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Research Article
Mechanism of Anti-Angiogenic and Renal Protective Activity of *Balanites aegyptiaca* Seeds Extract in Ehrlich Ascites Carcinoma-Bearing Mice

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Abstract

Background: One of the animal angiogenesis with high inflammation and rapid growth is Ehrlich ascites carcinoma. Balanites aegyptiaca seeds extract (BASE), a new synthesized compound has antioxidant and antidiabetic activity. The purpose of this study was to evaluate anti-angiogenic activity of BASE in ehrlich ascites carcinoma (EAC)-bearing mice. **Materials and Methods:** BASE was prepared and characterized using instrumental analysis and spectral data. Furthermore, the IC_{50} of BASE against the renal carcinoma cell line (RCC-949) was calculated. Adult albino mice weighing 25±5 g was used to assess the anti-angiogenic activity of BASE (100 and 200 mg kg^{-1} body weight) in EAC-bearing mice. **Results:** IC_{50} of BASE against the renal carcinoma cell line (RCC-949) was equal to 62.18 µg mL^{-1}. The daily oral administration of BASE at concentrations of 100 and 200 mg kg^{-1} body weight for 30 days to EAC-bearing mice resulted in a significant improvement in tumor volume and tumor weight, urea, creatinine, uric acid, TNF-α, NOx, TBARs, GSH, CAT, SOD, GPx and VEGF-C gene expression in EAC-bearing mice. Furthermore, BASE almost normalized these effects in renal histoarchitecture. **Conclusion:** The BASE has anti-angiogenic activity in EAC-bearing mice.

Key words: Anticancer, balanites aegyptiaca, ehrlich ascites carcinoma, mice, renal carcinoma cell line

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ehrlich ascites carcinoma cells (EAC) are a common tumour that is an undifferentiated carcinoma with high transplantable capability, no regression, rapid proliferation, shorter life span, 100% malignancy, and no tumor-specific transplantation antigen (TSTA)[1].

Oxidative stress participate in the progression stage of cancer and tumors process[2]. Because of this, ROS can cause damage or breach to the double chain macromollecules, alterations in Guanin and Thymine bases, and the synthesis of malondialdehyde mutations[3]. Antioxidants protect the body from the harmful effect of free radicals and ROS[4].

Furthermore, the Ehrlich carcinoma model is a breast cancer that is spread from one mouse to another via intraperitoneal passages[5,6]. In many chemotherapeutic research, Ehrlich models are utilized to create solid tumors by injecting tumour cells subcutaneously or ascetic tumors by injecting tumour cells intraperitoneally[7,8].

Despite considerable advancements in cancer treatment, the global prevalence of cancer continues to rise[9,10]. Cancer prevention using naturally occurring food components, on the other hand, has been deemed a feasible way to reduce the ever-increasing cancer incidence[11]. Some antioxidants produced in the body (endogens) and others derive from diet (exogenous) are included in these systems[12]. Vinca alkaloids, taxans, camptothecins and epipodophyllotoxins are some plants which have anticancer properties[13]. Balanites aegyptiaca contains high level of soluble tannins[14], polyphenols[15,16] and flavonoids[17], which are reactive and excellent antioxidant agents[18,19]. These reports prompted us to investigate other physiological and pharmacological functions of Balanites aegyptiaca. As an extension of our research on the biological value of neutral products[20-25], this study was designed to evaluate anti-angiogenic and renal protective activity of Balanites aegyptiaca seeds extract in Ehrlich ascites carcinoma-bearing mice.

MATERIALS AND METHODS

Materials: EAC cells were obtained from Cairo's Cancer Institute. In Swiss albino mice, the cells were maintained *in vivo* via intraperitoneal transplantation (2×10^6 cells per mice) into all groups except the first group[26].

Plant material: Balanites aegyptiaca seeds were purchased from the local market in Cairo. Authentication of the plant was carried out by prof. Heba A. Elgizawy, Faculty of Pharmacy, October 6 University.

Preparations of the water extract: Only 0.5 kg of air-dried crushed Balanites aegyptiaca seeds were extracted using hot water (in ratio 1:10). The extraction was carried out at 50°C for 2 h with stirring at regular intervals. It was then filtered and evaporated to dryness under reduced pressure to yield viscous mass. The extract was kept in airtight containers in a deep freeze maintained at 4°C until the time of further use.

