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Indian toad (*Bufo melanostictus*, Schneider) skin extract induces apoptosis and shows cytotoxic effect on Ehrlich ascites carcinoma (EAC) cells

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ABSTRACT

Indian toad (*Bufo melanostictus*) skin extract (TSE) was evaluated for cytotoxic and apoptogenic activities on Ehrlich ascites carcinoma (EAC) cells of EAC bearing Swiss albino male mice. LD50 of TSE was found to be 400 mg kg^{-1}, (i.v.) and 750 mg kg^{-1}, (i.p.) in mice. EAC cells (1-2 × 10^5 / mouse) were inoculated (i.p.) into mice (20.6 ± 0.14 gm) and proliferation and viability of EAC cell were determined in TSE treated and control groups. TSE (10, 20, 30 mg kg^{-1} d^{-1}, i.p. for 10 d) significantly inhibit EAC cell growth in dose dependent manner. TSE (50, 100 mg kg^{-1} d^{-1}, i.p. for 1 d) significantly reduced the viability of EAC cells and decreased the MTT values compared to control cells. TSE induced DNA breakage was reflected in DNA ladder and single cell gel electrophoresis (comet assay). TSE significantly (P<0.001) increased the number tailed cells and length-width ratio of DNA mass in EAC cells as compared to control. Fluorescent microscopy of TSE treated cells showed significant increase in number of early and late apoptotic cells compared to the control EAC cells. Apoptotic index of TSE treated cells was significantly (P<0.001) higher than that of the control cells. Changes in serum LDH, β_2-microglobulin in TSE treated EAC mice and our earlier studies indicated the involvement of immunomodulation which may indirectly be associated with the anticancer activity of TSE.

Keywords: Toad skin extract; *Bufo melanostictus*; Ehrlich ascites carcinoma; Cytotoxic agent;

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1. INTRODUCTION

Cancer, the second killer disease of the world and its management is a major socio-medical problem. Although, several chemotherapeutic approaches have been taken to tackle the complexity of different cancer, the incidence and mortality rate for most form of cancer still remain high. Toxicity and resistance may represent the potential obstacles to successful clinical development. Search for novel and safe drug from natural plant products are ventured worldwide, which are showing promising results. Very few animal sources are under research as therapeutics of malignancies; toad skin component is one of them. The toad, though ugly and venomous, wear yet a number of precious bioactive jewels in the skin. Several host defence peptides are highly selective towards cancer cells whereas, they have no toxicity on normal mammalian cells. These peptides generally have diverse structural array, cationic nature and a large portion of hydrophobic amino acids [1]. The skin secretion of toad or/and frog has been used in therapeutic purposes by many different cultures around the world. Toads are well-known to be an affluent source of granular gland secretions, containing large amount of biogenicamines, alkaloids, steroids, peptides and proteins [2-4]. These diverse biomolecules play a potent character in the molecular drug development for pharmacological and toxicological evaluations and even

for drug synthesis and medicinal chemistry [5]. Previously, it was bring into being that the skin extract of *Bufo asper* have significant antimicrobial activity. From the proteomic study of the skin secretions, 50 proteins were identified having molecular weight between 20 to 250 kDa [6]. In traditional Chinese medicine toad skin is used in human therapy and widely applied in China, Japan, Korea and other Asian countries as an alternative medicine [7, 8]. Many studies have reported that toad skin extract and its components can inhibit cancer cell growth, migration and invasion via different mechanisms [9-11]. The Chinese frog, 'Chan sue' is used as a cardiotonic agent, treating arrthythmia and other heart diseases and certain cancer [7, 12]. 'Kyushin' is a traditional Chinese medicine (Chan sue) frequently used in Japan and it has demonstrated digoxin like effects [13]. The anticancer activities of frog and toad skin have recently been investigated [14, 15]. Very few studies on the anticancer activities of Indian toad skin extract have been reported till date. Earlier report from the present laboratory, suggested that the Indian toad (*Bufo melanostictus*) skin extract reduces EAC cell growth and possess immunomodulatory activity [16]. Another report from our laboratory confirmed the fact that an active component of skin extract of Indian toad (*Bufo melanostictus*) (BM-ANF1) showed dose-dependent inhibition of U937, K562 and HepG2 cell proliferation arresting at G1 phase of the cell cycle [17,18]. An additional research from this laboratory established the fact that, BM-ANF1 in combination with curcumin exerts a marked inhibition of colon cancer proliferation [19]. Furthermore, a low immunogenic protein toxin (BMP1) showed anticancer activity against EAC bearing mice, leukemic and hepatoma cells [20, 21] The present study reports the cytotoxic and apoptogenic properties of Indian toad skin extract (TSE) on Ehrlich ascites carcinoma (EAC) cells of the experimental EAC bearing mice.

