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Cell Proliferation and Cytotoxic Studies of *Vernonia amygdalina* on Vascular Smooth Muscle Cells and HT 29 Cell Lines

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AAA and MAY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ATA, OAF and AAO managed the analyses of the study. Author TOO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The antiproliferative effects of the methanol leaf extracts of *Vernonia amygdalina* (*V. amygdalina*) Del (*Asteraceae*) on Vascular Smooth Muscle Cells (VSMC) and Human Colorectal Adenocarcinoma Cell line (HT 29) were investigated using the Cell Titer 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 the 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 (the mitogens) was also conducted at different time points. Concentrations of the extract (200, 400 and 700 μg/mL) were also added and incubated with the HT 29 cell line for 24, 48 and 72 h time points. The result showed that after 24 hours, the effect of the extract of the plant on the VSMC alone and in the presence of the mitogens was more of proliferation except the 200 μg/mL in which there was no proliferation in the presence of ET-1. At 48 hrs, the proliferation was even more marked. For instance, the 800 μg/mL of *V. amygdalina* in the presence of VEGF caused 153.3% increase. In the case of HT 29 cytotoxic study, the extract at all doses used caused cytotoxic effect. For instance, the 200 μg/mL concentration of *V. amygdalina* caused 69.2% cell inhibition at 72 hours time point. It must however be noted that it was at 24 hrs time point that the effect of the extract was most pronounced and consistent. It could be concluded from this study that while the extract has proliferative effects on the VSMCs, the reverse is the case for the HT 29 cell line. It thus showed that the extract caused inhibitory effects on HT 29 cell line indicating that the extract exhibits cytotoxic effect and could then serve as lead agents in the search for anticancer drug from natural products.

Keywords: V. amygdalina; HT 29; VSMC; anticancer; VEGF; ET-1.

1. INTRODUCTION

In cell biology and drug-discovery research, the characterizations of agents that will retard or promote cell proliferation are very important. During the last two decades or so, while more than 25% of drugs were derived from plant species, the other 25% were chemically altered natural products. It was also highlighted that only 5-15% of approximately 250,000 higher plants have been investigated for bioactive compounds [1,2].

Even though of recent synthetic chemistry has emerged 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 [3]. Plants contain almost unlimited capacity to generate compounds that fascinate researchers in the quest for new and novel chemotherapeutics [4].

Cancer is one of the most dangerous diseases in humans and presently there is a considerable scientific discovery of new anticancer agents from natural products [5]. Available management options such as surgery, radiation therapy, immunotherapy and chemotherapy are either toxic, expensive or both. This led to the search for alternative therapies in botanicals with anti-cancer activity. Medicinal plants have been used and are still being used to treat various chronic diseases thus natural products from plants have shown their potentiality in controlling cancer while exhibiting less or no side effects. For this reason, the use of plant extracts as complimentary to modern medicine is gaining increased popularity. In fact, in the developing countries, up to 80 % population is depending on herbal medicine to heal different diseases because of lack of access to allopathic medicine [6-9].

The use of chemotherapeutic drugs in cancer therapy involves the risk of life threatening host toxicity with serious side effect and they merely extend the patient's lifespan for a few years [10]. There is an increasing demand to use alternative concepts or approaches for the prevention of cancer and an increasing attention and realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanism of apoptosis. However, there are many cancers that are intrinsically resistant to apoptosis, making it vital to develop novel drugs for combination chemotherapy [11,12]. Measuring anti-proliferative properties against cancer cells using MTT assay provide useful insight on the chemo-protective potential of natural extracts especially that the

elimination of cancer in the early stages is an integral part of chemoprevention [13].

Vernonia amygdalina Del (family of *Asteraceae*) is a valuable medicinal plant that is widespread in East and West Africa [14,15]. It is known as bitter leaf and may be used as active anticancer agent [16] anti-bacterial, anti-malarial, and antiparasites [17]. This plant contains complex active components that are pharmacologically useful. The roots and the leaves are used in ethnomedicine to treat fever, hiccups, kidney problems, and stomach discomfort [18,19].

The anti-cancer effect of *Vernonia amygdalina* was first shown in human carcinoma of nasopharynx and later in leukemia cells P-388 and L-1210 using the chloroform extract of *Vernonia amygdalina* [20,21]. Izevbigie [16] has demonstrated that low concentrations (µg/mL) of water soluble leaf extracts potently retarded the proliferative activities of estrogen receptor positive (ER+) human breast cancer (MCF-7) cells *in vitro* in a dose-dependent manner. Other studies have shown that *Vernonia amygdalina*-treatment modulates phase 1 and phase 2 gene expressions in MCF- 7 cells in a dose and time-dependent fashion [22]. The aim of this study was to evaluate the effects of the methanol leaf extract of *Vernonia amygdalina* on VSMC and HT29 cell line.

