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# Anticancer Activity of *Balanitis aegyptiaca* Extract on Human Hepatoma Cells and Prostate Cell Line Culture

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**Abstract :** Cancer is a public health problem all over the world. Medicinal plants have been on the forefront whenever we talk about anticancer remedies, Herbal medicines have a vital role in the prevention and treatment of cancer. Large number of plants and their isolated constituents has been shown to potential anticancer activity. To establish an efficient protocol for cell suspension culture and growth of *Balanites aegyptiaca*, the effects of different plant growth regulators 2,4-D (2,4-Dicholorophenoxy acetic acid )and NAA (Naphthaline acetic acid ) at 1,2,3,4 and 5 mg/l on callus induction of three explants parts and cell suspension culture of *B. aegyptiaca* were evaluated in Tissue Culture Lab during years 2012-2014. The maximum percentage of callus induction (91.39%) and highly percentage of callus were obtained in MS medium supplemented with 3 mg/L 2,4-D and leaves explants (88.92 %), respectively. Elicitation process was obtained by applying $AgNO_3$ at 25, 50 and 75 µM, jasmonic acid at 15, 50 and 100 µM and tryptophan at 50,100 and 200 ppm to cell suspensions. The addition of 25 µM silver nitrate ($AgNO_3$) to the medium was effective in increasing amount of cell suspension dry weight (21.08 µg) for stem cell suspension. In cell suspension cultures, MS medium supplemented with 200 mg/l tryptophan gave maximum cell dry weight during two cell cycle 46.4 and 49.5 µg, respectively. Jasmonic acid was more effective than $AgNO_3$ and tryptophan for packed cell volume (PCV) and electrical conductivity (EC) of *B. aegyptiaca* cell suspension cultures. The diosginin yield of *B. aegyptiaca* cell suspension at 12 days after culture by spectrophotometric and HPLC were 0.760 mg/g and 0.801 mg/g dry weight, respectively which was significantly higher than that obtained from callus (0.69 mg/g dry weights).Methanol extracts of *B. aegyptiaca* cell suspension showed *in vitro* cytotoxicity against two different human cancer cell lines such as liver (Hep-G2), and prostate (PC-3). The 3-4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromides (MTT) viability was done using various doses 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml of the extract compared with methanol (control). Against hepatic carcinoma (Hep-G2) cell line plant extract at 12.5 showed 97.43 % growth of viability decreasing to 34.89 % for 100 ug. Where as in case of PC-3 cell line treated with plant extract at 3.125 ug showed maximum activity 98.47 decreasing to 26.74 % for 100%.
**Key words:** *B. aegyptiaca,* Cell suspension, Hepatic carcinoma, Prostat Carcinoma, Diosgenin, Callus.

## Introduction

Cancer is a complex multifactorial cell disease characterized by abnormal cellular proliferation. Cancer development and progression are dependent on the cellular accumulation of various genetic and epigenetic events[1,2], and is an aberrant net accumulation of typical cells arising from excess proliferation, insufficient

apoptosis, or a combination thereof[3]. Cancer development is normally caused by oncogene, tumor suppressor gene, and microRNA gene alterations[4]**.** It imposes a serious burden on the public health system, and its treatment and cure are scientifically challenging. In 2015, cancer is expected to claim nine million lives worldwide[5].

In 2006, prostate cancer is predicted to be the third leading cause of cancer-related deaths among men in the USA[6]**.** Traditionally, chemotherapy and radiotherapy have not proven to provide significant survival benefits to patients with advanced prostate cancer and most treatment options available are only palliative.

Hepatocellular carcinoma (HCC) is one of the malignancies with increasing incidence. Though there have been several curative methods for the disease, but the survival solely depends on the tumor location and the underlying liver disease, cirrhosis. There has been urgent need for the treatment of HCC to prevent its occurrence or its reoccurrence. Herbal compounds are known to play a major impact in all the stages of HCC. Therefore, there has been an increase in the research for the use of plant derived compounds as potential anticancer agents against HCC for a novel drug development[7,8].

*Balanites aegyptiaca* (L.) Del. Family: Balanitaceae is widely distributed along the tropical belt of Africa[9]. It is a small spinescent evergreen savanna tree, growing up to 4.5 -6 m with dark brown stem[10]. All the parts of that plant are traditionally used in several folk medicines. In Egypt, the fruits are used as oral hypoglycemic drug[11]**.** The oil is used in Nigeria against skin disease and rheumatism[12]**.** The stem bark is reputed for treatment of tape worm infection, stomach trouble, amoebic dysentery and less frequently for malaria in Sudan[10]**.** In Burkina Faso, the galls of that plant are used to treat the cattle brucellosis[13], sickle cell disease, whooping cough and mental disorders[14] while leaves are used to treat wounds, deramtosis, bilharziosis, cold and rheumatism.