2. MATERIALS & METHODS

2.1. Chemicals used

Acridine orange (Sigma, USA), DMSO (SRL, India), EDTA (Sigma, USA), Ethidium bromide (Sigma, USA), RPMI (GIBCO BRL, USA),Fetal bovine serum (GIBCO, BRL, USA), Penicillin-streptomycin (Biowest, Germany), Gentamicin (Nicholas, India), Heparin (Sigma, USA), Low melting point agarose (Promega, USA), Methanol (Spectrochem, India), MTT (Sigma, USA), Normal melting point agarose (Promega, USA), Triton X-100 (SRL, India), Trypan blue (SRL, India).

2.2. Animals used

Male albino mice (Swiss strain) having body weight 20.6 ± 0.14 gm were used for the experiments. The animals were housed in the departmental animal house (22 ± 2^{o}C, 60 ± 5% relative humidity) with food (Pellet, Ashirbad Industries, Chandigarh, India), soaked gram, water *ad libitum* and 12-12 h light and dark cycle. Animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and they were handled as per the guidelines in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Collection and preparation of Toad skin extract (TSE)

Adult toads (*Bufo melanostictus*, Schneider) of both sexes (80-100gm) were procured from M/S Reeta Ghosh & Company, Calcutta, India. Skin was collected from freshly pithed toads, washed in distilled water and kept immersed in methanol at room temperature for 30-40 days. The methanolic extract was then filtered, evaporated to become dry by rotary evaporator and the extract was stored at room temperature in a desiccator. The yield of the extract was 1.3 ± 0.46 % and was expressed in terms of dry weight. The extract (TSE) was suspended in 0.9% saline / phosphate buffer saline (pH 7.2) for the experiments.

2.4. Toxicity of TSE

Different concentrations (100, 200, 300, 400, 500, 600, 700, 800 mg kg^{-1}) of TSE were administered (i.v. / i.p.) in Swiss male albino mice (20-22 gm) and LD_{50} value was detected by plotting the mortality percentage of animals against the doses [16].

2.5. Transportation and maintainance of Ehrlich Ascites Carcinoma (EAC) Cells

Ehrlich ascites carcinoma (EAC) cells were collected from tumour bearing Swiss male albino mice at their respective log phase of growth. It was a gift from Indian Institute of Chemical Biology, Kolkata, India. A 5 ml syringe fitted with 20-gauge needle was used for this tumour cell aspiration. The freshly drawn fluid was diluted with phosphate buffer saline (PBS) (pH 7.4) and the number of tumor cells was adjusted to 1×10^{6} cells ml^{-1} by counting with a haemocytometer. The viability of tumour cells was checked by Trypan blue dye (0.4%) exclusion assay. Cell samples showing above 90% viability were used for transplantation. 0.1 ml of such suspension was injected intraperitoneally to each Swiss male mice. Aseptic condition was maintained throughout the transplantation process. The EAC cells were maintained with sub culturing each week.

2.6. EAC cell growth inhibition

Animals were inoculated (i.p.) with 0.1 ml of 2×10^{5} cells mouse^{-1} on day zero and TSE treatment was started after 24 hours of inoculation at the doses of 10, 20, 30 mg kg^{-1} d^{-1}, i.p. for 10 days. The control group was treated with the same volume of 0.9% saline. 5FU (10 mg kg^{-1} d^{-1}, i.p. for 10 days) was taken as standard. All the treatments were continued for 10 days. On the 11th day after transplantation, all the animals were sacrificed and tumour cells were collected by repeated intraperitoneal washing with phosphate buffer saline (pH 7.2). Tumour cell count was made from treated group and it was compared with that of the control group [23].

2.7. EAC cells viability assay

The procedure was a modification of the method of Fernandes and Klubes (1979) [22-23]. Control, treated and standard groups of mice (6 mice in each group) were inoculated with 0.1 ml of 2×10^{5} EAC cells on day zero.

On second day after intraperitoneal inoculation with EAC cells, mice received either (a) 0.9% saline (b) Toad skin extract (TSE) at a dose of (50, 100 mg kg^{-1} d^{-1}, i.p. for 1 d) and (c) 5FU (50 mg kg^{-1} d^{-1}, i.p. for 1 d). 24 hour after treatment, tumour cells from each group of mice were counted and harvested in cold saline, pooled, centrifuged and reinoculated (0.1 ml of 2×10^5 cells/ mouse) intraperitoneally into three groups containing 6 mice in each group. On the day seven after reinoculation, all experimental animals from each group were sacrificed and tumour cell count of each mice was estimated.

2.8. MTT assay

The chemosensitivity of the EAC cells from control (0.9% saline), treated (TSE 20, 30 mg kg^{-1} d^{-1} , i.p. for 5 days) and standard (5-FU, 10 mg kg^{-1} d^{-1}, i.p. for 5 days) group mice was determined by a quantitative colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT). The assay is based on the reduction of Tetrazolium salt by the mitochondrial dehydrogenase enzyme of intact cells to a purple formazan product [24]. The OD was recorded at 570 nm by a microtiter plate reader (Merck-MIOS mini, Model no.309).