2. MATERIALS AND METHODS

2.1 Collections and Identification of Plant Materials

Fresh leaves of *Vernonia amygdalina* were collected from the community around the University of Ibadan campus and then subsequently authenticated at the Department of Botany University of Ibadan where specimen voucher was also kept. The herbarium number is UIH-22640.

2.2 Preparation of Plant Extracts

2.2.1 Methanol leaf extraction of *Vernonia amygdalina*

The fresh plant leaves were washed with water to remove dirt and then dried under shade. The leaves were pulverized into powder form and 200 g of the powdered materials were separately dissolved in 2 L of methanol under cold maceration. After 72 hrs, the solution was filtered using Whatman filter paper. The black material in a slurry form was then placed in porcelain plate and put on hot water at about 40ºC to remove the remaining methanol in the extract.

2.2.2 MTT assay

Cells were cultured to confluence, trypsinized and plated in 96-well plates for cell proliferation assay. Twenty four hours after plating, cells were treated with various concentrations (25-100 µg/mL) of the extract along with the control in the presence or absence of mitogens and cultured for 24-48 hours to determine effects of treatment on cell growth.

MTT assay is based on the ability of cell to reduce MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to purple formazan in the mitochondria of living cells. The viable cells (VSMC and HT 29) 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 37ºC for 24 h to form a cell monolayer. MTT assay was performed over three days. On day one, the cells were trypsinized. After these have confluenced and on the second day, the cells were treated with *Vernonia amygdalina,* mitogens and *Vernonia amygdalina* + mitogens and 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. This was then incubated for three and half hours at 37ºC in culture hood. After this, the media was carefully removed and 150 µL of MTT solvent was added and covered with tin foil and cells agitated on orbital shakers for 15 minutes. Thereafter, the absorbance was read at 590 nm.

2.3 Statistics

Results are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), using Graph Pad Prism version 6. The level of statistical significance was considered as $\alpha<0.05$.

3. RESULTS AND DISCUSSION

3.1 Effects of the Extract of *V. amygdalina* on VSMC

The result of this study showed that after 24 hours, the effect of the extract on the VSMC alone and in the presence of the mitogens was more of proliferation. For instance, the 200 μg/mL of the extract alone and in the presence of

VEGF caused respectively 25 and 108.3% increase in cell proliferation but in the case of the 800 μg/mL alone and in the presence of VEGF the result was 83.3 and 41.7% increase in cell proliferation showing that in a way there is reduction in cell proliferation at this dose in the presence of VEGF. The result also showed that the extract at 200 μg/mL in the presence of ET-1 caused no cell proliferation whereas the 800 μg/mL in the presence of ET-1 caused 33.3% cell proliferation indicating that this mitogen also interfere with the extract's cell proliferation activity. At 48 hrs, the 200 μg/mL alone caused 73.3% increase in cell proliferation but in the presence of VEGF and ET-1 it caused 86.7 and 80% increase in cell proliferation. With respect to the 800 μg/mL alone and in the presence of VEGF and ET-1, there was increase in the proliferation of the cells at 126.7, 153.3 and 100%, respectively (Figs 1 and 2).

Studies with natural products involve biological activity and pharmacological assays for the screening of plant extracts, with subsequent evaluation of the fractions and isolated compounds were responsible for the activity observed [23].

In this present study *Vernonia amygdalina* alone and in the presence of VEGF resulted in a dose dependent and time dependent increase in cell proliferation which is similar to what Parenti et al. [24] demonstrated that VSMC proliferation induced by monocyte chemoattractant protein-1 was mediated by endogenous production of VEGF-A.

Vasoactive agents such as norepinephrine (NE), angiotensin II (ANG II), and endothelin-1 can induce hypertrophy and proliferation of vascular smooth muscle cell and thus are implicated in pathogenesis of hypertension [25-28]. Endothelins (ETs) are a family of three peptides (ET-1, ET-2, ET-3) that are implicated in the physiological control of vascular smooth muscle cell (VSMC) and myocardial contractility and growth. ET-1 is vasoactive peptide that acts via ET-A receptors coupling inducing vascular smooth muscle cell contraction [29-31].