Determination of BASE cytotoxicity on renal carcinoma (RCC-949) cell line: To form a full monolayer sheet, the 96 well tissue culture plate was inoculated with 1×10^5 cells mL^{-1} (100 uL $well^{-1}$) and incubated at 37°C for 24 h. The growth medium was decanted from 96 well microtiter plates after forming a confluent sheet of cells and washed two times with washed medium by the cell monolayer. The sample was double diluted in the medium of RPMI with 2% serum (maintenance medium). Only 0.1 mL of each dilution was tested in each well, with 3 wells serving as controls and maintenance. The plate was incubated and checked at 37°C.

Physical symptoms of toxicity, such as partial or total monolayer loss, rounding, shrinkage, or cell granulation, were examined in the cells. The MTT solution (5 mg mL^{-1} in PBS) was prepared (BIO BASIC CANADA INC). Each well received 20 uL of MTT solution. MTT was placed on a shaking table for 5 minutes at 150 rpm to thoroughly mix the it into the media. To allow the MTT to be metabolized, incubate for 1-5 h at 37°C and 5% CO_2. Excluding the media from the equation. (If possible, dry plate with paper towels to remove residue). In 200 uL DMSO, resuspend formazan (MTT metabolic product). Formazan was placed on a shaking table at 150 rpm for 5 min to thoroughly mix it into the solvent. At 560 nm, read the optical density and deduct the history at 620 nm. The optical density should be proportional to the number of cells.

Mice: The Animal Care and Use Committee of October 6 University developed guidelines for this experiment, which were followed. Adult mice weighing about 25 ± 2 g were purchased from Cairo University, Faculty of Veterinary Medicine. They were housed in air-conditioned cages at a temperature of 22°C, a relative humidity of 60%, and a light period of 8:00 to 20:00. Feed and water was provided *ad libitum* during the acclimatization period.

Experimental design: The animals were divided into 5 groups consisting of 6 animals, two controls groups and three treatment groups. Description of treatment group is presented in Table 1.

Six mice from each group were dissected on the 31st day, 24 h after the injection, all mice were sacrificed at the end of

Table 1: Description of treatment groups

Group	Group name	Treatment description
I	Normal control A	3 mL of distilled water, orally for 30 days
II	EAC control	Subcutaneous injection of 2×10^6 cells mice^{-1} in water
III	EAC+BASE	Subcutaneous injection of 2×10^6 cells mice^{-1}+Balanites aegyptiaca seeds extract (BASE) (100 mg kg^{-1} body weight) suspended in aqueous solution in a single daily dose[19,22] in drinking water, orally for 30 days
IV	EAC+BASE	Balanites aegyptiaca seeds (BASE) (200 mg kg^{-1} body weight) suspended in aqueous solution in a single daily dose[19,22] orally for 30 days
V	EAC+5FU	Intraperitoneal injection of 20 mg kg^{-1} body weight 5FU by on alternate days for 30 days in a single daily dose[27]

the experiment. Blood was collected, centrifuged, and plasma urea[28], creatinine[29], uric acid[30] were determined. The tumour mass was removed from each mice in groups (II-V) to estimate its weight. Also, ascites fluid was extracted from the peritoneal cavity and measured using a graduated centrifuge tube[27,31].

Renal TBARS[32], Nitric Oxide (NOx)[33], tumour necrosis factor (TNF-α)[34] were determined using enzyme-linked immunoassay (ELISA) (14780 Memorial Drive Suite 216, Houston, Texas). Also, renal GSH[35], superoxide dismutase (SOD)[36], glutathione peroxidase (GPx)[37] and (CAT)[38] activities were measured by commercial RANSEL kits (Randox Laboratories, Crumlin, Northern Ireland, UK).

Quantitative real-time PCR: The total RNA was extracted from the renal of the mice, and portions (10-15 µg) of the isolated RNA were subjected to quantitative PCR analysis in real time, using Sepasol-RNA1Super according to instructions of the manufacturer. The two-step RT-PCR gene expression was measured. The level of VEGF-C was quantified with the previously described quantitative real-time PCR. The tests were conducted in 50 mL single-plex reaction mixture. Conditions of reaction were a pre-incubation at 50°C in 2 min, followed by 10 min by 40 cycles at 95°C in 15 s and at 60°C in 1 min, respectively.