The use of plant cell and tissue culture methodology as a means of producing medicinal metabolites has a long history[15,16]. Since plant cell and tissue culture emerged as a discipline within plant biology, researchers have endeavored to utilize plant cell biosynthetic capabilities for obtaining useful products and for studying the metabolism[17,16]**.** In recent decades, interest in chemo-preventive plant natural products has grown rapidly. The etiology of several degenerative and aging-related diseases has been attributed to oxidative stress, and numerous studies have been undertaken to search for the most effective antioxidants[18,19,20]**.**

Callus culture can be done from different vegetative organs such as the leaf, root, node, stem, petiole, shoot tip, embryo and flower bud. Young vegetative organs are more effective for callus induction. According to[21] explant source is one of the most important parameters for successful long- term cell culture. Also Callogenesis was explants type dependent[22].

Recent studies on diosginin derivatives alone and in combination with other chemotherapeutic agents have demonstrated some limited benefit, but a need for more effective and less toxic means to target and/or prevent this disease clearly exists. Natural products have long proven to be a bountiful resource for identification of bioactive compounds used in the treatment of a variety of ailments and diseases, including cancer[23]. The diosginin derivatives currently being used for the treatment of hormone-independent prostate cancer are but one example among many of the importance of this resource.

However, systematic characterization of natural product and herbal therapies and identification of their mechanism(s) of action are crucial for the development of safe and efficacious therapies for prostate cancer prevention and treatment[24]**.**

## Material and Methods

### a. Plant material

The seeds were collected from Aswan Botanical Garden and cultured on MS after disinfected. The shootlets were taken from *in vitro* grown cultures on MS medium[25] supplemented with 0.5 mg/l benzyl amino purine (BAP) under 25ºC ±2 under florescent lamps with light intensity of 3000 lux at 16 hrs photoperiods.

**b. Callus induction**

For callus induction, the three types of explants (micronodes, leaves disc and root disc) of *B. aegypteaca* were transferred to MS medium supplemented with different concentrations of 2,4-D and NAA (1,2,3,4 and 5 mg/l) for each. The cultures were maintained in the dark at 20 °C for 6 weeks. The percentage of callus induction and callus fresh weights were recorded 6 weeks after culture.

***c.* Cell suspension**

Cell suspension culture was initiated by transferring friable callus obtained for various source (leaves, stem, root) (2g) to a 250 ml Erlenmeyer flask containing 50 mL of liquid MS medium supplemented with 3 mg/L 2,4-D and 3 mg/l NAA. The cells were separated by Tissue Sieving System®, 200 mesh of sieve was used to separate single cells in glass bottle collector. The suspension cultures were incubated on a rotary shaker at 120 rpm and 25 ± 2 °C under 16-h photoperiods with a light intensity of 900–1200 lx. The suspension cultures were established after 2 subcultures. Then the grown cells were transferred to 250-mL flasks containing 50 ml of liquid MS medium supplemented with 3 mg/L 2,4-D and 3 mg/l NAA and were subcultured every 6 days with an initial cell density of approximately 25% of PCV. The experimental design was a factorial based on completely randomized design with 3 replications.

**d. Elicitation treatments**

The suspension cells were cultured on MS medium containing 3 mg/l 2, 4-D and 3 mg/l NAA with different additives of elicitors for various types of explants.

For elicitation, two gram of callus and five ml of suspension were put in all flasks with treatments of jasmonic acid at 10, 50 and 100 µM, $AgNO_3$ at 15, 25 and 50 µM and tryptophan at 50, 100 and 200 ppm, respectively. Three replicates and fifteen explants were incubation for one month.

e. **Growth measurement**

Cell growth parameters such as packed cell volume (PCV), fresh cell weight (FW) and dry cell weight (DW) were determined at the stationary phase of growth (at the end of the experiment). The packed cell volume (PCV) was determined by centrifuging 10 mL of the suspension culture in a 15-mL graduated centrifuge tube at 2000 rpm for 5 min.

**f. Saponin extraction for determination of diosgenin**

One gram of callus powder of each treatment and control extracted three times by 30 ml methanol with agitation overnight on shaker, followed by centrifugation. At the end, all supernatants of methanol extracts were pooled and the methanol was evaporated using a rotary evaporator. Finally, a yellowish crystal powder of crude saponins was obtained[26]**.**

**g. Spectrophotometric of diosgenin**

Diosgenin was determined as described by[27,28], with some modification. Standard sapogenin (diosgenin and *p*-anisaldehyde (4-methoxybenzaldehyde) were purchased from Sigma. Sulfuric acid and ethyl acetate were both with analytical grade and were obtained from Frutarom. The diosgenin level was determined by measuring absorbance at 430 nm based on the color reaction with anisaldehyde, sulfuric acid and ethyl acetate. In brief, two color developing reagent solutions were prepared: (A) 0.5 ml *p*- anisaldehyde and 99.5 ml ethyl acetate. 200 ug of the methanol extract of callus was placed in a glass tube. To this, 1 mg of the methanol extract of callus powder or drying cell suspensions were first dissolved in 1 ml methanol, and 200 ul of this solution was placed in another tube; the methanol was evaporated under rs dissolved in 2 ml educed pressure. This residue was dissolved in 2 ml of ethyl acetate; 1 ml each of reagent A and B were added to the tube and stirred. The absorbance of the color developed solution was measured in a spectrophotometer (V-530- Uv/VIS, JASCO Corp., and Japan). Ethyl acetate was used as a control for the measurement of absorbance. As a reagent blank, 2 ml ethyl acetate was placed in a tube and assayed in similar manner. For tha calibration curve, 2 – 40 ug standard diosgenin in 2 ml ethyl acetate was used. Each sample was repeated three and the average was taken.

