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Bio-production enhancement of Natural diosgenin from *Balanites Aegyptiacae* by callus culture

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Abstract

Describes the effect of plant growth regulators (PGRs) on callus induction and using different elicitors *in vitro* to induce the production of (Diosgenin) in callus culture from different parts of *Balanites Aegyptiacae* and study the effect of diosgenin extract on different cell *In vitro* the production of natural diosgenin from callus culture became an alternative biotechnological approach to diosgenin production. This study lines. Root, stem, leaves callus calture of *B. aegyptiacae,* were raised on Murashing and skoog Medium (MS) supplemented with both NAA (Naphthaline Acetic acid) and 2,4D (2,4 – Dichlorophenoxy acetic acid) as plant growth regulators at different concentration. Then Elicitation process was applied by adding different amino acids and coconut milk at different concentrations to culture medium. Medium of root which supplemented with 300mg/Tryptophan gave the maximum diosgenin content in callus extract using spectrophotometric method and HPLC analysis. Then the cytotoxic activity of the root callus extract supplemented with tryptophan was determined against HepG2 (human hepatocellular carcinoma cell line), A549 (Human Lung Cancer cell line) and the result were 51.26 µm/ml, 28.9 µm/ml respectively. The bio safety of extract was detected on the normal human cells and the result was 287.3 µm/ml. Based on these findings, the production of bioactive secondary metabolites (Diosgenin) can be proceed in *vitro* from *Balanites Aegyptiacae* by callus culture to indicate the possibility to screen cultures with higher levels of Diosgenin which posses good potential for use as chemotherapeutic agent from natural source.

Keywords: *B.Aegypticae*, callus induction, diosgenin bio-production, Elicitation, hepatocellular carcinoma, Human lung cancer and normal cell lines.

INTRODUCTION

Medicinal plants are receiving a great attention from pharmaceutical industries for production of bioactive compound. Plant tissue cultures are alternative biotechnological controlled approach to bioactive compound production on demand.

Blanites Aegyptiacae (L.) Delile (Zygophlllaceae) popularly known as (Desert Date) is aspiny evergreen tree which widely distributed in arid regions of Africa, the Middle East, and India **[1].**

Balanites Aegyptiacae is a widely distributed African plant of medicinal interest containing a number of Cytotoxic and cytostatic compounds. It is a small spine scent evergreen savanna tree with dark brown stem which attain a highest of 4.5 to 6 **[2].**

In Egyptian folk medicine, the fruits are used as oral hypoglycemic and as an anthelmintic **[3],** an antidiabetic **[4].** An aqueous extract of the fruits mesocarp is used in Sudanese folk medicine as a treatment of jaundice. Indeed the plant used as to remove intestinal parasites with the root, branches, and bark fruits. Also extracts of Balanites display abortive and antiseptic properties. **[5].**

Diosgenin, a steroidal sapogenin occure abundantly in *Balanites Aegyptiacae* this bioactive phytochemical not only used as an important starting precursor for several steroidal drugs in pharmaceutical industry, but also revealed high potential in the treatment of various types of disorders as Cancer, hyperglycemia, inflammation, and many types of infections **[5].**

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Diosgenin as natural products including steroidal compounds have been growing not only as therapeutically active agent but also as lead in drug discovery approaches **[6, 7].** The production of cancer therapeutic from steroidal compounds has been attractive choice for medicinal chemists and many active molecules emerged **[7].**

Diosgenin has antiprolferative activity in prostate cancer (PC-3) and DU-145 cells) **[8]**. Colon Carcinoma (HCT-116 and HT-29 cells), erythroleukemia (HEL cells) **[9].** Squamous carcinoma (A431, Hep2), hepatocellular carcinoma (HepG2 and HCC cells), Lung cancer (A549 cells) **[10].** and several studies reported that the known anticancer mechanism of action of diosgenin are associated with modulation of multiple cell signalling which involved in cell growth/ proliferation, differentiation, epithelial mesenchymal transion migration and apoptosis **[11]**

However, the elicitation considered an important strategy to improve the production of plant secondary metabolites. Biotic and Abiotic compounds are able to acts as elicitors through a number of signal transduction pathways and the induction process regulated at the level of transcription **[12, 13].** Some amino acids could promote *in vitro* production of secondary metabolites, tryptophan helped to increase the formation of green spots and embryonic calli. **[14].** However, the bio production and determination of diosgenin specific targets is of major relevance to further validate its applications in the prevention and treatment of cancer **[15].**

This study aimed to the production of bioactive secondary metabolites (Diosgenin) *in vitro* from *Balanites Aegyptiacae* by callus culture and to indicate the possibility to screen cultures with higher levels of Diosgenin which posses good potential for use as chemotherapeutic agent from natural source.

