Cell count of viable and non-viable HT 29 cells after treatment with ethyl acetate extract of *A. muricata*

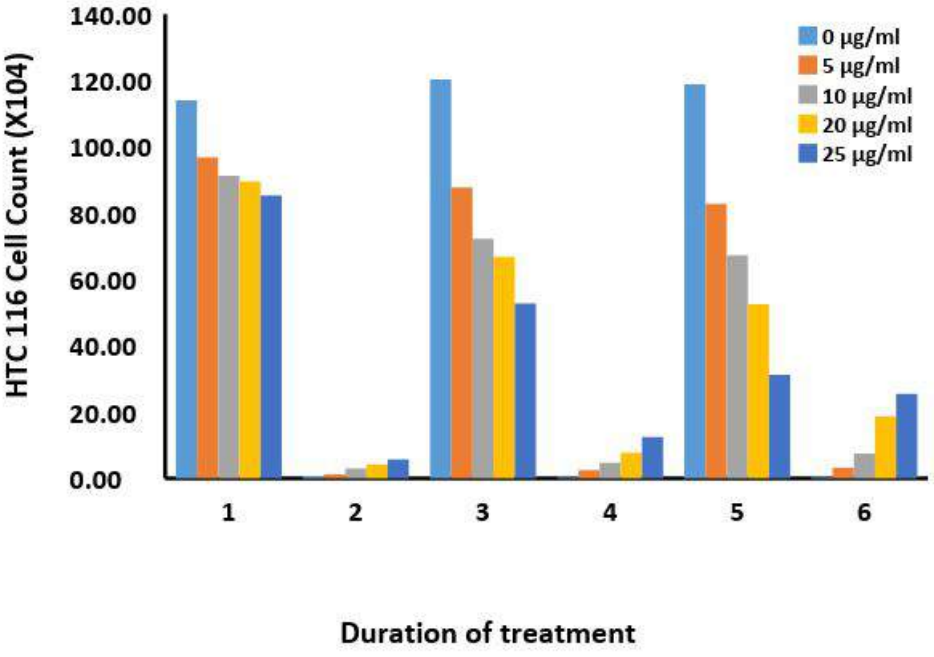

Figure 10: Cell count of viable and non-viable HCT 116 cells after treatment with ethyl acetate extract of *A. muricata*

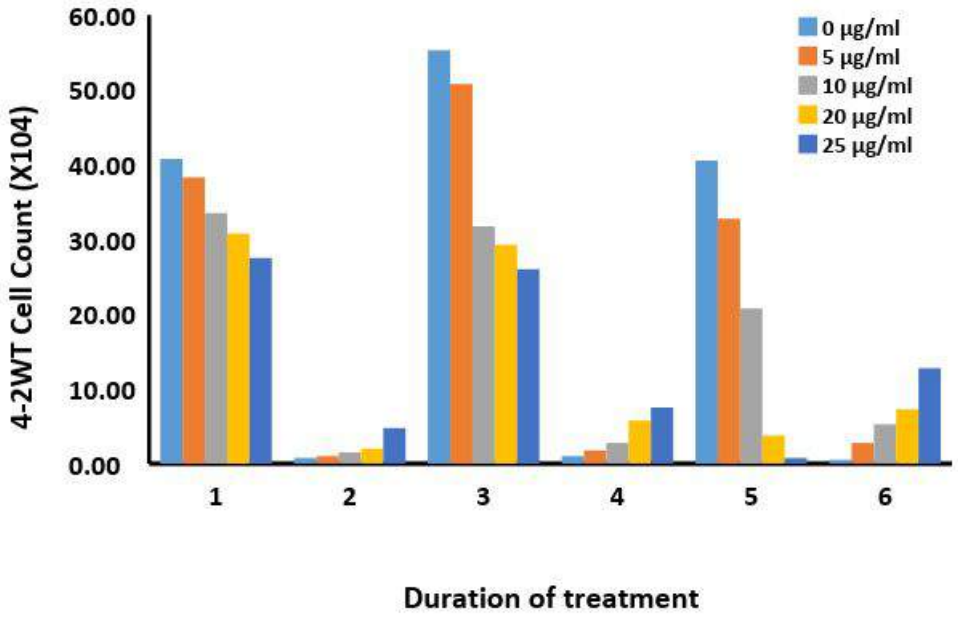

Figure 11: Cell count of viable and non-viable C4-2WT cells after treatment with ethyl acetate extract of *A. muricata*

4.4 Wound or Scratch Assay

Images from a wound or scratch assay experiment at different time intervals. Human breast adenocarcinoma cell line (MCF 7) were wounded with a p200 pipette tip and then imaged at different time intervals of 24, 36 and 48 hr using a microscope (X10) and a ZTE BLADE A910 camera.

