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Fish venom (*Pterios volitans*) peptide reduces tumor burden and ameliorates oxidative stress in Ehrlich's ascites carcinoma xenografted mice

M. Sri Balasubashini,[a] S. Karthigayan,[b] S. T. Somasundaram,[b] T. Balasubramanian,[b] V. Viswanathan,[c] P. Raveendran[d] and V. P. Menon[a,*]

[a]*Department of Biochemistry, Annamalai University, Annamalai Nagar 608 002, India*
[b]*CAS in Marine Biology, Annamalai University, Parangipettai 608 502, India*
[c]*Department of Pathology, Faculty of Medicine, Rajah Muthiah Medical College, Annamalai University, Annamalainagar 608 002, Tamil Nadu, India*
[d]*Department of Pathology, Regional Cancer Center, Trivandrum, India*

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Abstract—The present study was carried out to assess the effect of *Pterios volitans* venom (mixture of peptides) on Ehrlich's ascites carcinoma (EAC) and its influence on antioxidant status in the liver. Among six groups of albino mice, three were treated with sub-lethal doses of venom, along with the standard drug, 5-fluorouracil. In EAC-bearing mice, mean life span and antioxidants were significantly decreased, whereas, body weight, tumor volume, viable tumor cell count, lipid peroxidation and expression of proliferating cell nuclear antigen were significantly increased. These changes were brought back to near normal in treatment groups. The findings are further confirmed by histopathological observations.

Oxidative stress is potentially harmful to cells and reactive oxygen species (ROS) are implicated in the etiology and progression of many diseases including cancer. In normal conditions, antioxidant defense systems are able to detoxify ROS and prevent the damage to cellular macromolecules and organelles. Under conditions of excessive oxidative stress, however antioxidants are depleted and ROS can damage cellular components and interfere with critical cellular activities.[1]

Cancer cells have highly elevated protective mechanism to prevent lipid peroxidation. Several studies have demonstrated that lipid peroxidation is significantly decreased in tumor cells and tissues compared with that of corresponding normal cells.[2] The primary mechanism whereby cancer cells prevent lipid peroxidation is that, they have relatively low levels of the components of NADPH-cytochrome-P450 electron transport chain, which results in less favorable conditions for the initiation and propagation of lipid peroxidation.[3]

Cancer chemotherapy using antioxidant formulations is an exciting pharmaceutical research involving the use of either natural or synthetic components to delay, inhibit or reverse the development of cancer in normal or preneoplastic conditions. The approach of testing venom as antitumor agents dates back to the beginning of the past century when Calmette et al.[4] reported on the antitumour activity of snake venom (Naja species venom) on adenocarcinoma cells. Since then many reports have appeared on this subject and controversies still exist.[5] In addition to the snake venom, bee venom has been reported to have antiproliferative effect in vitro and reduction in tumor growth in vivo.[6] However, the marked curative properties of the snake venom are always hindered by their high toxicities; in most cases the venom are cytotoxic to normal cells same as they are toxic to that of malignant cells[7] surprisingly, it has been reported that a South Indian scorpion venom possesses superoxide dismutase (SOD) activity[8] and radical scavenging proteins have been reported recently in the tentacles of jellyfish toxin.[9]

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* Corresponding author. Tel./fax: +91 4144 238343; e-mail: cmrana@sify.com

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Thus, this study attempts to characterize and document the exact role of lionfish (*Pterios volitans*) venom on EAC-bearing mice to prolong the survival time by influencing the biochemical changes and antioxidant enzyme levels that are important for studying the anti-tumor effect. The biochemical markers chosen, namely reduced glutathione (GSH) content, glutathione peroxidase (GPx), glutathione reductase (GST) SOD, catalase (CAT) activities, and the extent of lipid peroxidation, are measured in liver, because it is well established that liver enzymes are more sensitive indicator of a distant neoplasm than blood.[10] The biochemical findings are further confirmed by the histopathological studies and immunohistological staining of proliferating cell nuclear antigen (PCNA) in liver.