The primer sequences were VEGF-C: F 5-AACGTGTCC AAGAAATCAGCC-3, R: 5-AGTCCTCTCCCGCAGTAATCC-3. The internal control used GAPDH-F: 5-CTCAACTACATGGTCTACA TGTTCCA-3 and -R:5 -CCATTCTCGGCCTTGA-CTGT-3'.

Histological assessment: The renal tissue was sliced and parts were fixed in histologic solution of 10% formaldehyde buffered. Only 5 µm thick renal tissue was stained with hematoxylin eosin (HE) and examined by light microscopic according to the method of Bancroft and Steven[39].

Ultrasound protocol: Mice were examined at Smart Scan Radiology Center-Cairo, Egypt; all experimental Ethics procedures were achieved. Once placed on the handling platform, each mouse was fixed in a supine recumbent position, the abdominal area was shaved to reduce imaging artifact. A conducting gel was applied to the area and the procedure was done using a multi-frequencies linear transducer (7-12 MHZ).

- The gel helps the transducer makes close contact with the body eliminating air pockets between the transducer and the skin that can block the sound waves to pass into the body. The probe was used on the abdomen and moved back and front over the abdomen until the interested images were captured
- Doppler study was also performed for diagnosis using the same transducer to get more details about the lesions vascularization
- Renal Images were stored on the ultrasound machine including images of all groups

Statistical analysis: Data were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test using the SPSS 18.0 Statistical Software Program (SPSS, Inc., IBM, Chicago, Illinois, USA). Differences of $p<0.05$ were considered statistically significant.

RESULTS

Figure 1 shows that the IC_{50} of BASE against renal carcinoma (RCC-949) cell line was equal to 62.18 µg mL^{-1}.

Oral administration of BASE (100 and 200 mg kg^{-1}) and intraperitoneal injection of 5-fluorouracil showed a significant decrease ($p< 0.05$) in tumour volume and weight compared to the mice bearing EAC (Table 2). The decrease was observed in tumour volume and weight in group of mice fed diet supplemented with BASE compared to 5-fluorouracil.

Table 3, 4 and 5 shows a significant increase in plasma urea, creatinine and uric acid, TNF-α, NOx, and TBARs, and a decrease in CAT, SOD, GPx, and GSH in renal tissue of mice with EAC compared to the standard nonbearing EAC group ($p<0.05$). Oral administration of BASE (100 and 200 mg kg^{-1}) and intraperitoneal injection of 5-fluorouracil significantly decreased urea, creatinine and uric acid and increased CAT, SOD and GPx, GSH levels ($p<0.05$) as compared to mice bearing-EAC.

Table 2: Effect of BASE and 5-fluorouracil (5FU) on tumor volume and tumor weight

No.	Groups	Tumor volume (mL)	Tumor weight (g)
I	Normal group (Non-tumor bearing mice (NTB)	0.00±0.0[a]	0.00±0.00[a]
II	EAC control (tumor bearing mice (TB)	2.10±0.11[e]	1.70±0.25[e]
III	EAC+BASE (100 mg kg^{-1} body weight)	1.34±0.15[d]	1.20±0.13[d]
IV	EAC+BASE (200 mg kg^{-1} body weight)	0.98±0.11[b]	0.52±0.08[b]
V	EAC+5-Fluorouracil (20 mg kg^{-1} body weight)	0.78±0.20[c]	0.65±0.22[c]

5-Fluorouracil was given i.p. as a daily dose of 20 mg kg^{-1} body weight. It was given to all groups except the normal and control one. The tested BASE was orally given daily for 4 weeks at concentration of 100 and 200 mg kg^{-1} body weight Results are presented as mean±standard deviation for groups of six animals each. Values followed by the same letter are not significantly different at $p \leq 0.05$

Table 3: Effect of BASE and 5FU on plasma urea, creatinine and uric acid in mice bearing ehrlich ascites carcinoma