2.9. Alkaline Comet assay

The alkaline comet assay of EAC cells was performed after TSE (30 mg kg^{-1} d^{-1}, i.p. for 5 days) treatment in treated group and saline (0.9%) treated control group [16, 25]. In brief, clean microscope glass slides were covered with 400µl of 0.75% normal melting point (NMP) agarose (Qualigen) in PBS pre warmed at 50^0C. A cover glass was positioned over the agarose solution, and allowed to be solidified. Then 85µl of cell-low melting point agarose (LMP) solution was placed over the first agarose layer and allowed to solidify under a clean cover glass. 100µl of 0.5% low melting point agarose was added further on NMP agarose layer again under a cover glass and allow to solidify at 4^0C. The slides were tenderly immersed in a freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10mM Tris, 10% dimethyl sulfoxide and 1% Triton X-100, pH adjusted to 10 with NaOH) and kept at 4^0C in the dark for 1 h. The slides were placed to the horizontal gel electrophoresis unit filled with fresh, cold electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13.5) for 20 min. Electrophoresis was conducted at 20Volt (1.0V/cm,175mA) for the next 20 min. The slides were then drained, and flooded slowly with three changes of neutralization buffer (0.4M Tris-HCl, pH 7.5), each for 5 min. The slides were stained with Ethidium bromide (10µg ml^{-1}), covered with a cover glass, and analyzed within 10 min at 100X magnification using a fluorescent microscope (Motic, Germany) with green filter. The photographs were taken through the attached CCD camera. The comet scores were recorded by random counting about 100 cells per slide, considering no overlap of counting and the DNA comet tail length and width was measured using Motic Images Plus 2.0 software.

2.10. DNA fragmentation study by ladder formation

EAC cells were quantitatively collected in TSE (30 mg kg^{-1} d^{-1}, i.p. for 5 days) treated and saline (0.9%) control group. It was then washed with cold phosphate buffer saline (pH 7.4) two to three times and fixed with 70% ethanol. The cells were then lysed with 1.2 ml of SDS lysis buffer (10 mM Tris·HCl, 10 mM EDTA, 100 mM NaCl, 2% SDS, pH 8.0) containing 20 µL Proteinase K (20mg/ml) and incubated in a water bath at 56^0C overnight. Next day lysate was centrifuged and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and recovered by ethanol–sodium aetate precipitation. The nucleic acid pellet was dissolved in 200µL of Tris-EDTA buffer (10 mM Tris-HCl,10 mM EDTA), adjusted to 1 µg ml^{-1} RNase A, and incubated for 10 min at 37°C. DNA was then recovered by phenol extraction and ethanol precipitation. The final DNA concentration was determined by the optical density at 260 nm. The DNA chain break was analyzed by agarose gel electrophoresis with the 2 % gel and stained with ethidium bromide [26].

2.11. Fluorescent microscopy

The EAC inoculated mice were divided into two groups. In one group, TSE (30 mg kg^{-1} d^{-1}, i.p.) was treated for 5 days. Second group was considered as saline (0.9%) control. After 24 h of the last treatment, the control and TSE treated cells were washed with chilled PBS (pH 7.2) and the stained with ethidium bromide and acridine orange (100 mg ml^{-1} each in PBS). The apoptotic cells could be visualized by acridine orange while the viable and nonviable cells could be differentiated by ethidium bromide. Two hundred cells were counted and the cellular states were recorded. The apoptotic index was calculated using the following formula [27].

Apoptotic index = (Apoptotic cells/ Total cells)× 100

2.12. Serum LDH and β2-microglobulin of EAC bearing mice

Swiss albino Male mice (20±1 gm) were divided into four groups (Group 1-Normal control, Group 2- EAC control, Group 3- TSE control and Group 4-EAC + TSE treated) in two sets (I & II).

After inoculating with EAC cells, 3rd and 4th group were treatment with TSE (20 mg kg^{-1} d^{-1}, i.p.) and the treatment continued for 7 days in set I and for 15 days in set II. Blood was collected aseptically from the retro-orbital plexus of mice and the following experiment was carried out in both the sets. Serum level of β2-microglobulin and lactate dehydrogenase (LDH) was estimated in all the groups of animals by enzymatic kinetic procedure using commercial kits (Boehringer-Manheim) and automated biochemical analyzer (Hitachi).

2.13. Statistical analysis

Values were expressed as mean ± SEM. Statistical analysis was done by one way Anova test and Student's *t* test, $P<0.05$ was considered significant. n = 6 in all the animal experiments, unless otherwise mentioned.

3. RESULTS

3.1. Toxicity of TSE

LD50 of TSE was found to be 400 mg kg^{-1} (i.v.) and 750 mg kg^{-1} (i.p.) in male albino mice.