Venom preparation. Specimens of *P. volitans* were obtained from the local aquarium, killed (by cooling), and the venomous spines were removed and stored in 10% glycerol solution at −80 °C, and the venom was prepared as described by Church and Hodgson.[11] The protein was estimated by Lowry et al.[12] The concentration was adjusted to 1 mg/ml; aliquoted and stored at −20 °C until use.

Toxicity assay. The LD_{50} was determined by the method of Litchfield and Wilcox.[13] Male albino mice of Swiss Webster strain (22 ± 2 g) were used and the study was approved by the Institutional Ethical Committee. LD_{50} was found to be 42.5 μg protein/kg bw.

Animal and ascites tumor. Adult male Swiss albino mice of 10- to 12-weeks old (22 ± 2 g) were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University. The animals were maintained under controlled conditions of temperature (23 ± 2 °C), humidity (50 ± 5%), and light (10 and 14 h of light and dark cycles, respectively) and were on commercial standard pellet diet and water ad libitum. The first inoculum of Ehrlich's ascites carcinoma (EAC) was kindly provided by the Amala Cancer Research Institute, Thirissur, Kerala. EAC were thereafter propagated by weekly intraperitoneal injection of 0.3 ml freshly drawn ascites fluid (diluted 1:5 in sterile saline) from a donor mice-bearing ascites tumor of 6- to 8-day-old, into three mice each with a mean body weight of 22 ± 2 g. Transplantation was carried out using a sterile disposable syringe under aseptic conditions.

Experimental design. In the present study 72 animals were divided into six groups of 12 animals.

Group 1:
Normal (received 100 μl of sterile saline by ip)
Group 2:
EAC control (received 100 μl of sterile saline by ip)
Group 3:
EAC induced mice + 1% of LD_{50} dose of venom in 100 μl of sterile saline (0.425 μg/kg bw by ip—low dose: LD)
Group 4:
EAC induced mice + 5% of LD_{50} of dose venom in 100 μl of sterile saline (2.125 μg/kg bw by ip—medium dose: MD)
Group 5:
EAC induced mice + 10% of LD_{50} dose of venom in 100 μl of sterile saline (4.25 μg/kg bw by ip—high dose: HD)
Group 6:
EAC induced mice + Standard 5-fluorouracil (5-FU-20 mg/kg bw)

Total experimental period was 10 days and after administering the last dose, 6 animals in each group were fasted overnight, anesthetized, and sacrificed by cervical decapitation. The liver tissues of animals were stored in −20 °C for further analysis. The remaining animals were left to calculate the mean survival time.

Antitumor effect of venom was assessed by observing the changes with respect to body weight, ascites tumor volume, packed cell volume, viable and non-viable tumor cell count, mean survival time (MST), and percentage increase in life span (%ILS). MST of each group containing six mice were monitored by recording the mortality daily for six weeks and % ILS was calculated using following equation.[14]

MST = (day of first death + day of last death)/2

$\%ILS = [(\text{Mean survival time of treated group/mean survival time of control group}) - 1] \times 10$

Preparation of tissue homogenate. The liver tissues were excised and homogenates were prepared in 3 volumes (w/v) phosphate buffer (0.1 M, pH 7.4), centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was used for assays.

For the estimation of lipid hydroperoxides (LOOH), 0.1 ml of tissue homogenate (supernatant) was treated with 0.9 ml of Fox reagent and incubated at 37 °C for 30 min. The color developed was read at 560 nm colorimetrically by the method of Jiang et al.[15] Lipid hydroperoxide levels are expressed as mmol/mg tissue.

Levels of thiobarbituric acid reactive substances (TBARS) in tissue homogenate were estimated by the method of Okhawa et al.[16] To 0.2 ml tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, and 1.5 ml of 0.8% TBA were added. The mixture is made up to 4 ml with distilled water and then heated in a water bath at 95 °C for 60 min. After cooling, 1 ml of water and 5 ml *n*-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 600*g* for 10 min, the absorbance of the organic layer was measured at 532 nm. 1,1,3,3-Tetramethoxypropane was used as the standard. The levels of TBARS were expressed as mmol/mg tissue.

