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Anti-proliferative and cytotoxic effects of methanol extract of the leaves of *Momordica charantia* L. (Cucurbitaceae) on vascular smooth muscle cells (VSMC) and HT-29 cell lines

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Momordica charantia **has been used traditionally for the treatment of several ailments. Some natural plant products are known to exhibit cytotoxic or anti-proliferative effects on cancer cell lines, such plants offer a promising therapeutic approach as an anti-tumor agent. In this study, the anti-proliferative effects of graded concentrations of methanol leaf extract of *M. charantia* (MEMC) at different point time was examined on vascular smooth muscle cells (VSMC), and human colorectal adenocarcinoma cell lines (HT-29) were investigated using the MTT proliferation assay. The result showed that after 24 and 48 h, the effect of MEMC on the VSMC alone and in the presence of the mitogens was more of proliferation. In the case of HT 29 cytotoxic study, the extract at all doses used caused a cytotoxic effect. The effect of the extract of *M. charantia* was more pronounced and consistent at 72 h time point exhibiting cytotoxic actions against cancer cell lines, the extract showed no toxic action to normal cells. This suggests a possible use of the plant *M. charantia* to identify compounds of possible interest in the treatment of cancer. While the extract possesses proliferative effects on the VSMCs, the reverse is the case, where it exhibited cell inhibitory effects on HT 29 cell lines indicating that the plant exhibit cytotoxic effects and could then serve as lead agents in the search for anticancer drugs from natural products.**

Key words: Anticancer activity, *Momordica charantia*, HT 29, VSMC, VEGF, ET-1.

INTRODUCTION

Cancer refers to a series of conditions whereby abnormal cells begin to divide uncontrollably. Sometimes, cancer begins in a part of the body and then spreads to other parts, a process known as metastasis (Lewandowicz et al., 2000; Kleihues et al., 2002). Cancer is one of the major causes of death globally, it is responsible for

millions of death each year (Rachet et al., 2010.); the incidence of cancer was 90.5 million with about 8.8 million deaths recorded in 2015 (Global Burden of Disease (GBD), 2015). The most common form of cancer includes lung cancer, prostate cancer, colorectal cancer, breast cancer and stomach cancer.

Cancer therapy including chemotherapy, radiation therapy, immunotherapy and stem cell transplantation is associated with various side effects (Abdel-Wahab and Levine, 2010); this implies that researches should focus on discovering novel anticancer agents that are effective with minimal side-effects. There are plant-derived formulations with potential anticancer effect (Chopra and Doiphode, 2002; Aggarwal et al., 2004); these compounds with potential anticancer activity has provided important leads for the development of clinically relevant anticancer drugs (Aggarwal et al., 2003). Antioxidant-rich foods are known to be beneficial in the prevention of cancer, cardiovascular diseases, diabetes, and other oxidative-stress-related chronic diseases (Kähkönen et al., 1999; Johnson, 2004), likewise some cancer patients use agents derived from different plants or nutrients as complementary or alternative medicines, exclusively or concurrently with chemotherapy and/or radiotherapy (Riboli and Norat, 2003).

These products if well researched can represent a new source of compounds with potential antioxidant and antiapoptotic activity. Scientific studies have identified various pharmacologically active and antioxidant compounds that have limited toxicity to normal cells (Riboli and Norat, 2003; Manach et al., 2004; Leung et al., 2009). *Momordica charantia* is a creeper belonging to the family Cucurbitaceae, with all its parts, including the fruit having a bitter taste (Basch et al., 2003; Abhishek et al., 2004). *M. charantia* contains biologically active phytochemicals including triterpenes, proteins, and steroids (Potawale et al., 2008). The triterpenes present in *M. charantia* can inhibit the enzyme guanylate cyclase which is one of the enzymes required for the growth of leukemia and other cancer cells. Physiological actions of *M. charantia* include hypoglycemia, hypolipidemia, anti-viral, antibacterial, immunomodulatory and anticarcinogenic effect which is the main scope of this study (Jilka et al., 1983).

Growth inhibitory properties of *M. charantia* whole plant extract were first reported by West et al. (1971). Thereafter, many growth inhibitors have been isolated from *M. charantia* and its antiproliferative activity has been demonstrated in a variety of tumor cell lines (Akihisa et al., 2007). The fruit extract of *M. charantia* has been proven to be cytotoxic to leukemic lymphocytes thereby inducing antitumor activity *in vivo* (Lee-Huang et al., 1995). A number of preliminary *in vitro* and *in vivo* studies with the water-soluble extract of *M. charantia* and its various purified fractions have shown anti-cancer activity against human bladder carcinomas and breast cancers (Zhu, 1990; Anila and Vijayalakshmi, 2000).

