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Ethyl Acetate Extract of *Senna alata* (L) Roxb Increases Cytotoxicity in the Human Breast, Prostate and Colorectal Cancer Cells

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Abstract: The objective of this study is to evaluate the effects of ethyl acetate extract of *Senna alata* (L) Roxb on some human carcinomas - MCF 7 (human breast), C4-2WT (prostate), HT 29 and HTC 116 (colorectal) cell lines. Screening assays carried out to determine cytotoxicity include: - MTT (3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), clonogenic cell survival, Trypan Blue exclusion and methylene blue assays. Evaluation of the results showed that the extract strongly decreased the proliferation of the carcinoma cells in a dose-dependent manner. 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 = 5.90 µg/ml, HCT 29 = 4.97 µg/ml, HCT 116 =11.86 µg/ml and C4-2WT = 9.48 µg/ml. Trypan Blue exclusion assay showed a decrease in the number of viable cells and an increase in the number of non-viable cells over 72 hrs post-treatment with the extract. Methylene blue assay showed that the number of viable cells, when their optical densities were measured over 72 hrs post-treatment, was reduced compared with the control. For clonogenic cell survival, there was an increase in cell proliferation and colony formation in the control cultures. However, cells treated with GI_{50} and 2X (twice) GI_{50} concentration of the extract showed a decrease in the number of colonies formed. The results indicate the cytotoxic potentials of the extract and therefore, suggests the use of ethyl acetate leaf extract of *Senna alata* (L) Roxb in preparing recipes for the management of cancer-related ailments.

Keywords: *Senna alata* (L) Roxb, Carcinoma Cells, Cytotoxicity, Clonogenic Cell Survival Assay

1. Introduction

Cancer is a public health problem affecting all ages. It is the second commonest cause of death in developed countries and among the three leading causes of death in developing countries. The World Health Organization (WHO) reported that about 24.6 million people live with cancer worldwide. There are 12.5% of all deaths are attributable to cancer and if the trend continues, it is estimated that by 2020, 16 million new cases will be diagnosed per annum out of which 70% will be in the developing countries [1]. Over the last five decades, natural products have proved to be very useful in anticancer chemotherapy drug development, particularly those derived from terrestrial microbes and higher plants. A review of the anticancer drugs introduced to the market in North America, Western Europe, and Japan since the 1940s has indicated that some 47% of a total of the 155 anticancer drugs approved up to 2006 were either natural products or directly derived by semi-synthesis from natural products [2]. Herbal medicine, also known as phytomedicine, or botanical medicine refers to using plants seeds, flowers, roots for medicinal purpose. Plants, herbs, and ethnobotanicals have been used since the early days of humankind and are still used throughout the world for health promotion and

treatment of disease. Plants and natural sources form the basis of today's modern medicine and contribute largely to the commercial drug preparations manufactured today. About 25% of drugs prescribed worldwide are derived from plants. Still, herbs, rather than drugs, are often used in health care [3]. For some, herbal medicine is their preferred method of treatment. For others, herbs are used as adjunct therapy to conventional pharmaceuticals. However, in many developing societies, traditional medicine of which herbal medicine is a core part is the only system of health care available or affordable [3, 4]. A recent study of cytotoxicity and anti-proliferative potentials of *Parquetina nigrescen* - a herbal medicine have indicated its prospect in cancer management [5]. These findings led to the search for more viable herbal plants with anti-cancer potentials. One of such plant is *Senna alata* (L) Roxb (*Cassia alata*). *Senna alata* (L) Roxb is a tropical perennial herb of medicinal value. Therapeutic evaluation of the use of *S. alata* leaves showed that it is used in treating abdominal pain, constipation, and liver abnormalities [6], skin inflammation, rashes on the skin, athlete's foot, and ringworm [7], lung cancer [8], hypoglycaemic and hypolipidaemic properties [9]. Phytochemical analysis of the leaf reveals the presence of flavonoids such as Quercetin, kaempferol, Luteolin and a phenolic acid - Caffeic acid [10]. These phytochemicals are known for their cytotoxic properties against various cell lines [11]. Natural products are generally regarded as possessing drug-like pharmacological qualities and they are biologically friendly molecular properties. These attributes make natural products an invaluable resource of chemical diversity and hence they have acted as excellent lead compounds for optimization by synthetic organic chemistry methods in anticancer agent discovery [12, 13]. Many studies have demonstrated antiproliferative, cytostatic and cytotoxic activities of phytochemicals against cancer cells [14]. Thus, chemoprevention by natural products may be considered a promising approach to cancer control and management [15]. This study reports an increase in cytotoxicity in the human breast, prostate and colorectal cancer cell lines using ethyl acetate extract of *Senna alata* (L) Roxb.

