



Anti-Cancer Effects of Methanolic Extract of Toad Egg on Experimental Model

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ABSTRACT

Cancer is a complex multi-factorial disease of the cell. Drug discovery against cancer is ventured throughout the world especially from the nature products. Various drugs have been used in the treatment of cancer, but all of them have advantages and also different disadvantages. So, nowadays it is trying to identify different compounds from natural products which have anticancer properties. The objective(s) of this present study was to identify the anti-cancer activity of *Bufo melanostictus* Schneider, 1799, egg methanolic extract [TEE(M)] on the Ehrlich ascites carcinoma (EAC) cells in mice and also on human leukemic cell line (U937 and K562). Various experimental studies such as mean survival time study, U937 and K562 Cell proliferation study, bright field microscopy study, fluorescent microscopy study and MTT assay of U937 and K562 cells were performed. The results of these studies established that TEE(M) not only decreases EAC cell count but also reduces the EAC cell size and causes the apoptosis of the EAC cells. TEE(M) also decreases human myeloid leukemic cell count (U937 and K562) significantly. So, it can be said that this exact has significant anticancer effects.

Keywords: Anti-cancer, Cancer model, Toad egg extract.

INTRODUCTION

According to WHO, cancer is the 2nd most dangerous and lethal disease. Cancer development and progression are dependent on cellular accumulation of various genetic and epigenetic events¹.

Chemicals from nature have been a part of human civilization even since over early ancestors began exploiting natural compounds to improve and enrich their own lives. There are three sources of natural products—plants, microorganisms and animals. Different bioactive molecules from natural sources contributed to the understanding of human physiology and led to the foundation for modern medical chemistry and pharmacology².

Organic compounds from terrestrial and marine organism have extensive past and present use in the treatment of many diseases and serve as compound of interest both in natural form and as templates for synthetic modification. Over 20 new drugs launched in the market between 2000 and 2005, originating from terrestrial plants, terrestrial microorganism, marine organism and terrestrial vertebrates are described. These approved substances, representative of very wide chemical trials, continue to demonstrate the importance of compounds from natural sources in modern drug development efforts.

There are various drugs for treatment of carcinogenic disease. But all of the drugs have their advantages as well as disadvantages. So, invention of new drugs in this area is very much desirable in cancer treatment. In the Middle Ages, the *Bufo* toad was celebrated as panacea and persecuted as a powerful poison. Bufotenine has been at

the centre of a scientific debate since its discovery in 1893³. Toads particularly members of genus *Bufo* are identified as a particularly convenient and useful source of various bioactive compounds having therapeutic purposes. Non-aqueous skin extract of *Bufo melanostictus* shows immune-modulatory and anti-neoplastic activities, but it has also toxic effects in rodents. TSE-LF induces neurotoxic effects on chick calcium ions. It also affects MAO activity and tryptophan hydrolase activity.

Recent work on the Indian toad *Bufo melanostictus* skin extract showed that whole extract has anti proliferative and apoptotic activity on leukemic cell line (U937 and K562) and HepG2 cell line⁴. A non-protein factor BM ANF-I from the toad skin extract have been Identified having anti proliferative and apoptotic activity on leukemic cell line (U937 and K562) and HepG2 cell line⁵. But there is no scientific evidence about anti-cancer activity on egg extract of *Bufo melanostictus* Schneider, 1799.

So, in this present study it has been tried to establish the anti-cancer activity of *Bufo melanostictus* Schneider, 1799, egg extract (methanolic extract) on the Ehrlich ascites carcinoma (EAC) cells in mice and also on human leukemic cell line (U937 and K562).

MATERIALS AND METHODS

Materials

All the materials were used are of analytical grade.

Animal conditioning

In this project, Swiss albino male mice (20±2gm body wt) were used. The animals were housed in standard condition of temperature (25±2°C), relative humidity (60±5%) and



light (12 hours light and dark cycle). They were fed with standard laboratory diet and libitum.

Mice Tumour Cells

Ehrlich ascites carcinoma (EAC) cells were maintained bi-weekly intraperitoneal (i.p.) inoculation of 10×10^6 cells/mouse.