Some proteins in bitter melon including MAP-30, MRK29, alpha-momocharin, beta-momocharin and momordicin have the ability to treat tumors and HIV (Yuan et al., 1999; El-Said and Al-Barak, 2011). Some proteins including alpha- and beta-momorcharins are known to inhibit HIV infections (Jjiratchariyakul. et al., 2001).

Clinical trials have found much evidence that *M. charantia* can improve immune cell function in patients with cancer (Cunnick et al., 1990; Yuan et al., 1999). In this work, the cytotoxic activity of methanol leaf extract of *M. charantia* on cancer cell lines as well as its effects on the normal cell *in vitro* was examined.

MATERIALS AND METHODS

Cells and reagents

Cell Titer 96 MTT (3-(4,5-dimethylthiazol-z-yl)-2,5-di-phenyl tetrazolium bromide microculture tetrazolium technique) (Promega Corporation Cat.# G3580), Human Colorectal Adenocarcinoma Cell lines (HT-29) (Sigma-Aldrich) and Vascular Smooth Muscle Cells (VSMC) (PromoCell, Germany), agarose (Bio-Rad), dimethylsulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM) (GibThai, Thailand), foetal bovine serum (FBS 20%) (Stem Cell Technology, Canada), penicillin (100 U/mL) and streptomycin (100 µg/mL), tween-80, sodium bicarbonate, trypsin/EDTA, propidium iodide (PI), 1 mM L-glutamine, in a 5% CO_2 humidified incubator were all used.

Collection and preparation of plant sample (*Momordica charantia*)

Fresh leaves of *M. charantia* were collected from the campus of the University of Ibadan, Nigeria, in May/June 2013. The leaves were properly identified and authenticated at the Department of Botany, University of Ibadan and the Voucher Specimen (VSN: UIH-22563) was deposited at the herbarium of the Department of Botany, University of Ibadan.

Preparation of plant extract

The leaves were dried at room temperature (27 ± 2°C) and pulverized to a fine powder using an electric blender. The fine powder (400 g) was soaked and extracted in 90% methanol (1 L) using Soxhlet extractor for 3 days until complete extraction. The extracts were filtered through Whatman no 1 filter paper and the filterate was evaporated to dryness by a rotary evaporator (Yamato, Rotary Evaporator, model-RE 801, Japan) at 190 to 220 rpm and 40 to 50°C for 24 h under reduced pressure to give amorphous solid mass. The extract yield was 12%.

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Cell culture

HT-29 and VSMC were procured from National Center for Cell Sciences, Pune, India. Cells were cultured and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 20% foetal calf serum (FCS, 20%), 1% glutamine and 1% penicillin-streptomycin (Gibco^R by life Technologies) in an adherent tissue culture plate at 37°C in a humidified incubator containing 5% CO_2. 96 wells microtiter plate were seeded with 5×10^3 cells per well and incubated again in a humidified atmosphere with 5% CO_2 at 37°C in an incubator. When the seeded plates achieved confluency, the cells were treated with graded concentrations of methanol leaf extract of *M. charantia*

Evaluation of anticancer activity

Cell viability and cell proliferation assay

The antiproliferative effects of the methanol leaf extract of *M. charantia* L. (Cucurbitaceae) on vascular smooth muscle cells (VSMC) and human colorectal adenocarcinoma cell lines (HT-29) were studied using the cell titre 96 MTT proliferation assay where the viable cells were seeded at a density of 5×10^4 (100 µL/well). For VSMC, log concentrations of each extract at 200 and 800 µg/ml were added and incubated for 24 and 48 h time points. Incubation of the extracts in the presence of VEGF and ET-1 was also conducted at different time points. Different concentrations of the extract (200, 400 and 700 µg/ml) were added and incubated with the HT 29 cell lines for 24, 48 and 72 h time points.

