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Assessment of the Anti-proliferative Effect and Preliminary Analysis of Cell Cycle Arrest and Pro-apoptotic Effects of *Balanites aegyptiaca* (L.) Delile on Colorectal Cancer Cells HCT-116 and HT-29

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Balanites aegyptiaca (L.) Delile (Zygophyllaceae), is used in traditional medicine for the treatment of intestinal worms, wounds, and inflammatory diseases. The purpose of this study is to assess the anti-proliferative effect and to analyse the pro-apoptotic and cell cycle arrest activities of

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B. aegyptiaca root bark extract and fractions against colorectal cancer cells HCT-116 and HT-29. The cytotoxicity of the crude extract and fractions were evaluated by MTS assay. The most active fractionwas subjected to crystal violet assay, Hoechst staining, cell cycle arrest, and annexin V/PI assays on cancer cells to highlight its mechanisms of action. The ethyl acetate fraction demonstrated the most cytotoxic effect on HCT-116 and HT-29 with IC_{50} values ranging between 3 and 4 µg/mL. At 10 µg/mL in the cell cycle arrest assay, the fraction increased G1 phase by 3.83% on HCT-116 and by 8.6% on HT-29 whilst G2/M phase was decreased by 5.63% on HCT-116 and by 6.62% on HT-29. Moreover, apoptotic cells were increased by 11.4% on HCT-116. The results suggest a potential source of anticancer molecules against colorectal cancer for isolation from the ethyl acetate fraction.

Keywords: Medicinal plants; natural products; cytotoxicity; anticancer; cytometry.

1. INTRODUCTION

The World Health Organization (WHO) defines cancer as an uncontrolled growth and spread of cells that can often invade surrounding tissue and can metastasize to distant sites in many parts of the body [1]. Despite the significant decrease of death rates in recent years in some developed countries [2,3], cancer remains the second leading cause of death globally and is estimated to account for 9.6 million deaths in 2018. Also in 2018, 18.1 million new cases were reported worldwide, and the number of new cancer cases per year is expected to rise to 23.6 million by 2030 [1].

According to the WHO, colon cancer occupies the third position among the most common cancers after lung and breast cancers, with 1.80 million cases reported in 2018 [1]. In the United States of America (USA), at least 200 000 new cases of colorectal cancer are reported each year according to the National Cancer Institute [4]. The treatment of this type of cancer depends on its size, location, and progression. Common treatments include tumor removal surgery, chemotherapy, and radiotherapy [5].

Due to the large scale of side effects and chemo-resistance of anticancer drugs, cancer research has focused on natural products, especially on medicinal plants as an alternative to find new natural anticancer molecules with less side effects and less chemo-resistance. Indeed, it is estimated that more than 60% of pharmaceutical anticancer drugs are of natural origin [6,7]. Accordingly, medicinal plants can be considered as an important source for the discovery of new anticancer molecules [6,7]. In Africa, the WHO estimates that more than 80% of the population of developing countries use traditional medicines, especially medicinal plants for their primary health needs. In these countries, the governments have been encouraged by the WHO to promote and integrate traditional medical practices into their national health system [8-10].

(A) (B)

Photo 1. *Balanites aegyptiaca* whole plant (A) and stem with leaves and fruits (B)

Balanites aegyptiaca (L.) Delile (Zygophyllaceae), known as the 'desert date,' is a common plant, widely distributed in dry land areas of Africa and South Asia. The traditional use of this plant includes treatment of intestinal worms, wounds, and inflammatory diseases [11]. Inflammation is implicated in the symptoms of colorectal cancer. Some previous investigations demonstrated interest of *B. aegyptiaca* in colorectal cancer management through antioxidant, analgesic, anti-inflammatory, and cytotoxic properties of extracts or isolated compounds from this plant [8,12-15].

The purpose of this study is to assess the anti-proliferative effects, to analyze by preliminary assays the pro-apoptotic and cell cycle arrest activities of *B. aegyptiaca* extracts against colorectal cancer cells HCT-116 and HT-29.

2. MATERIALS AND METHODS

2.1 Chemicals

The solvents were of HPLC grade from Fisher Scientific (Norcross, GA). Deionized water (18 MΩ-cm) from a Milli-Q water system (Millipore, Milford, MA, USA). McCoy's 5A, Leibovitz's L-15 medium, fetal bovine serum (FBS) from Mediatech, Inc. (Herndon, VA); Penicillin and streptomycin from Sigma Aldrich (St. Louis, MO, USA); an MTS assay kit, CellTiter 96 Aqueous Solution Cell Proliferation Assay from Promega (Madison, WI, USA); A FITC-Annexin V apoptosis detection kit from BD Biosciences (Rockville, MD, USA); PI/RNase staining buffer from BD Biosciences Pharmingen (San Diego, CA, USA); and the cell culture plastic materials were obtained from Falcon Labware (Franklin Lakes, NJ, USA).

2.2 Plant Material

The root bark of *Balanites aegyptiaca* (L.) Delile was collected by Sawadogo WR in August 2018 at Ouagadougou (Burkina Faso) with the following GPS position: N 12°39'43'' and W 1°49'14''. The plant was identified by Professor Amadé Ouedraogo, botanist at the Laboratory of Ecology, University Joseph Ki-Zerbo of Ouagadougou. A voucher specimen has been deposited at the herbarium of the "Centre National de la Recherche Scientifique et Technologique (CNRST)" under the specimen number 6901 and identification number 17262.

