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BALANITES AEGYPTIACA PROTECTION AGAINST PROLIFERATION OF DIFFERENT CANCER CELL LINE

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Abstract

Background: The aim of the present study was to evaluate *in vivo* anti-tumor activity of *Balanites aegyptiaca* fruits extracts in addition to its role in cell cycle and apoptosis.
Materials and Methods: Antitumor activities of ethylacetae extract (EAE), ethanol extract (EE) and chloroform extract (CE) were tested against different cell line Hep-2, MCF-7, HL-60 and HCV29T. Calculation of the IC50 values for these extracts confirmed that the most potent plant extract was EAE (40 ug/ml) followed by EE (55ug/ml), CE (61ug/ml)**.**
Results: The ethanolic and chloroform extracts showed lower difference in their potency, while the EAE was found to be more active indicating for nonpolar active principles responsible for the anti-proliferative activity. The ethanolic extract was three times higher active than the chloroform extract. The results obtained showed that EAE exert a significant anti-proliferative, enhancement of apoptosis and modulation of cell cycle phases compared with vincristine.
Conclusion: The presence of alkaloids, flavonoids and Phenolics increase its efficiency as anti-proliferative action. EAE has promising anti-cancer activity with higher activity than EE or CE extracts

Key words: antiproliferative, apoptosis, *Balanites aegyptiaca*

Introduction

Cancer is considered as one of the leading causes of death with increase mortality in human. The treatment protocol of cancer includes chemotherapy, immunotherapy, radiotherapy, and surgery in advanced grades and stages. However, the net therapeutic outcomes are still with high side effects or adverse effects (Mohan et al.,2011).Therefore, nowadays researches for cancer treatment are being developed with high efficacy with lowest side effects. Recently, plant-derived compounds have more attention due to their anti-cancer activities and their ability to improve the body's defense (Montgomery and, Dymock, 2001).

In traditional medicine, there has been a long history of herbal use to prevent and treat diseases, including diabetes, colic, diaheria, cancers, by enhancing the body's immune-defense (Paulo et al., 2006). The cell cycle phases include a G1phase in which the cell grows; an S , synthesis of DNA, a second gap phase called G2 and an M phase (for Mitosis) in which the cell divides into 2 daughter cells (Perumal etal.,2010).

Apoptosis (programmed cell death) is a way by which the cell can get rid of damaged cells from itself to prevent cancer. It is different from another process of death called necrosis, where damaged tissue dies for various reasons. The biochemical events responsible for apoptosis can be linked to the activation in cells of family of cycteine protease known as caspases, which cleave specific target protein in cells at aspartic acid residues (Peter etal., 2006).

Balanites aegyptiaca is an herbal medicine widely distributed in African, South Asia and the Middle East. The fruits are edible and used, in folk medicine, as an oral hypoglycemic agent. An aqueous extract of the fruit was used in the treatment of jaundice (Putul etal.,2000). Kernel extracts showed to be lethal to the miracidia and cercariae of Shistosoma mansoni and to Fasciola gigantica (Raushanara et al., 2010) and antiseptic (Ravid and Korean ,2003). The fruit mesocarp contains a large variety of chemicals amongst which are glycosides, coumarins, flavonoids, 6-methyldiosgenin and saponins (Raju and Mehta, 2005). The saponins are a structurally and biologically diverse class of glycosides of both steroids and triterpenes that are widely distributed in terrestrial plants and in some marine organisms (Ramos-Vara, 2005). In *Balanites aegyptiaca* plant diosgenyl saponins are the most abundant. The aim of the present study was to evaluate *in vivo* anti-tumor activity of *Balanites aegyptiaca* fruits extracts, in addition to its role in cell cycle and apoptosis.

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Materials and Methods

Extraction of Balanites aegyptiaca

Balanites aegyptiaca fruits were purchased from the local markets in Upper Egypt and were characterized by the stuff members of Botany Department, Faculty of Science, KAU, Saudi Arabia. The fruits were washed, kernels were removed and the fruits were suspended in ethylacatate (EAE) or ethanol (EE) or chloroform (CE) (100g/1000 ml) for 24 hours. The resulting extract centrifuged for 10 mins at 8000 *x* g then deprotienated with isopropanol (1:1) and concentrated by rotary evaporator at 4^0C. All the reagents used during this study were of high analytical grade.