MTT assay

Cell viability of HT 29 cells upon treatment with *M. charantia* fractions at different concentrations of 200 and 800 µg/ml, 200 µg/ml and VEGF, 800 µg/ml and VEGF, SPLC1, SPLC2 (VEGF 50 ng/ml) and SPLC3 (ET1 20 ng/ml) was assayed using MTT as described by Yedjou et al. (2006). Cells were cultured to confluence, trypsinized and plated in 96 well plates at an initial density of 10^5 cells/ml for cell proliferation assay. Twenty-four hours after plating, cells were treated with various concentrations (25 to 100 µg/ml) of the extract along with the control in the presence or absence of mitogens Ag II or LPS and cultured for 24 to 96 h to determine effects of treatment on cell growth. The blank sample contained medium only. MTT assay was performed at 24, 48 and 72 h. MTT assay works on the principle of the ability of the cell to reduce MTT to purple formazan in the mitochondria of living cells. The viable VSM cells were seeded at a density of 5×10^4 (100 µl/well) in 96-well plates and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 24 h to form a cell monolayer. MTT assay was performed over three days. On day one, the VSM cells were trypsinized after these have confluence and on the second day the cells were treated with GK, the final volume of the media was adjusted to 100 µl and the incubation continued. On day three, 20 µl of 5 mg/ml of MTT was added to each of the 96 wells but the well used as controls have no cell. After incubation for 72 h, the cells were centrifuged at 800 rpm for 5 min and the supernatant culture medium carefully aspirated. The cells were washed twice with PBS, with of 200 µl fresh medium containing added MTT (0.5 mg/ml). Further incubation was done for three and a half hour at 37°C and the medium was carefully removed. The cells were centrifuged at 800 rpm for 5 min with the supernatant aspirated, followed by addition of 100 µl DMSO to each well and then 200 µl of MTT solvent was added. This was covered with tin foil and cells agitated on orbital shakers for 15 min to solubilize formazan crystals. Thereafter, the absorbance was read using microplate reader (Tecan, Switzerland) at 590 nm. The amount of colour produced is directly proportional to the number of viable cells. This procedure was repeated for VSMC which is the normal cell and observed at 24 and 48 h point time.

Effect of *M. charantia* fractions on cell proliferation rate was determined by viable cell count using a hemocytometer (Lee et al., 2003). HL60 cells (10^5 cells/ml) were placed in a 24-well plate and incubated with different fractions of *M. charantia* at a final concentration of 20 µg/ml for 5 days. Viable cell counts were determined on each day post-treatment using trypan blue dye exclusion assay using a light microscope (Leica, Germany).

Statistical analysis

The results were expressed as mean ± SD. Cell viability was calculated using MTT absorbance of the control and treated cells: % survival = (mean value treated sample/mean value of the untreated sample) * 100. The results were treated to a one-way analysis of variance (ANOVA) and subsequently to the Tukey multi comparison post-test using the statistical package Graph Pad prism version 5 (Graph Pad software, San Diego CA, USA). Values of $\alpha_{0.05}$ were considered as significant (Betty and Jonathan, 2003).

RESULTS

Effects of methanol leaf extract of *M. charantia* on VSMC

The result show that after 24 h, the effect of each extract of the plants on the VSMC alone and in the presence of the mitogens was more of differentiation and proliferation with the proliferation pronounced by the 800 µg/ml concentration at 24 h. At 48 h, the proliferation was more marked (for instance the 800 µg/ml of *M. charantia* in the presence of vascular endothelial growth factor (VEGF) caused 153.3% increase) except for the 200 µg/ml *M. charantia* in the presence of VEGF that caused 14.3% decrease in cell proliferation (Figures 1 and 2).

Effects of methanol leaf extract of *M. charantia* on HT 29 cell line

In this study, the extract at all doses used caused a cytotoxic effect. For instance, there was 77.1% decrease in cell proliferation for 400 µg/ml of *M. charantia* at 24 h. At 48 h, the cell inhibitory or cytotoxic effect was more pronounced at 200 µg/ml concentration of the extract. At 72 h point time, *M. charantia* at all concentrations (200, 400 and 700 µg/ml) exhibited considerable cytotoxic effects on HT 29 cell lines (Figures 3 to 5).