2. Methods

2.1. Plant Material and Extraction

Fresh leaves of *Senna alata* (L) Roxb were plucked from an uncultivated piece of land, by the gate of the University of Port Harcourt, Abuja campus, Choba, Rivers State. The leaves, with voucher number UPH/V/1225, were identified and authenticated by Dr. Chimezie Ekeke of the Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers State and deposited in the herbarium. 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 S. alata (L) Roxb 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°C.

2.2. 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.

2.3. Cell Lines

The cell lines used for the present study are: 1) MCF-7 (breast carcinoma cells), 2) C4-2WT (prostate carcinoma cells, 3) HT 29 and 4) HCT 116 (Colorectal carcinoma cells). 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 37°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.

2.4. Preparation of Extract Stock and Working Solution

Ethyl acetate extracts of S. alata (L) Roxb were prepared as 50 mg stock solutions dissolved in dimethyl sulfoxide (DMSO) and stored at -4°C, for a maximum period of 4 weeks. Extract dilutions were made in culture medium immediately prior to use.

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.

2.5. Cytotoxicity Screening

2.5.1. Growth Inhibitory Assays: 3-(4,5-Dimethylthiazol-2-yl)-2,5 Phenyltetrazolium Bromide (MTT)

A confluent monolayer of test 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. Ethyl acetate fractions of *S. alata* (L) Roxb were dissolved in DMSO and diluted with complete DMEM medium to get a range of test concentration (0.1 µg to 100 µg/ml, 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 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 37°C 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 S. alata (L) Roxb were calculated for the four different cell lines - MCF7, C4-2WT, HT 29 and HCT 116 and compared statistically with the control [16]. 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.

2.5.2. Clonogenic Cell Survival Assay

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. This cell is then said to be clonogenic. A cell survival curve is therefore defined as a relationship between the dose of the agent used to produce an insult and the fraction of cells retaining their ability to reproduce [17]. Cells from an actively growing stock culture in monolayer were prepared in a suspension by the use of trypsin, which causes the cells to detach from the substratum. The number of cells per milliliter in this suspension was counted using a hemocytometer. From this stock culture, if 250 cells were seeded in duplicates into a 6- well plates using a sterile disposable pipette and allowed 24 h to adhere before extract was introduced. The concentration of ethyl acetate extract of *S. alata* (L) Roxb that caused 50% growth inhibition (GI_{50}) and twice (2X) the concentration of the GI_{50} as confirmed by the MTT assay as well as the DMSO controls were added to their respective labelled wells. After 24 h of adding the extracts, the culture medium in each well was removed by gentle vacuum aspiration using a Pasteur pipette with a fine angled tip. The medium was washed twice with PBS and 3 mls of fresh media introduced and changed every four days. The dishes were allowed to incubate between 7 – 14 days for colonies to grow. The length of time depends on the cell line eg HT 29 and HCT 116 approximately 7 days, MCF-7 was incubated for 7 days and while C4-2WT was approximately 14 days. Every single cell divides many times and forms a colony. Cells were fixed and counted when colonies in the control wells contain cells that were greater than 50 when viewed under an X10 microscope. All the cells that make up the colony are the progeny of a single cell [17].

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 colonies were washed twice with I ml of ice-cold PBS. The cell layer was then fixed by adding 1000 µl of 100% methanol to each well and let stand for 10min.

Cell staining: The fixative was removed by gentle vacuum aspiration using a Pasteur pipette and 500 µl of filtered 1% (w/v) Methylene Blue was added to each well. After 10 min, the excess dye was removed by another gentle vacuum aspiration using a Pasteur pipette. The remaining dye was then washed off thrice in tap water and air dried. The colonies, still stained with Methylene Blue, were examined microscopically and counted.

2.5.3. Trypan Blue Exclusion Assay

A modified method [18], was adopted for this study and has been reported previously [5]. Results were reported based on the 72 hrs assay.

2.5.4. Methylene Blue Proliferation Assay

A modified method [19] was adopted for Methylene Blue Proliferation assay and has been reported previously [5]. Results were reported based on the 72 hrs assay.

3. Results

3.1. Cytotoxic Effect of Ethyl Acetate Extracts of S. alata (L) Roxb

S. alata (L) Roxb extracts strongly decreased the proliferation of MCF-7, HT, 29 HCT 116 and C4-2WT when compared to the untreated control cells (Figures 1– 4). GI_{50} results calculated after MTT test showed the concentration of ethyl acetate extracts of S. alata (L) Roxb required for 50% inhibition of the different cell lines as follows: MCF-7 = 5.90 µg/ml, HT 29 = 4.97 µg/ml, HTC 116 =11.86 µg/ml and C4-2WT = 9.48 µg/ml.