Preparations of methanolic extract of egg [TEE(M)]

Bufo melanostictus Schneider, 1799, the toad (70 ± 10 g) procured the courtesy of experimental laboratory. After pithing the toad, the skin of lower portion of toad was removed and abdominal incision was done and egg was collected and weighed then was immersed in the methanol solvent. After 20 days the extract was collected and kept in the incubator at 37°C for one and half day and dried. In this way the crude extract was obtained. Then the crude extract was dissolved in 0.9% NaCl solution to prepare toad egg extract. The concentration of the prepared toad egg extract is expressed in terms of dry weight ($0.38 \pm 0.08\%$).

Estimation of protein content

The protein content of TEE(M) fraction was estimated by using the method of Lowry et. al. (1951) using BSA as standard⁶.

Minimal lethal dose (MLD)

TEE(M) was administered into male albino mice (20gm/mice) intravenously (n=4). The mortality was recorded up to 24 hrs of observation.

Transportation of Ehrlich ascites carcinoma (EAC) cells

Ehrlich ascites carcinoma (EAC) cells were collected from different tumour bearing Swiss male Albino mice at their respective log phase of tumour cells. The freshly drawn fluid was diluted with 0.9% NaCl solution and the tumour cell number was adjusted to 1×10^5 cells per ml by counting the number with a hemocytometer. The viability of the tumour cell was checked by Trypan Blue dye (0.4%) exclusion assay. Cell samples showing above 90% viability were used for transplantation. Aseptic condition was maintained throughout the transplantation process.

Tumor cell growth

Animals were inoculated intraperitoneally with $2-3 \times 10^5$ cells per mouse on Zero day and treatment was started with TEE(M) after 24hrs at a dose of 100miyu/20gm (i.p.). All treatment was continued for 10 days. On the 11th day after transplantation all the animals were sacrificed and tumour cells were obtained by intraperitoneal wash with 5ml 0.9% NaCl solution. Then this solution was diluted to 1:50 dilution and this diluted solution from treated group and it was compared with that of control group.

Bioassay of EAC cells

Control and treated groups of mice (6 in each group) were inoculated with $1-2 \times 10^6$ EAC cells on zero day. On the 1st day after intraperitoneal inoculation, treated mice received TEE(M) at the dose of 67.6mg/20mg \times 3 days (i.p.).

Twenty four hours after treatment, tumor cells from each group of mice were counted and harvested in cold 0.9%NaCl solution. Pooled, centrifuged and re-inoculated ($1-2 \times 10^6$ cells/mice) (i.p.) into two groups of 4 mice each per treatment. On 7th day after re-inoculation, all animals from each group were sacrificed and tumor cell counts were estimated.

Dose dependent assay of EAC cells

Control and two treated groups of mice (6 in each group) were inoculated with $1-2 \times 10^4$ EAC cells on zero day. On the 1st day after (i.p.) inoculation, treated mice received TEE(M) at two different doses [$0.1\text{mg}/20\text{mg} \times 10$ days and $0.05\text{mg}/20\text{gm} \times 10$ days], intraperitoneally. After full treatment all animals from each group were sacrificed and tumour cell counts were estimated.

Mean survival time study

Animals were inoculated (i.p.) with $2-3 \times 10^6$ cells/mouse on zero day and treatment was started with TEE(M) after 24 hrs at a dose of 19 mg/20mg (i.p.). All treatments were continued for 20 days. Mean survival time (MST) for each group containing 4 mice was noted. The animals surviving more than 60 days were considered to be cured. Mean survival time was noted with reference to control. Mean survival time of treatment groups were compared with the control group using the following equation⁷.