In this study at 24 hours, the 200 μg/mL dose of *Vernonia amygdalina* resulted in an inhibition of the proliferation of VSMC in the presence of ET-1, the higher dose (800 μg/mL) however showed a slight increase in cell proliferation. At 48 hours, however, there was an increase in the proliferation of the VSMCs in the presence of both *Vernonia amygdalina* and *Vernonia amygdalina*+ET-1. It can be deduced from this observation that the proliferative effect of the plant on VSMC is dependent on time and dose. VSMCs have been viewed as directly responsible for generating the atherosclerotic plaque, via proliferation, migration from the media and synthesis of matrix proteins [32]. Consequently huge efforts have been made to inhibit the accumulation of these cells, which in the case of stent stenosis have largely been successful. However, reviews of atherosclerosis have emphasized the advantageous protective function of VSMCs in atherosclerosis [33]. This is based on findings that plaques in humans that have undergone plaque rupture, and directly led to heart attacks, show a paucity of VSMCs compared with stable lesions [34]. Indeed VSMCs are the only cells capable of synthesizing components of the fibrous cap in plaques (the structure that separates the blood from the thrombogenic plaque interior), and whose rupture or erosion may trigger myocardial infarction.

Apoptosis of VSMCs has been recognised in atherosclerosis. In early lesions, apoptotic frequencies are minimal but peak in advanced plaques with both VSMCs and macrophages showing features of apoptosis [35]. This observation raised the possibility that VSMC apoptosis could promote plaque rupture by thinning the fibrous cap. Indeed, plaques from patients with unstable symptoms show higher levels of apoptosis than those with stable lesions [36]. VSMC apoptosis has also been associated with numerous other features within plaques including inflammation, calcification, thrombosis [37] and both negative remodeling (vessel shrinkage) [38,39] and aneurysm formation [40]. VSMC apoptosis causes release of IL-1 and up-regulation of monocyte-chemoattractant protein 1 (MCP-1) [41] and interleukin (IL) 8, causing infiltration of macrophages in vivo [42]. *In vitro,* VSMC apoptosis can promote both thrombin generation [43] and vascular calcification [44], and apoptotic vascular cells are thrombogenic both locally [45] and systemically [46]. Therefore the proliferation of VSMC by *Vernonia amygdalina* at higher dose in this study might be a useful therapeutic target to prevent VSMC apoptosis in stable and unstable atherosclerosis.

3.2 Effects of the Extract of *V. amygdalina* on HT 29 Cell Line

In the case of HT 29 cytotoxic study, at 24 hrs, the 700 μg/mL concentration caused the greatest

cytotoxicity at 61.8% cell inhibition and this was followed by the 400 μg/mL concentration at 60.5% decrease in proliferation. The 200 μg/mL concentration of the extract only caused 52.6% inhibition of the HT 29 cell line. At 48 hrs time point, the 200 μg/mL concentration caused 50% inhibition followed by that of 400 μg/mL causing inhibition at 37.5% while the 700 μg/mL caused 12.5% cell inhibition. At 72 hrs, the 200 μg/mL concentration caused the greatest cytotoxic effect at 56.1% cell inhibition and this is followed by 400 and 700 μg/mL at 51.2 and 31.7%, respectively. It is to be noted that the antiproliferative effect of the extract increases with time with the 200 μg/mL concentration showing some form of consistency with time (Figs. 3 and 5).

It has been known that plants have a long history of use in the treatment of cancer [47] and herbal medicines have a vital role in the prevention and treatment of cancer [48]. The use of plant derived natural compounds as part of herbal preparations and alternative sources of drugs continues to play major roles in the general wellness of people all over the world [47,49]. Agents capable of inhibiting cell proliferation, inducing apoptosis or modulating signal transduction are currently used for the treatment of cancer. The use of multiple targets on cancer cells is considered to be more effective in cancer treatment.

Studies on cancer treatments reveal that most, if not all, chemotherapeutic agents kill cancer cells through the induction of apoptosis. In this study, we examined the cytotoxic efficacy of *Vernonia amygdalina* on HT 29 cell line using MTT assay where it was shown that *Vernonia amygdalina* caused inhibition of cell proliferation at all doses tested and at all time points. However, the 200 μg/mL dose showed the most consistent effect. This report is similar to several reports of the anti- cancer activity of *Vernonia amygdalina* against several cell lines [13,50,51]. Yedjou *et al.* [52] reported that the toxicity of *Vernonia amygdalina* extract to human breast adenocarcinoma (MCF-7) cells is associated with apoptotic and secondary necrotic cell death resulting from phosphatidylserine externalization due to loss of membrane integrity. The anticancer activity of this plant has been adduced to its high phenol content [53].