Culture Cell lines

Hep-2 (larynx carcinoma), MCF7 (breast adenocarcinoma), (HL-60) human leukemia and (HCV29T) bladder cancer cell lines were kindly provided by Dr. Ehab Mostafa (Department Biochemistry (Tanta University) with 10% fetal bovine serum , 20 µg/ml L-glutamine, and 30 µg/ml Gentamicin. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C.

Counting Cells and Viability

Total number of cells / ml and viability (%) were calculated in 100 µl of cells suspension mixed with 100 µl of vital dye (trypan blue). A drop of mixture was transferred as hemocytometer and observed under the microscope. The living cells did not stain with the dye, while the dead cell stained with blue color. The viable cells and dead cells were counted in WBCs squares of hemocytometer and the results were calculated [Rathee et al.,2009].

Assessment of Anti-Proliferative Activity

The anti-proliferative potency of plant *Balanites aegyptiaca* extracts was measured using MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide) assay. The assay depends on the reduction of MTT by mitochondrial dehydrogenase to form blue formazan product, which a good index of normal mitochondria function and cell viability (Reich and Schibli, 2005).

After 24 h incubation, a mono layer of cell was removed and 300 µl of the medium containing different concentrations of either EAE or EE or CE (25, 50, 75 and 100 µg/ml). In addition, vincristine sulphate (10 ug) (Sigma-Aldrich, St Louis, MO) was used as positive control. The cultures were incubated for 48 hrs, and then 100 µl of the medium were added to 20 µl MTT solutions. Reduced MTT was assayed at 550 nm. Untreated cells were used as a negative control while, cells treated with 100nM vincristine sulfate were used as a positive control. Extracts that cause 50% inhibition of proliferation were selected for further investigations.

Assessments of Cell Cycle and Apoptosis in Cell Culture

The cell lines in experiment 1 were exposed to 75 ug/ml of EAE for 72 h at 37°C in an atmosphere containing 5% CO2. The distribution of cells among the different phases of the cell cycle and apoptosis rate were evaluated by flow cytometric analysis of the DNA content. Cells were collected, washed twice with PBS, fixed by ethanol 70%, and kept at _20°C for at least 4 h. Propidium iodide (10 ug/ml) in PBS containing 100 U/ml DNase-free RNase was added to the cells for 15 min at room temperature. Cells were acquired by a FACS flow cytometer, and then analysis was performed using Software House, Inc (Ruby et al., 1995).

Analysis of Caspase-8 and-9 Activity in Cell Line

The cells were washed twice with 2 ml PSA and subsequently treated with lysis buffer for 10 min on ice bath to lyse cells (25µl of lysis buffer / $1x10^6$cells). The cell lysate was centrifuged at 10.000 xg for 5 min. The enzymatic reaction for caspase activity was carried out using ELISA kit from Biomedica, England.

Statistical Analysis

All values were expressed as the mean ± SD. Data were evaluated by using SPSS for windows. The one way ANOVA test was used to examine whether there are any significant differences between the different groups, the value of $P < 0.05$ was considered significant.

Results

In the present study, the cytotoxic effect of different extracts from *Balanites aegyptiaca* on different cell line cells was characterized by conducting cell viability assay. Cultures of cells line were treated with different extracts concentration from 0 to 100 ug /ml.

The most potent extracts with anti-proliferative activity were EAE compared with EE or CE. Fig 1-4 showed the cytotoxic effect of different extracts against different cell line, with maximum effect at 60 ug EAE compared with vincristine as control. Calculation of the IC50 values for these extracts confirmed that the most potent plant extract was EAE (40 ug/ml) followed by EE