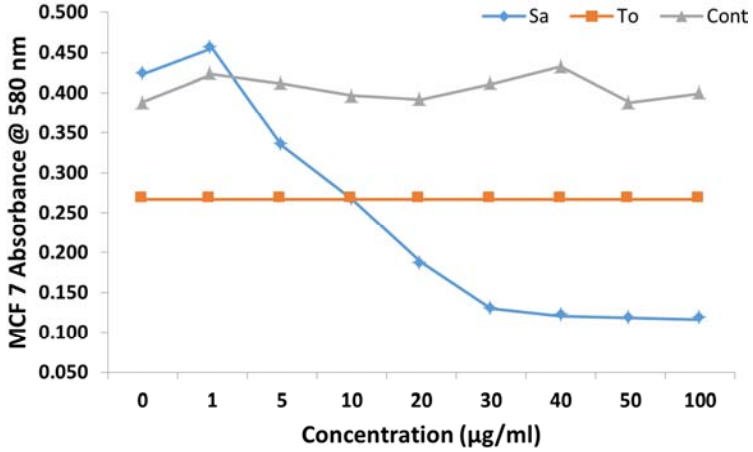

Figure 1. *Cytotoxic activity of ethyl acetate extract of S. alata (L) Roxb on MCF 7 after 72 hr treatment.*

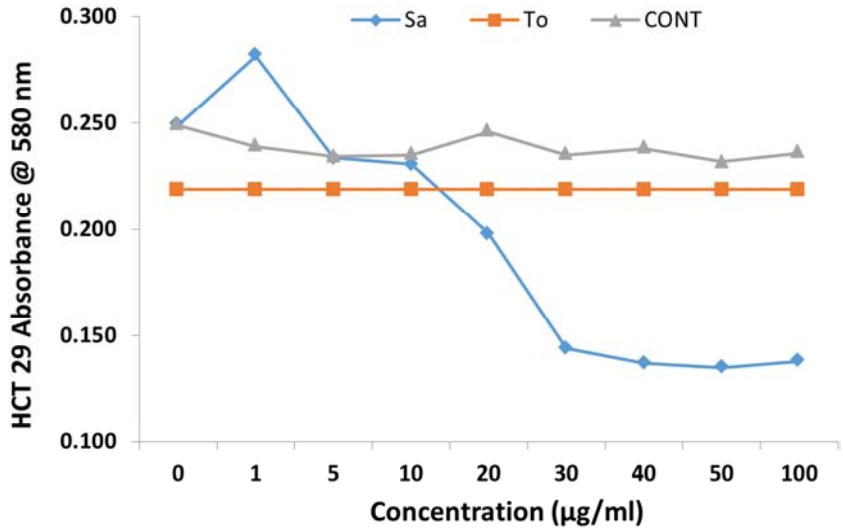

Figure 2. *Cytotoxic activity of ethyl acetate extract of S. alata (L) Roxb on HT 29 after 72 hr treatment.*

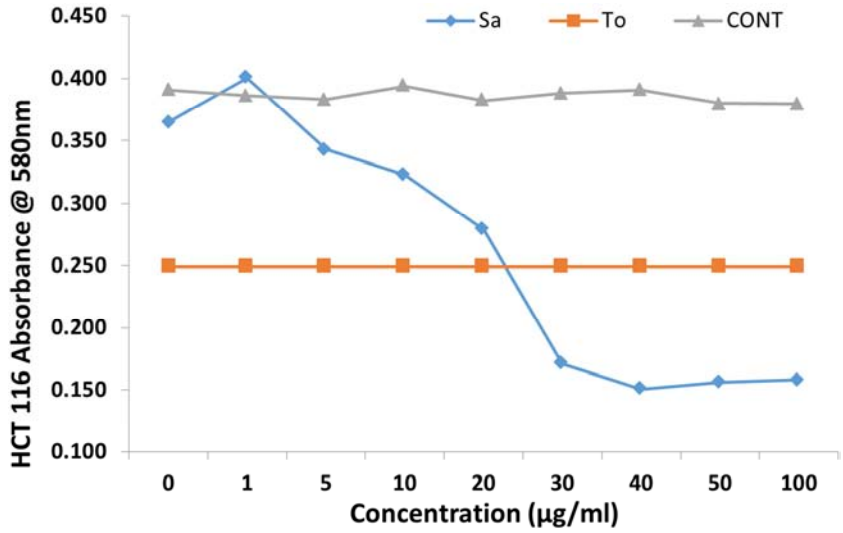

Figure 3. *Cytotoxic activity of ethyl acetate extract of S. alata (L) Roxb on HCT 116 after 72 hr treatment.*