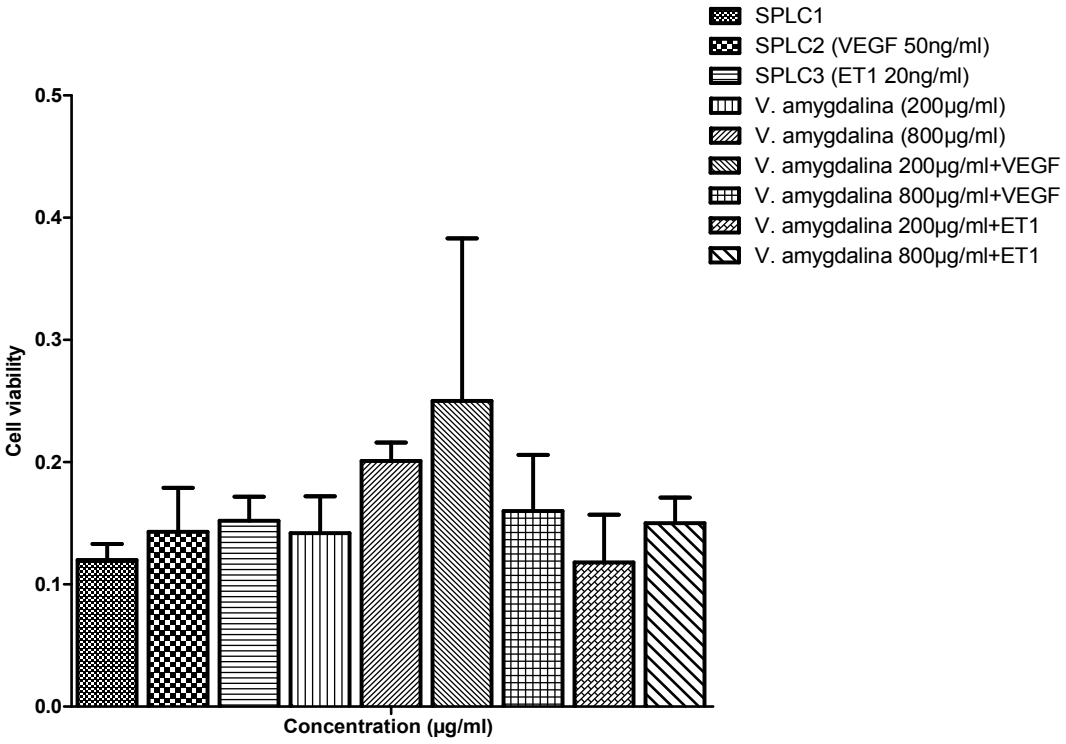

Fig. 1. Effect of the extract of *V. amygdalina* on VSMC cell viability at 24 hrs time point