Groups	Treatment description	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})	Uric acid (mg dL^{-1})
I	Normal group (Non-tumor bearing mice (NTB)	35.4±4.98[a]	0.56±0.11[a]	3.11±0.31[a]
II	EAC control (tumor bearing mice (TB)	72.6±7.65[d]	1.54±0.32[c]	5.63±0.41[b]
III	EAC+BASE (100 mg kg^{-1} body weight)	38.70±3.22[b]	0.99±0.08[b]	3.26±0.28[b]
IV	EAC+BASE (200 mg kg^{-1} body weight)	33.76±3.87[a]	0.68±0.09[a]	3.00±0.12[a]
V	EAC+5-Fluorouracil (20 mg kg^{-1} body weight)	48.09±4.09[c]	0.93±0.08[b]	3.29±0.43[a]

Results are presented as mean±standard deviation of number of observations within each treatment. Values followed by the same letter are not significantly different at $p \leq 0.05$

Table 4: Effect of BASE and 5FU on levels of renal tumour necrosis factor (TNF-α), Nitric Oxide (NOx), thiobarbaturic acid reactive substances (TBARs) and reduced glutathione (GSH) in mice bearing ehrlich ascites carcinoma

Groups	Treatment description	TNF-α (pg mg^{-1} protein)	iNOs (pg mg^{-1} protein)	TBARs (nmol mg^{-1} protein)	GSH (mg%)
I	Normal group (Non-tumor bearing mice (NTB)	97.50±6.44[a]	42.78±4.5[a]	4.26±0.42[a]	6.90±0.41[d]
II	EAC control (tumor bearing mice (TB)	198.45±8.70[e]	84.33±6.21[d]	9.38±0.30[e]	3.11±0.22[a]
III	EAC+BASE (100 mg kg^{-1} body weight)	124.40±10.96[d]	65.43±5.52[c]	5.28±0.51[c]	5.32±0.40[b]
IV	EAC+BASE (200 mg kg^{-1} body weight)	110.54±9.75[b]	49.80±4.23[b]	4.71±0.22[a]	6.23±0.52[c]
V	EAC+5-Fluorouracil (20 mg kg^{-1} body weight)	118.30±8.74[c]	64.87±8.34[c]	8.27±0.63[d]	5.36±0.23[b]

Results are presented as mean±standard deviation of number of observations within each treatment. Values followed by the same letter are not significantly different at $p \leq 0.05$

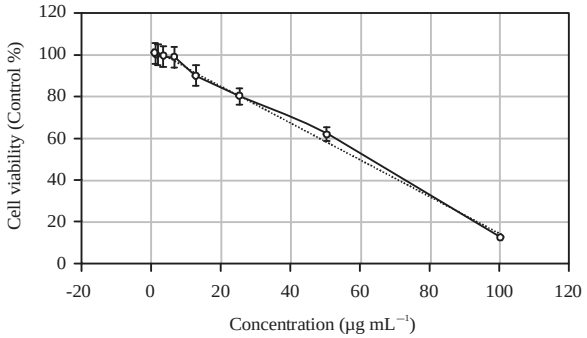

Fig. 1 : IC_{50} of BASE against renal carcinoma (RCC-949) cell line

Subcutaneous EAC implantation significantly ($p<0.05$) decreased the levels of renal vascular endothelial growth factor C (VEGF-C) gene expression in mice as compared to normal control group. BASE at concentrations of 100 and 200 mg kg^{-1} and intraperitoneal injection of 5-fluorouracil significantly increased the levels of VEGF-C gene expression ($p<0.05$) in non-bearing EAC mice as compared to EAC-bearing mice (Fig. 2).

Histopathological examination of renal sections of the normal group (I) showed normal renal parenchyma; note the normal renal glomeruli (g) and renal tubules (t), (H and E×400) (Fig. 3a).