3.2. Tumour cell growth inhibition

Mean percentile inhibition of EAC cell count after treatment with TSE (10 mg kg^{-1} d^{-1}, 20 mg kg^{-1} $d^{-1}$and 30 mg kg^{-1} d^{-1}, i.p. for 10days) treated groups were 33.1%, 51.4% and 73.2% respectively as compared to the control. Where as standard 5-FU (10 mg kg^{-1} d^{-1}, i.p., for 10 days) produced 55.2% inhibition of EAC cell count compared to control (Fig 1, Table 1)

Table 1: Effect of TSE on Body weight, EAC cell growth, EAC cell viability, Tumor volume, MTT value of EAC bearing mice.

Parameters	EAC Control	TSE (10mg/Kg) + EAC	TSE (20mg/Kg) + EAC	TSE (30mg/Kg) + EAC	5-FU (10mg/Kg) + EAC
Body weight (g)	28.6±0.17	26.4±0.13	24.5±0.18[a]	23.1±0.17[b]	22.4±0.12[b]
EAC cell count ($\times10^6$)	23.5±0.56	15.7±0.42[b]	11.4± 0.37[c]	6.3±0.23[c]	10.5±0.31[c]
Mean percentile inhibition of EAC cell growth (%)	00.0	33.1	51.4	73.2	55.2
EAC cell viablity (count$\times10^6$)	14.4±0.43	11.7±0.29[a]	8.9±0.16[c]	--	9.8±0.12[c]
*Reduction in viability (%)	00.0	18.5	38.0	--	31.4
Tumor volume (ml)	4.70±0.11	4.12±0.08	3.54±0.06[a]	2.20±0.03[b]	1.84±0.07[c]
MTT value (OD at 570nm)	0.104±0.002	--	0.068±0.006[b]	0.043±0.002[c]	0.069±0.005 [c]
Reduction in MTT value(%)	00.0	--	21.9	34.6	35.3

Values are expressed as Mean ± SEM, n=6, [a]P<0.05, [b]P<0.01, [c]P<0.001, TSE treated groups compared with EAC control group. *Viability was done at the dose of 50mg/Kg and 100 mg/Kg TSE and 50mg/Kg 5-FU, per day for 1 day, i.p. (Body weight of normal mice was 20.6 ± 0.14)

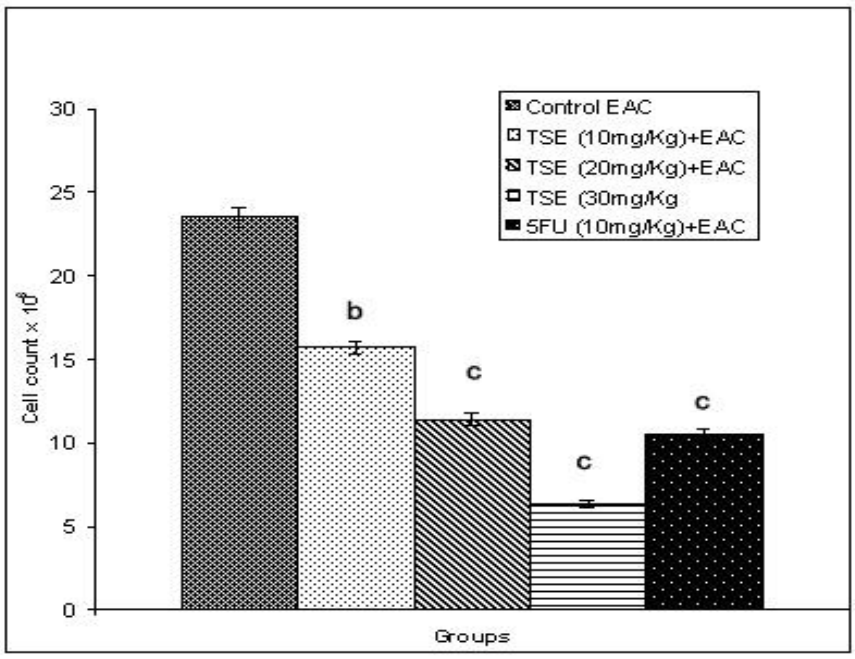

Figure 1: Effect of TSE on EAC cell growth inhibition of EAC bearing mice.

[b]P<0.01, [c]P<0.001, significant.

3.3. EAC cell viability

TSE treatment significantly decreased the EAC-cell viability. On 7th day after reinoculation the TSE (50 and 100 mg kg^{-1} d^{-1} , i.p. for 1 d) treated group showed 18.5% (P<0.05) and 38.0% (P<0.01) significant reduction in viability of EAC cells respectively compared to the control. Where as standard 5-FU (10 mg kg^{-1} d^{-1}, i.p., for 1 d) reduced the viability of EAC cells was about 31.4% (P<0.01) (Fig.2 ,Table 1).