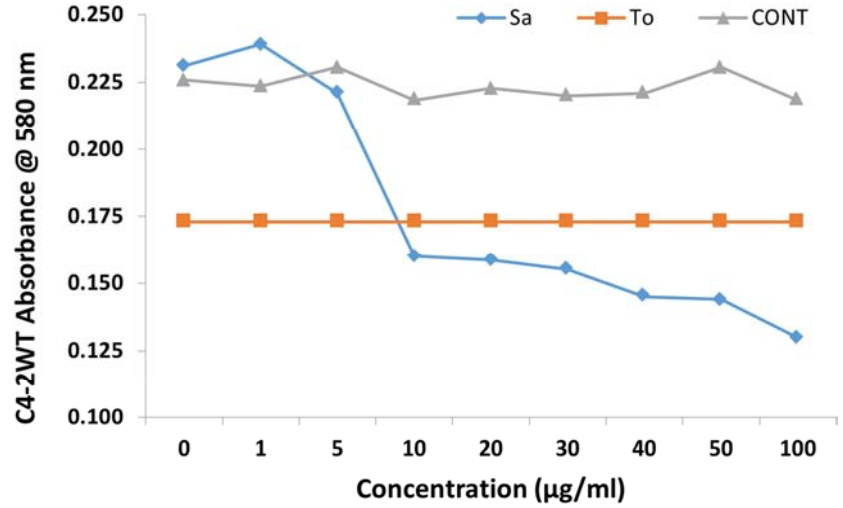

Figure 4. *Cytotoxic activity of ethyl acetate extract of S. alata (L) Roxb on C4-2WT after 72 hr treatment.*

3.2. Clonogenic Survival Fraction of the Carcinoma Cells

The relationship between the percentage of cells that survived in the control and extract treated cells is shown in Figures 5 -

8. Cells treated with GI_{50} and 2X GI_{50} of the extract when compared with the control cells showed a percentage decrease in the number of colonies formed.

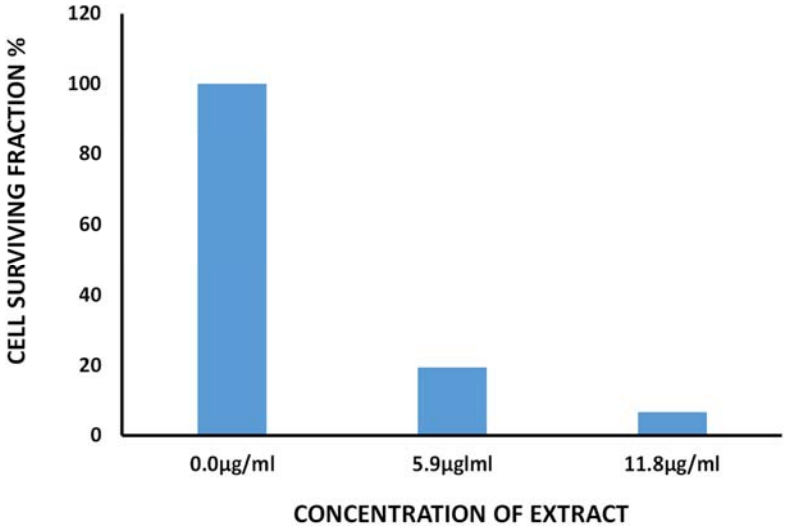

Figure 5. *Cell survival fraction of MCF 7 carcinoma cells 9 days after treatment with ethyl acetate extract of S. alata (L) Roxb.*

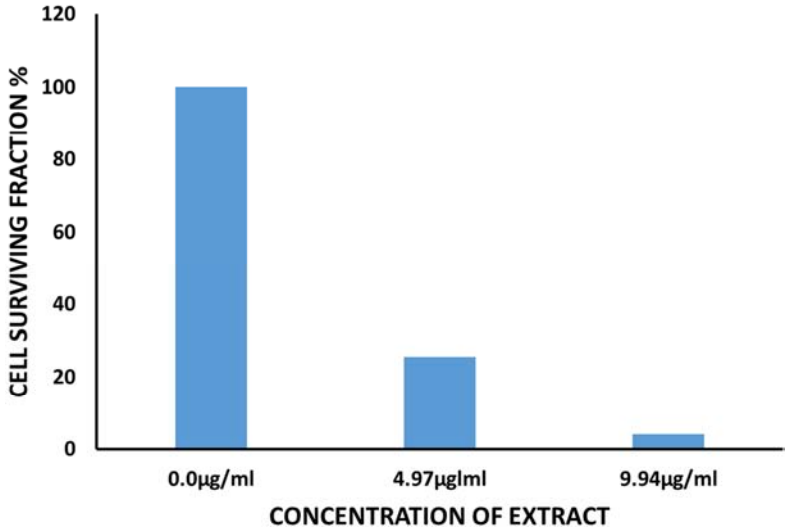

Figure 6. *Cell survival fraction of HT 29 carcinoma cells 7 days after treatment with ethyl acetate extract of S. alata (L) Roxb.*