**h. HPLC detection of diosgenin**

Diosgenin was purchased from Total Herb Solution Pvt. Ltd., Mumbai (India). Divya Madhunashini Vati (Divya Pharmacy, Haridwar) were procured from the local market. Methanol, acetonitrile and water of HPLC grade were purchased from Qualigens, Mumbai, India. All the other solvents and reagents used were of analytical grade.

**Preparation of standard solution**

10 mg of diosgenin was weighed and dissolved in 5 ml methanol by means of sonicated in UI Trasons-H J.P. selecta, S.A. apparatus for 15 min. The solution was diluted up to 10 ml with methanol (1 mg/ml). Pipette out 1 ml solution from stock solution and diluted up to 10 ml with methanol (100 µg/ml).

**Chromatographic conditions**

Chromatographic estimation was performed using an HEWLEH PACKARD *Hp* 1047A -C18 column in Central Analysis Lab. (particle size 5 µm; 150mm X 4.6 mm ID), a mobile phase consisting of acetonitrile: water: in the ratio of 90:10 v/v at flow rate of 1 ml/min with IR detection at 203 nm.

**i. Anti-cancer activity**

**Cell culture studies a. Prostate:** The androgen-sensitive human prostate cancer cell line PC-3, was obtained from the Al-Azhar University, The Regional Center for Mycology & Biotechnology and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (Gibco® Invitrogen Corp.). Erythromycin was included in the medium at a concentration of 100 mg/l (Sigma). The cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO2 according to **Alley *et al.* (1998).**

**b. Liver:** Human cancer cell lines namely liver (hep-2) were grown in RPMI-1640 with 2 mM L-glutamine medium pH 7.2. Penicillin was dissolved in PBS and sterilized by filtering through 0.2µ filter in laminar air flow hood. The media was stored in refrigerator (2-8oC). Complete growth medium contains 10 % FCS. The medium for cryopreservation contains 20 % FCS and 10 % DMSO in growth

***Balanites aegyptiaca* extract preparation:** *Balanites aegyptiaca* suspension cells extract was supplied in powdered form successive culture treated with elicitors and has highest amount of digosinine ($AgNO_3$ at 25 uM). A calculated amount ranged for (0.5 – 1.0 g) of extract was used for all studies here, and the digosinine of this extract was determined by[27,28]**.** For testing in *in vitro* studies, the extract was completely dissolved in deionized distilled water and filtered with a 0.2-µm membrane for sterility. Control cells for all experiments were treated with deionized distilled water filtered for sterility.

**Cell growth assays:** Cells were seeded in 96-well plates at a density of 5000 cells/well in a final volume of 100 µl. Twenty-four hours after seeding, the growth medium was replaced with fresh medium containing the vehicle control (sterile $dH_20$) or increasing concentrations of *B. aegyptiaca* from 3.13 to 100 µg/ml. Eight wells were prepared for each *B. aegyptiaca* concentration and time-point (24- h). The WST-1 cell proliferation assay (Roche Diagnostics, Indianapolis, IN) was conducted as described by the manufacturer. This assay measures overall mitochondrial dehydrogenase activity in a cell population which correlates to the number of metabolically active cells in the culture[29].

**Flow cytometric analysis of cell cycle and cell death:** Following exposure to *B. aegyptiaca* extract (0, 3.125, 6.25, 12.5, 25, 50 or 00 µg/ ml) for 24 h, cells were collected, washed in PBS and fixed in a 2:1 (vol/ vol) solution of chilled ethanol overnight before staining with propidium iodide (PI) in the presence of 100 µg/ml RNAse (DNAse-free). Cell cycle distribution was analyzed for 10000 cells per condition on a Becton-Dickinson flow cytometer (San Jose, CA) and data analysis was performed using 'CellQuest PRO' analysis software. The percentage of total cells partitioning out in Sub-G0 (sub-genomic DNA) fraction was considered the dead cell fraction[29].

## Results And Desiccation

### 1. Callus induction

Callus initiation occurred from the cut ends of the explants during 4 weeks of culture in most of the leaf explants. The induction of callus from leaves, roots and stem were different on different concentrations of hormone (Table 1). The concentration of 3 mg/l 2,4-D and NAA was the first to induce production after 4 weeks with 91.39 % and 89.11 %, respectively. The MS media with 3 mg/l 2,4 –D alone had the highest callus production 80.87 % after 4 weeks from roots. This value decreased to the minimum level 31.40 %. These treatments reported that the best part of explants produce callus was leaves from quantity and quality as shown the next explanation.