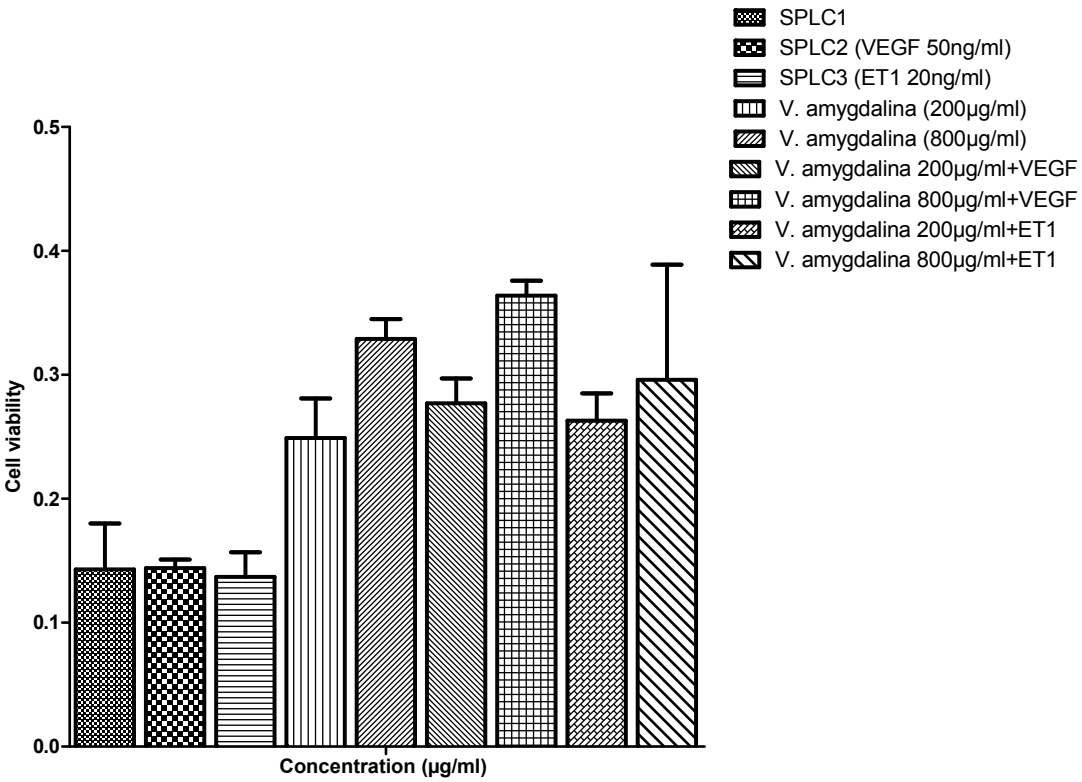

Fig. 2. Effect of the extract of *V. amygdalina* on VSMC viability at 48 hrs time point

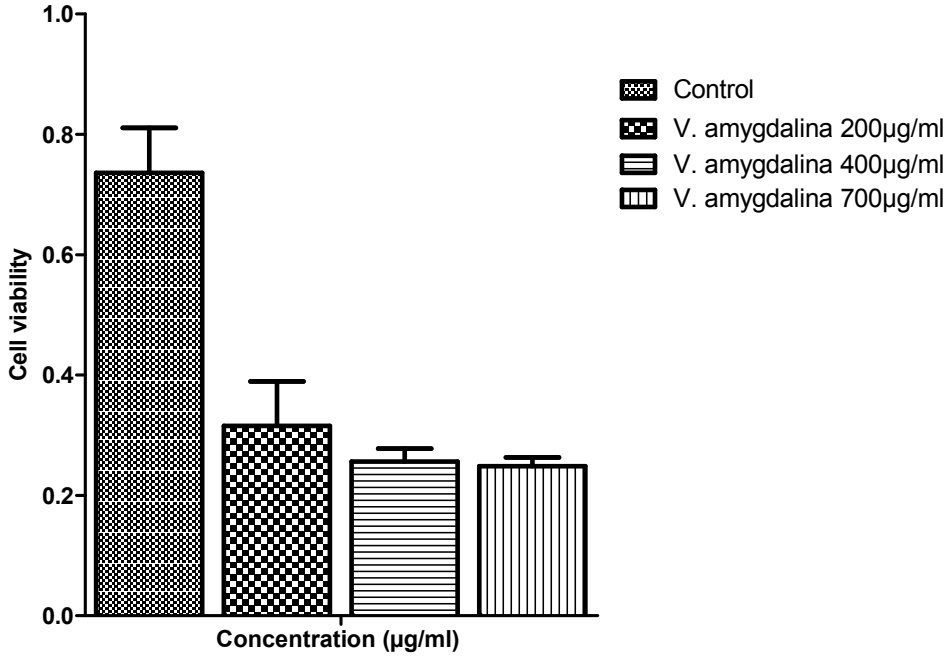

Fig. 3. Effect of methanol leaf extract of *V. amygdalina* on HT 29 cell line at 24 hrs time point

