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**NOVEL CELLULAR STAINING PROTOCOL AND ANTIPROLIFERATIVE
 EFFECT OF VERNONIA AMYGDALINA DELILE ON LUNG AND PROSTATE
 CANCER CELLS**
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ABSTRACT

There is a critical need for more effective therapeutic approaches for cancer. *Vernonia amygdalina* Delile (VAD) has been used in African traditional medicine for the prevention and/or treatment of several diseases including diarrhea, intestinal illnesses, and cancer. However, the effects of VAD on human lung cancer and human prostate cancer cells remain largely unknown. The aim of this study was to explore a novel cellular staining protocol using acridine orange/propidium iodide (AO/PI) and to test the antiproliferative activity of VAD against human lung cancer (A-549) cells and human prostate cancer (PC-3) cells. Our studies demonstrate that VAD inhibits the proliferation of both A-549 and PC-3 cells in a dose-dependent manner. This finding suggests that VAD may be useful in lung and prostate cancer prevention. However, further research is needed to elucidate the chemopreventive effects of VAD against cancer

Keywords: *Vernonia amygdalina* Delile; cellular staining; antiproliferative effect; lung cancer; prostate cancer

I. INTRODUCTION

Medicinal plants are commonly used as complementary and alternative therapies for the prevention and/or treatment of many diseases including cancer. It was reported in 2002 that approximately 1 in every 5 Americans uses medicinal plants or herbal products for the prevention/or treatment of a disease¹. Interestingly, the number of people who use complementary and alternative therapies continues to grow in the United States and other developed countries^{2,3,4}. Over the past decade, the use of VAD either as food or as chemopreventive agent against cancer prevention and/or treatment has received increasing attention^{5,6}. VAD is commonly called bitter leaf in English. It is a species of *Vernonia amygdalina* (family of asteraceae) and a valuable medicinal plant that is widespread in some African countries⁷. It has been used in African traditional medicine for the prevention and/or treatment of several diseases including stomach discomfort, diarrhea, and intestinal illnesses, but the most documented species is *Vernonia amygdalina*^{8,9,10,11}. Although several studies have reported that VAD is used for the treatment of many diseases in many African countries, its antiproliferative activity on human cancers is poorly elucidated. Therefore, the aim of this study was to explore a novel cellular staining protocol using the acridine orange/propidium iodide (AO/PI) dye and test the antiproliferative activity of VAD against human lung cancer (A-549) cells and human prostate cancer (PC-3) cells.

II. RESULTS
1. Fluorescent Microscopy Images Showing the Antiproliferative Effect of *Vernonia amygdalina* Delile on human Lung Cancer (A-549) cells and human Prostate Cancer (PC-3) Cells

In the present study, we tested the antiproliferative activity of *Vernonia amygdalina* Delile (VAD) leaf extracts against human lung cancer (A-549) cells and human prostate cancer (PC-3) cells by the means of double staining (acridine orange/ propidium iodide). We observed a strong dose-response relationship with regard to VAD treatment, showing a significant increase in the percentage of dead cells compared to the percentage of live cells in the control (Figures 1 and 2). Acridine orange (OA) is a nuclear dye that is permeable to both live and dead cells. OA stains both live and dead nucleated cells to emit green fluorescence. Propidium iodide (PI) is a nuclear dye that is impermeable to live cells and permeable to dead cells. PI stains all dead nucleated cells to emit red fluorescence. As seen in Figures 1 and 2, the percentage of dead cells (red) increases with increasing doses of VAD.