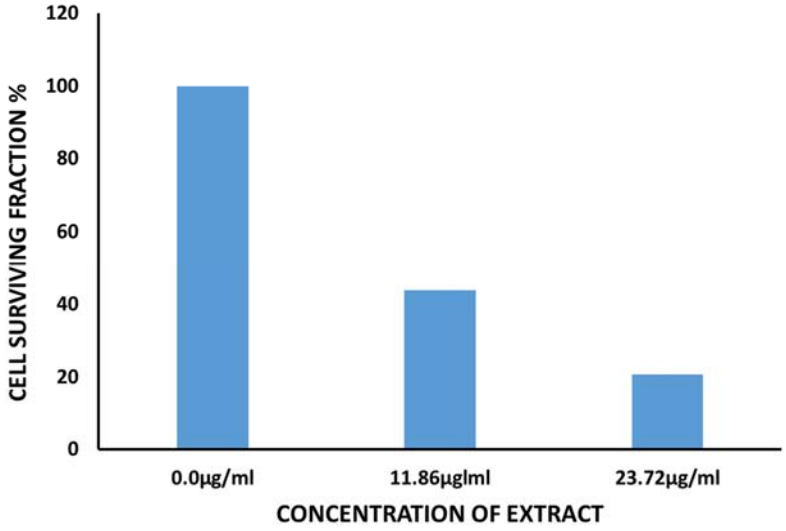

Figure 7. *Cell survival fraction of HCT 116 carcinoma cells 7 days after treatment with ethyl acetate extract of S. alata (L) Roxb.*

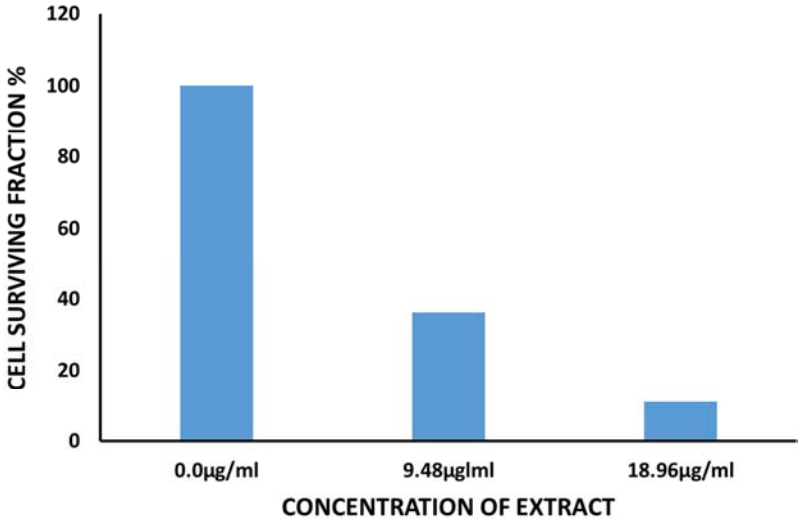

Figure 8. *Cell survival fraction of C4-2WT carcinoma cells 14 days after treatment with ethyl acetate extract of S. alata (L) Roxb.*

3.3. Effect of Ethyl Acetate Extracts of S. alata (L) Roxb on Viable Cell Growth

Results obtained for Trypan blue exclusion assay showed that inhibition of cell growth and proliferation of cells by ethyl acetate extract of *S. alata* (L) Roxb occurred in a concentration-dependent manner (figures 9 - 12). There was a decrease over a period of 72 hrs in the total number of viable cells (VC) and an increase in the total number of non-viable cells (NVC) in a dose dependent concentration of the extract.

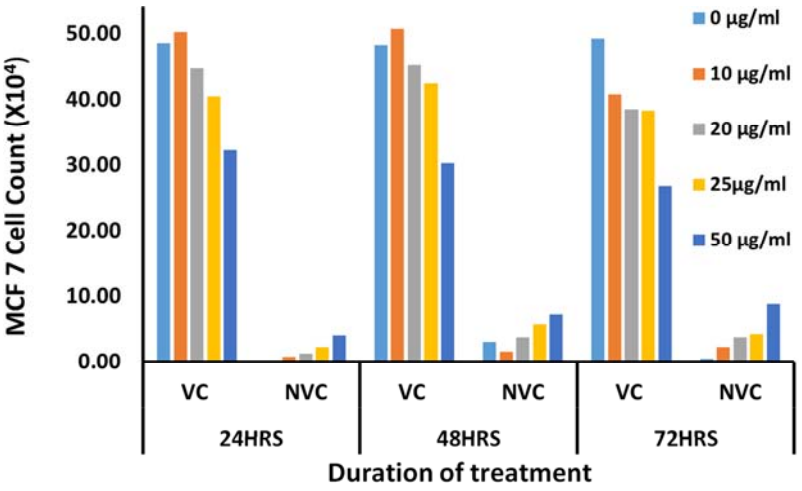

Figure 9. *Cell count of viable and non-viable MCF 7 cells after treatment with ethyl acetate extract of S. alata (L) Roxb.*