Percentage increase in life span (%ILS) = $(\text{MST of treated group} \div \text{MST of controlled group}) \times 100$

U937 and K562 Cell proliferation study

To observe cell proliferation inhibitory effects, 100 miyu I of myeloid leukemia cell (U937 and K562) suspension containing 1×10^5 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum in each well were added in 96 well micro titer plate separately. TEE(M) was dissolved in sterile demonized water and added at different concentration (9.15 mg and 18.3 mg/100 miyu I). The culture plate was incubated in a humidified 5% carbon dioxide incubator for 24 hrs and 48 hrs at 37°C . *In-vitro* growth of the culture was monitored using trypan blue and haemocytometer. The cell count was compared with the untreated control cell growth⁸.

Bright field microscopy study

Morphological changes of TEE(M) treated K562 cells were examined after Giemsa Staining under bright field microscopy. Morphological changes of TEE(M) treated EAC cells were examined after Giemsa Staining under bright field microscopy.

Fluorescent microscopy study

The myeloid leukemia cell (U937 and K562) suspension was also treated with TEE(M) [9.15mg/100 miyu I] and after 24 hrs. They were stained with Ethidium bromide and Acridin orange (100 miyu g/ml each in phosphate buffer saline). Acridine orange visualizes the cells, which have



undergone apoptosis while ethidium bromide differentiates between viable and non-viable cells.

MTT assay of U937 and K562 cells

100 miyu I of myeloid leukemic cell (U937 and K562) suspension in RPMI-1640 and supplemented with 10% fetal calf serum containing, 1.5×10^5 cells were added to each well of a channel plate separately. TEE(M)[3.45mg/100 miyu I] was added to each group of cells in wells. 20 miyu I MTT (5mg/ml) was then added in each well and allowed to incubate at 37°C in 5% carbon dioxide incubator for 3 hrs. 100 miyu I DMSO was Addy to each well to dissolve the Formazan Crystal formed. The optical density was recorded at 570 NM by strip reader (Merck-MIOS no.309)⁹.

Growth inhibitory rate (%) = $[1 - (\text{OD of treated} / \text{OF of control})] \times 100$

Statistics

Each experiment was repeated at least three times. Data are presented as mean±SE. Significance of mean values of different parameters between the incubated mitochondria were analyzed using one way post hoc tests (Tukey’s HSD test) of analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the incubations. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Estimation of protein content

Protein content of the TEE(M) found to be 2.1 ± 0.23 mg/gm dry weight.

Minimal lethal dose (MLD)

Minimal lethal dose of TEE(M) was found to be 0.75 gm (i.p.) per 20 gm of male Swiss Albino mice.

Identification of the physical features

The EAC cell bearing mice were found with rough and yellowish body surface but in case TEE(M) treated mice, they had a smooth body surface (Fig. 1A and 1B).



Figure 1: Control [EAC cell bearing] (A) and TEE(M) treated (B) male Swiss Albino mice.

Tumour Cell growth

TEE(M) [100 Miyu g/20gm ×10 days i.p.] treated group produced 97.80% inhibition of EAC cell compared to control group; whereas 5-FU treated (0.2mg/20 gm ×10 days; i.p.) standard group produced 55.20% inhibition of EAC cell (Table 1).

Bioassay of EAC cell

TEE(M) [100 miyu g /20 gm×3 days; i.p.] treated group differ significantly from the control group in EAC Cell viability. TEE(M) treated group produced 52.87% inhibition of EAC cell compared to control group, whereas 5-FU treated (0.2 mg/20gm ×10 days; i.p.) standard group produced 31.40% inhibition of EAC cell (Table 1).

Mean survival time study

The effect TEE (M) on the survival of tumour – bearing mice (Fig.8). The MOST for the control group was 45 ± 2.10 day, whereas it was more than 90 ± 3.20 days for the TEE(M) treated group [100miyu g /20 gm ×20 days; i.p.]. The increase in the life span of tumour– bearing, mice with TEE(M) treatment was found to be 200-00% as compared to the control group; whereas the survival days for 5-FU treated (0.1mg/20 gm ×25 days, i.p.) group are 81 ± 3.50 days. Here ILS % is 184.10% (Table 1).

Table 1: Effect of TEE(M) on EAC cell count of male albino mice.