2.3 Extraction and Fractionation

An amount of 100 g of *B. aegyptiaca* root bark powder was macerated and stirred in 500 mL of ethanol for 24 hours. After filtration, the extract was evaporated and lyophilized to obtain 10.5 g of dried extract. A quantity of 2 g of this extract was dissolved in 10 mL of DMSO and 90 mL of distilled water were added to obtain final volume of 100 mL which was used for the liquid-liquid fractionation with the following solvents: Petroleum ether (PE), methylene chloride (MC), ethyl acetate (EA), and 1-buthanol (1B). The five fractions were dried by evaporation and lyophilization.

2.4 Cell Culture

HCT-116 and HT-29 cells were obtained from the American Type Tissue Collection (Rockville, MD, USA) and maintained in McCoy's 5A supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C and subcultured every two days [16].

2.5 Cell Proliferation Analysis

HCT-116 and HT-29 cells were seeded in a 96-well plate and incubated at 37°C plus 5% CO_2 for 24 hours. The extract and fractions (dissolved in DMSO) were added to the tested wells at different concentrations and incubated for 24, 48, and 72 hours. The final concentrations of DMSO in tested wells did not exceed 1%. Controls received the same quantity of DMSO without drugs. After 48 h of exposure, the medium was replaced by 100 µL of fresh medium and 20 µL of CellTiter 96 aqueous solution. This solution in the presence of viable cells is bio-reduced by dehydrogenase enzymes in metabolically active cells into a formazan product which can be measured by the amount of absorbance at 490 nm. Accordingly, the quantity of formazan in each well is directly proportional to the number of living cells. After 2 h of incubation, 60 µL of medium from each well was transferred to an ELISA 96-well plate, and the absorbance was measured at 490 nm. The absorbance of the medium without cells was used as blank. All experiments were performed at least in triplicate and the results were expressed as percent of control [16]. The crude extract was tested at concentrations ranging from 10 to 300 µg/mL after 48 hours of exposure in order to verify the level of its cytotoxicity against colorectal cancer

cells. The fractions of this crude extract were tested at 5 and 50 µg/mL after 48 hours of exposure to select the most cytotoxic fractions. After this selection, the antiproliferative effect was performed at 1, 2, 4, 5, 8, 10 µg/mL after 24, 48 and 72 hours of exposure in order to determine the IC_{50} values of these most active fractions.

2.6 Crystal Violet Staining Assay

The crystal violet staining method [17] allows the observation of morphological changes in the vacuoles of treated cells. HCT-116 and HT-29 were seeded in 24-well plates for 24 h and then, treated with different concentrations of the plant fraction for 48 h. The tested concentrations (5, and 10 µg/mL) were chosen according to the IC_{50} values of the cytotoxic fractions. Cells were stained with 0.2% crystal violet in 10% phosphate-buffered formaldehyde. After 2 min of exposure, the cells were washed with PBS and photographed under the microscope. The morphological changes of the vacuoles are the signs of toxicity of the tested sample.

2.7 Preliminary Cell Cycle Analysis

The effect of the plant extract on the cycle cell was analyzed on the G1, S, and G2/M phases as described by Wang et al, 2006 [16] HCT-116 and HT-29 cells were seeded in 24-well plates for 24 hours and treated with the most cytotoxic fraction (ethyl acetate fraction) at 5 and 10 µg/mL for 48 hours. Cells were collected by centrifugation (2000 rpm / 5 min), fixed gently with 100% ethanol at 4°C for 2 hours and then treated with 0.25% Triton X-100 for 5 min in an ice bath. After that, the cells were re-suspended in 300 µl of PBS added by 40 µg/ml propidium iodide (PI), 0.1 mg/ml RNase and incubated in the dark at room temperature for 20 min. Cell cycle analysis was performed using a LSRII flow cytometer 585 nm (BD Biosciences, Franklin Lakes, NJ) and FlowJo 10.1.0 software (Tree Star, Ashland, OR, USA). In order to increase the level of accuracy, at least 20,000 cells were counted for each measurement [17].

2.8 Preliminary Pro-apoptosis Analysis

2.8.1 Hoechst 33258 Staining Assay

The Hoechst 33258 is a fluorescent stain commonly used as preliminary test to analyze the nuclear fragmentation in treated cells. It is excited from 350 to 461nm by ultraviolet light and then emits blue/cyan fluorescent light [7, 16]. Briefly, cells were treated at 5 and 10 µg/mL of the ethyl acetate fraction for 48 hours in a 24-well plate. Then the cells were removed by trypsin, collected by centrifugation (2000 rpm/5 min), and stained for 10 min in the dark with the solution containing 0.01 mg/mL Hoechst 33258, 33 mg/mL formaldehyde, and 5 mg/mL NP-40 in PBS. Cell morphologies were examined and photographed using a fluorescence microscope at 365 nm.

2.8.2 Annexin V/ Propidium Iodide Staining (PI) Assays

Cell apoptosis can be analyzed by flow cytometry after staining with annexin V. Briefly, Cells were seeded in 24-well plate (2×10^5) and treated with the ethyl acetate fraction at 5 and 10 µg/mL for 48 hours. Cells were collected by centrifugation (2000 rpm/5 min) and stained with annexin-V FITC and propidium iodide (PI) [16]. Cells in culture medium without extract were used as a control for double staining. Stained samples were analyzed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and Flow Jo software (Tree Star, Ashland, OR). At least 20,000 cells were counted for each measurement in order to increase the accurate.