DISCUSSION

This study demonstrates for the first time the differentiation and regeneration ability of the *M. charantia* methanol leaf extract on VSMC which is the normal cell. The test concentrations did not exert a cytotoxic effect on VSMC even in the presence of mitogens. This result

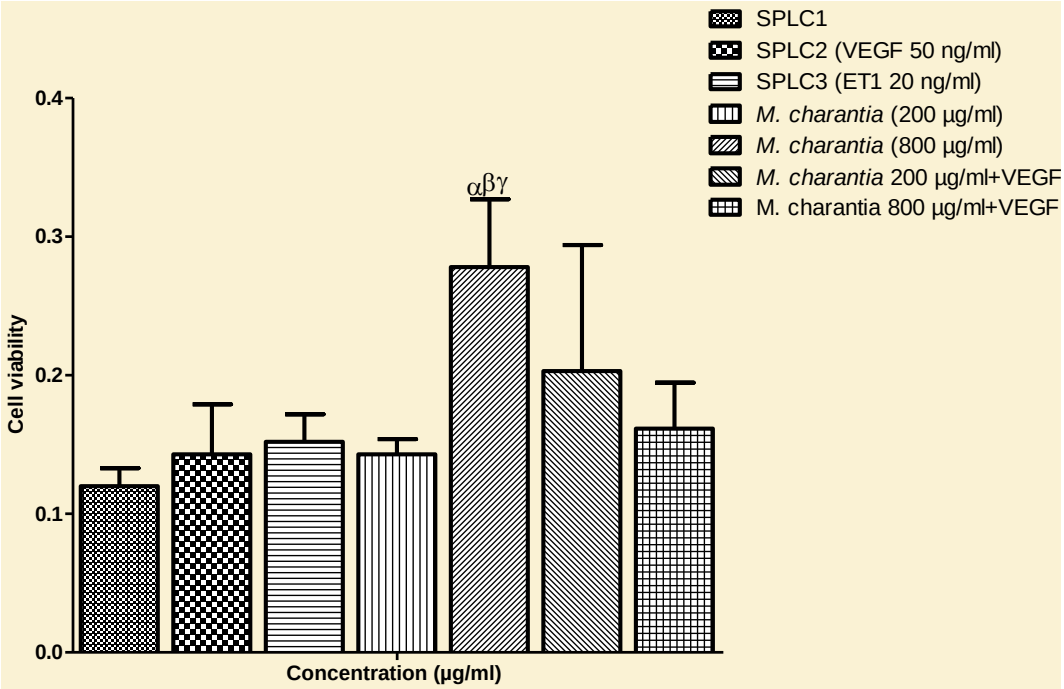

Figure 1. Effect of methanol leaf extract of *M. charantia* on VSMC cell viability at 24 h time point. α: $\alpha_{0.05}$ when compared with SPLC 1; β, $\alpha_{0.05}$ when compared with SPLC 2 (VEGF 50 ng/ml); γ: $\alpha_{0.05}$ when compared with SPLC 3 (ET1 20 ng/ml).