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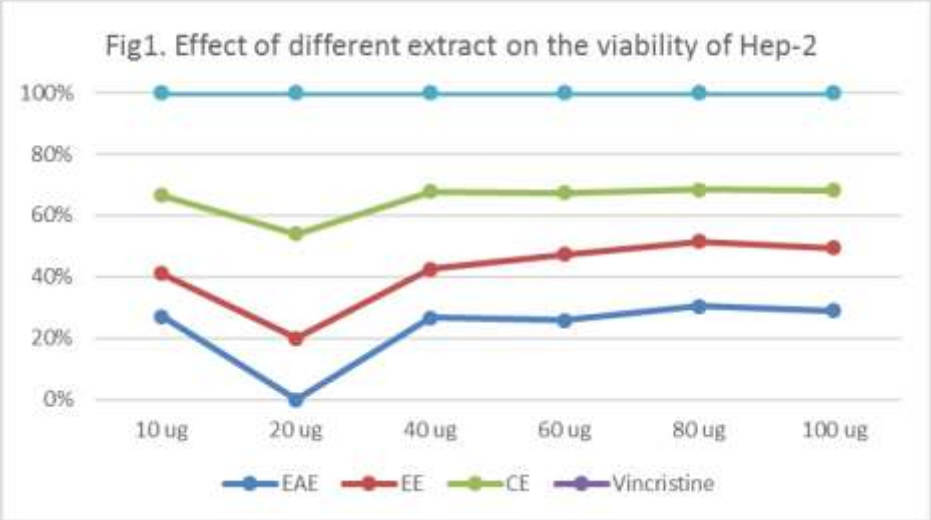
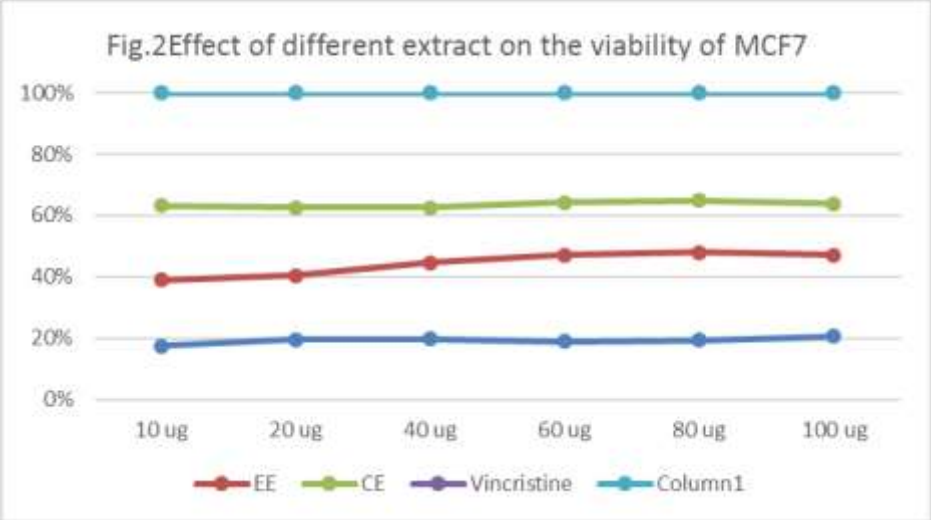
(55ug/ml), CE (61ug/ml**).** The ethanolic and chloroform extracts showed lower difference in their potency, while the chloroform extract of was found to be more active than the ethanolic extract indicating for non-polar active principles responsible for the anti-proliferative activity. The ethanolic extract was three times higher active than the chloroform extract.

MTT assays revealed that treatment with *Balanites aegyptiaca* extracts affected the viability of different cell lines. We used doses of 0–100 ug/ml different extracts (EAE or EE or CE) for 24 h. We observed >90% reduction in viability of all cell line used with the highest tested concentration (60 ug of EAE).

The results of our study show that *Balanites aegyptiaca* extracts has a cytotoxic effect on Hep-2, MCF7, HL-60 and HCV29T in a concentration dependent manner but the extract showed better therapeutic value against MCF7 cell lines with IC50 = 60 µg/ml. Flow cytometric analysis of cell cycle showed that addition of EAE elevate the Go/S phases compared with untreated cell line (fig 5, 6).