Experimental

a-Plant preparation

This experiment performed at Tissue culture lab, Medicinal and aromatic plant research department. Horticulture research institute, agriculture research center. All chemicals used purchased from El-Gomhouria Co. for trading drugs, chemicals & medical supplies and Sigma company.

The seeds were collected from Aswan Botanical garden and cultured on MS media after disinfecting by colorex 20% for 10 min and washing three times with distilled water. The shootlets were taken from *in vitro* grown culture on MS media supplemented with 0.5mg/Benzyl amino purine (BAP) at 25˚C under florescent lamps with light intensity of 3000 lux at 16 hrs photoperiods **[16].**

b- Callus induction (control) :

The three parts of Explants used for callus induction as (leaves, root and stem discs) of *B. Aegyptiacae* were transferred to three different concntrations of Plant growth regulators (PGRs) NAA(Naphthaline Acetic acid) and 2,4D(2,4 –Dichlorophenoxy acetic acid) at (1,2,3mg/L) for each on MS culture media. The cultures were maintained in the culture chamber at 20˚C with dark condition and 40-60% humidity used throughout the experiment then the callus fresh weight were recorded after 5 weeks as shown in **table (1)**.

b- *c- Elicitation Treatment of Callus :*

The callus weights which cultured on MS Media containing 3mg/L2, 4 D and 3 mg/LNAA subcultures to different MS Media supplemented with four different elicitors phenyl alanine, glutamine , tryptophan at (100,200,300 mg/L) and coconut milk at (10,20,30 ml/L) respectively .Three replicates and 36 tube for each type were incubated for one month. The experimental design was a factorial based on completely randomized design with three replicates.

c- *d- Saponin Extraction for determination of diosgenin:*

One gram of dry weight callus powder of each treatment and control extracted three times by 30 ml Methanol with agitation overnight on shaker, and then followed by centrifugation, finally the supernatant of methanol extracts were pooled and methanol evaporated using a rotary evaporator and yellowish crystal powder of crude saponins was obtained finally **[17].**

Diosgenin was determined spectorophotometric as describes by **[18]** with some modifications. Standard sapogenin (Diosgenin) and p- anisaldehyde (4-methoxybenzaldehyde) were purchased from sigma. Sulfuric acid and ethyl acetate were both analytical grade and obtained from Frutarom industries LTd. 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 m p-Anisaldehyde and 99.5 ethyl acetate, and (B) 50 ml concentrated sulfuric acid and 50 ml ethyl acetate. 200µg of methanol extract of callus powder (drying cell) firstly dissolved in 1ml methanol then 200µl of this solution placed in another tube, the methanol was evaporated under reduced pressure. Then the residue was dissolved in 2 ml of ethyl acetate; 1 ml each of reagents A and B were to the tube and stirred. The test tube was placed in a water bath maintained at 60˚C at 10 min to develop color, samples taken out allowed to cool then placed for 10 min in 25˚C water bath. The absorbance of the color developed solution was measured in a spectrophotometer (V-530-UV/VIS, Jasco Corp., JAPAN). Ethyl acetate was used as control for the measurement of absorbance as a reagent blank; 2ml ethyl acetate was placed in a tube and assayed in a similar manner. For the calibration curve, 2-40µg standard diosgenin in 2 ml ethyl acetate was used. The Calibration curve was prepared by using original diosgenin at 10, 20, 30,40,50,60,70,80,90 and 100 ppm). Each sample was repeated thrice and the average was taken.

e-Diosgenin Quantification by HPLC Analysis:
Performed by waters 2690 Alliance HPLC system equipped with a waters 996 photodiode array detector at Nawah Scientific Company.

HPLC analysis condition:
Column C18 Kromasil: 4.6x150mm, 5µmMobile phase: Acetonitrile: water 90:10%Mode of elution: Isocratic Flow rate: 1ml/mi Temperature: Ambien Wavelength: 203 nm.