On the other hand, in the renal of EAC-bearing control group (II), histological examination of complete renal showed diffuse degeneration of the renal tubules (arrow) as well as leucocytic cells infiltrations (*), (H and E×400) (Fig. 3b). Histopathological examination also showed regression of the renal lesions and normal renal parenchyma; note the normal renal glomeruli (g) and renal tubules (t), (H and E×400) of EAC-bearing mice treated with BASE (100 mg kg^{-1} body weight) as compared to the EAC-bearing control group (Fig. 3c). Also, renal of EAC-bearing mice treated with BASE (200 mg kg^{-1} body weight) (group IV) showed apparently healthy renal glomeruli with slight congestion in the renal glomerular blood capillaries (arrows) and normal renal tubules with intact lining epithelium, (H and E×400) (Fig. 3d). In addition, all samples of EAC-bearing mice treated with 5-fluorouracil (20 mg kg^{-1}, i.p) (group V) showed moderate inflammation (×200H and E) (Fig. 3e).

Renal ultrasound of the normal group (I) showed that the renal parenchyma was regular, homogenous with normal echogenicity (Fig. 4a).

Also, the renal ultrasound imaging of EAC-bearing control group (II) showed a renal mass that appear as irregular, heterogenous, ill-defined with mixed contents, also ascites can be evaluated in some captions (Fig. 4b).

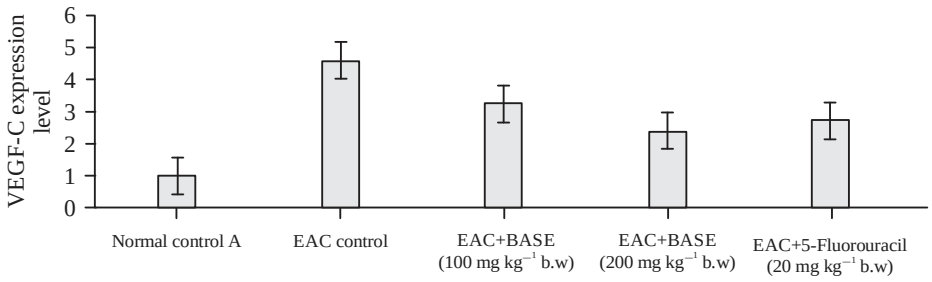

Fig. 2: Effect of BASE and 5FU on levels of renal vascular endothelial growth factor C (VEGF-C) gene expression in mice bearing ehrlich ascites carcinoma. Representative bar diagram of three independent experiments are presented

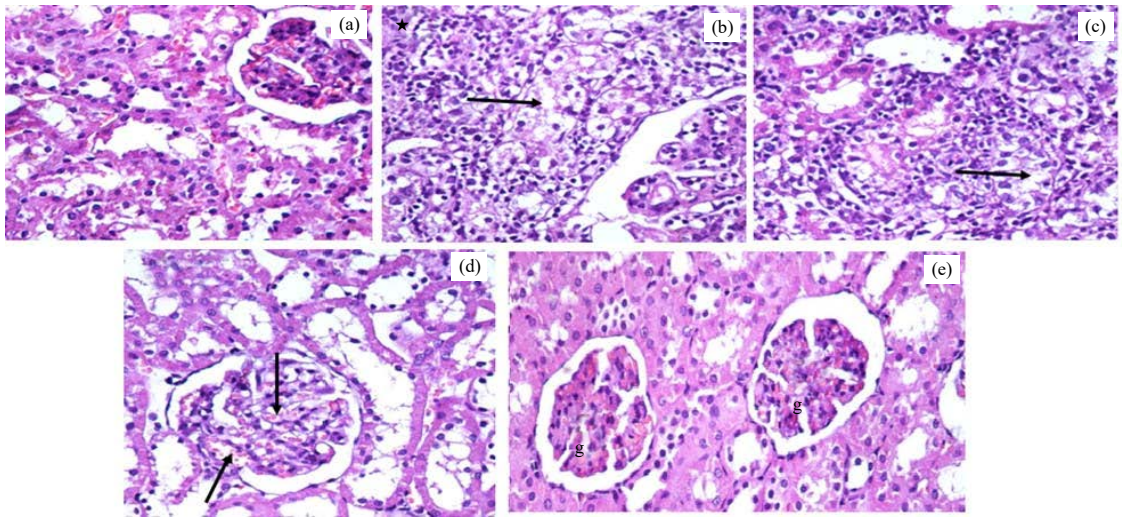

Fig. 3(a-e): Sections stained with hematoxylin and eosin (H and E, 400×) histological examination of mice' renal of different groups compared to control group, (a) Group I: Normal control, (b) Group II: EAC group, (c) Group III: EAC+BASE (100 mg kg^{-1} body weight) was administrated, (d) Group IV: EAC +BASE (200 mg kg^{-1} body weight) was administrated, (e) Group V: 5FU (20 mg kg^{-1} body weight)+EAC was administrated