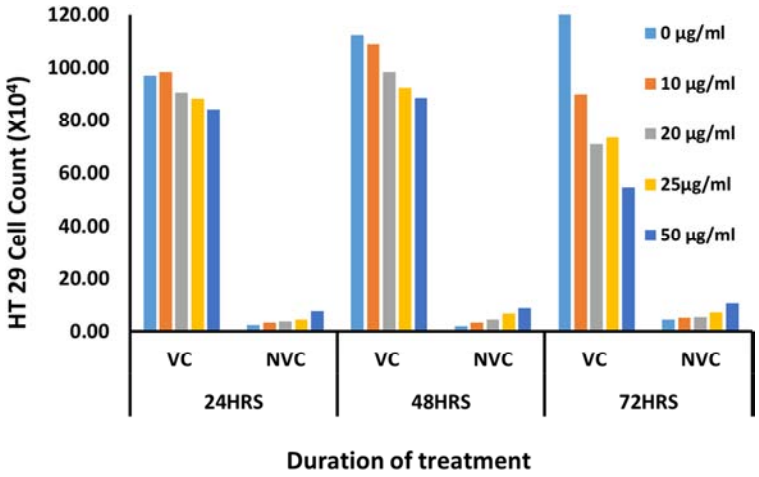

Figure 10. *Cell count of viable and non-viable HT 29 cells after treatment with ethyl acetate extract of S. alata (L) Roxb.*

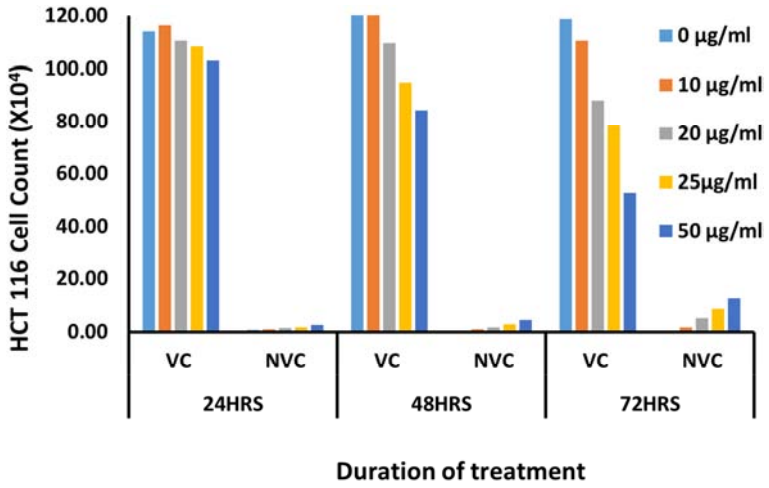

Figure 11. *Cell count of viable and non-viable HCT 116 cells after treatment with ethyl acetate extract of* *S. alata (L) Roxb.*

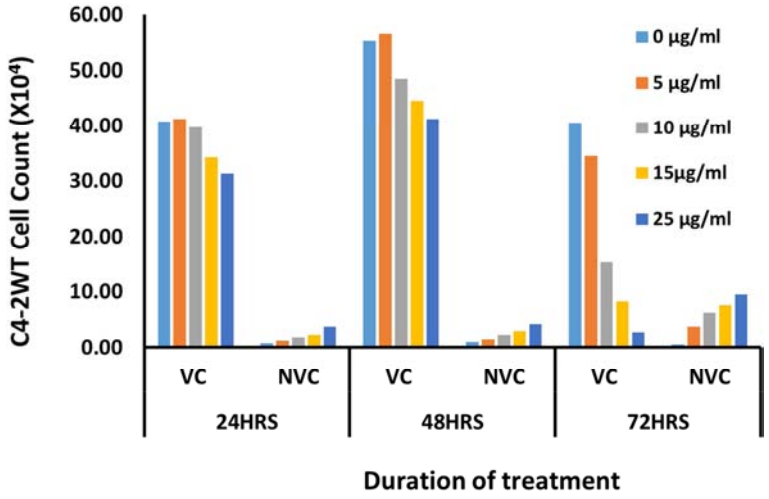

Figure 12. *Cell count of viable and non-viable C4-2WT cells after treatment with ethyl acetate extract of* *S. alata (L) Roxb.*

3.4. Effect of Ethyl Acetate Extracts of S. alata (L) Roxb on Cell Growth and Proliferation

Results obtained for Methylene Blue proliferation assay showed that 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 obvious (Figures 13 -15). The response of monolayers of the carcinoma cell lines to ethyl acetate extract of *S. alata* for methylene blue colorimetric microtiter 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.