**Table (1). Effect of 2,4-D , NAA and explants type on callus formation percentage of *Balanites aegyptiaca***

| Treatments \ source | Root | Leaves | Stem | Mean |
|---|---|---|---|---|
| **Control** | 75.88 | 89.95 | 99.70 | 88.51 |
| **2,4 – D 1 mg/l** | 70.57 | 100.0 | 89.76 | 86.78 |
| **2,4 – D 2 mg/l** | 64.04 | 34.48 | 100.0 | 66.17 |
| **2,4 – D 3 mg/l** | 80.87 | 96.40 | 96.90 | 91.39 |
| **2,4 – D 4 mg/l** | 55.94 | 90.30 | 99.80 | 82.01 |
| **2,4 – D 5 mg/l** | 64.22 | 100.0 | 93.10 | 85.77 |
| **NAA 1mg/l** | 33.30 | 100.0 | 49.28 | 60.86 |
| **NAA 2mg/l** | 31.40 | 85.25 | 25.28 | 47.31 |
| **NAA 3mg/l** | 68.72 | 98.62 | 100.0 | 89.11 |
| **NAA 4mg/l** | 66.34 | 94.22 | 100.0 | 86.85 |
| **NAA 5 mg/l** | 33.33 | 22.22 | 11.11 | 22.22 |
| **Mean** | **60.83** | **88.92** | **85.38** | |
| **LSD5% type of explants** | **1.9810** | | | |
| **Hormone** | **2.0921** | | | |
| **Interaction** | **0.9107** | | | |


**Fig. 1. From right to left callus elicitors treatments $AgNO_3$ , Jasmonic acid and tryptophan**

### 2. Effect of elicitation treatment callus growth

According to the data in Table (2) using additives of $AgNO_3$ to MS medium at 50 uM increase leaves dry weight to 14.72 ug from 0.64 g of fresh weight compared with MS without any additives (control) which decreased dry weight to 3.47 ug from 0.98 g fresh weight. On the other hand, on roots callus there is a significant increasing in fresh weight for all treatments except with jasmonic acid 100 uM and tryptophan at 100 mg/l (0.85 ug and 0.81 ug, respectively) while, 1.59, 1.28, 1.36, 1.32, 1.23, 1.02, 1.09 ug for control $AgNO_3$ at (25, 50 and 100 uM), Jasmonic acid at 15 and 50 uM, respectively have non-significant effect. The callus obtained from bark when exposed to $AgNO_3$ at 25 uM gave the highest significant effect of dry weight 21.08 ug produced from 0.89 g fresh weight.

On water contents a little amount of water was recorded for leaves control 55.53 % while all treatments have water contents ranged between 95.98 % and 99.94%.

**Table (2). Effect of some elicitors and explants type on fresh material, dry material and water contents of *Balanites aegyptiaca* cell suspension**

| Source / weight / Treatments | Leaves | | | Root | | | Stem | | |
|---|---|---|---|---|---|---|---|---|---|
| | **F.W. gm** | **D.W. ug** | **M.C %** | **F.W. gm** | **D.W. ug** | **M.C %** | **F.W. gm** | **D.W. ug** | **M.C %** |
| **Control** | 0.98 | 3.47 c | | 0.9 abc | 1.59 a | | 0.99 b | 15.63 ab | 98.43 ab |
| **$AgNO_3$ 25µM** | 0.71 | 10.42 | 55.53 | 1.00 a | 1.28 ab | 99.82 c | 0.89 b | 21.08 a | 97.64 c |
| **$AgNO_3$ 50 µM** | 0.64 | abc | 98.43 | 1.03 a | 1.36 ab | 99.87 abc | 1.01 b | 20.95 a | 97.94 bc |
| **$AgNO_3$ 75 µM** | 1.55 | 14.72 a | 97.62 | 0.90 abc | 1.32 ab | 99.87 abc | 1.00 b | 13.03 b | 98.69 a |
| **Jas. acid 15 µM** | 0.64 | 5.97 abc | 95.98 | 1.00 a | 1.23 ab | 99.85 bc | 0.93 b | 11.86 b | 98.74 a |
| **Jas. Acid 50 µM** | 1.33 | 5,68 bc | 99.15 | 1.00 a | 1.02 abc | 99.88 abc | 0.49 c | 16.31 ab | 96.68 d |
| **Jas. acid 100 µM** | 1.31 | 7.61 abc | 99.22 | 0.85 bc | 1.09 abc | 99.90 abc | 1.24 a | 13.55 b | 98.85 a |
| **Tryp. 50$mgl^{-1}$** | 1.81 | 6.87 abc | 99.48 | 0.96 ab | 0.84 bc | 99.87 abc | 0.97 b | 13.79 b | 98.57 ab |
| **Tryp. 100 $mgl^{-1}$** | 1.66 | 11.74 | 98.74 | 0.98 ab | 0.88 bc | 99.91 ab | 1.07 ab | 15.70 ab | 98.54 ab |
| **Tryp. 200 $mgl^{-1}$** | 1.66 | abc | 98.13 | 0.81 c | 0.51 c | 99.91 ab | 1.03 b | 15.43 ab | 98.52 ab |
| | | 8.34 abc | 99.01 | | | 99.94 a | | | |
| | | 14.00 ab | | | | | | | |
| **LSD5%** | **NS** | **8.851** | **NS** | **0.1435** | **0.6065** | **0.07671** | **0.1799** | **6.07** | **0.6883** |