Standard preparation:
Diosgenin was purchased from total Herb Solution Pvt. Ltd., Mumbai (India).Stock solution of 1 mg/ml in Ethanol was prepared, and 4 dilutions were prepared in concentrations of 50µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml. Each of the dilutions was filtered using 0.22 µm syringe filter then 20 µl were injected **[15].**

***Sample preparation*:**
The sample was filtered using 0.22 µm syringe filter then 20 µl was injected.

f- Biological Study:
Cell Culture for Cancer Cell Lines
Hepatocellular Carcinoma HepG2 Cells and A549 NSCLC (Human Lung Cancer) were obtained from VACSERA (Cell Culture Unit, Cairo, Egypt).This cell lines originally purchased from the American Tyoe Culture Collection (ATCC).

HepG2 cell culture:
cells were cultured in RPMI medium and supplemented with 10% fetal bovine serum, 2µm/ml L-glutamine, 250ng/ml fungi zone, 100 units /ml Penicillin G sodium, and 100 units /ml streptomycin sulphate at 37C in a humidified 5% CO2 incubator. Then the culture were passaged every four days by trypsinization using 1ml trypsin/EDTA solution for 5 min at 37C.the cells were used when confluence had reached 75% **[20].**

A549 NSCLC cell culture: cells were cultured in RPMI Medium and supplemented with amixture of 0.6mg/ml glutamine (Gibco BRL, Invitrogen, MerelbeKe, Belgium), 200IU/ml Penicillium (Gibco BRL), 200IU/ml streptomycin (Gibco BRL) and 0.1 mg/ml gentamycin (Gibco BRL). The fetal calf serum (FCS) was heat-inactivated for one hour at 56C. Then the cells were incubated at 37C in sealed (Air-tight) falcon plastic dishes in 5% CO_2 atmosphere.

Cytotoxic Assay:
Cytotoxicity of diosgenin extract against different cancer cell lines measured using MTT cell viability assay. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5diphenyl tetrazolium bromide). This assay based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT (5mg/ml of MTTin 0.9% NaCl) and from blue insoluble to mazan crystal. Solubilization of the crystal by acidified isopropanol **(**0.04 N HCL in absolute isopropanol) was carried out. The extent of the reduction of MTT was quantified by measuring the absorbance at 570nm **[20].**The number of viable cells is directly proportional to the level of soluble formazan dark blue color.

Cell growth Assay:
Cells were placed in a flat bottom 96-well micropalte (10^4-10^6 cells) in 200µl PBS (phosphate buffer solution pH 7.0). Then add 20µl of MTT solution and mix well. The growth medium was replaced with fresh medium containing vehicle control (Sterile distilled water) or increasing concentrations of *B. Aegyptiacae* from (18.75 to 300µg/ml). Eight wells were prepared for each concentration. After incubation for 24 h at 37C in dark remove aliquot for analysis by adding 200µl of acidic isopropanol and incubate for 1 hour at 37C in dark then read plate in ELISA reader at 570nm **[21].**Data expressed as the percentage of relative viability compared with the untreated cells. The cytotoxicity indicated by < 100% relative viability. The percentage of relative viability was calculated using the following equation:
Absorbance of treated cells /Absorbance of control cells X100.
The half maximal inhibitory concentration of cell viability (IC_{50}) was calculated from linear equation of sample concentration and cell viability%. **[22].**

Statistical analysis:
The data of callus induction and diosgenin determination from the callus induction by various parts of explants were analyzed by complete randomized design (CRD) using LSD at 5%to determine the variation between means of treatments.

Results and Discussion:

A. Callus induction

1. Effect of PGRs
In all parts, the callus fresh weight was found to increase with increasing the concentration of PGR (2,D and NAA) on MS basal media and no significance difference in callus fresh weight between 1mg/Land 2mg/L (2,4 D and NNA) on MS basal media ; however in all parts (root, stem and leaves) the highest fresh weight was found with 3 mg/L(2,4D and NAA) which preparing callus for the second step of elicitation treatment. These results are in agreement with the observation of *Champagain et al.,* **[1]** similarly, cell culture of *Balanites Aegyptiacae* which explain the role of varying plant growth regulator (PGR)(2,4D and NAA)to enhance the secondary metabolites production and their results was found that the highest callus dry weight was found with 3mg/l 2,4D among all explants.