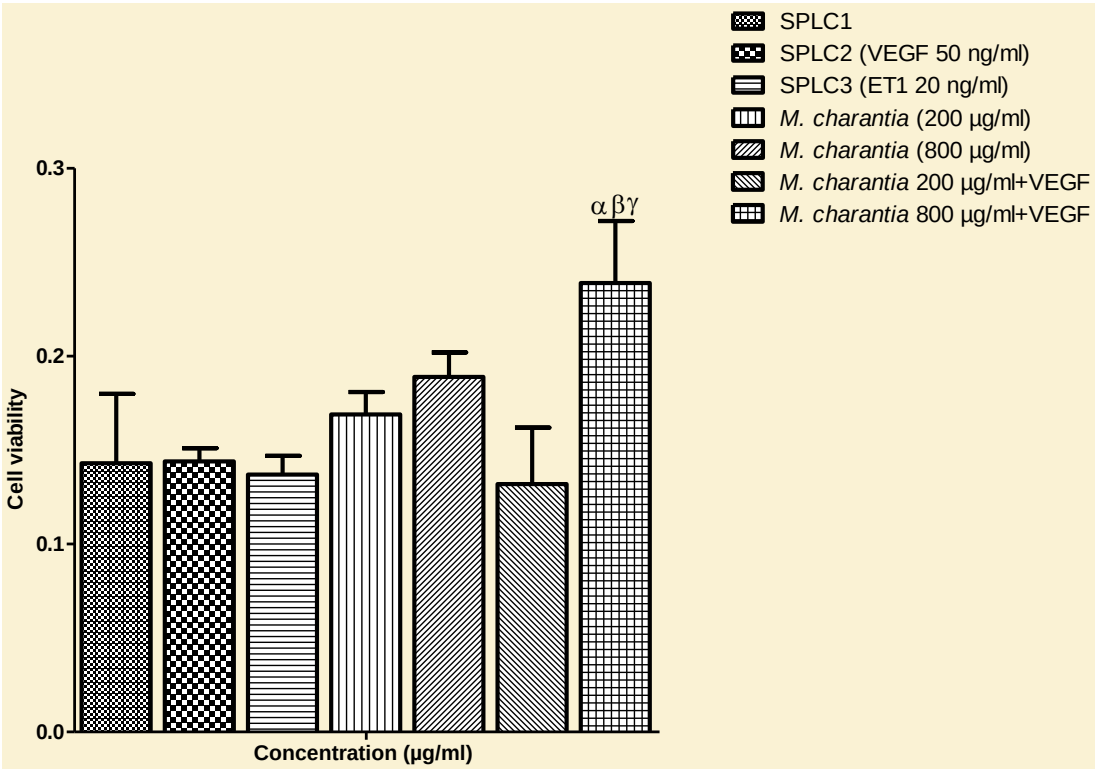

Figure 2. Effect of the methanol leaf extract of *M. charantia* on VSMC cell viability at 48 h time point. α: $\alpha_{0.05}$ when compared with SPLC 1; β: $\alpha_{0.05}$ when compared with SPLC 2 (VEGF 50 ng/ml); γ: $\alpha_{0.05}$ when compared with SPLC 3 (ET1 20 ng/ml).

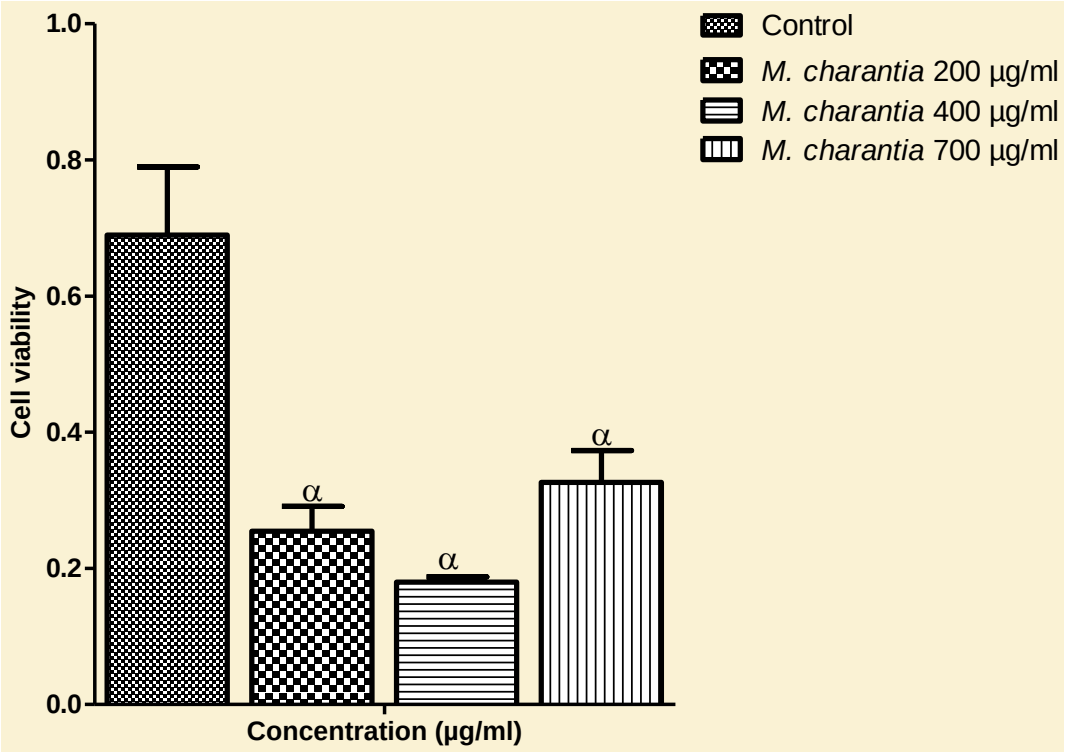

Figure 3. Effect of methanol leaf extract *M. charantia* on HT 29 cell lines at 24 h. **α**: Significant values when compared with control at $\alpha_{0.05}$.