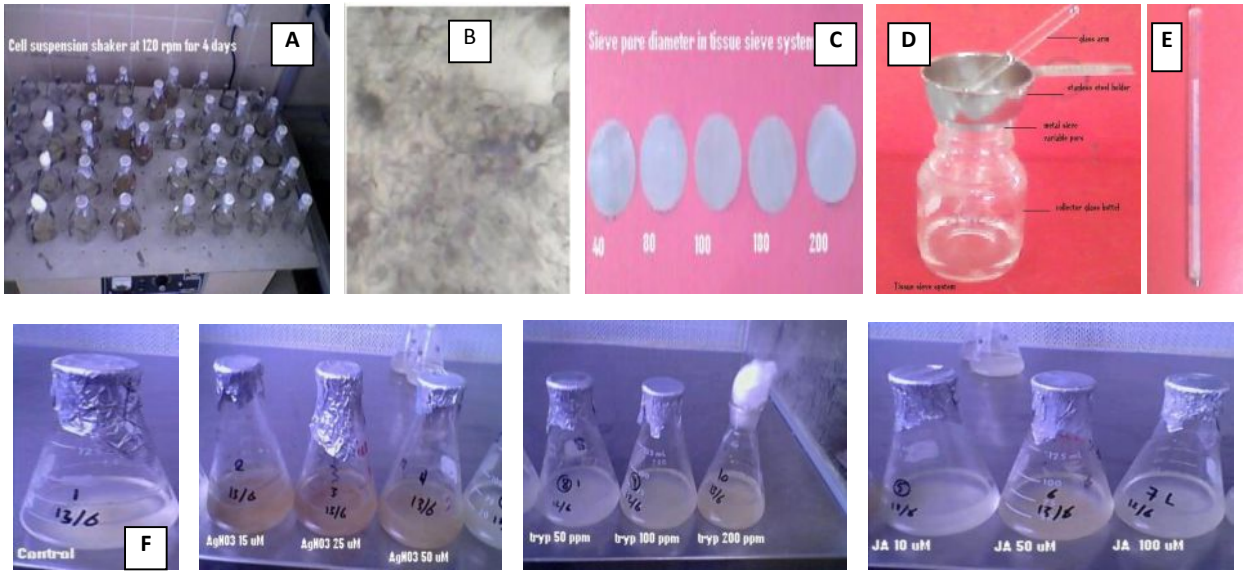


**Fig. 2. (A) Cell suspension system (B) cell aggregates (C) Various sieve pores for cell separation (D) Cell separator system (E) Wintrope tube for PCV determination (f) Cell suspension treatments in flasks.**

### 3. Growth cycle

Among the elicitors, 50 mg/l tryptophan showed a good stimulatory effect on cell growth with a cell dry weight 46.40 during the first cycle of culture and scored 49.50 ug after the second period, which were significantly higher than those of hormone-free MS medium (control) and media containing $AgNO_3$ and Jasmonic acid at various concentrations. Therefore, it could be concluded that tryptophan was more effective than $AgNO_3$ and Jasmonic acid for growth of *Balanites aegyptiaca* cell suspension cultures. Growth of *Balanites aegyptiaca* cell suspension cultures also varied significantly depending on the subcultures (Table 3). The maximum cell density PCV (35.40%) for cells treated with jasmonic acid 50 um and electrical conductivity

(EC) was increasing to the maximum value 4.19 were recorded for MS medium supplemented with 15 uM jasmonic acid.

**Table (3). Effect of some elicitors on fresh material, dry material, packed cell volume (PCV) and electrical conductivity (EC) of *Balanites aegyptiaca* leaves cell suspension**

| Source | Suspension I | | Suspension II | | | |
|---|---|---|---|---|---|---|
| | F.W. | D.W. ug | F.W. | D.W. ug | PCV | EC |
| **Control** | 0.41 c | 2.23 b | 0.62 c | 3.37 b | 20.00 bc | 2.40 b |
| **$AgNO_3$ 25µM** | 0.89 ab | 3.97 b | 0.81 bc | 4.07 b | 1.17 d | 2.38 b |
| **$AgNO_3$ 50 µM** | 0.96 ab | 8.83 b | 1.00 ab | 9.13 b | 18.33bc | 2.68 b |
| **$AgNO_3$ 75 µM** | 0.83 b | 2.13 b | 0.97 abc | 2.30 b | 13.27 cd | 3.19 ab |
| **Jas. acid 15 µM** | 1.04 ab | 31.50 ab | 1.29 a | 32.27 ab | 18.73 bc | 4.19 a |
| **Jas. Acid 50 µM** | 1.06 ab | 14.43 ab | 1.10 ab | 14.33 b | 35.40 a | 3.03 ab |
| **Jas. acid 100 µM** | 0.97 ab | 10.00 b | 1.00 ab | 12.67 b | 27.37 ab | 2.79 ab |
| **Tryp. 50$mgl^{-1}$** | 1.09 a | 46.40 a | 0.96 abc | 49.50 a | 12.17 cd | 2.78 ab |
| **Tryp. 100 $mgl^{-1}$** | 0.97 ab | 14.10 b | 0.99 ab | 20.37 ab | 6.60 cd | 2.46 b |
| **Tryp. 200 $mgl^{-1}$** | 0.99 ab | 6.77 b | 1.00 ab | 7.80 b | 7.60 cd | 3.08 ab |
| **LSD5%** | **0.2486** | **32.03** | **0.3598** | **31.63** | **14.01** | **1.497** |