2.9 Statistical Analysis

Data are presented as the mean of at least three independent assays with standard deviation (SD). A two-way ANOVA of multiple comparisons from GraphPad Prism 6 was employed to determine whether the results had statistical significance. In some cases the Student's t-test was used for comparing two groups. The difference is statistically significant at $p < 0.05$ [(*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$, and (****) = $p < 0.0001$].

3. RESULTS

3.1 Anti-proliferative Effect

B. aegyptiaca ethanol extract exhibited significant antiproliferative effect in a dose dependent manner (Fig. 1) against colorectal cancer cells HCT-116 and HT-29. When testing the five fractions from the crude ethanol extract, ethyl acetate fraction showed the most cytotoxic effect against both cell lines and its effect is in dose dependent manner (Figs. 2 and 3). Through the crystal violet assay, ethyl acetate fraction induces morphology damages on both cancer cells until cell death (Fig. 4). The crude extract is less toxic against HCT-116 and HT-29 when compared to the ethyl acetate fraction of this extract. The concentration that inhibits 50% of

cancer cell proliferation (IC_{50}) ~~is~~ ranged from 49 to 52 µg/mL for the crude, whilst the IC_{50} value of the fraction is less than 4 µg/mL (Table 1).

3.2 Preliminary Cell Cycle Arrest and Pro-apoptosis Analysis

At 10 µg/mL in the cell cycle arrest assay, the ethyl acetate fraction increased the G1 phase by 3.83% on HCT-116 and by 8.6% on HT-29 whilst the G2/M was decreased by 5.63 % on HCT-116 and by 6.62% on HT-29 (Figs. 5 and 6). No significant change was observed on the S phase. In the Hoechst staining assay, the results revealed that the majority of treated cells have their complete nucleus (Fig. 7). In the Annexin V/PI assay, ethyl acetate fraction increased apoptotic cells by 11.4% on HCT-116 whilst, at the same condition, no significant apoptotic effect on HT-29 (Figs. 8,9) was observed.

4. DISCUSSION

4.1 Anti-proliferative Effect

The results of anti-proliferative effect of *B. aegyptiaca* extract and fractions on colorectal cancer cells using MTS method revealed that the ethyl acetate fraction of this plant is sixteen times more toxic than the crude extract, meaning that this fraction contains the most cytotoxic compounds of this plant and can be used for the isolation of new anticancer molecules. Indeed, the IC_{50} values of the crude ethanol extract and its ethyl acetate fraction on HCT-116 are 52.51 ± 2.25 and 3.91 ± 0.51 respectively while on HT-29 these values are 49.71 ± 1.39 and 3.67 ± 0.41 respectively. Interestingly, some previous studies demonstrated that the extracts of *B. aegyptiaca* can be classified as lowly toxic products. Indeed, the extracts of *B. aegyptiaca* may be safe since its LD_{50} is greater than 3000 mg/kg [18]. Moreover, the consumption of the crude oil of *B. aegyptiaca* seed at 5g/100g body weight do not cause any serious damage to the liver and kidney [19]. Ethyl acetate is a polar aprotic solvent and its fraction contains polar compounds like saponins and polyphenols which are well known to induce anticancer properties through different mechanisms of action including cell cycle arrest and apoptosis [20-25]. Most of the studies on the anticancer properties of *B. aegyptiaca* have been done on its fruit, kernel, and stem bark and have demonstrated its capability to inhibit cancer cell proliferation, including HCT-116, U937, K562, and MCF-7 [26] and to induce cell death by apoptosis [26-29]. Other studies on *B. aegyptiaca* are mostly linked to its anti-diabetic and anti-ulcer effects [18,30-34]. Our current research is focused on the antiproliferative, pro-apoptotic and cell cycle arrest of root bark crude extract and fraction of *B. aegyptiaca*. The results demonstrated the potential of the ethyl acetate fraction of this plant for colorectal cancer management, and moreover the interesting perspectives about the isolation of new anticancer molecules from this fraction. Our results constitute scientific basis for the traditional uses of *B. aegyptiaca* as a healing source of old wounds, inflammation, and infectious diseases.

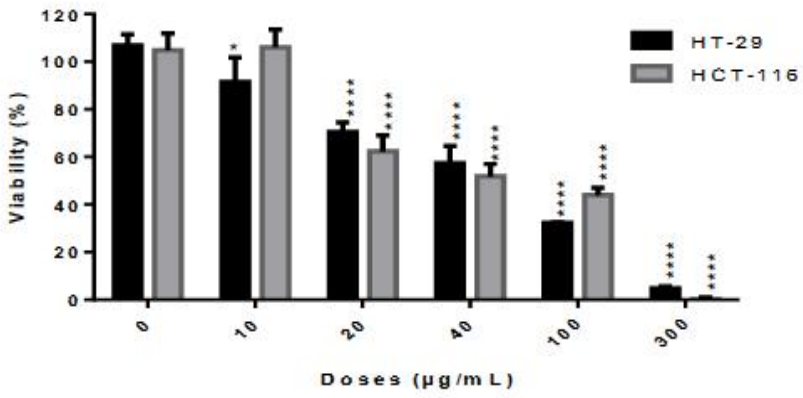

Fig. 1. Antiproliferative effect of *B. aegyptiaca* ethanol extract on HCT-116 and HT-29 at concentrations ranging from 10 to 300 µg/mL after 48 hours of exposure. The results are the mean ± SD of three independent assays. The level of statistical significance was set at p < 0.05. In each figure, one star (*) corresponds to the *p* < 0.05, 2 stars (**) for *p* < 0.01, 3 stars (***) for *p* < 0.001, and 4 stars (****) for *p* < 0.0001