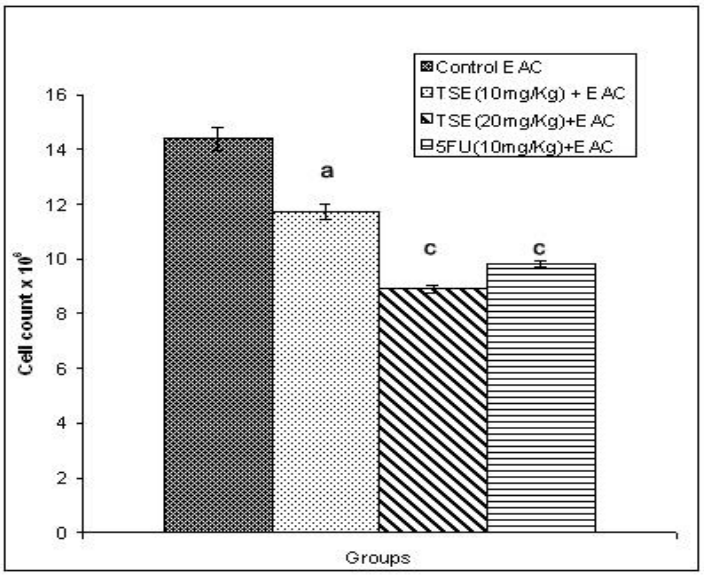

Figure 2: Effect of TSE on EAC cell viability of EAC bearing mice.

[a]P<0.05, [c]P<0.001, significant.

3.4. MTT assay

It was observed at 7th day that, standard 5 FU (10 mg kg^{-1} d^{-1}, i.p. for 5 days) could be able to reduced the MTT value of about 35.3% and TSE (20 and 30 mg kg^{-1} d^{-1}, i.p. for 5 days) showed 21.9 % and 34.6 % reduction in OD value respectively as compared with untreated control EAC cells. The OD of TSE treated cells was significantly greater than the control (Fig.3, Table 1).

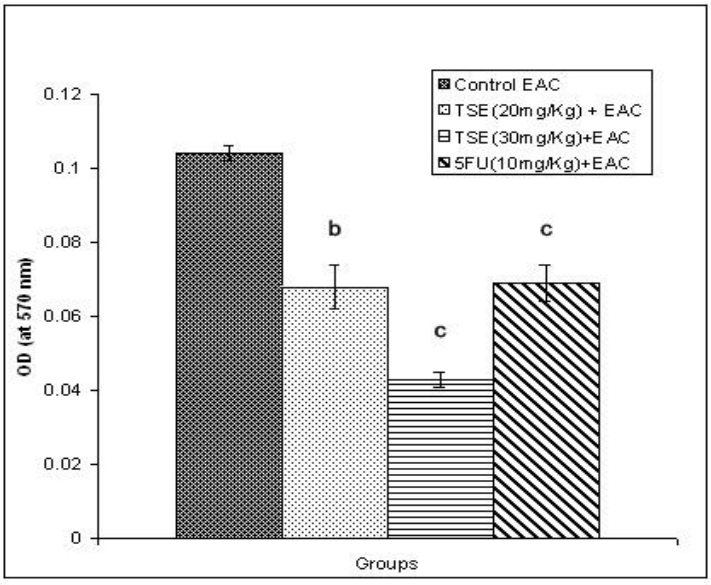

Figure 3: Effect of TSE on MTT assay of EAC cells.

[b]P<0.01, [c]P<0.001, significant.

3.5. Alkaline Comet assay

Mean number of tailed cells (TC) (%) and mean of length:width ratios (L:W) were analyzed in control and TSE treated (30 mg kg^{-1} d^{-1}, i.p. for 5days) EAC cells at 7th days of tumour cell inoculation. The mean length to width ratio of the DNA mass observed in TSE treated EAC cells was significantly greater (*P*<0.001) compared to that of normal control cells. The mean length-width ratio of control cells was 1.14 ± 0.06 where as in TSE treated EAC cells, the ratio was 2.27 ± 0.10. Similarly, the mean frequency of tailed cells was (92.4±0.26) in TSE treated EAC cells, which was significantly different (*P*<0.001) from the normal control (8.7 ± 0.13) (Fig 4, Table 2).