## 4. Diosgenin contents

The extracted diosginin from cell suspension cultures was detected using the HPLC method with a C-18 column. Figure 3a-b shows the chemical structure of diosginin and HPLC spectrum of diosginin in samples obtained from cell suspension cultures.

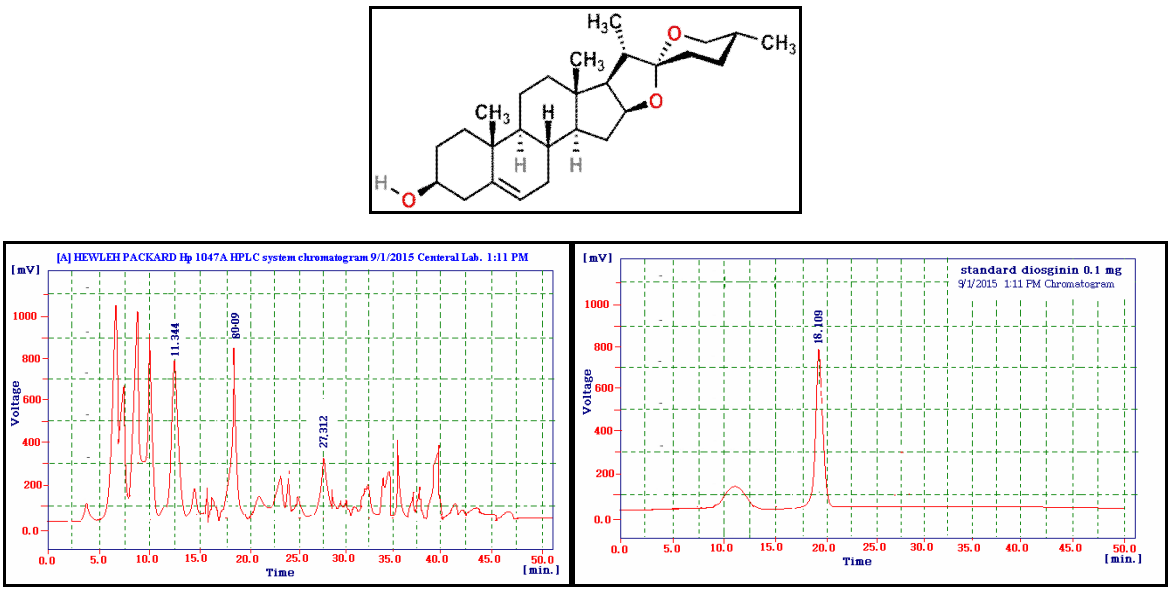


**Fig. 3. (B) HPLC spectrum of standard** diosginin **(A)** diosginin **purified from B. aegyptiaca cell suspension culture**

Diosgenin content of the cell culture samples of *B. aegyptiaca* was analyzed by spectrophotometry and HPLC method Table (4). The analysis results were presented in Table 4. The diosgenin content determined by spectrophotometry was 0.760 mg/g, while diosgenin content determined by HPLC was 18.09 mg/g. The diosgenin yield of *B. aegyptiaca* cell suspension on day 12 after culture was 0.760 mg/g dry weight for suspension and 0.691 mg/g for callus treated with $AgNO_3$ 15 µM which was significantly higher than that without treatment (control) (0.014 mg/g for suspension and 0.016 mg/g for callus of dry weight. The diosgenin content of *B. aegyptiaca* cell suspension culture reported here was higher than that previously reported by[30] who found that Diosgenin content of the cell culture samples of *D zingiberensis* was analyzed by the validated

microplate-spectrophotometry and HPLC method. The diosgenin content determined by microplate-spectrophotometry was 0.157 ± 0.006 mg/g, while diosgenin content determined by HPLC was 0.151 ± 0.002 mg/g. Similarly, Cell culture of *D. zingiberensis* has been regarded as an alternative means for efficient and controllable production of diosgenin[31]. [32,33]explain the role of chemical stresses including elicitation, varying plant growth regulators, and mineral nutrient concentrations can enhance the secondary metabolite production in plants or plant cell cultures[16,34,35]. Furthermore, secondary metabolite production can also be influenced by environmental factors such as light and temperature[36,37]**.** In conclusion, Plant cell culture is the most useful and convenient experimental system for examining effects of various factors on the biosynthesis of desired products and for exploring effective measures to enhance their production. In order to screen appropriate elicitors or high-yield cell strains, it is very important to determine diosgenin content of the cell culture samples of *B. aegyptiaca* quantitatively and rapidly. we here describe a reliable protocol for callus induction from various explants and fast growing cell suspension cultures of *B. aegyptiaca* MS medium supplemented with $AgNO_3$ at 25, 50 and 75 uM showed the maximum cell growth and proliferation, and therefore can be used to elicit the production of disogenine or other secondary metabolites, and to scale-up for mass production in bioreactors.