By checking apoptosis through measuring the caspases- 8 and 9 activities (table 1), it was found that, addition of EAE to the medium enhance the enzymes activity compared with EE or CE but still lower than vincristine ($p<0.001$)





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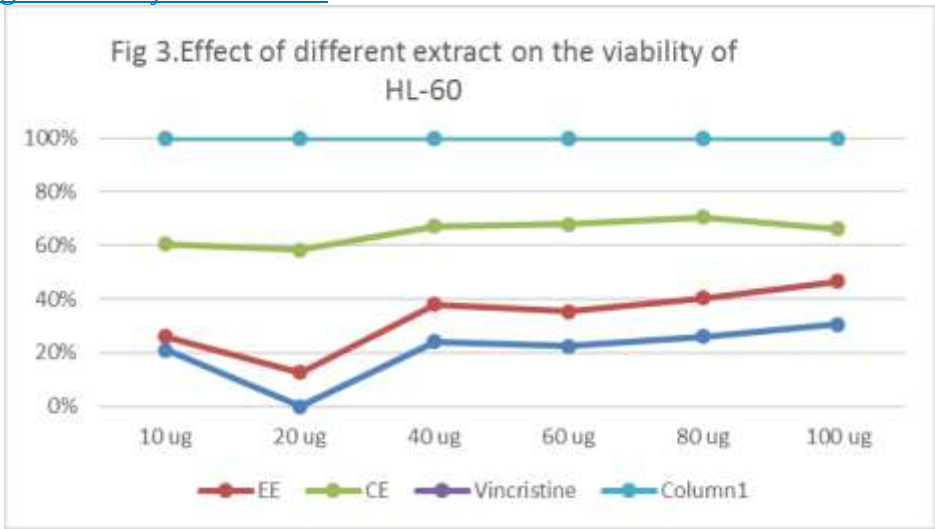

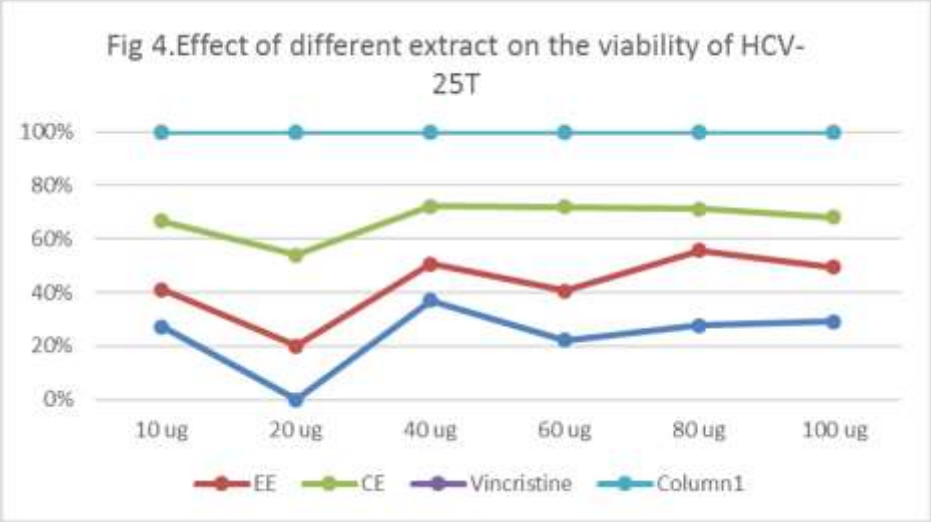

Flow Cytometric Analysis

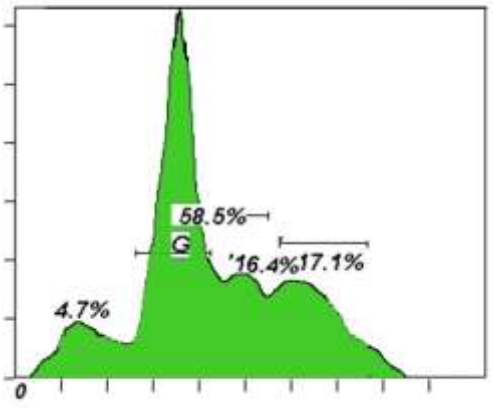

Figure 5: Flow cytometric analysis of cell cycle untreated Hep2. The results showed that, preG1 was 4.7%, G0/G1 phase was 58.5%. S phase was 16.4% and G2/M was 17.1%