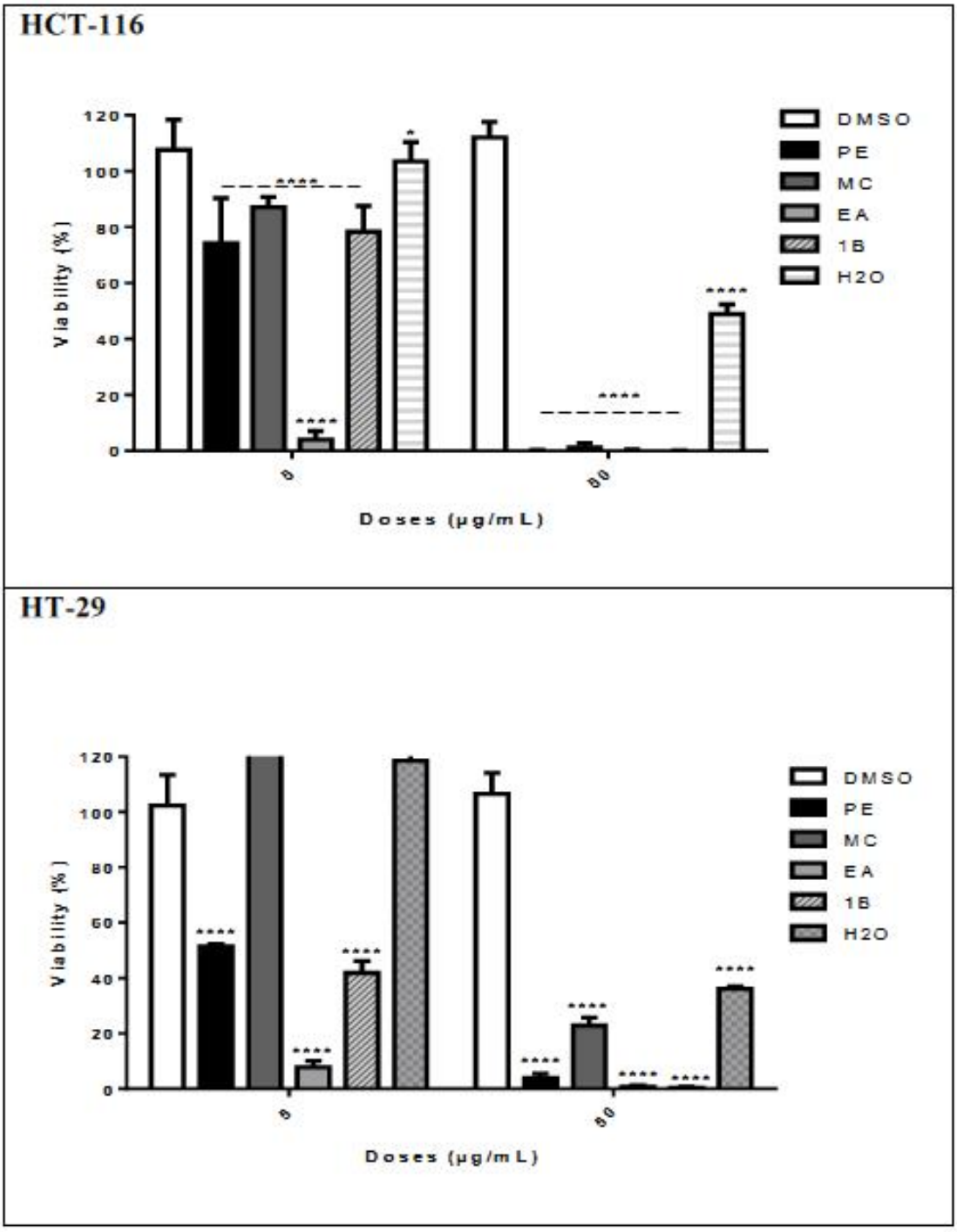

Fig. 2. Antiproliferative effect of five fractions from *B. aegyptiaca* ethanol extract on HCT-116 and HT-29 at 5 and 50 µg/mL after 48 hours of exposure. PE=Petroleum ether, MC= Methylene choride, EA= Ethyl acetate, 1B= 1-Butanol, H2O= Water, DMSO=Dimethylsulfoxyde. The results are the mean ± SD of three independent assays. The level of statistical significance was set at p < 0.05. In each figure, one star (*) corresponds to the *p* < 0.05, 2 stars (**) for *p* < 0.01, 3 stars (***) for *p* < 0.001, and 4 stars (****) for *p* < 0.0001