**Table (4). Effect of some elicitors on diosginin of *Balanites aegyptiaca cell* suspension and callus culture**

| Source | diosginin µg/100 g f.w. after 12 days | | |
|---|---|---|---|
| | Suspension mg/g (spectrophotometric) | Suspension HPLC mg/g | Callus |
| **Control** | 0.014 d | -- | 0.016 d |
| **$AgNO_3$ 25µM** | 0.760 a | 0.801 | 0.691 a |
| **$AgNO_3$ 50 µM** | 0.510 ab | -- | 0.413 ab |
| **$AgNO_3$ 75 µM** | 0.440 ab | -- | 0.314 ab |
| **Jas. acid 15 µM** | 0.085 c | -- | 0.076 c |
| **Jas. Acid 50 µM** | 0.073 c | -- | 0.043 c |
| **Jas. acid 100 µM** | 0.082 c | -- | 0.091 c |
| **Tryp. 50mgl$^{-1}$** | 0.101 bc | -- | 0.067 c |
| **Tryp. 100 mgl$^{-1}$** | 0.091 c | -- | 0.012 c |
| **Tryp. 200 mgl$^{-1}$** | 0.102 bc | -- | 0.081 c |
| **LSD5%** | **0.4321** | | **0.3910** |

## 5. Inhibitory effect of diosgenin on Hep-G2 and PC-3 cells activity

MTT reduction is usually performed to study mitochondrial/non mitochondrial dehydrogenase activity as a cytotoxic test for a variety of chemical compounds. The effect of the methanolic extract of *Balanitis aegyptiaca* dry cells extract on the growth of Hep-G2 cells were assessed by the MTT assay. Methanolic extract in a concentration range of 3.125 ug to 100 ug showed a dose responsive curve after 24hrs of treatment. The results are shown in Table (5) and Figure 4. The cell suspensions treated with $AgNO_3$ 25µM induced diosgenin amounts to the maximum quantity which used in biological assay.

**Table (5). Effect of methanolic extracts of cell suspension of *Balanities aegyptiaca* on cell viability of Hepatocellular carcinoma cells and prostate carcinoma cells by MTT methods**

| Source | MTT Cell viability % | |
|---|---|---|
| **Sample concentrations** | ***Hepatocellular carcinoma cells*** | ***prostate carcinoma cells*** |
| 100 | 34.89 | 26.74 |
| 50 | 78.67 | 39.85 |
| 25 | 90.26 | 68.71 |
| 12.5 | 97.43 | 82.16 |
| 6.25 | 100 | 90.89 |
| 3.125 | 100 | 98.47 |
| 0 | 100 | 100 |
| **$IC_{50}$** | **82.7 µg** | **41.2 µg** |

The IC50 value was calculated as 0.1mg. Further, when 0.1mg of the plant extract was treated to the cells in the presence of the standard chemotherapeutic drug, etoposide (mg), the percent viability was further decreased when compared to the drug alone as seen in Figure 4.