Table 2: Effect of TSE on the single cell electrophoresis of EAC cells

Cell Groups	Tailed Cells (%)	Length-width ratio
EAC Control (0.9% Saline)	7.5 ± 0.08	1.23 ± 0.035
TSE (30mg/Kg/day for 5days)	91.0 ± 0.25*	2.12 ± 0.104*

*P<0.001, significant

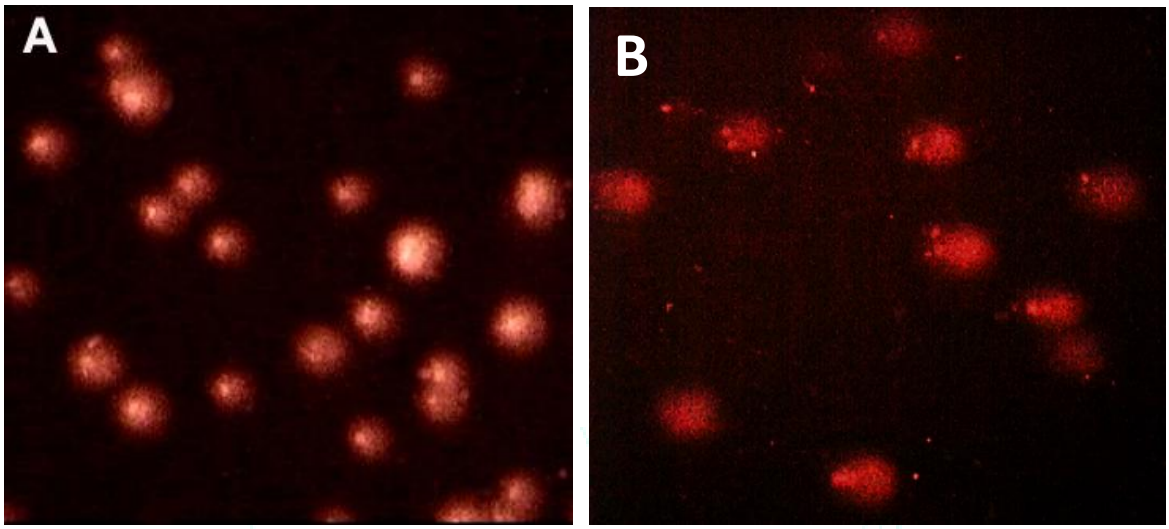

Figure 4: Effect of TSE on alkaline comet assay of EAC cells.

A = EAC control cells, B = TSE treated EAC cells

3.6. DNA fragmentation study by ladder formation assay

TSE (30 mg kg^{-1} d^{-1}, i.p. for 5 days) produced ladder type of DNA fragmentation in Ehrlich ascites carcinoma cells where as control cells produced single DNA band on the agarose gel (Fig.5).

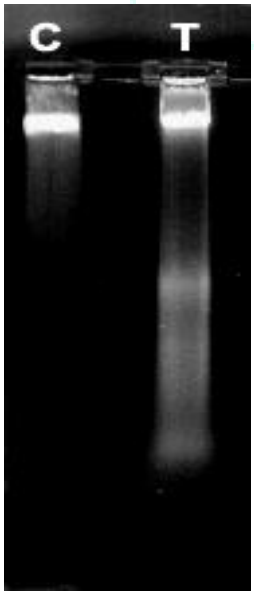

Figure 5: Effect of TSE on DNA fragmentation of EAC cells

C = DNA of EAC control, T = DNA of TSE treated EAC cells.

3.7. Fluorescent microscopy

TSE (30 mg kg^{-1} d^{-1}, i.p. for 5 days) treatment produced 16.5%±0.24 live cells with normal nuclei (LN), 43.5%±0.28 apoptotic cells (A), 13.0%±0.19 dead cells with normal nuclei (DN) and 26.5%±0.31 dead cells with apoptotic nuclei (DA). Whereas the control EAC cells showed 85.5%±0.34 live cells with normal nuclei, 6.0%±0.13 apoptotic cells, 5.5%±0.10 dead cells with normal nuclei and 3.0%±0.05 dead cells with apoptotic nuclei (Fig. 6, Table 3). The apoptotic index in TSE treated EAC cells was 69.7 whereas the apoptotic index of control EAC cells was 9.0 (Table 4).