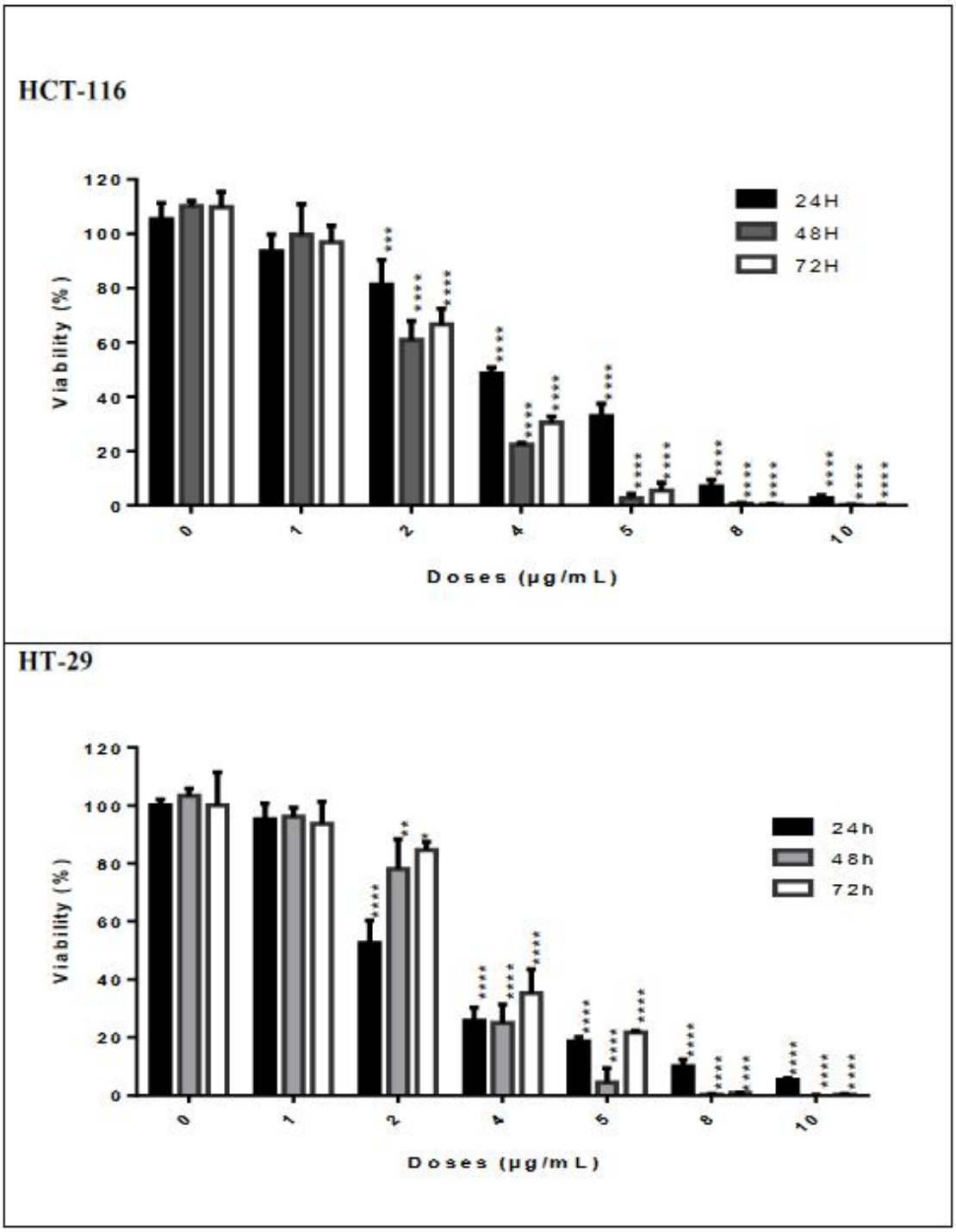

Fig. 3. Antiproliferative effect of Ethyl acetate fraction from *B. aegyptiaca* ethanol extract on HCT-116 and HT-29 at 1, 2, 4, 5, 8 and 10 µg/mL after 24, 48, and 72 hours of exposure. The results are the mean ± SD of three independent assays. The level of statistical significance was set at $p < 0.05$. In each figure, one star (*) corresponds to the $p < 0.05$, 2 stars (**) for $p < 0.01$, 3 stars (***) for $p < 0.001$, and 4 stars (****) for $p < 0.0001$

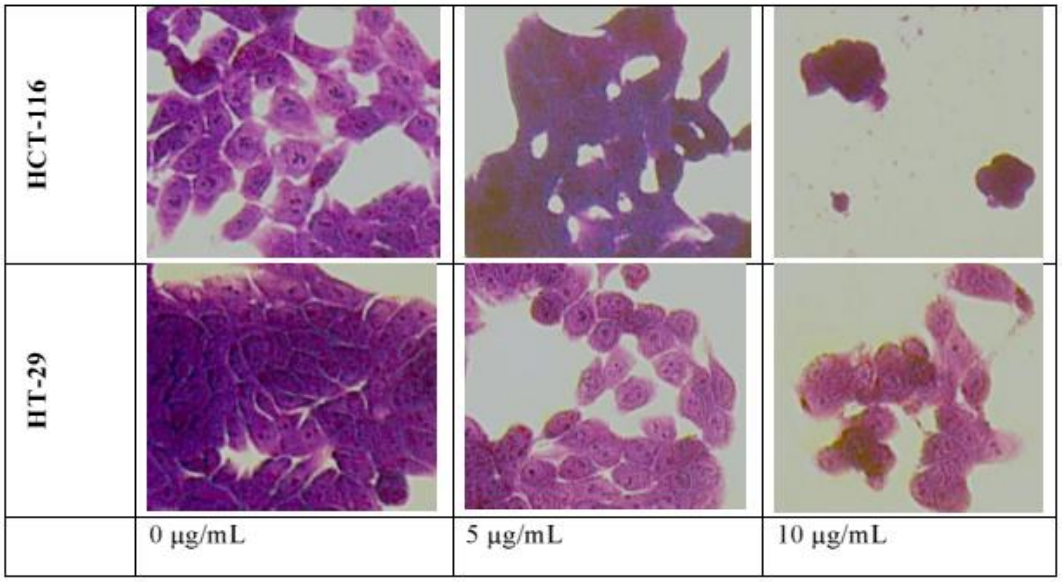

Fig. 4. HCT-116 and HT-29 cells treated with the ethyl acetate fraction of *B. aegyptiaca* at 5 and 10 µg/mL for 48 hours. The cells were stained by crystal violet, and pictures were made using Olympus IX70 microscope (X20). Morphological damages are signs of cytotoxicity of the fractions