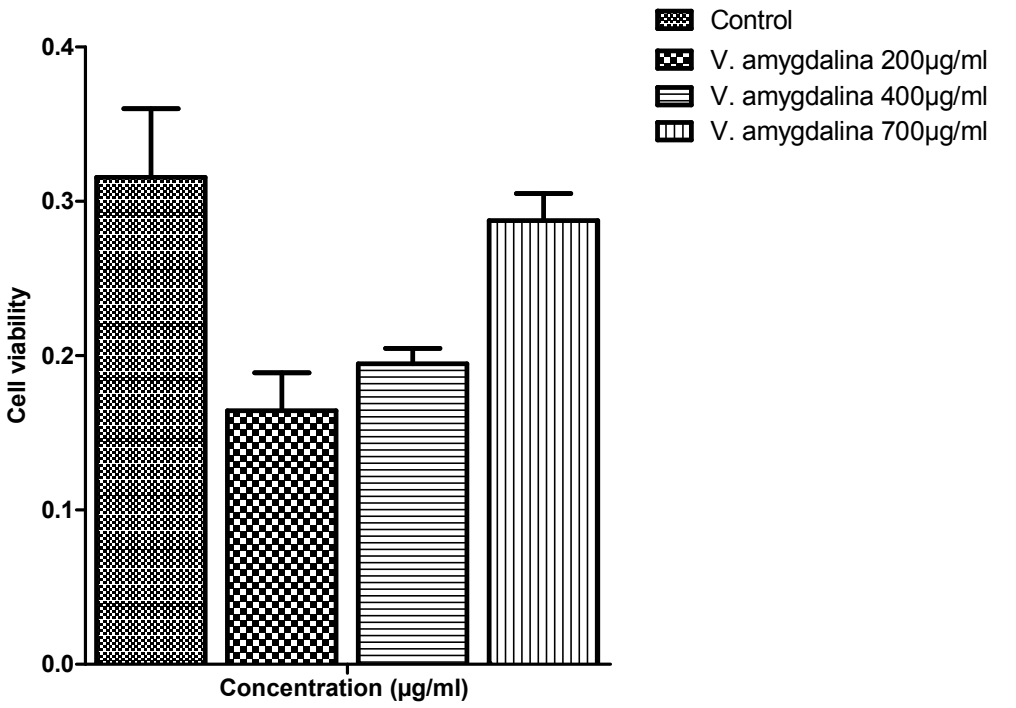

Fig. 4. Effect of methanol leaf extract of *V. amygdalina* on HT 29 cell line at 48 hrs time point

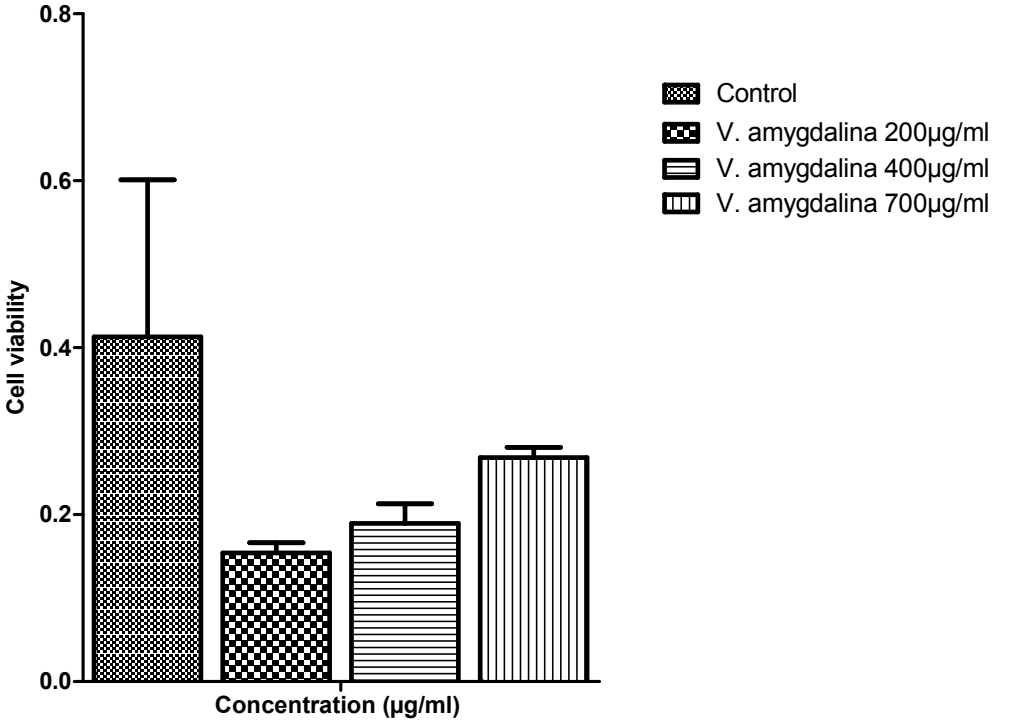

Fig. 5. Effect of methanol leaf extract of *V. amygdalina* on HT 29 cell line at 72 hrs time point

4. CONCLUSION

The present data demonstrate that *Vernonia amygdalina* has protective and proliferative effects on VSMC at higher concentration and may inhibit the proliferation of cancer cells possibly through apoptosis. The anticancer property of the plant may be attributable to its high phenol content. We can conclude that *Vernonia amygdalina* extract has a good potential to be used as a new cancer therapeutic agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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