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Antitumor Activity of Balanitoside Extracted from *Balanites aegyptiaca* Fruit

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ABSTRACT

The current study aimed at investigating the antitumor efficacy of balanitoside extracted from *Balanites aegyptiaca* fruit against Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice. The extracted balanitoside was proved by chemical analysis and LD50 of balanitoside was determined. Then, mice were injected intraperitoneally with balanitoside (10mg /kg b.wt) before and after EAC inoculation, to achieve preventive and therapeutic effects daily, for 9 days. The effects of balanitoside on the count of EAC cells and life span prolongation were studied; malondialdehyde (MDA), nitric oxide (NO) levels as well as catalase (CAT) and caspase 3 activities were estimated. Cytological studies on EAC cells and histopathological examination of liver tissue were carried out. Treatment with balanitoside decreased EAC cell count for preventive and therapeutic groups. MDA and NO levels were decreased in liver and serum in preventive and therapeutic groups compared to positive control group. While CAT activity was increased in liver and plasma of preventive and therapeutic groups in comparison with positive control group. Caspase 3 activity in EAC cells, was increased in preventive and therapeutic groups in comparison with positive control group. Survivin expression in liver was decreased in preventive and therapeutic groups in comparison with positive control group. The present work indicates that balanitoside isolated from fruit extract of *Balanites aegyptiaca* may possess significant antitumor and antioxidant activity *in vivo*.

INTRODUCTION

Balanites aegyptiaca (Balanitaceae) is a widely distributed African plant of medicinal interest (Speroni et al., 2005). The fruits are edible and known as desert dates. It is a small evergreen savanna tree with a dark brown stem which usually attains a height of 4.5-6 m (Koko et al., 2005a). In Egyptian folk medicine, the fruits are used as an oral hypoglycemic and an anti-diabetic (Sarker et al., 2005). An aqueous extract of the fruit mesocarp is used in Sudanese folk medicine in the treatment of jaundice (Sarker et al., 2005). Indeed the plant is used as a purge to remove intestinal parasites with the root, branches, bark, fruit and kernel extracts shown to be lethal to the miracidia and cercariae of Shistosoma mansoni and to Fasciola gigantica (Koko et al., 2005a). Additionally extracts of the tree display abortive and antiseptic properties (Speroni et al., 2005). The roots and bark of *Balanites aegyptiaca* contain numerous steroidal saponins and yamogenin or diosgenin glycosides (Speroni et al., 2005). The fruit mesocarp contains a large variety of chemicals amongst which are the pregnane glycosides, coumarins, flavonoids, 6-methyldiosgenin and saponins (Koko et al., 2005b).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 (Deng et al., 1999). 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 balanitoside extracted from *Balanites aegyptiaca* fruits and study some of its biochemical, cytological and histological effects.

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MATERIALS AND METHODS

Saponin isolation

Balanitoside have been isolated from *Balanites aegyptiaca* fruits using a procedure described by Wiart et al. (2004). Powdered fruits (2 kg) from *Balanites aegyptiaca* were extracted with petroleum ether (3 times with 1.5 L) over 3 days with shaking at

ambient temperature and the combined extracts filtered. Then the precipitate extracted with methelene chloride (3 times with 1.5 L) over 3 days with shaking at ambient temperature and the combined extracts filtered then the precipitate extracted with ethyl acetate(3 times with 1.5 L) over 3 days with shaking at ambient temperature and the combined extracts filtered. The precipitate extracted with methelene chloride (3 times with 1.5 L) over 3 days with shaking at ambient temperature and the combined extracts filtered the precipitate extracted with butanol (3 times with 1.5 L) over 3 days with shaking at ambient temperature and the combined extracts filtered and evaporated to dryness in a rotary evaporator under reduced pressure.

Test for saponins

Saponins were identified according to the method described by Ayoola1 et al. (2008), also Foam test was applied according to the method described by Arulpriya et al. (2010)

Elucidation of Chemical structure of saponin

The yielded paste chromatographed on thin layer chromatography using CHCl3/MeOH (v/v: 95/5) as the eluent according to Reich and Schibli,(2007***).*** The structure of balanitoside was identified using mass spectroscopy according to Sparkman, (2000), NMR spectroscopy according to (James, 2007) and IR according to Laurence and Christopher, (1989).

Electrochemical behaviour of balanitoside

The cyclic voltammogram (Cv), anodic cathodic behaviour of balanitoside in aqueous solution on pt –electrode was done using Potentioscan, wenking POS73 combined with X-Y recorder. The electrochemical cell are described by Khedr et al.(1989), the working electrode was pt gauze and the reference electrode is pt wire. The electrode potentials were measured relative to a saturated calomel electrode (SCE).

Determination of median lethal dose (LD_{50}) of balanitoside

Studies carried out for determination of the median lethal dose are important to help us to assess the limit dose recommended, We use procedure described by Meier and Theakston, (1986) to calculate the (LD_{50}).

Dose-response curve

Dose-response curve was done to know which is the most effective dose on the reduction of EAC count, according to the procedure described by Tiaseer, (1999)

Effect of balanitoside on life span prolongation

Life span calculation was carried out according to the method described by Mazumdar et al**.,** (1997).

Increase in life span = (T – C) / C × 100

T = number of days the treated animals survived

C = number of days the control animals survived.

Experimental design : experimental design was done according to Al-Shimaa et al. (2008); 72 female Swiss albino mice at 8 weeks of age were weighed and divided into 4 different groups, 18 mice /group as follow:

Group 1: Negative control group, Mice injected i.p. with saline only daily.

Group 2: Positive control group, Mice injected i.p. with EAC cells by the concentration of (2.5×10^6 cells / 0.3 ml/mouse), once at the beginning of the experiment.

Group 3: Preventive group, Mice were injected i.p with balanitoside 10 mg/kg at the beginning of the experiment, at the second day mice were injected i.p. with EAC cells by the concentration of 2.5×10^6/0.3ml/mouse once, then the day after mice were injected i.p with balanitoside 10 mg/kg.

Group 4: Therapeutic group, Mice were injected i.p. with EAC cells by the concentration of 2.5×10^6/0.3ml/mouse once at the beginning of the experiment, at the second day mice were injected i.p with balanitoside 10 mg/kg.

Mice in group 3 and 4 injected i.p with balanitoside 10 mg/kg daily for 9 days, till the end of the experiment.

A) Sampling of blood and EAC cells: At the end of the experiment, the blood samples (about 3 ml) were collected from the retro-orbital venous plexus under light ether anesthesia divided to 2 parts to obtain serum and plasma. Serum was prepared by centrifuging blood at 4000 r.p.m for 10 minutes. Serum samples were aliquoted and stored at -20°C until biochemical analysis. Plasma was prepared by collecting the blood using anticoagulant as ethylene diamine tetra acetic acid "EDTA". The blood was centrifuged at 4000 r.p.m for 10 minutes and the top yellow layer (plasma) was pipetted off and freezed at -20°C until using. EAC cells were harvest from each mouse in centrifuge tube contains heparinized saline to prevent clotting. Each sample of EAC cells were subjected for counting and viability tests according to the method of McLiman et al.(1997). Liver tissues were excised from each mouse and divided into 2 parts, part of liver tissue from each group was collected in centrifuge tube contains phosphate buffer saline (PBS) pH 7.4; for biochemical analysis , the second part of liver tissue from each group was preserved in 10% neutral formalin solution for histological examination.