Table 1: effect of plant growth regulator (PGR) and elicitation on callus induction:

	Callus fresh weight (g)		
	Root	**Stem**	**Leaves**
PGR mg/L(control)			
1mg/L *NAA+2,4D*	**0.165 ^{e}**	**0.169 ^{d}**	**0.174 ^{d}**
2mg/L *NAA+2,4D*	**0.161 ^{e}**	**0.161 ^{d}**	**0.158 ^{d}**
3mg/L *NAA+2,4D*	**0.203 ^{d,e}**	**0.290 ^{e}**	**0.236 ^{e}**
100 mg/L PAL	**0.684 ^{a,b,c}**	**0.804 ^{a}**	**0.545 ^{b,c}**
200mg/L PAL	**0.461 ^{b,c,d}**	**0.507 ^{b,c}**	**0.642 ^{b}**
300mg/L PAL	**0.392 ^{c,d,e}**	**0.480 ^{c}**	**0.580 ^{b,c}**
100 mg/L Glu	**0.603 ^{b,c}**	**0.520 ^{b,c}**	**0.638 ^{b}**
200mg/L Glu	**0.531 ^{b,c}**	**0.497 ^{c}**	**0.615 ^{b}**
300mg/L Glu	**0.587 ^{b,c}**	**0.457 ^{c}**	**0.806 ^{a}**
100 mg/L Tryp	**0.524 ^{b,c}**	**0.578 ^{b}**	**0.533 ^{b,c}**
200mg/L Tryp	**0.578 ^{b,c}**	**0.467 ^{c}**	**0.601 ^{b}**
300mg/L Tryp	**0.886 ^{a}**	**0.506 ^{b,c}**	**0.527 ^{b,c}**
10 mg/L CM	**0.744 ^{a,b}**	**0.477 ^{c}**	**0.566 ^{b,c}**
20mg/L CM	**0.491 ^{b,c}**	**0.459 ^{c}**	**0.575 ^{b,c}**
30mg/L CM	**0.571 ^{b,c}**	**0.526 ^{b,c}**	**0.468 ^{c}**
LSD at 0.05	**0.258**	**0.072**	**0.105**

Means (n=3) in each column followed by different letters are significantly different at P<0.05.

2. Effect of elicitation

According to data in **Table (1)** addition of different elicitors to MS media increase callus fresh weight when compared with media with plant growth regulator only (2,4D and NAA) as control **[23].** Which explain the role of chemical; stresses including elicitation, varying plant growth regulators, and mineral nutrient concentrations can enhance secondary metabolites production in Cell Cultures of *D. zingiberensis*. On root callus there was significant difference increase in fresh weight for all treatments. Callus obtained from root when supplemented with tryptophan at 300 mg/L concentration gave the highest callus induction in this part. On the other hand, on Stem part also there was significant increase in fresh weight for all treatments but callus obtained from stem when supplemented with phenyl alanine at 100 mg/L gave the highest significant effect. On Leave Part there was significant difference between all treatments but the highest callus induction obtained from glutamine elicitation at 300 mg/L.

B- Diosgenin content

1- Spectrophotometric method

Diosgenin content of the cell culture extract samples of *B. Aegyptiacae* was analyzed by spectrophotometric method for all treatments at all concentrations to detect the highest content.

The Diosgenin concentration (based on dry weight) was found to be the highest in 3mg/Lof 2,4D and NAA for all three parts calli(root, stem and leaves) (**Table 2**).

According to data represented in table (2) using additive elicitors to MS media increase diosgenin content in dry weight when compared to media without elicitation additives.

On root callus there was increase in diosgenin content after elicitation and there was significant difference between all treatments but the highest level of diosgenin content obtained from tryptophane at 300 mg/L concentration was (3.11 mg/100g dry weight) when compared with that without treatment (control) (0.164 mg100/g). the diosgenin content of Balanites Aegypticae cell culture extract reported here was higher than that previously reported by **[25].** who found that diosgenin content of cell culture samples of *D zingiberensis* was detected by spectrophotometer and HPLC method was (0.157 and 0.151 mg/g) respectively , so cell culture of *D zingiberensis* has been regarded as an alternative means for efficient and controllable method for production of diosgenin.