Table 5: Effect of BASE and 5FU on levels of renal catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in mice bearing ehrlich ascites carcinoma

Groups	Treatment Description	CAT (Umol H_2O_2 consume mg^{-1} tissue)	SOD	GPx
I	Normal group (Non-tumor bearing mice (NTB)	23.67±3.22^{d}	2.84±0.27^{d}	13.56±1.44^{d}
II	EAC control (tumor bearing mice (TB)	11.76±0.87^{a}	1.66±0.21^{a}	3.22±0.36^{a}
III	EAC+BASE (100 mg kg^{-1} body weight)	18.70±1.87^{b}	2.25±0.32^{c}	8.58±0.86^{c}
IV	EAC+BASE (200 mg kg^{-1} body weight)	21.88±3.22^{c}	2.66±0.27^{d}	11.87±1.05^{cd}
V	EAC+5-Fluorouracil (20 mg kg^{-1} body weight)	10.76±1.43^{a}	1.98±0.18^{b}	5.70±0.74^{b}

Results are presented as mean±standard deviation for groups of six animals each. Values followed by the same letter are not significantly different at $p \leq 0.05$. SOD: one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min/mg protein; GPx: µg of GSH consumed min^{-1} mg protein.

Ultrasound also showed coarse texture and the regressed abnormal focal lesion of EAC-bearing mice treated with BASE (100 mg kg^{-1} body weight) as compared to the EAC-bearing control group (III) (Fig. 4c). In addition, renal ultrasound imaging of EAC-bearing mice treated with BASE (200 mg kg^{-1} body weight) (group IV) showed marked improvement with no inflammation which can be evaluated in some captions (Fig. 4d).

In addition, all samples of EAC-bearing mice treated with 5-fluorouracil (20 mg kg^{-1} i.p) showed regressed and moderate focal lesion (group V) (Fig. 4e).

DISCUSSION

The anti-angiogenic efficacy of BASE was studied in Ehrlich ascites carcinoma-bearing mice. A formazan test was

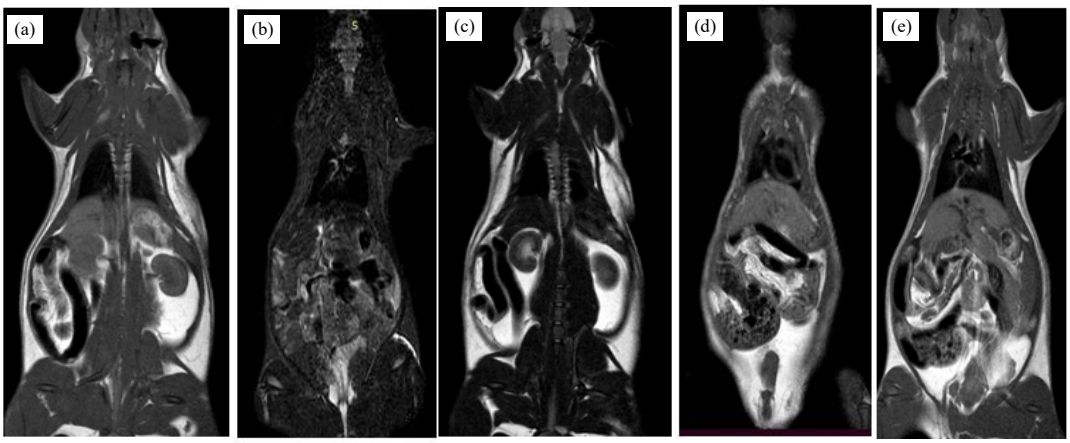

Fig. 4(a-e): Ultrasound examination using a multi-frequencies linear transducer (7-12 MHZ) of mice' renal of different groups compared to control group, (a) Group I: Normal control; (b), Group II: EAC group; (c), Group III: EAC+BASE (100 mg kg^{-1} body weight) was administrated, (d) Group IV: EAC+BASE (200 mg kg^{-1} body weight) was administrated, (e), Group V: 5FU(20 mg kg^{-1} body weight) +EAC was administrated.

used to test BASE's cytotoxic activity against the renal cancer (RCC-949) cell line. The BASE concentration versus cell viability curve was created by incubating the cell line with varied concentrations (0-100 μg mL^{-1}). At the end of the 48 h incubation period, the response parameter (IC_{50}) for each cell line was computed (Fig.1).