Parameters	Groups		
	10 days treatment		
	Control	5-FU	TEE(M)
EAC Cell CountX10 ⁴	208.56±37.43	50.68±4.24*	4.56±1.41*
% inhibition	-----	55.20	97.80
	3 days treatment		
	Control	5-FU	TEE(M)
EAC Cell CountX10 ⁴	93.81±6.52	64.35±4.29*	44.21±5.39*
% inhibition	-----	31.40	52.87
	Control	5-FU	TEE(M)
Mean survival time	45.00±2.10	81.00±3.50*	90.00±3.20*
% life span	-----	184.10	200.00
Value shown are mean±SE (n=4)			*p<0.1

Dose dependent assay on EAC cells

TEE(M) [(100 miyu /20 gm ×10 days; i.p.)] treated group differ significantly from the control group in EAC Cell in dose dependent assay . High dose treated group produced 86.50%inhibition of EAC compared to control group; whereas low dose treated group produced 45.80 % inhibition of EAC cells compared to control group (Table 2).

Table 2: Dose dependent effect of TEE(M) on EAC cell count

Group	Cell count ×10 ⁴	% inhibition
Control Group	123.10±19.33	
High dose treated group	16.50±5.66*	86.50
Low dose treated group	66.69±9.56*	45.80
Value shown are mean±SE (n=4)		*p<0.1

Dose dependent assay on U937 cells

A significant percentile inhibition of 15.01%, 20.28%, 34.64% and 49.02% was produced by TEE(M) [1miyu g /miyu l], [2.5 miyu g /miyu l], [5 miyu g /miyu l] and [10 miyu g/ miyu l] respectively after 24 hours treatment on U937 cells as compared with control. Similarly, 71.95 % inhibition of cell count was observed when U937 was treated with Arac (Table 3).

Table 3: Effect of TEE (M) on U937 cell count

Group	Cell count ×10 ⁴	% inhibition
Control Group	102±14.43	
Arac	23±5.23**	71.95
TEE (M)		
1.0 miyu g/miyu l	86.69±4.99**	15.01
2.5 miyu g/miyu l	81.31±4.98**	20.28
5.0 miyu g/miyu l	66.67±3.87**	34.64
10.0 miyu g/ miyul	52.00±3.27**	49.02
Values shown are mean±SE(n=4)		**p<0.001

Dose dependent assay on K562 cells

TEE(M) at dose of 1 miyu g /miyu l produced 33.72% inhibition and at dose of 2.5 miyu g/miyu l produced 48.90% inhibition on K562 cells. On the other hand, TEE (M) at the dose of 5 miyu g/miyu l produced 64.00% inhibition and at the dose of 10 miyu g/miyu l produced 86.22% inhibition respectively compare to the control group. Similarly, 31.53% inhibition of cell count was observed when K562 was treated with Imatinib Mesylate (IM) (10 miyu g/ 100 miyu l) (Table 4).

MTT assay of U937 and K562 Cell

U937 when treated with TEE(M) at different doses such as 2.5 miyu g/miyu l, 5miyu g/miyu l and 10 miyu g/ miyu l, produced 40.69%, 51.73%, 83.83% of significant inhibition as compared with the control group. Whereas when the

cells were treated with AraC (10 miyu g/100 miyu l), it produced 35.29% of significant inhibition (Figure 2).

Table 4: Effect of TEE(M) on K562 cell count

Group	Cell count ×10 ⁴	% inhibition
Control Group	58.42±8.43	
IM	40.00±5.41**	31.53
TEE (M)		
1.0 miyu g /miyu l	38.72±4.25**	33.72
2.5 miyu g /miyu l	29.85±2.98**	48.90
5.0 miyu g/miyu l	21.03±1.25**	64.00
10.0 miyu g/ miyul	8.05±.025**	86.22
Values shown are mean±SE(n=4)		**p<0.001