On stem Callus there was a significant difference between all treatments and higher increase in diosgenin content when compared to that callus without elicitation the highest diosgenin content in this part was obtained from elicitation with phenylalanine at 100 mg/L to be (0.299 mg/100g) when compared with control at (0.069 mg/100g). While on leaves callus the highest level of diosgenin obtained from elicitation with glutamine at 300mg/L when compared to control at (0.100 mg/100g). This finding is consistent with **[14,26].** Who investigated the effect of some amino acids and reported that it could promote in vitro production of secondary metabolites in *Mentha longifolia* and increase the mass of emberyogenic callus in immature embryo culture of rice.

Table 2: Effect of elicitation treatments on Diosgenin content in *Balanites Aegyptiacae* callus dry weight.

	Diosgenin (mg/100g) callus dry weight		
Part of explant	**Root**	**Stem**	**Leaves**
Control	**0.164** ^{f}	**0.069** ^{b}	**0.100** ^{c,d}
100 mg/L PAL	**0.274** ^{b}	**0.293** ^{a}	**0.350** ^{b,c}
200 mg/L PAL	**0.14** ^{f}	**0.080** ^{b}	**0.106** ^{c,d}
300 mg/L PAL	**1.00** ^{d}	**0.099** ^{b}	**0.583** ^{a}
100 mg/L Glu	**1.90** ^{c}	**0.129** ^{b}	**0.300** ^{b,c}
200 mg/L Glu	**1.79** ^{c}	**0.121** ^{b}	**0.160** ^{b,c,d}
300 mg/L Glu	**2.05** ^{b,c}	**0.137** ^{b}	**0.733** ^{a}
100 mg/L Tryp	**0.920** ^{d,e}	**0.087** ^{b}	**0.363** ^{b}
200 mg/L Tryp	**0.430** ^{e,f}	**0.177** ^{a,b}	**0.333** ^{b,c}
300 mg/L Tryp	**3.11** ^{a}	**0.180** ^{a,b}	**0.310** ^{b,c}
10 ml/L CM	**0.322** ^{f}	**0.060** ^{b}	**0.136** ^{b,c,d}
20 ml/L CM	**0.871** ^{d,e}	**0.074** ^{b}	**0.103** ^{c,d}
30 ml/L CM	**0.093** ^{f}	**0.083** ^{b}	**0.026** ^{d}
LSD at 0.05	**0.5633**	**0.1563**	**0.2200**

Means (n=3) in each column followed by different letters are significantly different at P<0.05.

2- HPLC method

The extracted diosgenin from cell culture was detected using HPLC method with Column C18 Kromasil.

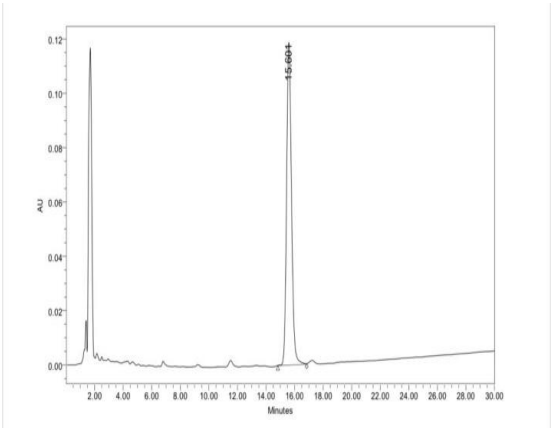

Fig. 1. HPLC spectrum of standard Diosgenin

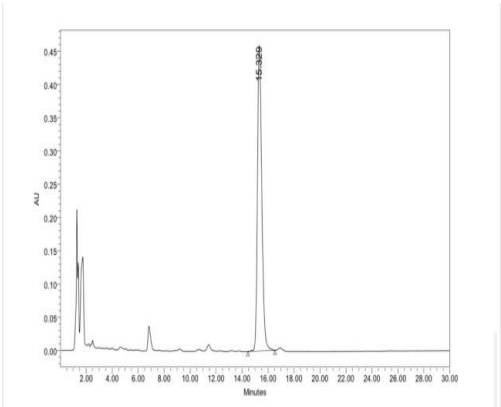

Fig. 2. HPLC Spectrum of diosgenin purified from B. Aegyptiacae cell culture extract (tryptophan 300ppm)