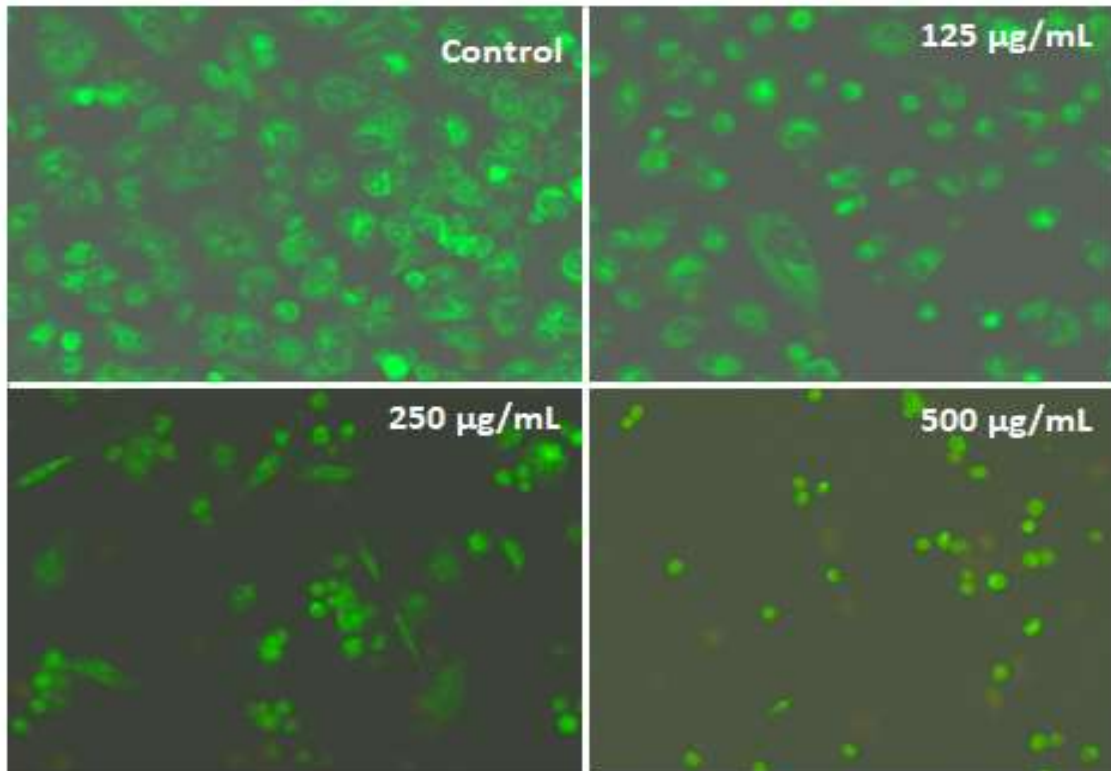


Figure 1: Human lung cancer (A-549) cells stained with the live/dead assay kit. Live cells (Green) and dead cells (Red). A-549 cells untreated (A-control) and A-549 cells treated with *Vernonia amygdalina* Delile at 125 µg/mL (B), 250 µg/mL (C), and 500 µg/mL (D). Live cells (green fluorescent) and dead cells (red fluorescent) were determined based on the acridine orange and propidium iodide assay using the fluorescence microscope.

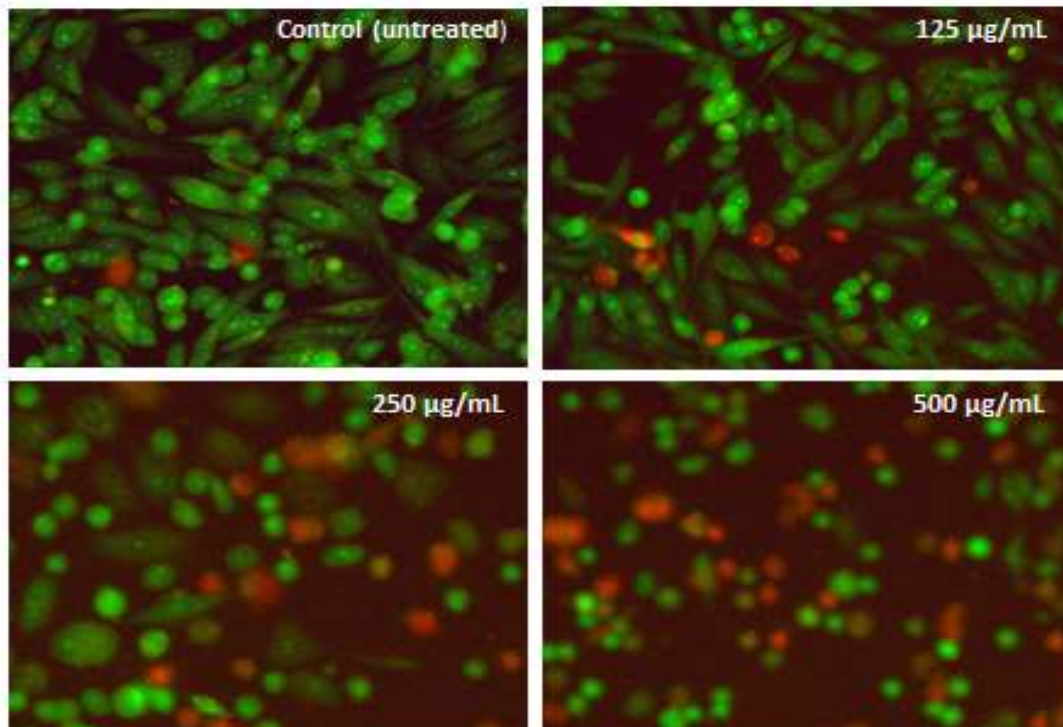


Figure 2: Human adenocarcinoma (PC-3) cells stained with the live/dead assay kit. Live cells (Green) and dead cells (Red). PC-3 cells untreated (A-control) and PC-3 cells treated with *Vernonia amygdalina* Delile at 125

µg/mL (B), 250 µg/mL (C), and 500 µg/mL (D). Live cells (green fluorescent) and dead cells (red fluorescent) were determined based on the acridine orange and propidium iodide assay using the fluorescence microscope.