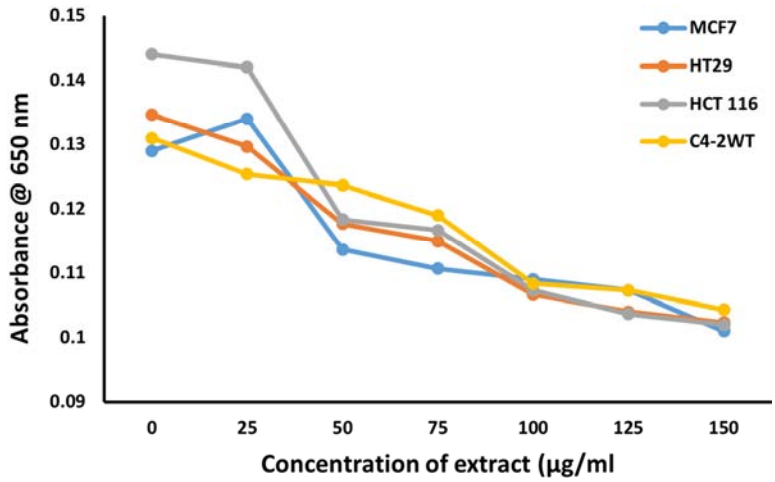

Figure 13. *Optical density of viable cell lines 24 hrs after treatment with ethyl acetate extract of S. alata (L) Roxb.*

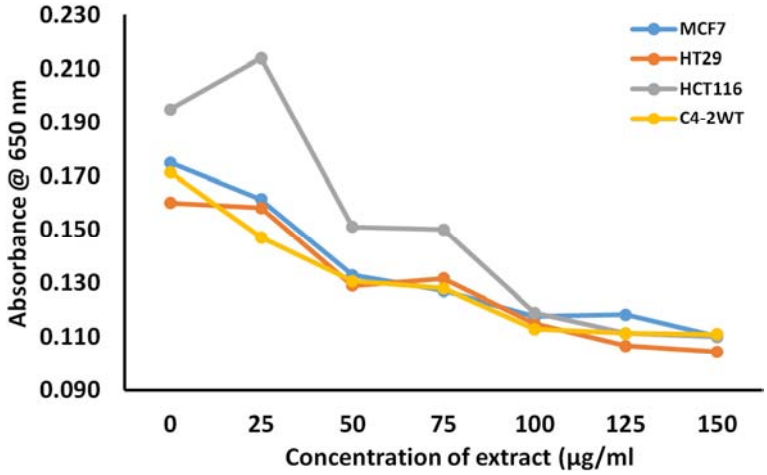

Figure 14. *Optical density of viable cell lines 48 hrs after treatment with ethyl acetate extract of S. alata (L) Roxb.*

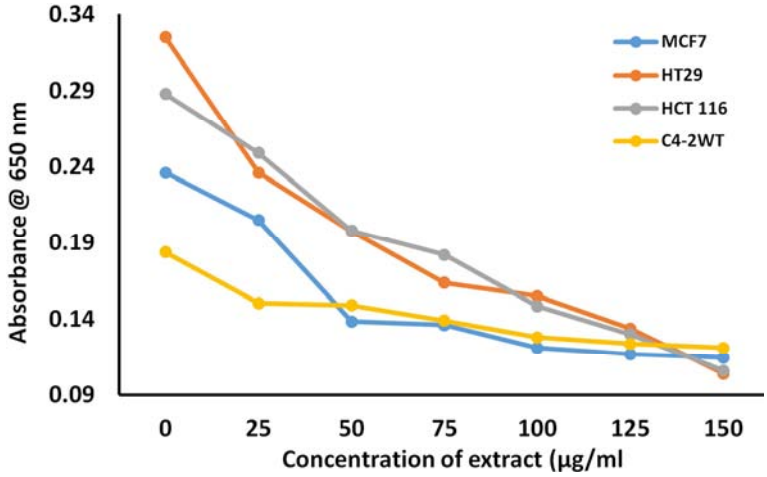

Figure 15. *Optical density of viable cell lines 72 hrs after treatment with ethyl acetate extract of S. alata (L) Roxb.*