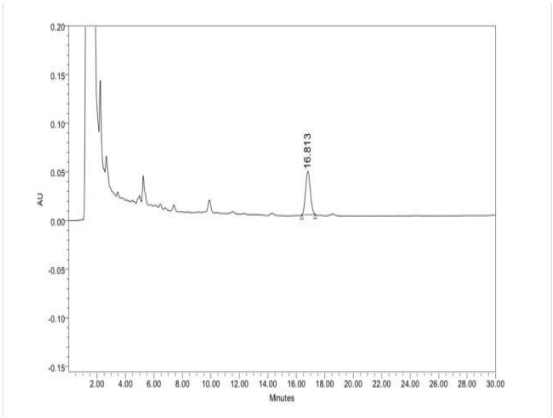

Fig. 3. HPLC Spectrum of diosgenin purified from B. Aegyptiacae cell culture extract (glutamine 300ppm)

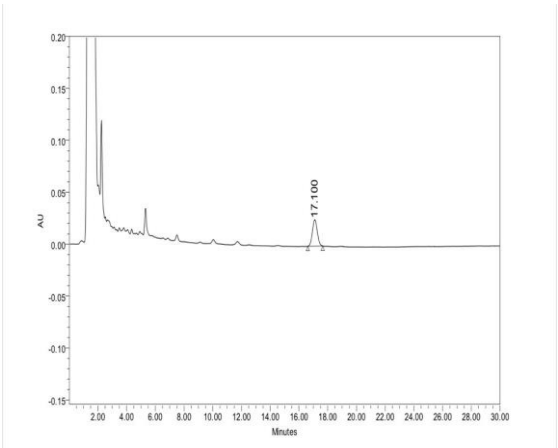

Fig. 4. HPLC Spectrum of diosgenin purified from B. Aegyptiacae cell culture extract (phenylalanine 100ppm)

The highest diosgenin content determined by spectrophotometeric method obtained from tryptophan at 300 mg/L concentration was (3.11 mg/100g dry weight) while when determined by HPLC was (3.12 mg/100g) figure (2) for Root callus extract. Diosgenin content obtained from Glutamine at 300 mg/Lwas (0.733 mg/100g) by spectrophotometer while by HPLC was (0.880 mg/100g) figure (3) For Leave callus extract, and for Stem callus extract diosgrnin

content determined by spectrophotometer was (0.299 mg/100g) while by HPLC was (0.481mg/100g) figure (4) .which agree with cell culture of *D.zingiberensi*s which has been acts as alternative and efficient controlled production of diosgenin **[23, 27].** Which explain the important role of chemical stresses including elicitation, different plant growth regulators and mineral nutrient to enhance secondary metabolite production in plant cell cultures which consider an alternative important biotechnological approach to diosgenin production **[28, 29]**

a. Apoptotic effect of diosgenin

1. On MTT cell line

The results of MTT assay indicate that diosgenin significantly inhibits the proliferation of HepG2 and A549 cells in a dose dependent manner, suggesting that diosgenin induces hepG2 and A549 cell death.these results was in agreements with *Gautam et al., Kim et al.,and with samy selim and soad Al-jaouni* **[30, 31,32].**which demonstrated that Diosgenin showed high potent anti-cancer activity against hepG2 and A549 and induce cytotoxicity and significantly inhibited the growth and proliferation of these cancer cells in adose – and time-depentent manner.

Apoptosis is an important pathway of cell death. The results demonstrated that the percentage of apoptotic diosgenin treated cells increased significantly in a dose dependent manner, suggesting that diosgenin inhibits proliferation by promoting apoptosis in tumor cells.

According to data in Table (3) the effect of methanolic extract of *Balanites Aegyptiace* dry cells extract on the growth of HepG2 and A549 carcinoma were assessed by MTT assay which usually performed to study the mitochondrial /non mitochondrial dehydrogenase activity as cytotoxic test for a variety of chemical compounds. Methanolic extract in a concentration range of 18.75 to 300 µg showed a dose responsive chart after 24hrs of treatment. The diosgenin amount were treated and induced by elicitation (Tryptophan 300 mg/l) to the maximum quantity which used in biological assay.

The effect of methanolic extract of *Balanites Aegypticae* were also assessed on a Normal lung cells to detect and emphasis the safety of our extract as illustrated in table (3).

The present study shows the effect of diosgenin on autograph that means a cell death process that is distinct from apoptosis. It refers to the process that is eukaryotic cells in which a double membrane wraps around a portion of cytoplasm and intracellular organelles and proteins to be degraded from an autophagosome. This fuses with endosomes to form an amphisome, which eventually fuses with lysosomes to form an autophagolysosome that degrades the packaged contents. Under physiological conditions, autophagy occurs at low levels.