B) Biochemical Parameters

1) Determination of plasma and liver catalase activity was assayed by using Biodiagnostic kit method (Biodiagnostic Company, Giza/Egypt) according to Aebi, (1984)
2) Determination of serum and liver malondialdehyde level was assayed by using Biodiagnostic kit method (Biodiagnostic Company, Giza/Egypt) according to Satoh, (1978)

3) Determination of serum and liver nitric oxide level was assayed by using Biodiagnostic kit method (Biodiagnostic Company, Giza/Egypt) according to Montgomery and Dymock,(1961)
4) Determination of EAC cells caspase 3 activity was carried out as indicator for apoptosis using (R & D systems) kit according to Casciola et al. (1996).

C) Immunohistochemical study was carried to detect survivin according to Ramos-Vara (2005).

D) Histopathological Study: Histopathological examination of liver tissue fixed in formalin was carried out according to the method described by Culling, (1983) using hematoxylin and eosin staining.

E) Cytological Study: Cytological study on EAC cells was carried out according to the method described by Amer, (1986) using Giemsa staining.

F) Statistical Analysis: Statistical analysis of data was performed by using SPSS 14.0 version using one way analysis of variance (ANOVA) according to the method described by Levesque, (2007) numerical data were expressed as mean + SD , P values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Balanites aegyptiaca fruit (2 kg) after undergoing extraction yielded a yellow paste (4.6 g) which was further identified to be saponin as emulsion appeared in saponin test; also foam appeared in the foam test. Thin layer chromatography shown in Fig 1 revealed that only one band appeared which suggested that the extracted compound was pure saponin.

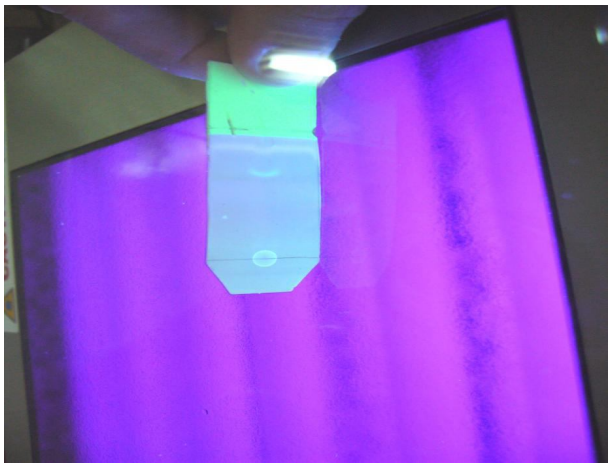


Fig. 1: Thin layer chromatography of extract.

Saponins to which balanitoside belongs to are a large family of structurally-related compounds of steroid or triterpenoid aglycone (sapogenin) linked to one or more oligosaccharide moieties by glycosidic linkage, the carbohydrate moiety consists of pentoses, hexoses, or uronic acids. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provide saponins with strong surface-active properties which distinguish these compounds from other glycosides (Yu et al., 2010). They readily dissolve in water to form colloidal solutions that foam upon shaking therefore they are added to shampoos, liquid detergents, toothpastes and beverages as emulsifier and long-lasting foaming agent (Sajadi et al., 2007). Saponins have a broad range of pharmacological effects most are haemolytic and toxic to cold-blooded animals, while certain saponins also display molluscicidal, anti-inflammatory, anti-fungal/anti-yeast, anti-bacterial/antimicrobial, anti-parasitic and anti-viral activity (Sparg et al.,2004). Numerous reports have also highlighted the highly cytotoxic properties of many saponins Saponins can act as potential anti-cancer agents by preventing carcinogenesis (Valsala et al., 2001) or by exerting direct anti-proliferative and/or cytotoxic effects against cancer cells (Kerwin, 2004) even *in vivo* (Drissi et al., 2006), and it is notable that there are only two *in vivo* studies of *Balanites aegyptiaca* that have been carried on mice until now (Yau et al., 2011).

To prove the saponin structure IR NMR and mass spectroscopy were used which showed the structure to be balanitoside as cleared from **Graphs** 1, 2 and 3 as well as Fig 2 and Graph 4. The cyclic voltammogram (anodic and cathodic curves) of balanitoside on pt – electrodes was carried between -2V (SCE) and 2V(SCE) at a scan rate 0.05V/s was shown in Graph 4 The data of this graph indicates presence of two oxidation peaks on the anodic branch and no any reduction peak on the reverse scan. This behaviour was compared with ascorbic acid as a standard antioxidant (Aurelia et al., 2009). In our study, it is cleared that we extracted saponin as confirmed by test for saponins as emulsion appeared and foam appeared in the foam test. From TLC it was found that the extracted saponin was pure as one band appeared as shown in Fig 2 and the saponin structure was proved by NMR, mass spectroscopy and IR analysis as shown in Graphs 1 , 2, 3 and 4 respectively also confirmed that the extracted saponin was balanitoside as follow :