III. DISCUSSION

Novel Cellular staining Protocol and Cellular Viability

In the present study, we explored a novel cellular staining protocol with acridine orange/propidium iodide (AO/PI) and tested the antiproliferative activity of VAD against human lung cancer (A-549) cells and human prostate cancer (PC-3) cells. As result, we successfully developed a new cellular staining protocol to detect and distinguish cellular morphology, cellular damage, live cells, and dead cells that are clearly visible under a fluorescent microscope. In addition, we observed that VAD extract inhibits the proliferation of both A-549 and PC-3 cells in a dose-dependent manner. Assessing cellular morphology and cellular viability is an important component in the development and discovery of cancer drugs. As seen on Figures 1 and 2, there is a strong dose-response relationship with regard to VAD treatment, showing a significant increase in the percentage of dead cells compared to the percentage of live cells in the control. Treatment of A-549 and PC-3 cells treated with VAD resulted in morphological alterations showing signs of apoptosis and/or necrosis. Apoptosis is characterized by a series of typical morphological features such as cell shrinkage, blebbing of plasma membrane, chromatin condensation, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells^{12,13}. Necrosis is often characterized by irreversible cellular damage which includes cytoplasmic swelling, cell membrane damage, organelle breakdown, and release of cellular content into the surrounding medium^{14,15}. As seen on Figures 1 and 2, acridine orange (OA) stains both live and dead nucleated cells to emit green fluorescence. Propidium iodide (PI) stains all dead nucleated cells to emit red fluorescence. The loss of cellular membrane integrity in non-viable cells allows the PI dye to freely diffuse into the cells and bind to their DNA. Because PI dye can only enter cells with compromised cell membranes, healthy cells/live cells were not stained while dead cells/dying cells were stained by the PI. A recent report in our laboratory indicated that leukemia (HL-60) cells treated with VAD and stained with AO/PI only emit green fluorescent detecting live cells and red fluorescent detecting dead cells when cells are analyzed by the cellometer vision¹⁶. The use of the fluorescence microscope allows the researcher to not only detect live and dead cells, but also detect and distinguish cellular morphology and cellular damage. In addition, the fluorescence microscope reveals morphological alterations showing signs of apoptosis and/or necrosis.

IV. METHOD

Chemicals and Media

Culture plates, flasks, test tubes, acridine orange and propidium iodine (AO/PI) were purchased from Sigma-Aldrich INC (St. Louis, MO).

Vernonia amygdalina Delile Preparation

The preparation of *Vernonia Amygdalina* Delile (VAD) extract was done in the Department of Chemistry and Biochemistry at Jackson State University as previously described⁵.

Tissue/Cell Culture

The human lung cancer (A-549) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA) were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), and 0.1% penicillin/streptomycin solution (Sigma-Aldrich, Inc., St. Louis, MO) and grown in an incubator at 37°C in 5% CO₂. The human prostate cancer (PC-3) cells obtained from the ATCC were cultured in Kaign's Modification of Ham's F-12K medium, supplemented with 10% fetal bovine serum (FBS), and 0.1% penicillin/streptomycin solution (Sigma-Aldrich, Inc., St. Louis, MO) and grown in an incubator at 37°C in 5% CO₂.

Cell treatment

Approximately 85 percent of A-549 cells and PC-3 cells seeded in a polystyrene 6 well-plate (Nunc Brand Products, USA) were treated with three doses (125, 250, and 500 µg/mL) of VAD for 48 hours.

Novel Cellular Staining with the AO/PI and Fluorescence Microscopy Imaging

After treatment, untreated (control) and treated cells were washed twice with PBS. Cells seeded in each polystyrene 6 well-plate were stained by adding 50 µL of AO/PI into 1 mL of fresh medium and incubated overnight at 37°C. Cells were examined and photographed under Olympus fluorescent microscope. This novel approach is a modified staining protocol using AO/PI that we developed in our laboratory. This technique is a

rapid, sensitive, and successful method to examine cellular morphology, live cells, cellular damage, apoptosis and/or necrosis (Fig. 1 and 2).

Data Analysis

All the experiments were performed in triplicates and repeated three times. The means of the different groups were compared using one-way of variance (ANOVA) followed by student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

V. CONCLUSIONS

Cancer is commonly one of the leading causes of death in both men and women worldwide. It is believed that medicinal plants have the potential to cure cancer and other diseases. Our aim in the present study was to explore a novel cellular staining protocol with acridine orange/propidium iodide (AO/PI) and to test the antiproliferative activity of VAD extract against human lung cancer (A-549) cells and human prostate cancer (PC-3) cells. A novel finding was that we successfully developed a new cellular staining protocol to detect and distinguish cellular morphology, cellular damage, live cells, and dead cells under a fluorescent microscope. In addition, our finding demonstrates that VAD inhibits the proliferation of the human lung cancer (A-549) cells and human prostate cancer (PC-3) cells in a dose-dependent manner.

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Author Contributions

CGY, SST, and KW conceived, designed, and drafted the manuscript. PBT participated in the implementation of the study, acquisition, analysis and interpretation of data. All the authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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