In this concern, the tumour cells are different with normal cells in there metabolic products and genes. Some tumor cells have in their nucleus some genes like P53 protein gene which significantly increased in cells treated with diosgenin which further supports in induction of apoptosis by diosgenin.

Surviving fraction at each concentration and the inhibition concentration IC_{50} which is the concentration of treatment was determined and calculated using Sigma plot program.

Table (3). Effect of methanolic extract of dry cells of *Balanites Aegyptiacae* on cell viability of Hepatocellular carcinoma, Human Lung Carcinoma cell lines and Human normal cell line.

Concentration (µg/ml)	MTT cell viability%		
	HNC	HepG2	A549
0	100	100	100
18.75	97.19	72.06	76.03
37.5	93.95	62.20	25.32
75	88.40	43.54	22.20
150	87.97	12.17	15.57
300	45.90	11.43	13.20
IC_{50}	***287.3µg***	**51.26 µg**	**28.89 µg**

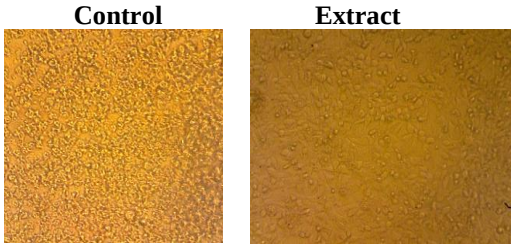

Fig.5. Inhibitory effect against Hepatocellular Carcinoma (HepG2) control and in the presence of extract.

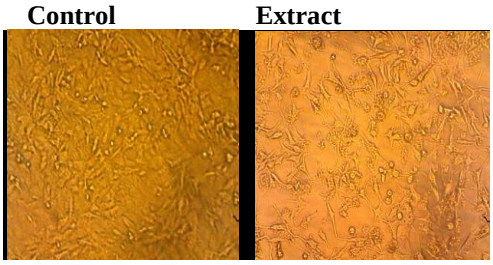

Fig.6. Inhibitory effect against (Human lung Carcinoma A549) control and in the presence of extr

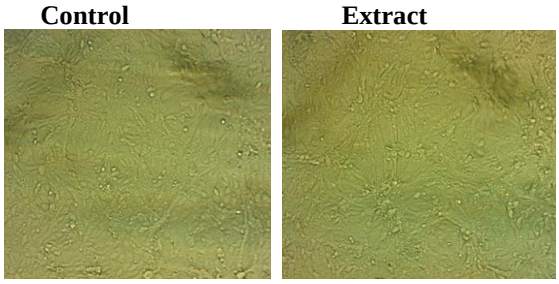

Fig. 7. Inhibitory effect against HNC (Human Normal cells) control and in the presence of extract.

1. Morphological Changes

Inverted Microscope observation of the *Balanites Aegyptiacae* methanolic extract-treated HepG2 and A549 lung carcinoma cell lines after 24 hrs of exposure, the treated cells started showing gradual cell shrinkage, cell rounding and detaching from the surface of the tissue culture flask, and following by cell swelling and rupture (the morphological obvious gives an indicator for cell apoptosis) .and some cell treated with diosgenin contained numerous autophagic vacuoles of various size.

A dose ranging from 0 µg to 300 µg was effective in inducing cytotoxicity in the cancer cells by the values 51.26 and 28.89 respectively figure (5,6). While the effect on human normal cells there is no obvious affect which emphasis the biosafty of the extract normal cell. Many studies on methanolic extractof the leaves of *Sanseviera roxburghiana* and *costus malortieanus* reported that potent cytotoxicity against HepG2 liver cancer cell line in a dependent dose **[33,34].**Results showed that the morphological changes were typical of apoptic feature indicating the anticancer activity of *Artemisia vulgaris*. Detailed analysis using MTT assay and morphological observations by fluorescence microscope and inverted microscope confirmed that the aquas extract of *Moringa olifera* have cytotoxic effects on HeLa cells. Methanolic extract of *Oroxylum indicum* also showed good cytotoxicity against Dalton lymphoma cells **[35, 36].**

Conclusion:

Here in this work the results indicated that, the *in vitro* production of bioactive secondary metabolites is not limits by seasonal or regional restrictions and can be accomplished by controlled conditions and environment where the possibility exists to screen cultures with higher levels of diosgenin production which posses good potential for use as cancer chemotherapeutic agent as important natural source.

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