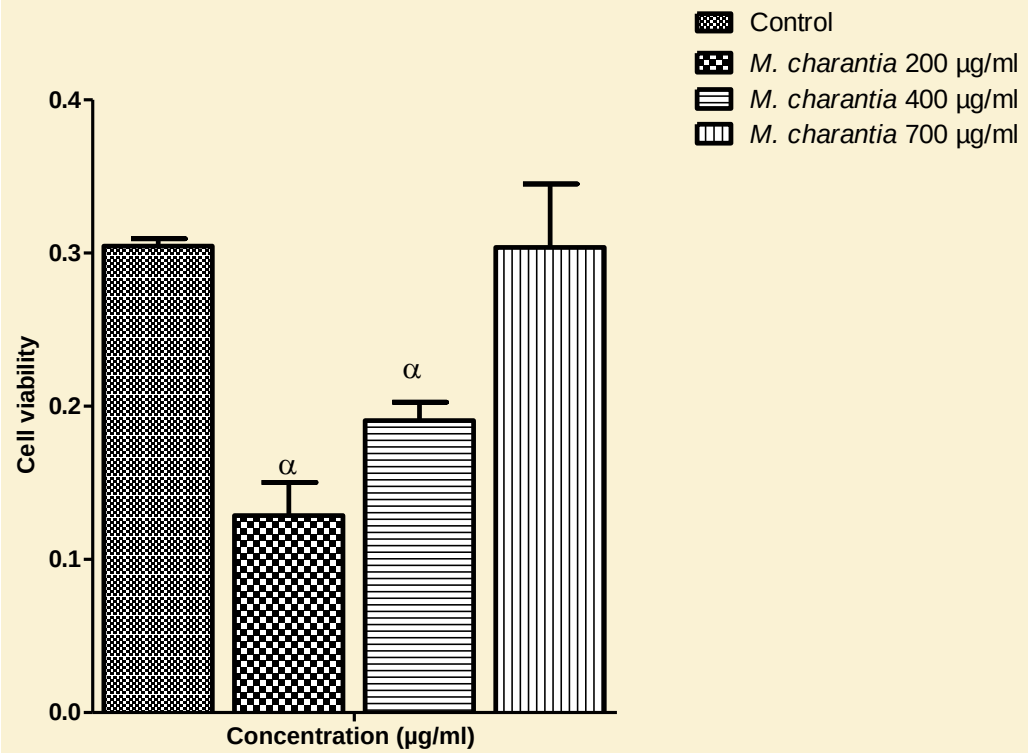

Figure 4. Effect of methanol leaf extract of *M. charantia* on HT 29 cell lines at 48 h. α: Significant values when compared with control at $\alpha_{0.05}$.