4. Discussion

Researchers are focusing their attention on extracts and biologically active compounds isolated from plant species used in herbal medicine, due to the fewer side effects. Medicinal plants are playing an important role as anticancer agents and it is significant that many of currently used anticancer agents are derived from a natural source such as plants [20]. The cytotoxic effect of ethyl acetate extract of *S. alata* (L) Roxb was studied using different cytotoxicity screening assays. MTT result of the present study showed a potent cytotoxic effect on the carcinoma cells with *S. alata* (L) Roxb extract in a dose-dependent manner. Thy GI_{50} value obtained were: MCF-7 = 5.90 µg/ml, HT 29 = 4.97 µg/ml, HCT 116 =11.86 µg/ml and C4-2WT = 9.48 µg/m. Their values were found to be lower than that specified by NCL, USA for categorization of a pure compound as anticancer agent The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation inhibition of less than 30µg/ml after the exposure time of 72 hours [21]. However a crude extract with IC_{50} less than 20µg/ mL is considered highly cytotoxic [22]. Similar studies on cytotoxic activity of *C. alata* leaves extracts reveals that the Hexane fraction of *C. alata* leaves exhibited cytotoxic activity against parental A549 (lung cancer cells) and OV2008 (ovarian cancer cells) cell lines [23], also the hexane fraction and f6l (a mixture of polyunsaturated fatty acid esters) exerted a cytotoxic effect on MCF-7 (breast carcinoma cells), T24 (bladder carcinoma cells) and Col 2 (colorectal carcinoma cells) cell lines in a dose-dependent manner, but were not effective against A549 (non-small cell lung adenocarcinoma) and SK-BR-3 (breast carcinoma cells) cell lines [24]. On the other hand, petroleum ether extract possessed anticancer activity against HCT-15 (colon carcinoma) and Hep2 (Human cervix carcinoma) cell lines [25], while the Chloroform fraction of *Cassia alata* L showed remarkable cytotoxicity against HepG2 cells [8].

In clonogenic assay method, the ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce. Some cells may form large colonies, indicating that the cells have survived the treatment and have retained the ability to reproduce indefinitely. Some cells may remain single, not divide, and, in some cases, may show evidence of nuclear deterioration as they die by apoptosis. These cells would be scored as dead. On the other hand, some cells may go through one or two divisions and form small colonies of

just a few cells. These cells would be scored as dead [17]. In this study, there was a significant loss of the ability to proliferate and form large colonies when the GI_{50} and 2X GI_{50} concentrations of the extract were used, this was due to the cytotoxic effect of the extract on the different carcinoma cell lines.

Result obtained for Trypan Blue exclusion and methylene blue assay showed a remarkable reduction in the total number of proliferating cells and an increase in the total number of non-viable cells over 24, 48 and 72 hr post-treatment with the leaf extract of S. *alata* (L) Roxb in a dose-dependent manner. The decrease in the number of proliferating cells is as a result of cytotoxicity induced by varying concentrations of the extract used.

A preliminary phytochemical screening of the leaves of *S. alata* (L) Roxb by High Performance Liquid Chromatography (HPLC) revealed that 100g of the leaves contains substances tentatively identified as Quercetin 232.315 ± 1.821/100g (13.17%), Kaemferol 148.568 ± 4.133mg/100g (8.42%)**,** Caffeic acid 80.312 ± 4.710/100g (4.55%) and Luteolin 19.284 ± 5.758/100g 1.1 (%) [9]. Flavonoids such as Quercetin, kaempferol, Luteolin and a phenolic acid - Caffeic acid are very common dietary phytochemicals and are known for their cytotoxic properties against various cell lines [11, 26-28]. This result suggests that the cytotoxic effect of *S. alata* extract on cancer cell lines may be derived from the synergistic effects of these phytochemical constituents present in the leaves of the plant. Some researchers have reported the synergistic activity of phenolic compounds found in propolis (caffeic acid phenethyl ester (CAPE), quercetin, chrysin) in the cytotoxic and antiproliferative effect of propolis on cancer cell lines [29-31].

5. Conclusion

Plants extracts have been the source of most drugs used in ethnomedicine for combating various ailments. *Senna alata* (L) Roxb is a tropical perennial herb of medicinal value. Its leaves contain active phytochemicals that were found to contain the important constituents needed to combat various kinds of disease in human.

The results from this study have provided information on the *in vitro* cytotoxicity potential of ethyl acetate extract of leaves of this plant on MCF 7, HT 29, HTC 116 and C4-2WT carcinomas, suggesting a possible use of this natural plant product as a promising anticancer agent.

Phytochemical compounds, identified in a previous study showed that the leaves contain Quercetin, kaempferol, Caffeic acid and Luteolin. It is quite interesting that these compounds have been reported as anticancer agents. Therefore, the presence of these compounds working in synergy could be responsible for the cytotoxic activity of this extract.

Further *in vivo* studies are needed to ascertain its efficacy and to elucidate its possible mechanism (s) of action at molecular and biochemical levels.

Acknowledgements

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Conflict of Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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