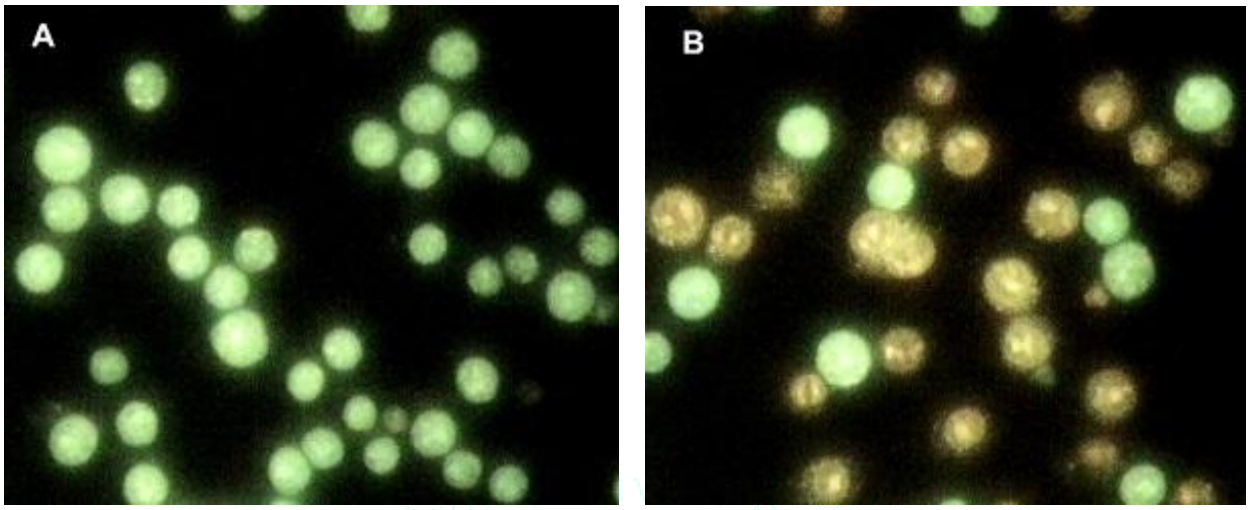

Figure 6: Effect of TSE on fluorescent microscopic analysis of EAC cells

A = Control EAC cells, B = TSE treated EAC cells

Table 3: Effect of TSE on cell architecture modulation and apoptosis induction on EAC cells

Cell architecture	EAC Control (0.9% Saline) Cells (%)	TSE (30mg/Kg/day for 5days) Treated EAC cells (%)
Live cells with normal nuclei (LN)	85.5	16.5
Apoptotic cells (A)	06.0	43.5
Dead cells with normal nuclei (DN)	05.5	13.0
Dead cells with apoptotic nuclei (DA)	03.0	26.5

Total cell counted = 200

Table 4: Effect of TSE on apoptotic index of EAC cells

Cell Groups	Total cells	Apoptotic cells	Apoptotic index
EAC Control (0.9% Saline)	200	18.0 ± 0.47	9.0
TSE (30mg/Kg/day for 5 days)	200	139.4 ± 1.06*	69.7

Apoptotic index = Number of apoptotic cells/ Total cells (×100), *P<0.001, significant

3.8. Serum LDH and β2-microglobulin of EAC bearing mice

The serum biochemical analysis of EAC bearing mice revealed that TSE (20 mg kg^{-1} d^{-1}, i.p., for 7 days and 15 days) showed that the LDH level was significantly increased in TSE treated group at 7 days as compared with normal control as well as EAC bearing mice but at 15 days it came towards normal. The serum β2-microglobulin level also significantly increased in TSE treated EAC bearing mice at 7^{th} day. The raised β2-microglobulin level came towards normal and no significant different was observed at 15^{th} day (Table 5).

Table 5: Effect of TSE on serum LDH and β2-microglobulin level of EAC bearing mice.

Mice group	LDH level (U/L)	β2-microglobulin (mg/L)
Normal control	376±0.28*	1.8 ± 0.06*
EAC control	384±0.42	2.1 ± 0.08
TSE control	391±0.37	2.0 ± 0.10
EAC + TSE treated (7th day)	416±0.54*a	3.2 ± 0.05*b
EAC + TSE treated (15th day)	389±0.48	2.2 ± 0.07