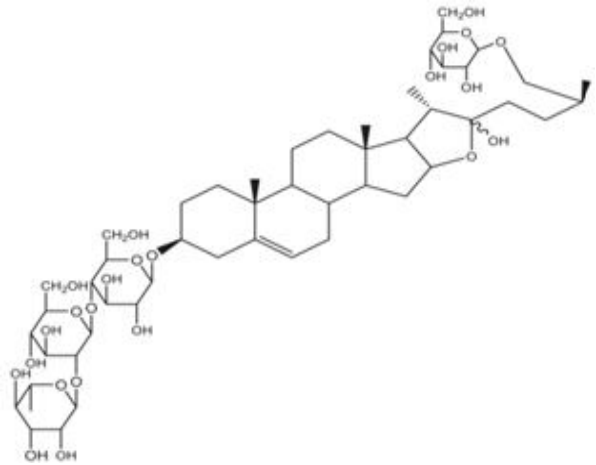
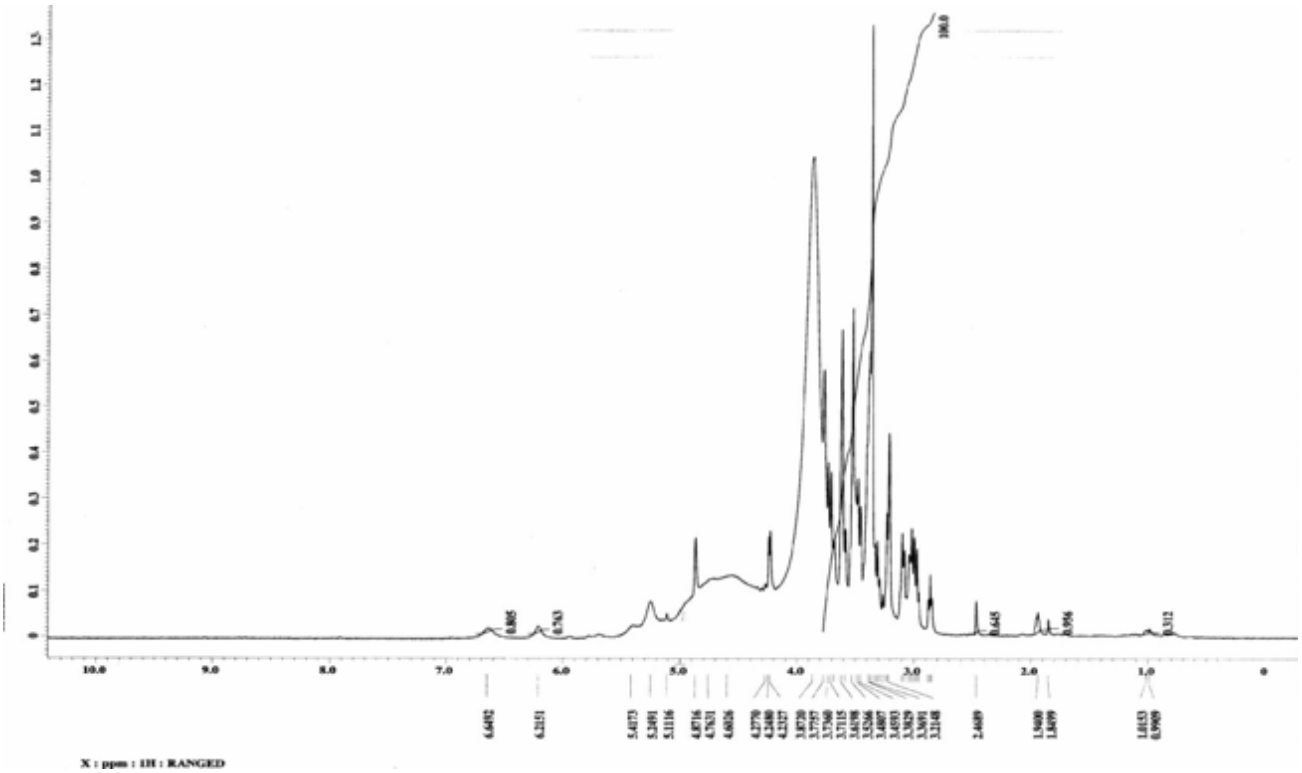
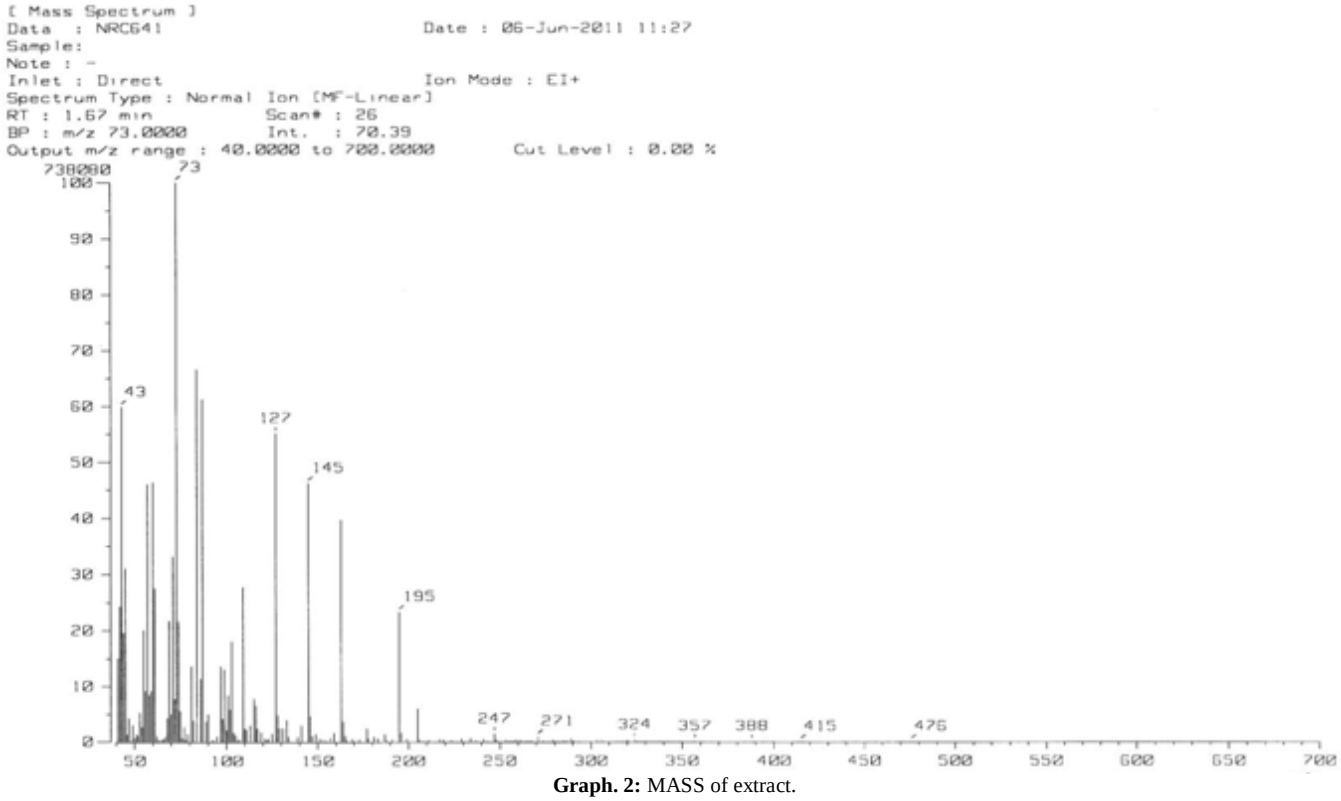


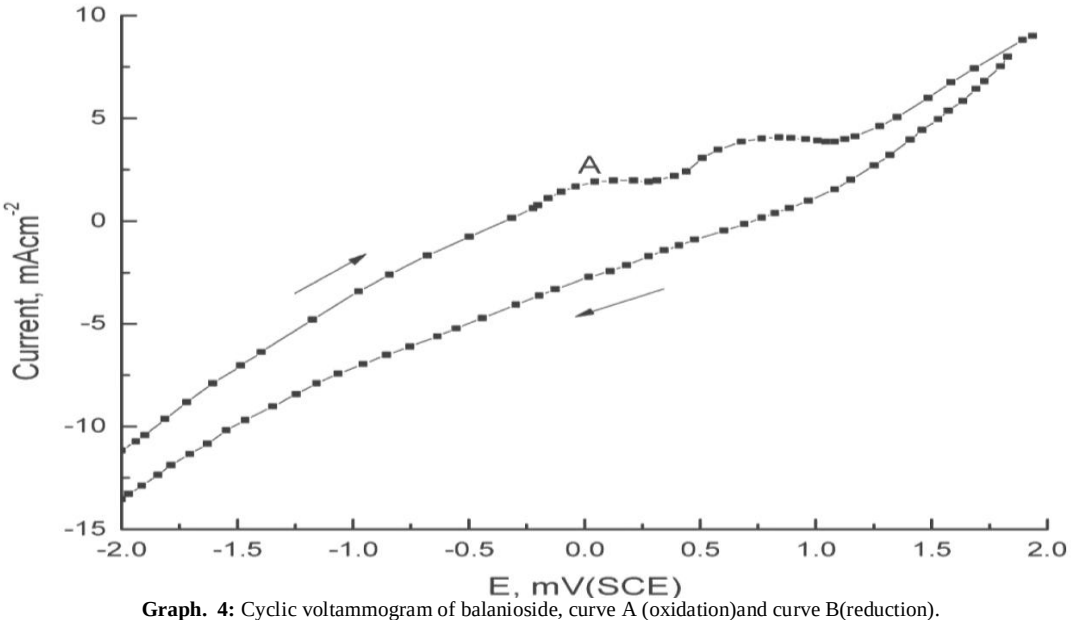
Fig. 2: Balanitoside.