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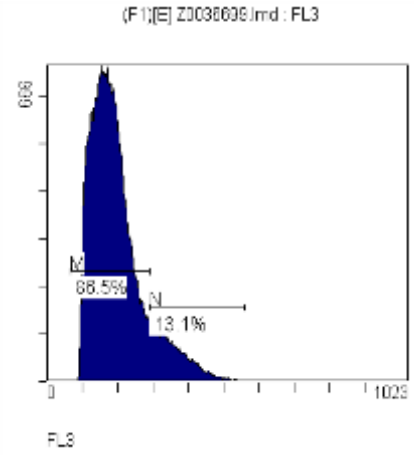

Figure 6: Cell cycle phases after one day of treatment of Hep2 with 60 ug EAE.
The results showed that, preG1 was 86.5%, G0/G1 phase was 13.1%. Delay cell- cycle progression and cell cycle arrest at the S phase and G2/M phase.

Table 1: The activities of caspase-8 and caspase-9 in Hep2 cell line treated with *Balanites aegyptiaca* or vincristine (Mean ±SD).

Parameter	Untreated	EAE (60 ug)	EE (60 ug)	CE (60 ug)	Vincristine (10 ug)
Caspase-8 (U/ml extract) Mean ±SD	0.11±0.03	0.65±0.06^{a,b,c}	0.49±0.06 ^{a,b,c}	0.33±0.04 ^{a,b,c}	0.93±0.08 ^{a,b,c}
Caspase9 (U/ml extract) Mean ±SD	0.17±0.05	0.55±0.07 ^{a,b,c}	0.48±0.06 ^{a,b,c}	0.56±0.04 ^{a,b,c}	0. 96±0.08^{a,b,c}

a, b,c represent P<0.05 was significant *a*, value treated versus untreated, *b*; Vincristine versus extracts.
c,EAE versus EE and CE.

Discussion

Cancer is the second leading cause of death after cardiovascular diseases (Said and Hanafy, 2006). The use of complementary and alternative medicine, represented mainly by plants, ranges between 30-75% (Sajadi et al., 2007) .This in turn justifies the interest in search of possible anticancer agents from the flora of different countries. In accordance with this worldwide trend, *Balanites aegyptiaca* fruit contain s aponins which balanitoside belongs to are a large family of steroid like substances or triterpenoid aglycone. Saponins have a broad pharmacological effect as anti-inflammatory, antibacterial and anti-viral activity (Salzman etal., 2009). It was reported that it has highly cytotoxic properties as potential anti-carcinogenesis (Samia and Fatma, 2009). It also had anti-mutagenic activity (Ayoola et al.2008).It was found that saponins from *Balanites aegyptiaca* have potent anti-cancer activity against MCF-7 human breast cancer cells and HT-29 human colon cancer cells.

The results obtained showed that the increase in viability and cell count in different cell line tested treated with different extracts as EAE, EE or CE was found that EAE is more potent as anti-proliferative than EE or CE.

This is in agreement with Gnoula et al. (2008) who stated that a mixture of saponins isolated from *Balanites aegyptiaca* significantly increased the survival of mice bearing leukemia by 30% over the survival time of mice bearing leukemia, without treatment.

Elie et al. (2010) stated that treatments of HT-29 human colon cancer cells with 0.5,3, and 5 mMol saponin obtained from *Balanites aegyptiaca* for 24h generated caspase-3 cleavage, thereby inducing apoptosis activation. In current study, in Hep-2 the caspase-8 and 9 activities were significantly elevated following treatments with EAE, EE or CE comopared with 10ug vincristine as positive control. Therefore, EAE induces apoptosis through caspase activation in Hep-2. It is notable that anti-proliferative effects of these extracts are mediated through cell cycle arrest as indicated with flow cytomeric analysis, the activation of caspases (apoptosis), which may be the same mechanism probably works for balanitoside. This is in agreement with Arindam et al (2003) who reported that apoptosis is characterized by cell shrinkage, membrane blebbing, DNA fragmentation and apoptosis.

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We concluded that organic solvent extracts (EAE, EE or CE) have a potential cytotoxicity activity on Hep-2, MCF-7, HCV29T and HL-60 cell lines but the effect was more significant on Hep-2 cell lines. EAE has promising anti-cancer activity with higher activity than EE or CE extracts.

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