The best cytotoxic results were obtained with BASE because it contains high levels of soluble tannins[14], polyphenols[15,16] and flavonoids[17], which are reactive and promising antioxidant[18,19] and excellent in their use. A mixture of steroidal saponins [balanitin-6 (28%) and balanitin-7 (72%)], isolated from B. aegyptiaca kernels, demonstrated appreciable anticancer effects on human cancer cell lines in vitro by using against A549 non–small-cell lung cancer (IC_{50}, 0.3 μM) and U373 glioblastoma (IC_{50}, 0.5 μM) cell lines. Balanitin 6/7 (bal6/7) displayed higher antiproliferative activity than etoposide and oxaliplatin, markedly less active than taxol. It indicated that balanitin 6/7 mixture is more cytotoxic compound than cytostatic one. In vitro anticancer activities are due to partial depletion of ATP, leading to major disorganization of actin that does not induce an increase in intracellular reactive oxygen species and makes BASE work as DNA alkylators and produces its anti-angiogenic activity[40]. *In vivo*, bal6/7 increased the survival time of mice bearing murine L1210 leukemia grafts to the same extent reported for vincristine[41]. Our *in-vivo* results proved that the administration of BASE decreased the tumor volume and weight as compared to the EAC control group. On the other hand, reduction of tumor volume and weight indicated a decrease in abnormal cell divisions, *i.e.* tumor proliferation[42,43]. In this study, we observed that BASE can revert or inhibit EAC induced tumor[44] which may be due to its free radical scavenging properties[19,22]. Subcutaneous EAC implantation significantly increased NO, TNF-α, and TBARs, as well as significantly decreased GSH, GPx, SOD, and CAT in mice as compared to the standard control group. These findings agree with a previous study conducted by Elumalai and Arunakaran[45] who reported that the consumption of free amino acids for the protein synthesis of rapidly dividing tumour cells can disrupt renal enzyme activity.

The inhibitory effect of BASE due to presence of saponins showed significant anti-inflammatory, antinociceptive activity in the carrageenin-induced edema in rat, and acetic acid-induced writhing test in mice and antioxidant action by using a method based on the Briggs-Rauscher oscillating reaction. The samples, extracts and pure substances, were intragastrically administered to animals[46-48].

Furthermore, BASE inhibited the expression of VEGF and reduced tumor angiogenesis *in vivo*, which was associated with the decrease in NO, TNF-α, in renal tissues[49]. According to the structure activity relationship of BASE and their anti-inflammatory effects, Hussein *et al.*,[18,22] demonstrated that the antioxidant and antitumor activity of BASE is dependent on its structure function.

On the basis of the results of the present study, it can be stated that BASE has anti-angiogenic and antioxidant potential in EAC-bearing albino mice, which can be attributed to its structure-activity relationship.

The antioxidant activities were significantly correlated with the total phenolic and flavonoid contents. The study also showed that B. aegyptiaca galls and leaves fractions exhibited a moderate xanthine oxidase inhibitory activity compared to the acetylcholinesterase which was weakly inhibited by the tested extracts and fractions[50,51].

BASE have a renal-protective effect, according to histological examination and ultrasound imaging. Because renal inflammation is an early event in a cite condition, the administration of BASE improved the renal tissue in mice that could be associated with a reduction in inflammatory response in EAC bearing mice.

To the best of my knowledge, the prophylactic effect of BASE against EAC-induced renal toxicity has never been reported, and this study may be the first of its kind. This could pave the way for the development of newer, safer, and more powerful antitumor agent.

CONCLUSION

Biochemical analysis, histological assessment and ultrasound imaging discovered that BASE have anti-angiogenic activity in EAC-bearing mice by normalizing the levels of inflammatory mediator and VEGF gene expression.

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