Graph. 1: NMR of extract.



Graph. 2: MASS of extract.



Graph. 4: Cyclic voltammogram of balanioside, curve A (oxidation)and curve B(reduction).

1H NMR (DMSO-d6, ppm)

δ = 0.97 (s, 3H, CH3-19), 1.01 (s, 3H, CH3-18), 0.99 (s, 3H CH3-21), 2.46 (m, 1H, C3-βH), 4.20 (t, 1H, OH, D2O-exchangeable), 5.41(t, 1H, C6-αH),

M.S (EI): m/z (%)

Molecular weight 1065.2 g/mol ,415 (C27H43O3, 0.02), 397 (C27H43O2, 0.03), 309 (C12H21O9, 0.03), 279 (C11H19O8, 0.21), 163 (C6H11O5, 27), 147 (C6H11O4, 0.76), 145 (C6H9O4, 32), 115 (C6H11O2, 5), 109 (C3H9O4, 19), 87 (C4H7O2, 32), 84 (C5H8O, 46), 73 (C3H5O2, 70), 43 (C2H2O, 42),

IR (KBr, cm^{-1})

υ = 3414 (OH), 2965, 2934 (CH-aliphatic), 1641 (C=C),1260(C-O-C).

For determination of median lethal dose (LD_{50}) of balanitoside, all doses up to 1000 mg were safe which suggests that balanitoside may be slightly toxic.

As to toxicity study balanitoside extracted from *Balanites aegyptiaca* was found to be a safe compound upto 1000 mg/kg in mice as none of the mice died our results were confirmed by Yau et al. (2011) who stated that the LD_{50} of *Balanites aegyptiaca* is 1131.3 mg/kg which mean it is slightly toxic.

It is cleared that 10 mg/kg of balanitoside was found to be the most effective dose as it reduced the number of EAC cells in treated mice group to about half the number of EAC cells in positive control mice group as shown in dose response curve (Fig 3).

A dose-response curve is a simple x-y graph relating the magnitude of a stressor (e.g. concentration of a pollutant, amount of a drug, temperature, intensity of radiation) to the response of the receptor (organism under study), the response may be a physiological or biochemical response, or even death (Altshuler ,1981) therefore dose-response curve is done to know which is the most effective dose on the reduction of EAC count, it is shown from Fig 3 that the dose of 10mg/kg is the most effective dose in lowering the number of EAC count by 45% in comparison with positive control ones which matches with Gnoula et al. (2008) who reviewed that the maximal tolerated dose [MTD in mice was 10 mg/kg].

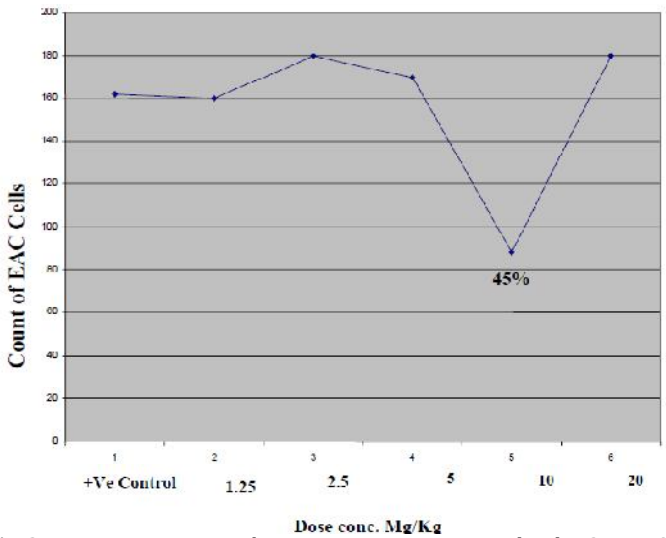


Fig. 3: Dose response curve where group 1 is positive control and 2, 3, 4, 5, 6 are mice injected with 1.25, 2.5, 5, 10, 20 mg balanitoside/kg mice respectively.

The increase in the life span of EAC bearing mice and treated with balanitoside in preventive and therapeutic groups was found to be 36.72%, 13.63% respectively in comparison with positive control group as shown in **Table (1) .**

In the current study, it has been demonstrated that balanitoside isolated from *Balanites aegyptiaca* displays anti-cancer activity as it is obvious from Fig 4 that there was highly

significant decrease in the EAC count in mice bearing EAC and treated with balanitoside compared to positive control mice as it is cleared that balanitoside has lowered the number of EAC by 62.76% and 61.17% in preventive and therapeutic group respectively.

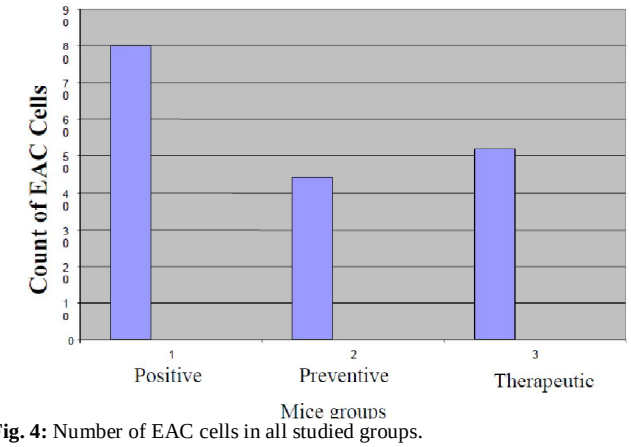


Fig. 4: Number of EAC cells in all studied groups.

It is reported by Gnoula et al. (2008) that a mixture of steroidal saponins: balanitin-6 and balanitin-7, isolated from *Balanites aegyptiaca* kernels, demonstrated appreciable anticancer effects in human cancer cell lines *in vitro* by using against A549 non-small-cell lung cancer (IC50, 0.3 μM) and U373 glioblastoma (IC50, 0.5 μM) cell lines.

It is reviewed that fixed oil of *Balanites aegyptiaca* exhibited anticancer activity against lung, liver and brain human carcinoma cell lines. It also had antimutagenic activity against *Fasciola gigantica* induced mutagenicity (Hanan et al., 2010). Elie et al. (2010) showed that saponins extracted from *Balanites aegyptiaca* have potent anti cancer activity against MCF-7 human breast cancer cells and HT-29 human colon cancer cells.

For diosgenyl saponins the presence of an α-l-Rhap-(1 → 2)-β-d-Glcp sequence at C-3 was considered beneficial for cytotoxicity activity (Irma et al.,2010).