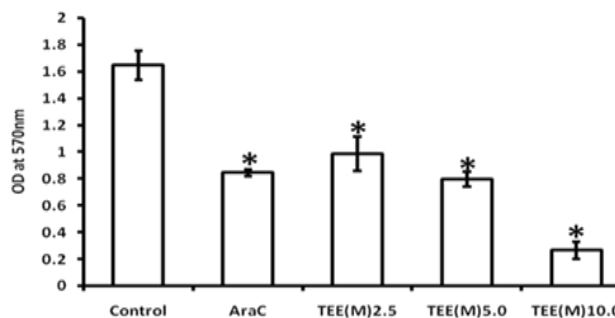


Figure 2: Effect of TEE(M) and standard drug AraC for U937 on optical density (O.D) at 570 NM after 24 hrs MTT assay of human myeloid leukemic cell suspension as compared to the O.D. of control. Values shown are mean±SE(n=4), *p<0.001

Morphological study

Fluorescent microscopy study (Ethidium bromide and Acridine orange staining)

- A) **EAC cells:** TEE(M) [100 miyu g/10 gm × 10 days; i.p] caused EAC cell size to decrease. Various apoptotic cells and necrotic cells were observed under microscope (Data not shown).
- B) **U937 cells:** TEE(M) [2.5 miyu g/ miyu l] caused membrane blebbing accompanied with the nuclear fragmentation of the cells as compared with the control (Data not shown).
- C) **K562 cells:** TEE(M) [5.0 miyu g/ miyu l] caused nuclear fragmentation of the cells accompany with early and late apoptotic cells when compared with the control (Data not shown).

Bright field study (Giemsa Staining)

- A) **EAC cells:** TEE(M) [100 miyu g / 20 gm × 10 days; i.p] caused EAC cell size to decrease, which is accompanied with the visualisation of maximum number of apoptotic and necrotic cells with membrane blebbing and nuclear fragmentation as compared with the control (Figure 3).



B) K562 cells: TEE(M) [5.0 miyu g/ miyu l] caused nuclear fragmentation and nuclear movement towards periphery and membrane disruption in the cells when compared with the control (Figure 3).

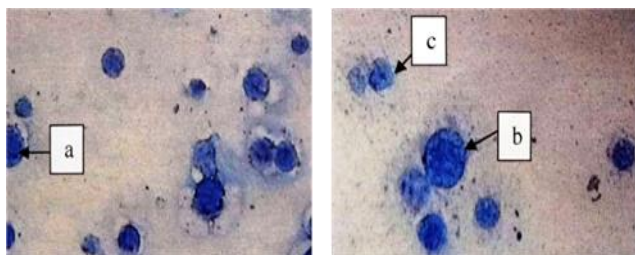


Figure 3: Effect of TEE(M) on morphological characteristics of EAC cells (Giemsa staining). (A) Non-treated EAC cells (a), B) TEE(M)-treated EAC cells [Nuclear fragmentation (b), Dead cell (c)].

DISCUSSION

The life cycle of the toads and frogs, like that of other amphibians consist of four main stages: egg, tadpole, metamorphosis and adult. Toad's eggs are present in translucent gelatinous strings in stagnant water, on weeds or around the stem of water plants. Before, the formation of tadpole from eggs, eggs are present in water. So, there are various compounds in the egg can protect it from the external environment. The eggs of toads are characterized by a rich variety of bioactive peptides and dextrose¹⁰. Gallinacean preparation know to the Chinese as chan'su consist of dried skin of a local toad (*Bufo ggargarizans*) and has been used to treat tooth ache, sinusitis of gum. Other traditional Chinese medicine formulations containing dried toad skin include jiuixin (called Kyushin in Japan)¹¹. Chinese doctors sometimes recommended Lou Shen Wan containing toad secretion for leukemia¹¹. A number of different types of bioactive peptides have been identified from the glandular skin secretions of Australia anurans of the *Litoria* genus including wide spectrum antibiotics.

Frog eggs were thought to cure rheumatism and to cure a sore eye. Egg's yolk was applied to preparation of ointment which is beneficial against hemorrhoids, toothaches and tumour. Amphinase, a proteinoous enzyme isolated from eggs of Northern Leopard frog has role in treatment of brain tumour¹². Crinia Frog eggs contain Riparins which effects on cancer cell membrane and destroy the cells¹².