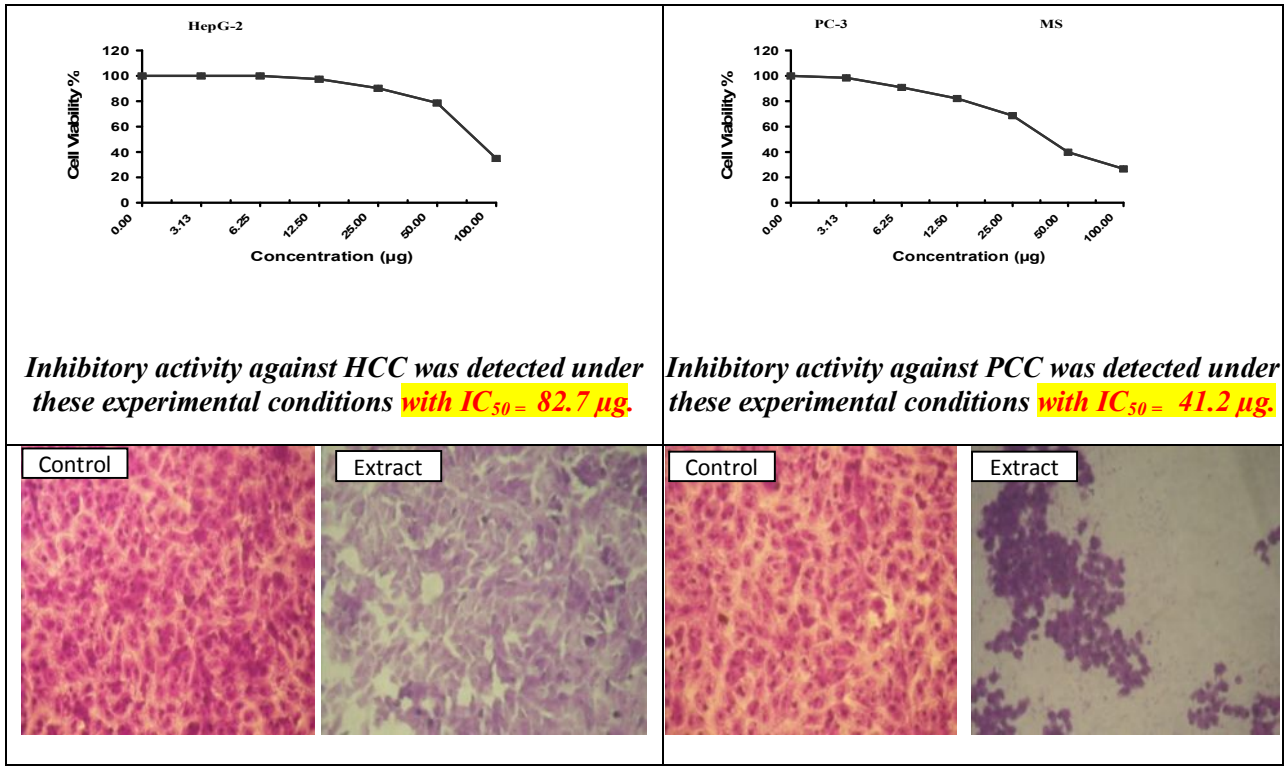


**Fig. 4. Inhibitory activity against Hep-G2 and PC-3 down: control and extract for cell treated and untreated of Hep-G2 and PC-3**

**Morphological observation**

Light microscopic observation of the *Balanitis aegyptiaca* methanolic extract-treated HepG2 and PCC cell line after 24 hours of exposure showed typical morphological features of apoptosis. Characteristic apoptotic features like cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs were observed[38]. The apoptotic cell count was noted in the presence or absence of the plant extract. The plant extract induces apoptosis in the cancer cells as shown in Table 5 when stained with giemsa. Our study describes the potential use of *Balanitis aegyptiaca* as a source of anti-cancer drug. The methanolic extract of the cell suspension was tested for its cytotoxicity and apoptotic properties against the hepatocellular carcinoma (Hep-G2) cells and prostatic cellular carcinoma cells (PC-3) *in vitro*. A dose ranging from 0 mg ml-$^{1}$ to 100 mg mL-$^{1}$ was effective in inducing cytotoxicity in the cancer cells. IC50 value was calculated to be 82.7 for Hep-G2 and 41.2 µg/l for PC-3 which was used in further validation assays. Studies on the methanolic extract of the leaves of *Sansevieria roxburghiana* and *Costus malortieanus* showed potent cytotoxicity against Hep-G2 liver cancer cell line in a dose dependant manner[39,40]. The key feature of a potential antitumor drug accepted widely is the apoptosis-induction capacity rather than the necrosis induction. Cancer cells treated with any drug show morphological features of apoptosis that includes cell shrinkage, membrane blebbing, nuclear condensation and apoptotic body formation[41]. Results showed that the morphological and nuclear changes were typical of apoptosis indicating the anticancer activities of *Artemisia vulgaris*. Similarly, detailed analysis through MTT and morphological observation with the help of fluorescence microscopy and inverted microscopy, confirmed that the aqueous extract of *Moringa olifera* [42] as well as *Cassia occidentalis* [43] to have cytotoxic effects on HeLa cells. Methanolic extract of *Oroxylum indicum* also showed to possess good cytotoxicity against Dalton's lymphoma cells[44]. Analysis of the methanolic extract of *Buddleja asiatica* and compounds isolated from it showed significant cytotoxic activity against HepG2 cell line as determined by the SRB assay[45]**.** Among various methanolic extracts of plants screened for cytotoxicity against HepG2 cells by the MTT method, extracts of *Melaleuca leucadendron* L. and *Callistemon rigidus* R.Br were considered to be most promising[46]. Methanolic extract of *Grewia hirsute* possessed significant antioxidant and anti-proliferative potential when tested against

HepG2 cell lines[47]. Also, the methanolic extract of the dried fruit extract of *Nycanthes arbour-tristis* showed potent anticancer activity when tested against MDA-MB-231 breast cancer cell lines[48] whereas methanol fraction of *Rubia cordifolia* extract showed considerable inhibition on the growth of the Hep2 and HeLa cells[49]. *Philodendron selloum* and *Terminalia bellerica* plant methanol extracts treated HepG2 showed hepatocyte denegenration, decrease in the number of cancer cells and necrotic debris as observed in light microscope[50]. Ethanolic extract of *Piper sarmentosum* was shown to trigger cell death in HepG2 through apoptosis, through morphological analysis using Giemsa and AO/EtBr staining procedures[51] which is on par with the results obtained in our study. Thus, our results show that the extract of *B.aegyptiaca* leaves cell suspension treated $AgNO_3$ with possess good potential for use as cancer chemotherapeutic agent, as well as a supportive therapy during etoposide treatment, to augment its effect.

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