Our data showed that balanitoside posses an α-l-Rha*p*-(1 → 2)-*β*-d-Glc*p* sequence at C-3 as shown in Fig 2 which mean that balanitoside have cytotoxicity properties , that was confirmed by Muhammed et al.(2001) and Yadav and Manju,(2010) who stated that balanitoside posses an α-l-Rhap-(1 → 2)-β-d-Glcp sequene at C-3 .

The increase in the life span (T/C) index of EAC bearing mice and treated with balanitoside in preventive and therapeutic groups was found to be 36.72%, 13.63% respectively as shown in Table 1.

This is in agreement with Gnoula et al.(2008) who reviewed that Bal6/7 (a mixture of saponins isolated from *Balanites aegyptiaca*) weakly but significantly increased the survival of mice bearing L1210 leukemia by 30% over the survival time of mice bearing L1210 leukemia, without treatment by Bal6/7 . There was significant decrease in the level of MDA (Table 2) and NO (Table 3) associated with significant increase in catalase activity (Table 4) in preventive and therapeutic groups compared to positive control group. Moreover, EAC cells caspase activity (Table 5) showed significant increase while, EAC viable cell count (Fig. 4) revealed significant decrease in preventive and therapeutic groups compared to positive control group.

Antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated such as cancer (Mayo Clinic., 2007).

Generally there is a relationship between antioxidative activity and oxidative potentials, the lower the oxidative potential of extracts the higher would be the antioxidant capacity and the lower the oxidative potential, the higher is the ability to donate electron easily to the system generated free radicals. The antioxidant properties of the extracts were assessed by cyclic voltammetric method from its oxidation potential values (Arulpriya et al., 2010).

It was observed from Fig. 2 (Graph 4) that balanitoside have antioxidant properties as for the cyclic voltammogram, shows the variation of current with potential [anodic and cathodic] at scan rate 50mv/s for balanitoside in aqueous solution, the experiament is carried on pt – electrode between -2500 mv and + 2500 mv versus saturated calomel electrode (SCE).

It was observed that the anodic branch is characterized by the presence of anodic peaks at 1250 mv (vs SCE) which may be related to oxidation of primary alcoholic group -CH2OH on the balanitoside. It was shown that the cathodic branch was free from any reduction peak.

Aurelia et al. (2009) reviewed that the electrochemical oxidation of ascorbic acid, is an irreversible process, as no reduction peak appears.

Typically it reacts with oxidants such as reactive oxygen species such as hydroxyl radical formed from hydroxyl peroxide and reduce it and thereby neutralize reactive oxygen species, such radicals are damaging to animals and plants at the molecular level due to their possible interaction with nucleic acids, proteins, and lipids which can further initiate or propagate the development of many diseases, such as cancer(Raushanara et al., 2010). So we assume that the same mechanism probably works for balanitoside.

Malondialdehyde (MDA) is used routinely as a marker of oxidative stress as elevated malondialdehyde correlates with the extent of primary tumor (Salzman et al., 2009). Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals which is accepted as an indicator of lipid peroxidation (Yazar et al., 2004). MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in non diseased organ (Sujata et al., 2010).

Lipid peroxidation mediated by free radicals is considered as a primary mechanism of cell membrane destruction and cell damage, and the enhanced lipid peroxidation leads to tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals (Perumal et al., 2010).

Table. 1: Ehrlich cell count and (T/C) index of studied groups.

Mice no.	Positive control group EAC cells count ($x10^6$)	Therapeutic group EAC cells count ($x10^6$)	Preventive group EAC cells count ($x10^6$)
1	75	18	22
2	54	22	24
3	62	26	24
4	72	33	38
5	65	33	22
6	85	22	23
Mean ±S.D.	68,83±10.87	25.66***±6.21	25.50***±6.18
% Change	-	61.17%	62.76%
Increase life span T/C%	-	13.63%	36.72%

Numerical data were expressed as mean ± SD. Preventive and therapeutic groups were compared to positive control group.
*P value <0.05 was considered significant. ** P value <0.01 was considered highly significant. *** P value <0.001 was considered very highly significant.
N.S. P value >0.05 was considered non significant

Table. 2: Malondialdehyde level in serum and liver of studied groups.

Mice no.	Negative control group		Positive control group		Therapeutic group		Preventive group	
	MDA (serum) n mol/ml	MDA (liver) n mol/g. tissue	MDA (serum) n mol/ml	MDA (liver) n mol/g. tissue	MDA (serum) n mol/ml	MDA (liver) n mol/g. tissue	MDA (serum) n mol/ml	MDA (liver) n mol/g. tissue
1	9.90	104.90	24	210.50	7.66	117.24	8.00	11.3
2	10.50	106.00	17.48	212.60	9.66	115.17	9.66	106.12
3	9.70	105.50	0.22	211.50	7.00	116.00	10.20	108.00
4	6.16	105.40	21.17	206.00	8.10	117.23	6.44	106.00
5	11.00	105.30	22.85	207.21	8.30	116.43	13.00	109.10
6	9.00	105.50	22.00	212.50	10.00	114.40	10.00	110.00
mean±S.D.	9.37±1.71	105.37±0.35	21.28±2.27	210.00±2.80	8.48***±1.15	116.12** ± 1.15	9.55***±2.21	108.60**±1.43
%Change	-	-	-	-	60.15%	44.70%	55.12%	48.289 %

Numerical data were expressed as mean ± SD. Preventive and therapeutic groups were compared to positive control group.
*P value <0.05 was considered significant. ** P value <0.01 was considered highly significant. *** P value <0.001 was considered very highly significant.
N.S. P value >0.05 was considered non significant

Table. 3: Nitric oxide level in serum and liver of studied groups.

Mice no.	Negative control group		Positive control group		Therapeutic group		Preventive group	
	No (serum) Umol/l	No (liver) Umol/g. tissue	No (serum) Umol/l	No (liver) Umol/g. tissue	No (serum) Umol/l	No (liver) Umol/g. tissue	No (serum) Umol/l	No (liver) Umol/g. tissue
1	10.5	8.20	17.10	16.0	13.80	16.00	17.00	12.50
2	11.18	12.90	36.18	18.20	12.50	12.80	13.00	12.80
3	8.55	11.80	29.53	18.00	14.00	15.80	7.89	12.60
4	10.07	11.7	26.64	14.00	9.86	11.7	12.60	11.20
5	9.96	10.7	30.54	20.00	15.80	11.8	15.74	10.70
6	9.50	11.9	27.99	23.00	13.19	16.1	13.34	9.80
Mean ±S.D.	10.05±1.07	11.21±1.70	25.99±6.84	18.22±3.07	12.63±3.11***	14.01±2.15*	11.99**±1.72	11.60±1.21*
%change	-	-	-	-	51.40%	23.10%	53.86%	36.33%

Numerical data were expressed as mean ± SD. Preventive and therapeutic groups were compared to positive control group.
*P value <0.05 was considered significant. ** P value <0.01 was considered highly significant. *** P value <0.001 was considered very highly significant.
N.S. P value >0.05 was considered non significant

Table. 4: Catalase activity in plasma and liver of studied groups.