There is no Schneider evidence about antineoplastic activity ok egg extract of *Buffo melanostictus* Schneider, 1799. So, in my present study it has been tried to show the anti proliferative activity of *Bufo melanostictus* Schneider, 1799, egg extract on the Ehrlich ascites carcinoma (EAC) cells in mice and also on human leukemia cell line (U937 and K563) when treated with *Bufo melanostictus's* methanolic egg extract. Anti-proliferative activity has been described through EAC cell count, EAC cell viability assay and cell count of U937 and K563 (myeloid leukemia cell), EAC cell does dependent assay on the other hand cytotoxic activity has been described by MTT assay and by different staining

technique e.g. Ethidium bromide and Acridin orange and Giemsa staining.

Minimal lethal dose of TEE(M) after injecting into the mice (i.p.) has been calculated by recording the mortality up to 24 hrs observation. The further doses of TEE(M) treatment in the present investigation has been accordingly taking care that the total summation of the doses at the time of treatment should not crosses MLD value. The aqueous egg extract shows PAS staining positively, so glycoprotein is present in this extract.

Earlier study had explored that the effect of toad skin extract on the peritoneal EAC cells of the male albino mice is an indirect method of evaluating it's inhibitory effect on tumour cells. Similarly, in this present study TEE(M), on treatment in EAC cell count significantly. These results demonstrate the direct inhibitory effect of TEE(M) on EAC cell count.

It had been established that the application of any anti-proliferative drug to EAC cell bearing mice decrease the viability of the cells when they were transferred from one mice to another. Same experiment is tried in this present study and it is found that TEE(M) decreases the viability of the EAC cells, which again confirms that TEE(M) may have some proliferating effect on EAC cell by Which it can improve the condition of cancerous mice.

Earlier studies had established that the application of graded does of anti-proliferative drug to EAC cell shows the graded response in the case of decreasing the EAC cell. Same experiment is tried in this present study and it is found that TEE(M) on treatment in EAC cell with graded does reduces EAC cell count in a graded manner. These results demonstrate the direct and graded inhibitory effect of TEE(M) on EAC cell count.

The morphological cell changes incurred during apoptosis are unique and should be a deciding factor concerning the mechanism of cell death. Cell death by apoptosis is characterized by cell chromatin condensation together with visible nuclear fragmentation and formation of apoptotic bodies. These changes may be used as markers for apoptosis¹³. In this present study Giemsa staining of EAC cells that are treated with TEE(M) causes the visualization of nuclear fragmentation and membrane disruption as compared to the non-treated EAC cells. On the other hand similarly Giemsa staining of K562 cell that are treated with TEE(M) causes the visualization of membrane disruption and nuclear fragmentation in comparison to the non-treated cells. Mosmann et Al., (1983)¹⁴ described MTT assay 3-(4,5- dimethylthiazol-2-tl)-2,5- dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and formation dark blue formazan crystals which is largely impermeable to cell membranes causing in it's accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the level of the crystals that are solubilized. Percentile inhibition of cells is inversely proportional to the level of the formazan product created

which can be quantified by measuring optical density of the medium, while exploring the effect of non-protein compound, isolated from the methanolic skin extract of *Bufo melanostictus*, on U937 and K562 cell suspension on TEE(M) treatment decreases as compared with the non-treated U937 and K562 cell. Thus, it inferred that TEE(M) treatment decreases the number of viable U937 and K562 cells.

CONCLUSION

From the above study it can be concluded that TEE(M) not only decreases EAC cell count but also reduces the EAC cell size and causes the apoptosis of the EAC cells. TEE(M) also decreases human myeloid leukemic cell count (U937 and K562) and also some way signals that apoptotic mechanism of these leukemic cell line. The other works that should be done on future study is the isolation and purification of anti-proliferating fraction from crude TEE(M) and it's further analysis. The whole study was made only to observe the anti-cancerous effect of toad egg methanol extract TEE(M) on the cancerous cells but other bioactivity of TEE(M) was not studied.

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