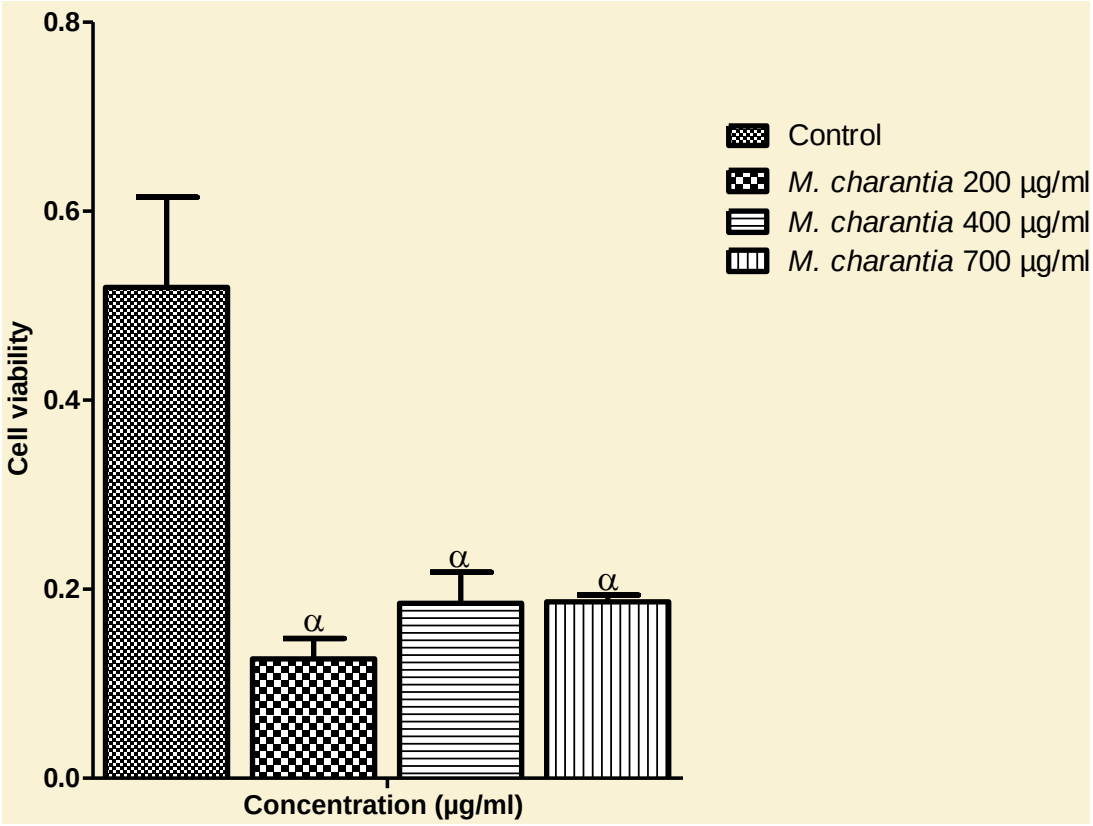

Figure 5. Effect of methanol leaf extract *M. charantia* on HT 29 cell lines at 72 h. α: Significant values when compared with control at $\alpha_{0.05}$.

supports our claims in a previous study that the methanol leaf extract of *M. charantia* caused regeneration and proliferation of pancreatic beta cells.

In the case of HT 29 cytotoxic study, the extracts at all doses used caused a cytotoxic effect. There was 77.1% decrease in cell proliferation for 400 µg/ml of *M. charantia* at 24 h. The effect of the extract of *M. charantia* was more pronounced and consistent at 72 h time point. Chia-Jung et al. (2012) reported that *M. charantia* extract causes mitochondria- related cell death in human cancer cells through Caspase- and mitochondria-dependent pathways. Thus methanol leaf extract of *M. charantia* exhibited cytotoxicity on HT 29 cells, due to its ability to induce cell death in cancer.

The result from this study showed that while the extract had proliferative effects on the VSMCs, the reverse is the case, where it exhibited cell inhibitory effects on HT 29 cell lines indicating its cytotoxic effects. This study is consistent with the reports of Jutamas et al. (2015) which explained that plumericin isolated from *M. charantia* vine exerts antiproliferative effects against leukemic, breast and liver cancer cell lines.

Terpenes, a phytochemical present in *M. charantia* (Chang et al., 2008) have been reported to have anti-proliferative effect (Akhisa et al., 2007). This suggests that terpenes contributed to the anti-proliferative effect of *M. charantia* observed on the cancer cell lines.

The findings of this study negate the report of Soundararajan et al. (2012) which explained that *M. charantia* seed fractions did not exhibit any antiproliferative activity. However, Soundararajan et al. (2012) reported that the differentiation-inducing fraction in M. charantia was non-proteinaceous in nature, showing that the differentiation-inducing factor of M. charantia is different from its antitumor factors. Thus, the present study for the first time describes the differentiation inducing action of the methanol leaf extract of *M. charantia* which can be further studied as an inducer of differentiation and regeneration of normal cell, either alone or combined with the suboptimal concentrations of other known inducers of differentiation.

As a result of this study, it can be recommended that the methanol leaf extract of *M. charantia* can be combined with the known anticancer agent in order to act synergistically for a more effective treatment of cancer. The rationale being that the commercially available anti-cancer drugs in high concentrations not only kill cancer cells but also healthy normal cells in the body (Nagasawa et al., 2002). Therefore, a low to moderate dose of the anticancer drug (either temozolomide or vinblastine) can

be combined with a high dose of the crude methanol extract of *M. charantia* to produce a maximal anti-cancer effect, without killing healthy cells.

Conclusion

This study has demonstrated that the crude methanol leaf extract of *M. charantia* can cause a significant decrease in cancer cell viability (an increase in cell death) without being harmful or injurious to healthy cell lines like smooth muscle cell line. These effects were both time and dose-dependent with maximal effect occurring after 72 h at a dose of 200 µg for the methanol leaf extract of *M. charantia*. Therefore, *M. charantia* is cytotoxic to cancer cell lines (that is, it has anticancer property since it induces cell death) while it has no cytotoxic but caused regeneration of normal cells. Thus, *M. charantia* represents a promising candidate that could be developed for cancer prevention and treatment in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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