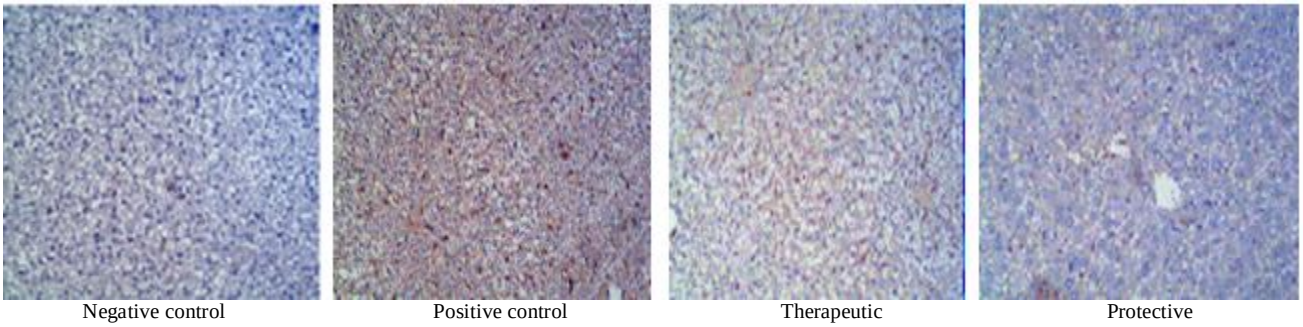
No.	Negative control group		Positive control group		Therapeutic group		Preventive group	
	Cat (pasma) U/ml	Cat (liver) U/gm	Cat (pasma) U/ml	Cat (liver) U/gm	Cat (pasma) U/ml	Cat (liver) U/gm	Cat (pasma) U/ml	Cat (liver) U/gm
1	304.00	3.30	83.00	1.50	174.00	3.20	176.00	3.60
2	351.00	4.60	91.00	0.90	182.00	1.80	165.00	2.00
3	330.50	3.90	112.00	1.60	191.00	2.40	183.00	2.30
4	323.00	2.70	94.00	1.90	205.00	3.00	151.00	3.30
5	301.00	4.20	73.00	0.80	155.00	2.90	165.00	3.00
6	335.00	3.40	54.00	1.80	141.00	2.80	154.00	3.20
Mean ±S.D.	3241.12±18.9	3.70±0.68	84.76±19.78	1.41±0.48	174.40±23.53	2.68±0.51	165.62±12.15	2.91±0.64
%Change					105.74%	90.07%	95.39%	106.38 %

Numerical data were expressed as mean ± SD. Preventive and therapeutic groups were compared to positive control group.
*P value <0.05 was considered significant. ** P value <0.01 was considered highly significant. *** P value <0.001 was considered very highly significant.
N.S. P value >0.05 was considered non significant

Table. 5: Caspase-3 in plasma and liver of studied groups.

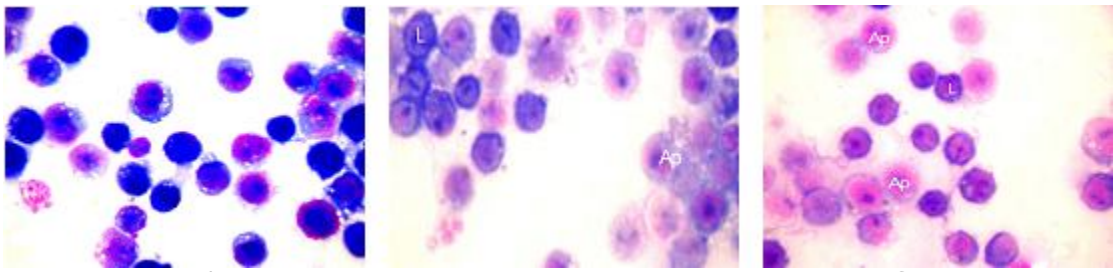
Mice no.	Positive control group caspase-3 (ng/ml)	Therapeutic CG caspase-3 (ng/ml)	Preventive CG caspase -3 (ng/ml)
1	0.40	0.33	0.80
2	0.20	0.50	0.80
3	0.32	0.40	1.00
4	0.20	0.31	0.70
5	0.40	0.43	0.90
6	0.20	0.42	0.80
mean±S.D.	0.28±0.09	0.39±0.06**	0.83±0.10***
%Change	-	39.28%	196.42%

Numerical data were expressed as mean ± SD. Preventive and therapeutic groups were compared to positive control group.
*P value <0.05 was considered significant. ** P value <0.01 was considered highly significant. *** P value <0.001 was considered very highly significant. N.S. P value >0.05 was considered non significant



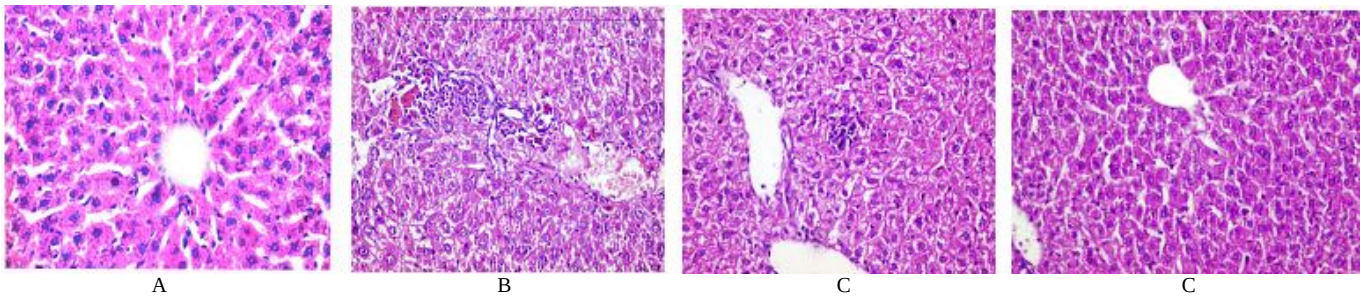
Negative control Positive control Therapeutic Protective

Fig. 5: Photograph represents survivin expression in liver of all studied groups.



A B C

Fig.(6): A: PHOTOMICROGRAPHS OF EAC CELLS OF POSITIVE CONTROL GROUP
B:Photomicrographs of EAC cells of Therapeutic group(L= Life cell ; Ap = Apoptotic cell).
C:Photomicrographs of EAC cells of Preventive group(L= Life cell ; Ap = Apoptotic cell).



A B C C

Fig. 7: A: Negative control group: Normal blood vessel and normal nucleus and hepatocytes are radiating outward from a central vein in the center, Hx & E X 200. B: Positive control group:Cellular inflammatory infiltration, congestion in blood vessels, hyperchromatinea ,nuclear hypertrophy , debris in the central vein ,haemorrhage and wide sinusoids. Hx & E X 200. C: Therapeutic group: Cytoplasmic degeneration have been reduced, mild cellular inflammatory infiltration and the nuclei of hepatic cells are better, Hx & E X 200. D: Preventive group: Normal array of the hepatic cords radiating from the central vein, there is no appearance of cellular inflammatory infiltration ,the cytoplasm is intact with normal eosinophelia and the nuclei is similar to control ones with usual chromatinophelia, Hx & E X 200.