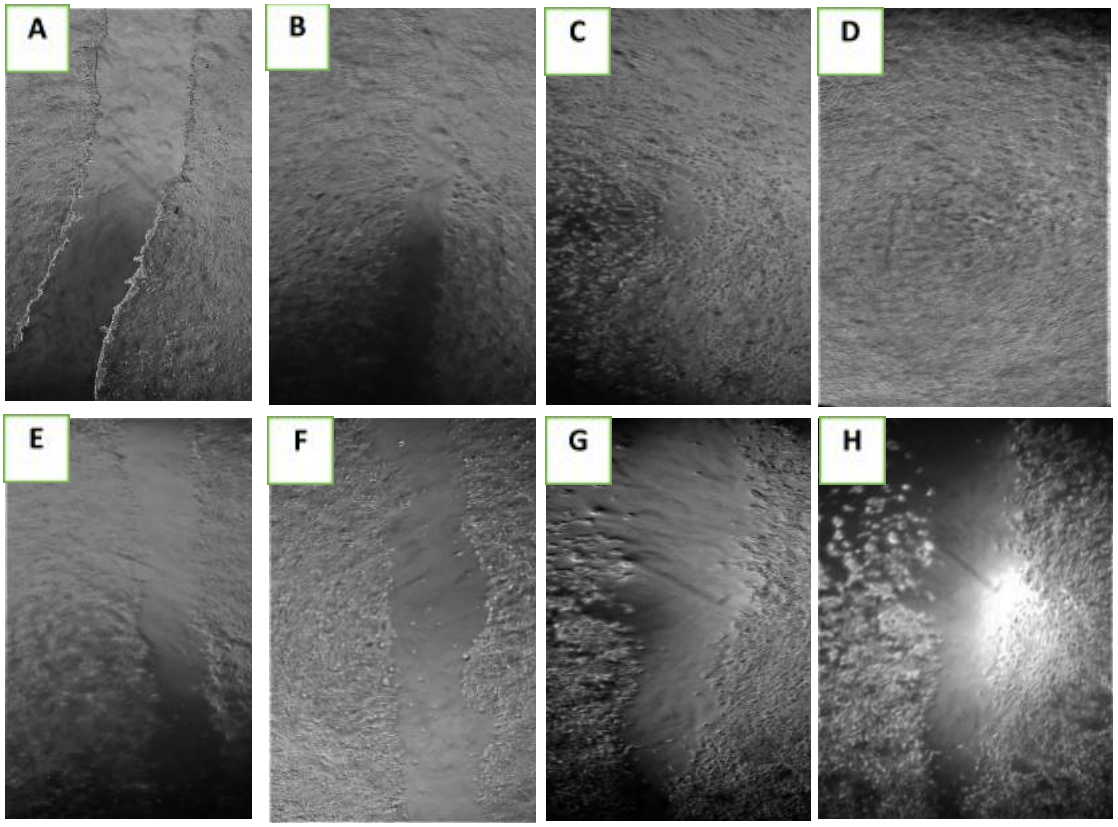

Plate 1: A: MCF 7 Control @ 0 h. B: MCF 7 Control @ 24 h showing proliferation of cells to about 50% confluence. C: MCF 7 Control @ 36 h showing proliferation of cells to about 75% confluence. D: MCF 7 Control @ 48 h showing proliferation of cells to almost 100% confluence. E: MCF 7 scratched cells @ 0 hr before addition of GI50 concentration of A. muricata extract. F, G & H: MCF 7 scratched cells @ 24, 36 and 48 hr after addition of GI50 concentration of extract showing non-proliferation of cells.

V. DISCUSSION

Numerous studies have been conducted on *Annona muricata* L. Apart from it being an important source for the food industry and an indigenous medicinal plant, *A. muricata* is proven to possess a wide spectrum of biological activities. In addition to all previous studies on this plant, the most promising activities include the anti-proliferative activity of ethyl acetate leaf extract of *Annona muricata* L. on selected carcinoma human cell lines of MCF 7, HT 29, HCT 116 and C4-2WT.

The result of the cytotoxic activity of ethyl acetate leaf extract of *Annona muricata* L. on selected carcinoma human cell lines of MCF 7, HT 29, HCT 116 and C4-2WT using MTT assay showed a significant ($p<0.05$) cytotoxic effect of the extract in a concentration-dependent manner. Increase in the concentration of the extract produced a significant decrease in the number of proliferating cells. MTT assay measures the number of viable cells in a medium through the formation of formazan. The amount of formazan produced is directly proportional to the number of viable cells. The 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 leaf extract of *Annona muricata* L as a potential anticancer agent against these carcinoma human cell lines. Cytotoxic activity observed may be due to loss of cellular function and viability either through necrosis or by apoptosis caused by extract of *Annona muricata* L. Recently, series of *in vitro* studies were conducted to determine the mechanism of action of ethyl acetate extract of *A. muricata* leaves against colon cancer cells (HT-29 and HCT-116) and lung cancer cells (A-549). Results obtained showed that the leaf extract was able to induce apoptosis in colon and lung cancer cells through the mitochondrial-mediated pathway. This

anti-proliferative effect was associated with cell cycle arrest in the G1 phase (Moghadamtousi *et al.,* 2014b; Moghadamtousi *et al.,* 2014c). The study also showed that treatments of HCT116 and HT-29 cells resulted in the up-regulation of the apoptotic pathway, as suggested by an increase in the production of ROS, an increase in detectable cytochrome *c*, and an increase in initiator and executioner caspases in both of the tested cell lines. Furthermore, an increase in the levels of Bax protein was also observed by flow cytometry, which further suggested the activation of the apoptotic pathway by extracts of *A. muricata* Yang *et al.*, 2015).