*aP<0.05, *bP<0.01, significant

4. DISCUSSION

The present investigation demonstrated that the Indian toad (*Bufo melanostictus*) skin extract (TSE) significantly inhibits Ehrlich ascites carcinoma cells growth in EAC bearing Swiss albino mice in a dose (20, 30, 40 mg kg^{-1} d^{-1}) dependent manner. TSE not only reduced the EAC cell count, it also decreased the accumulation of ascites fluid in the peritoneum and increased the percentage of dead cells as observed in trypan blue dye exclusion study. The viability of cancer cells also significantly decreased dose dependently. Earlier study from this laboratory showed that TSE significantly increased the life span of EAC bearing mice [16]. In the present study, TSE significantly reduced the MTT values in cancer cells as compared to control in both 50 and 100 mg/Kg of doses. MTT assay is the most common assay employed for the detection of cytotoxicity or viability following the exposure of cytotoxic substances. MTT assay shows more sensitivity in detecting early cytotoxicity compared to the LDH leakage assay and other assay [28]. The cytotoxic and antiproliferative drugs may disrupt the mitochondrial succinate dehydrogenase system thus reduces the MTT value. In the present study, the reduced MTT values and reduced viability of EAC cells by the TSE is mainly due to its antiproliferative and cytotoxic nature. It is also evident that TSE induce apoptosis in various human cancer cell lines such as T24 human bladder carcinoma cells, A549 human lung carcinoma, HepG2 hepatocarcinoma cells, SP53 mantle cell lymphoma and non-Hodgkin's lymphoma [29-31]. In this study, comet assay is employed for the detection of different genotoxic agent and also be utilized for the determination of DNA fragmentation in the apoptotic cells. As the extent of apoptosis increases, the tail movement and tail length of the comet formed increases, which are correlated with the degree of DNA damage [32]. Here, TSE induced DNA fragmentation was observed in single cell gel electrophoresis. TSE produced significant increase in length width ratio in EAC cells as compared to the control (Fig 5, Table 2). This is due to the movement of fragmented DNA mass of the apoptotic tumor cells during electrophoresis. In apoptotic cells there are approximately 60,000 DNA strand breaks generated per cell [33]. With the comet assay, 100 DNA strand breaks per cell are sufficient for DNA damage to be detected [34]. The TSE induced ladder pattern of DNA fragmentation in the agarose gel again supported its apoptogenic activity. DNA fragmentation at 180-200 bp by endonuclease is the most characteristic phenomena of apoptosis [33]. DNA fragmentation produces a ladder pattern of arrangement reflected in agarose gel electrophoresis, thus employed for the detection of apoptosis. Observation on the studies on Chinese toad (*Bufo Bufo gargarizans)* skin preparation (Chan Sue) with flow cytometry, agarose gel electrophoresis of DNA and fluorescent staining in human bladder carcinoma cell line, indicated its apoptogenic nature [29]. Chan Sue induced apoptosis in human leukemic cells (U937) was also reported [35]. In previous study from our lab it has already been reported that TSE of Indian toad exert significant antiproliferative, cytotoxic and apoptogenic activities on U937 and K562 leukemic cell lines. Microscopic observations of the altered fragmented nuclei, formation of apoptotic bodies, cellular membrane blebbing and cell surface shrinkage in TSE treated leukemic cells strongly support the apoptogenic properties of TSE of Indian common toad [17]. Here the apoptotic, necrotic and dead cells were detected and counted in the fluorescent microscope with acridine orange – ethidium bromide double staining. The double staining of the EAC cells indicated that TSE produced significantly greater number of apoptotic cells as compared to control (Fig 6). The calculated apoptotic index due to TSE in EAC cells was significantly high as compared to control. From these observations it may be opined that the EAC cell growth inhibition, reduced viability and reduced MTT values in the TSE treated cancer cells is associated with its antiproliferative and cytotoxic nature and the increased comet length-width ratio, DNA ladder formation and the increased apoptotic index indicated the apoptogenic nature of TSE.

Interestingly, the serum lactate dehydrogenase (LDH) level was significantly increased in TSE treated EAC bearing mice at 7th day but it returned towards normal at 15th day of treatment. It has been reported that LDH is generally elevated in patients with certain aggressive malignancies [36]. Though, initially TSE treated EAC mice showed elevated LDH level, continued treatment restore the LDH level, which was supported and correlated with the cancer progression arrest in EAC bearing mice. β2-microglobulin level has been found to correlate with the tumour growth and progression kinetics [37, 38]. TSE mediated restoration of elevated β2-microglobulin at 15 days indicated that TSE might reduced the tumour burden or tumour progression. Again, LDH uplifting agent is responsible for interferon induction and enhancement of natural killer cell activity by inoculation of Ehrlich ascites carcinoma cells into mice [39]. Interferon-γ and NK-cells inducing agents have

anticancer activity on renal cell carcinoma in mice [40]. Interferon (IFN-γ) may activate macrophage which in turn up and regulate the MHC II molecule [41]. The TSE induced elevation of β2-microglobulin further supported the involvement of MHC I molecule in the TSE induced antitumor and immunomodulatory activities. The rise in serum β2-microglobulin is related to the immunomodulation properties of interferon [42]. MHC plays a central role in the development of both humoral and cell-mediated immune responses. The class I MHC α chain expression level on cell membrane suppressed in absence of β2-microglobulin. It is evidenced in Daudi tumour cells which do not synthesize β2-microglobulin [43]. Previous report from this laboratory have demonstrated that Indian toad (*Bufo melanostictus*) skin extract increased peritoneal macrophage count, reduced carbon clearance time from blood and increased lymphocyte chemotaxis in EAC bearing mice [16]. From this result it was presumed that TSE may be involved in the induction of interferon and NK-cell, which in turn involved in the macrophage mediated antitumour activity and the induction of cell mediated immunity. It is widely recognized that macrophages are one of the major effector cells in the anti-tumor immune defense system of the host. There are certain reports that macrophage activating agent exerts antitumour activity against wide variety of tumours [44]. It has been evident that chemotherapy in association with immunotherapy produced better results against cancer and showed lesser resistance against drug [45-47]. The present investigation suggested that toad skin extract (TSE) induced immunomodulatory activity may be associated with its antitumour activity.

It may be concluded that Indian toad (*Bufo melanostictus*) skin extract possess antiproliferative, cytotoxic and apoptogenic activity on Ehrlich ascites carcinoma (EAC) cells of EAC bearing mice, which may be connected with its immunomodulatory activity.

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