Table 1. IC_{50} values of ethanol extract and ethyl acetate fraction of *B aegyptiaca* on HCT-116 and HT-29 at 48H

Samples	IC_{50} (µg/mL)	
	HCT-116	HT-29
EtOH Extract	52.51 ± 2.25	49.71 ± 1.39
Ethyl acetate fraction	3.91 ± 0.51	3.67 ± 0.41

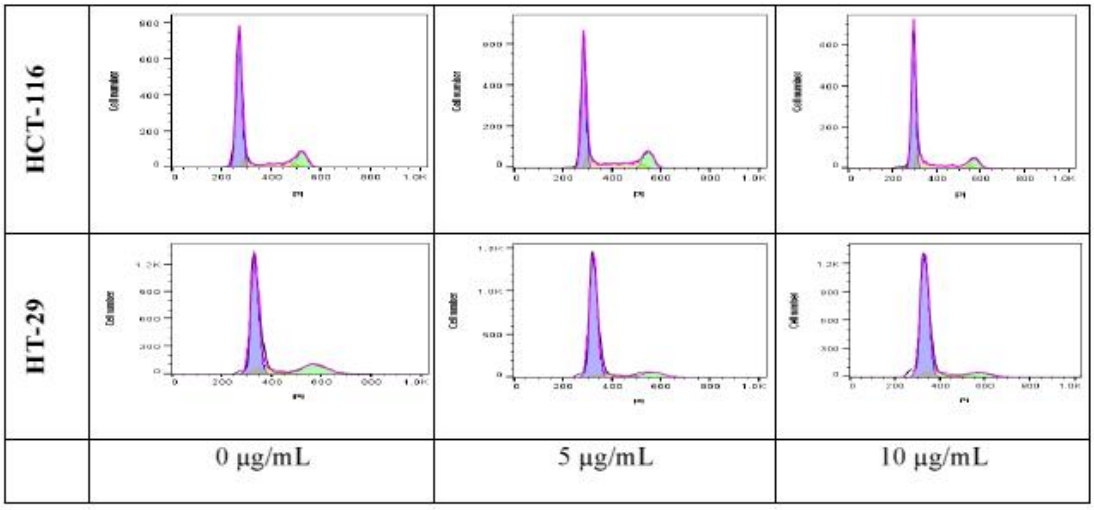

Fig. 5. Cell cycle analysis by cytometry of HCT-116 and HT-29 cells treated with *B. aegyptiaca* ethyl acetate fraction at 5 and 10 µg/mL for 48 hours. G1 phase is represented by the first pic in blue color, S phase is the middle one in yellow and G2/M the last pic in green color.