Also, Gavamukulya *et al.,* 2014; Eka Prasasti, 2012 and Retno, 2012 used MTT assay for the evaluation of anticancer activity of ethanolic leaves extracts of *A. muricata* against two human breast cancer cell lines MDA and SKBR3. Gavamukulya reported that ethanol extract of the soursop plant leaves was found to be highly cytotoxic in vitro against the two human breast cancer cell lines MDA and SKBR3. Similarly, a study conducted by Yang *et al.*, (2015) suggested that Graviola leaf extract, flavonoid-enriched extract and acetogenin-enriched extract all showed the capacity to down-regulate prostate cancer, with Graviola leaf extract being the most efficient at doing so. This study not only showed the efficacy of *A. muricata* extracts at inhibiting prostate cancer but also the importance of using whole-leaf extracts to achieve the highest inhibitory efficacy in combating cancer.

Result obtained for TBEA showed 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 h post-treatment with the leaf extract of *A. muricata*. This is an indication of the non-proliferation of the cells due to the cytostatic or cytotoxic activity of the extract. According to Yahaya Gavamukulya *et al.,* 2014; Eka Prasasti, 2012 and Retno, 2012, the *in vitro* anticancer activities of the methanolic and aqueous extract of *A. muricata* leaves was done by using Trypan blue-exclusion assay (TBEA) against EACC and normal spleen cells. Similarly, Lali, (2012) carried out a study on the anticancer activity of ethyl acetate fraction of *A. muricata* against DLA cell lines using Trypan blue-exclusion assay and reported that 82% cell death occurred within a 200µg/ml concentration of the extract used. A study by Kashii *et al.* (1994), quantified toxicity on retinal cultures of foetal rats by the trypan blue dye exclusion assay. They were able to concomitantly visualize cell toxicity due to a cell fixation step after staining with trypan blue and photographed the cells under a Hoffman modulation microscope. Similarly, Takeuchi *et al.* (1993) studied cytotoxicity on lymphocytes by the trypan blue method and also employed a cell fixation step which enabled visualization. Since we did not fix the cells for the trypan blue dye exclusion assay, the photomicrography was not optimal. Due to cytotoxicity, most cells had lost their adherent properties, which was a deterrent to acceptable focal clarity. Uzuner, (2018), proposed a protocol for the development of a direct Trypan Blue Exclusion assay to detect cell viability of adherent cells into ELISA Plate wells. This protocol briefly includes paraformaldehyde (PFA) fixation of trypan blue stained cells, however, counting process via trypan blue exclusion method is highly subjective. This study showed that the TBEA method can be used to monitor the anti-proliferative potentials of ethyl acetate extract of *A. muricata* on some carcinoma cells.

Viable cells are able to absorb methylene blue and stain blue when viewed under the microscope. This dye is washed into a medium containing 1:1 (v/v) ethanol and 0.IM-HCI as the elution solvent. Results obtained for the methylene blue assay showed that the intensity of the medium decreases with increase in the concentration of extract used in treating the cells. 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. 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. The decrease in the number of proliferating cells is as a result of cytotoxicity induced by varying concentrations of the extract used.

Cell migration plays a central role in many complex physiological and pathological processes, therefore, the wound healing or scratch assay is employed by researchers to study cell proliferation, tissue matrix remodelling, or estimate cell proliferation and migration rates of different cells and culture conditions *in vitro*. Photomicrographs obtained after 48 hrs using MCF 7 as the experimental model showed an increase in cell proliferation and wound healing up to approximately 100% confluence in control cultures not treated with the extract. However, cells treated with GI_{50} of the extract showed non-proliferation, expansion and deterioration of the wound or gap created by the scratch. According to Horwitz and Webb, (2003); Ridley *et al.*, (2003), wounded tissue initiates a complex and structured series of events in order to repair the damaged region. These events may include increased vascularization by angiogenic factors, an increase in cell proliferation and extracellular matrix deposition, and infiltration by inflammatory immune cells as part of the process to destroy necrotic tissue. The wound healing process begins as cells polarize toward the wound, initiate protrusion, migrate, and close the wound area. These processes reflect the behaviour of individual cells as well as the entire tissue complex. Liang *et al.*, (2007), noted that one of the major advantages of this simple method is that it mimics to some extent *in vivo* migration of cells.

VI. CONCLUSION

This study has demonstrated linearity in the relationship between different concentrations of the extract used against some human carcinoma cells. It further reported a strong dose-dependent inhibition in treated cell lines and at a lower concentration. The ethyl acetate fraction of leaf extract of *A. muricata* was thus found to be highly cytotoxic *in vitro* against the MCF 7, HT 29, HCT 116 and C4-2WT carcinoma cells thereby leading to non-proliferation of the cells. The GI_{50} limit of the crude extract was lower than 30 µg/ml and therefore, may be considered promising and for further purification as an anti-proliferative agent against human carcinoma cells,

Conflict of Interests

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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