From the results showed in Table 2 it was cleared that EAC induced oxidative stress caused enhanced lipid peroxidation. Preventive and therapeutic groups showed significant degree of protection against oxidative damage caused by EAC by decreasing lipid peroxidation in comparison with positive control mice, as it was observed that balanitoside has lowered the MDA level by 48.28% in liver and 55.12% in serum of preventive group in comparison with positive group and have lowered the MDA level by 44.70% in liver and 60.15% in serum of therapeutic group in comparison with positive group. Hence, it may be possible that balanitoside decreases lipid peroxidation level due to its antioxidant effect. Our results are in agreement with Mayba et al. (2011) who reported that the increase in malondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals in CCl4 induced hepatotoxicity in rats . Treatment with ethanol extract of *Balanites aegyptiaca* significantly reversed these changes, and it may be possible that the mechanism of hepatoprotection by ethanol extraction of *Balanites aegyptiaca* is due to its antioxidant effect.

Another study was carried by Sivakumar et al. (2011) who stated that methanol extract of *Triumfetta rhomboidea* exhibited significant antitumor and antioxidant activity *in vivo* in EAC bearing mice, biochemical parameters (lipid peroxidation, antioxidant enzymes) were estimated . Treatment with *Triumfetta rhomboidea* decreases the levels of MDA. Methanol extract of *Lippia nodiflora* (MELN) was evaluated for antitumor activity against Ehrlich's ascites carcinoma (EAC) bearing Swiss albino mice . MELN significantly decreased the levels of lipid peroxidation that indicate MELN exhibited significant antitumor activity (Ashokkumar et al., 2009).

The effect of *Symplocos racemosa* also decreases the level of lipid peroxidation in Ehrlich ascites carcinoma (EAC) bearing swiss albino mice which indicates that the *Symplocos racemosa* exhibited antitumor effect by modulating lipid peroxidation in EAC bearing mice (Vijayabaskaran et al., 2010).

Also the enhanced antioxidant status of *Ziziphus mauritiana* was evident from decline in level of lipid peroxidation (Tulika et al., 2011) as well as *Artemisia nilagirica* decreases lipid peroxidation level as compared to EAC control mice. This indicates that the *Artemisia nilagirica* showed defense mechanism against free radicals (Ashokkumar et al., 2009).

Also Mohan et al. (2011) stated that the saponins of ethanol extract of *Balanites aegyptiaca* was able to stabilize reactive oxygen species by reacting with them and oxidizes subsequently to more stable and less reactive radicals. In this respect saponins in *Balanites aegyptiaca* play an important role as antioxidant for prevention of oxidative damage.

The excessive production of nitric oxide (NO) in the cell may be due to viral or bacterial infections and can promote pathogenesis by promoting oxidative stress, tissue injury and even cancer. There is some evidence that NO production inhibitory activity of saponins is correlated with their cytotoxicity and anti-inflammatory activity (Rathee et al., 2009). Our data showed in Table 1 and Fig 5 a and b that balanitoside has significant inhibition on NO formation that might be due to its anti-inflammatory effect, as it was observed that balanitoside lowered the NO level by 36.33% in liver and 53.86% in serum of preventive group in comparison with positive group and lowered the NO level by 23.10% in liver and 51.40% in serum of therapeutic group in comparison with positive group.

As in another plant study, it is indicated by Hyun et al. (2004) that methanol extract of *A. quinata* showed potent activity against the cancer cell lines. Further examination on their inhibitory effect on the formation of NO showed that it exhibited a significant inhibitory effect on nitric oxide production which confirms A. *quinata* is a predominant anti-inflammatory saponin.

Also, it was reviewed by Sei et al. (2005) that saponin in *P. kamtschaticum* is used traditionally to treat cold, atherosclerosis, arthritis and to overcome fatigue. Since that time it was suggested that those diseases are primarily associated with excessive NO formation, and that *P. kamtschaticum* has inhibitory effect on NO formation which is associated with its putative anti-inflammatory effect.

Catalase is a hemoprotein and it protects cells from the accumulation of H_2O_2 and able to prevent the tissue from reactive free oxygen and hydroxyl radicals, by catalysing the reduction of H_2O_2 to form H_2O and O_2 (Perumal et al., 2010) One of the well-known biochemical changes that occur in cancer patients and tumor-bearing animals is a marked decrease in catalase activity (Yasmineh and Theologides., 1993). Therefore we studied the catalase activity in negative,positive, preventive and therapeutic groups to know the effect of balanitoside in the recovery of tumored mice. It was revealed from Table 1 and Fig 7 a and b that balanitoside extract from *Balanites aegyptiaca* was able to prevent effectively the decrease in CAT activities, in preventive and therapeutic mice as compared to positive control mice, as it is cleared that balanitoside has raised the CAT activity by 106.38% in liver and 95.39% in plasma of preventive group in comparison with positive group and have raised the CAT activity by 90.07% in liver and 105.74% in plasma of therapeutic group in comparison with positive group. That may be directly correlated to scavenging or neutralizing of radicals by balanitoside resulting in protection of this important defense enzyme. Sivakumar et al. (2010) reported that methanol extract of *Triumfetta rhomboidea* increased the level of catalase (CAT) in EAC bearing mice which mean that *Triumfetta rhomboidea* exhibited significant antitumor and antioxidant activity *in vivo*. Also it is reviewed that EAC inoculation markedly suppressed catalase level, treatment with 2-nitroimidazole showed antitumor activity through their significant recovery, as it significantly increased CAT level (Abu-Zeid et al., 2000). Methanol extract of *Lippia nodiflora* (MELN) significantly increased the level of CAT in EAC bearing Swiss albino mice which indicates that MELN exhibited significant antitumor activity (Ashokkumar et al.2009). *Symplocos racemosa* increased the level of catalase (CAT) in Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice which indicates that the *Symplocos racemosa* exhibited antitumor effect by augmenting anti-oxidant

defense system in EAC bearing mice (Vijayabaskaran et al., 2010). The enhanced antioxidant status of *Ziziphus mauritiana* was evident from increased level of catalase in EAC bearing mice (Tulika et al., 2011). *Artemisia nilagirica* increases catalase (CAT) level as compared to EAC control mice. This indicates that the *Artemisia nilagirica* showed defence mechanism against free radicals (Perumal et al., 2010). It was reported that plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells (Putul et al., 2000) and antitumor activity in experimental animals. Anti-tumor activity of these antioxidants is either through induction of apoptosis or by inhibition of neovascularization (Ravid et al., 2003).

Saponins are potential anticancer agents, with different mechanisms of action. Their cytotoxic effects may be due to either apoptosis inducement or non-apoptotic cell death stimulation (Sun et al., 2009).

Immunohistochemical investigation of survivin expression in liver showed that there was an extremely significant difference between the preventive group and the positive control group. The therapeutic group showed a significant difference with the positive control group. Finally, the preventive group demonstrated no significant difference with the negative control group as shown in Fig (5).

Survivin is a unique member of the inhibitor of apoptosis (IAP) protein family that interferes with post-mitochondrial events including activation of caspases , survivin is abundantly expressed in the embryonic tissues and in most of the tumors ,but in normal differentiated cells it is almost absent (Sah et al ., 2006).

Irma et al. (2010) reviewed that one of the well-known mechanisms of saponins as cytotoxic agents is Caspase 3 activation and survivin downregulation as an apoptosis intrinsic pathway.