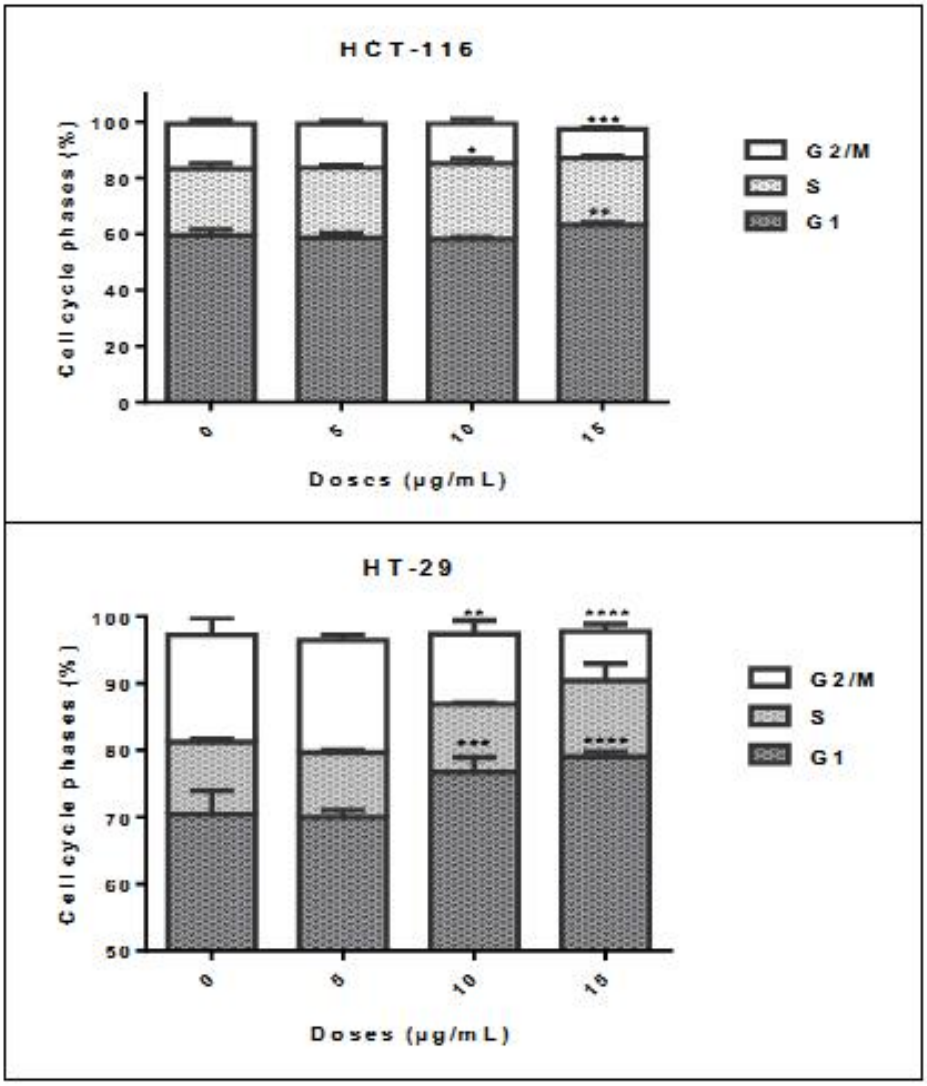

Fig. 6. Quantitative analysis of cell cycle phases S, G1, and G2/M in HCT-116 and HT-29 cells treated with B. aegyptiaca ethyl acetate fraction at 5 and 10 µg/mL for 48 hours. The results are the mean ± SD of three independent analysis. The level of statistical significance was set at $p < 0.05$. In each figure, one star (*) corresponds to the $p < 0.05$, 2 stars (**) for $p < 0.01$, 3 stars (***) for $p < 0.001$, and 4 stars (****) for $p < 0.0001$

4.2 Cell Cycle Arrest and Apoptosis Preliminary Analysis

Our study demonstrated the higher cytotoxicity of the ethyl acetate fraction on HCT-116 and HT-29 with the IC_{50} values less than 4 µg/mL. It is well known that apoptotic process and cell proliferation are linked by cell-cycle regulators, and that apoptotic stimuli affect both processes. Indeed some cell-cycle genes including p53, RB, and E2F are mostly involved in the balance between apoptosis and proliferation, which is strictly necessary to sustain tissue homeostasis [35]. In this study, the antiproliferative effect of the plant fraction does not work only through apoptotic or cell cycle arrest pathways. Indeed, the percentage of apoptosis when using cytometry assay is very low compared to the cytotoxicity demonstrated in the MTS assay. Indeed, at 10 µg/mL, the ethyl acetate fraction induces more than 90 % of cell death while at the same dose, the percentage of apoptotic cells is about 11%. This difference should be due to the fact that many cells are damaged and their nuclei

disintegrated due to the high level of cytotoxicity of the plant fraction, therefore these cells can no longer be observed by Hoechst staining. Indeed, we observed with the Hoechst method that at the higher concentration (10 µg/mL), many cells are damaged whilst their nuclei are still in normal morphology. It is well known that some apoptotic cells rapidly lose the integrity of their plasma membrane and then turn to a status of secondary necrosis. These results are confirmed by the Annexin VP/I assay where the fraction did not induce significant apoptosis against HT-29. Moreover, in the cell cycle analysis, the fraction has no significant effect on the different phase of the cell cycle at tested concentrations (5-10 µg/mL). All these results show that the mechanism of action of this fraction against HCT-116 and HT-29 mostly follows other pathways than apoptosis or cell cycle arrest processes. Our future research will clarify this pathway and will focus on the isolation of cytotoxic molecules of the ethyl acetate fraction of *B. aegyptiaca*.

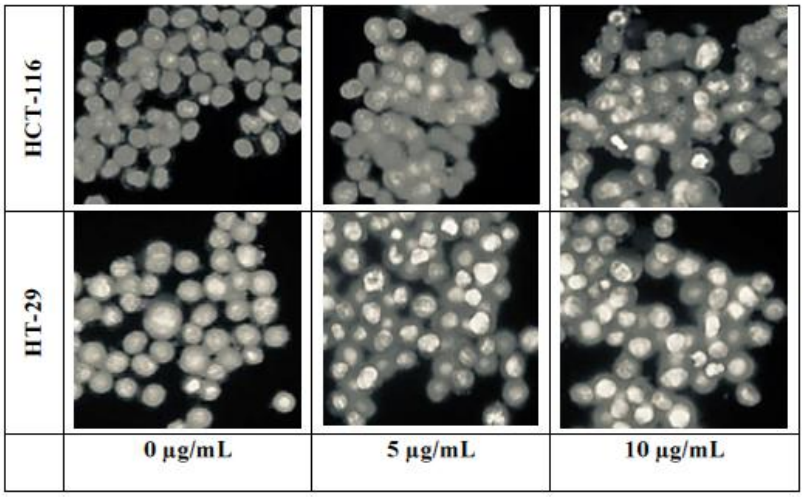

Fig. 7. HCT-116 and HT-29 cells treated with *B. aegyptiaca* ethyl acetate fraction at 5 and 10 µg/mL for 48 hours. The cells were stained by Hoechst, and pictures were made using an Olympus IX70 microscope (X20). Nucleus fragmentation is the sign of apoptosis.