Our results showed in Fig. 5 that there is down regulation of survivin in both therapeutic and preventive group in comparison with positive control group as there was an extremely significant difference between the preventive group and the positive control group. The therapeutic group showed a significant difference with the positive control group. Finally, the preventive group demonstrated no significant difference with the negative control group.

This is in agreement with Peter et al (2006) who stated that DMC (2,5-Dimethyl-celecoxib) might exert its cytotoxic anti-tumor effects via the down-regulation of survivin, also Tang et al (2009) stated that Ursolic acid is a potent anticancer agent, acting through activation of caspase-3, and is highly correlated with inactivation of survivin pathway and Xiao-Dan (2010) reviewed that Tan-I (Tanshinone I) demonstrates apoptosis-inducing effects on monocytic leukemia cells by activation of caspase-3 as well as down-regulation of survivin, these results indicate that Tan-I may be an effective anti-leukemia reagents. These data suggested that balanitoside is a potent anticancer agent exert its effect by down-regulation of survivin. Diosgenin, a naturally occurring steroidal sapogenin, has been suggested to play an important role in anticancer protection. Cytotoxic activity of the compound is mainly due to its inhibition of cell cycle signaling and induction of apoptosis (Raju and Mehta, 2009). Our data in Table 1 and Fig 8 showed that balanitoside has inducible effect on caspase 3 as it was observed that balanitoside has increased the activity of caspase 3 by 196.42% and 39.28 % in preventive and therapeutic mice groups respectively in comparison with EAC bearing mice which may be due to the induction of apoptosis by balanitoside. So we assume that balanitoside as a diosgenin may induce cell cycle arrest and apoptosis in EAC cells.

Elie et al., (2010) stated that treatments of HT-29 human colon cancer cells with 0.5,3, and 5 mMol/L SAP-1016 (saponin extracted from *Balanites aegyptiaca*) and dioscin (a known potent cytotoxic spirostane saponin which is a well known anti-cancer agent), for 24h generated caspase-3 cleavage , thereby inducing apoptosis activation. In HT-29, the caspase-3 activation that was observed following treatments with SAP-1016 resembled the activation observed following treatment with 5 mM dioscin. It has been concluded; therefore that SAP-1016 induces apoptosis *via* caspase-3 activation in HT-29cells as dioscin.

It is notable that antiproliferative effects of diosgenin are mediated through cell cycle arrest, the activation of p53, release of apoptosis-inducing factor, and modulation of caspase-3activity (Corbiere et al., 2004)which may be the same mechanism probably works for balanitoside.

The cytological examination of EAC cells (positive control group) revealed a significant increase in number of mitotic cells Fig (6a) while the cytological examination of EAC cells in both preventive and therapeutic groups Figs (6b) and (6c) respectively revealed a significant decrease in number of mitotic cells compared to positive control group. Also, the microscopic examination showed that the number of apoptotic cells were higher in the preventive and therapeutic groups. Moreover, there is no significant difference between preventive and therapeutic groups in the appearance and morphology of EAC cells.

The mentioned results were further supported by cytological examination. The cytological examination of EAC cells (positive control group) revealed a significant increase in number of mitotic cells Fig 6a .While the cytological examination of EAC cells in both preventive and therapeutic groups Fig 6b and 6c respectively, revealed a significant decrease in number of mitotic cells compared to positive control group. Also, the examination showed that the number of apoptotic cells were higher in the preventive and therapeutic groups. Moreover, there is no significant difference between preventive and therapeutic groups in the appearance and morphology of EAC cells. This is in agreement with Arindam et al. (2003) who stated that apoptosis is characterized by cell shrinkage, chromatin compaction, plasma membrane blebbing, DNA fragmentation and collapse of the cell into small intact fragments (apoptotic bodies). Also Paulo et al. (2006) reviewed that during apoptosis, cells displaying extreme condensation of the chromatin, fragmented or non-fragmented nuclei, preserved cytoplasm organelles were considered to be in early apoptosis, cells in late apoptosis presented similar

condensation of the chromatin, but without organelles and nuclear wrapper.

Histopathological results showed that negative control mice liver showing normal blood vessel and normal nucleus and hepatocytes are radiating outward from a central vein in the center as presented in Fig (7a). Positive mice liver showed cellular inflammatory infiltration, congestion in blood vessels, hyperchromatinea, nuclear hypertrophy debris in the central vein haemorrhage and wide sinusoids as presented in Fig (7b) Interestingly, treatment with balanitoside extract reduced most of the pathological alterations induced by EAC cells in mice.In preventive mice group, as a whole, the liver tissue showed a normal appearance to a large extent as reflected by normal array of the hepatic cords radiating from the central vein, there is no appearance of cellular inflammatory infiltration, the cytoplasm is intact with normal eosinophelia and the nuclei is similar to negative control ones with usual chromatinophelia as shown in Fig (7c). The therapeutic mice group liver showed enhancement in the histology of the liver and the cytoplasmic degeneration have been reduced. The mild cellular inflammatory infiltration and the nuclei of hepatic cells were better as shown in Fig (7d).

As for the histopathological examination, there was diminishing in pathological structure to a great degree, towards normal intact histological structure, as for control mice liver shows normal blood vessel and normal nucleus as presented in Fig 7a. Positive mice liver showing cellular inflammatory infiltration, congestion in blood vessels, hyperchromatinea, nuclear hypertrophy, debris in the central vein haemorrhage and wide sinusoids as shown in Fig 7b. This is in agreement with Said and Hanafy, (2006) who stated that histopathological studies showed that EAC cells caused fatty degeneration, enlargement of liver cells nuclei and presence of necrosis. Also Amal et al. (2010) stated that microscopal examination of liver of Ehrliched mice, revealed thickening in hepatic capsule with inflammatory and pigmented cells as well as diffuse kupffer cells, proliferation in between the degenerated cytomegalic hepatocytes. Samia and Fatma, (2009) stated that liver section of mouse after EAC cells inoculation displayed hypertrophy of liver cells, necrosis and most of the hepatocytes have vacuolated cytoplasm with deeply stained pyknotic nuclei. For preventive mice group, as a whole, the liver tissue showed to a large extent a normal appearance reflected by normal array of the hepatic cords radiating from the central vein, there is no appearance of cellular inflammatory infiltration, the cytoplasm is intact with normal eosinophelia and the nuclei is similar to control ones with usual chromatinophelia as shown in Fig 7c. As for the therapeutic mice liver shows enhancement in the histology of the liver the cytoplasmic degeneration have been reduced, mild cellular inflammatory infiltration and the nuclei of hepatic cells are better as shown in Fig 7d. In another study, ehrliched mice treated with *Zizyphus* showed that there was a diminishing in pathological structure, to a great degree, towards normal intact histological structure (Mosaad et al., 2007). Also it is showed that animals treated with *Zizyphus* extract had a significant improvement in histological feature of liver because of containing antioxidant principles that were cytotoxic towards tumor cells (Ruby et al., 1995). And we suggest that balanitoside may enhance the liver condition according to the same mechanism.

The present study suggested that balanitoside exhibits potential antitumor and antioxidant activities *in vivo* and it possess the apoptosis mechanism, which is preferable because eliminating tumor cells by apoptosis is helpful to lower the side effects in patients by avoiding necrosis (Shuli et al., 2010).

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