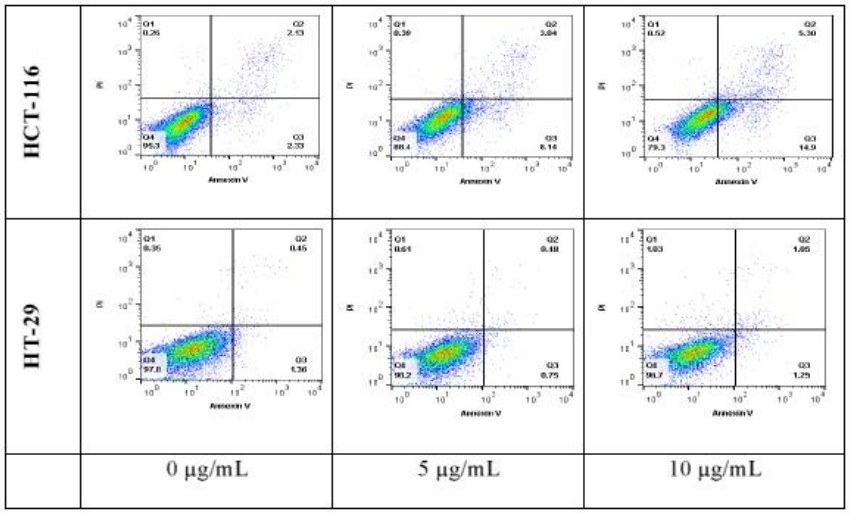

Fig. 8. Apoptosis analysis by cytometry of HCT-116 and HT-29 cells treated with *B. aegyptiaca* ethyl acetate fraction at 48 hours. The apoptotic cells are observed in the right side of the square, including early apoptosis at the bottom, and late apoptosis in the upper, whilst the living cells are at the bottom left side of the square and necrosis in the upper.

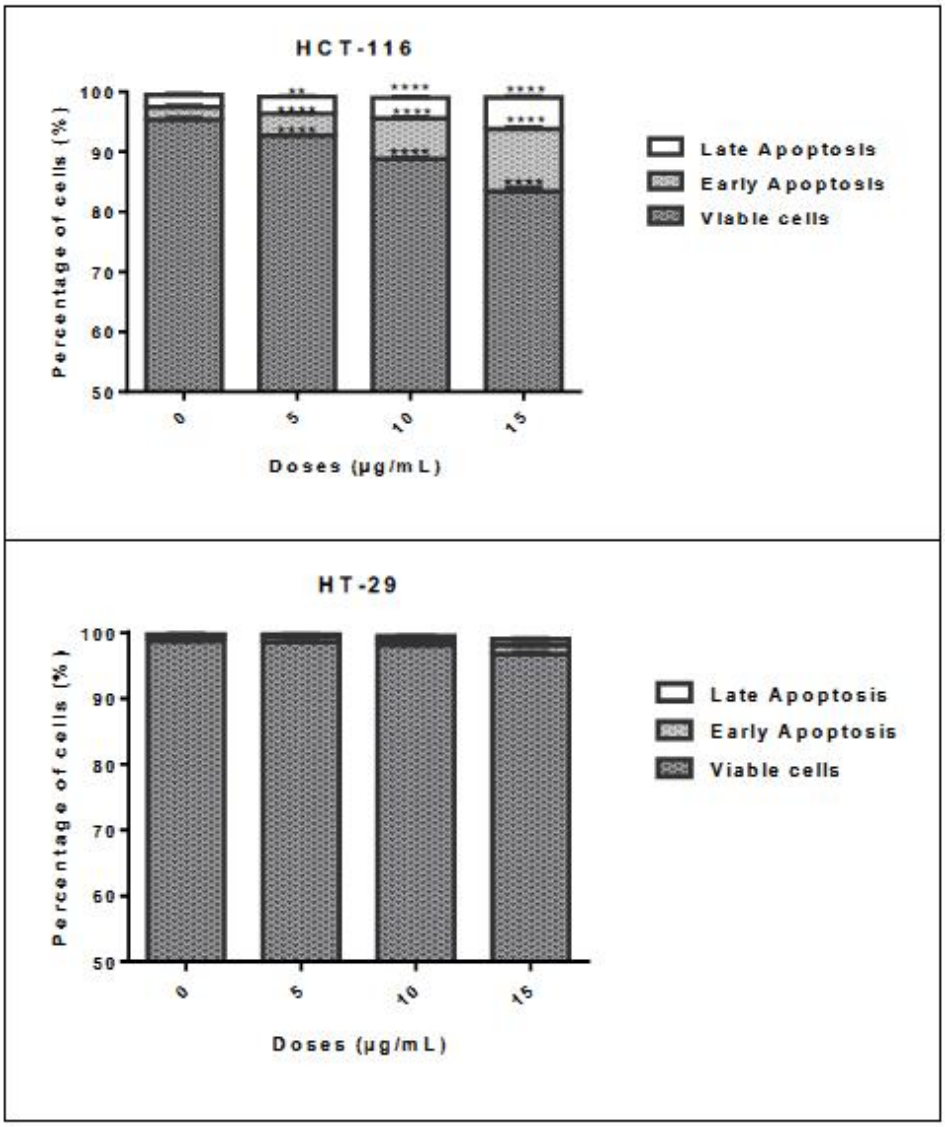

Fig. 9. Quantitative analysis of early and late apoptosis in HCT-116 and HT-29 treated with *B. aegyptiaca* ethyl acetate fraction at 5 and 10 µg/mL for 48 hours. The results are the mean ± SD of three independent analysis. The level of statistical significance was set at $p < 0.05$. In each figure, one star (*) corresponds to the $p < 0.05$, 2 stars (**) for $p < 0.01$, 3 stars (***) for $p < 0.001$, and 4 stars (****) for $p < 0.0001$

5. CONCLUSION

Balanites aegyptiaca exerted important cytotoxicity against colorectal cancer cells HCT-116 and HT-29 with moderate effect on cell cycle arrest and apoptosis according to our preliminary analysis. The results of this study constitute a scientific basis for the uses of *B. aegyptiaca* in traditional medicine and for the isolation of natural anticancer molecules against colorectal cancer.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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