Determination of SOD and CAT enzyme activities. SOD was assayed according to the technique of Kakkar et al.[17] based on the reduction of NBT^{2+} to a blue for-

mazan (MF^+) by $O_2^{\cdot -}$. The color developed was measured spectrophotometrically at 550 nm and was expressed in terms of 50% NBT reduction/min/mg protein.

CAT was assayed as described by Sinha.[18] The reaction mixture contained 1.0 ml phosphate buffer (0.01 M, pH 7.0), 0.1 ml tissue homogenate, and 0.4 ml H_2O_2 (0.2 M). The reaction was stopped by the addition of 2.0 ml dichromate–acetic acid reagent (5% potassium dichromate and glacial acetic acid, 1:3 ratio). The absorbance was measured colorimetrically at 620 nm and the values were expressed as μmol of H_2O_2 utilized/min/mg protein.

Determination of glutathione and glutathione dependent enzymes. A tissue level of reduced GSH was determined by the method of Boyne and Ellman.[19] Homogenates were immediately precipitated with 0.1 ml of 25% trichloroacetic acid and the precipitate was removed after centrifugation. Free SH groups were assayed in a total volume of 3.0 ml by the addition of 2.0 ml DTNB (0.6 mmol), 0.9 ml phosphate buffer (0.2 M, pH 8.0), and 0.1 ml homogenate. The absorbance was recorded at 412 nm. GSH values were expressed as mmol GSH/mg tissue.

GPx activity was measured by the method of Flohe and Gunzler.[20] Briefly, the reaction mixture contained 0.2 ml phosphate buffer (0.4 M, pH 7.0), 0.1 ml sodium azide (10 mmol), 0.2 ml tissue homogenate, 0.2 ml GSH (30 mmol), and 0.1 ml H_2O_2 (0.2 mmol). The contents were incubated at 37 °C for 10 min, and the reaction was arrested by addition of 0.4 ml of 10% TCA and the reaction mixture centrifuged. Supernatant was assayed for GSH content by using Ellman's reagent and expressed as μmol of GSH utilized/min/mg protein. The protein was estimated by the Lowry et al.[12]

GST activity was determined spectrophotometrically by the method of Habig and Jakoby.[21] The reaction mixture contained 1.7 ml phosphate buffer (100 mmol, pH 6.5), 0.1 ml GSH (30 mmol), and 0.1 ml CDNB (30 mmol). After preincubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 ml tissue homogenate and the absorbance was followed for 5 min at 340 nm. The specific activity of GST was expressed as μmol of GSH–CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 mmol/cm.

Histopathological analysis. For histopathological study, liver of two animals from each group was stored in 10% formalin. They were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section, and stained with hematoxylin and eosin (H&E), and viewed under microscope.

Immunohistological studies. Briefly, cut tissue sections were deparaffinized and endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide in methanol for 30 min. Non-specific binding was blocked with normal sheep serum (51%) in PBS (containing 0.14 N NaCl and 0.005 N $Na_2H_2PO_4$) and then the tissue sections were incubated at 4–8 °C overnight with 1:50 diluted PCNA mouse monoclonal antibody (Dako, CA, USA). Immunostaining was performed using biotin–streptavidin peroxidase method. The staining intensity has been graded as: −, no staining; +, mild staining; ++, moderate staining; +++, intense staining. The staining in percentage was done by counting about 300 cells in different fields of the tissue.

Statistical analysis. Values are expressed as means ± SD of 6 animals in each group. Data within the groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A value of $P < 0.05$ was considered statistically significant.

Effect of fish venom on mean survival time and tumor growth. The toxicity assay of *Pterios volitans* venom extract on the albino mice was used to arrive at sublethal concentrations required for the present study. Based on the values, three different concentrations of the fish venom were fixed (1%, 5%, and 10% of LD_{50} values as given in the experimental design). Table 1 shows the changes in the body weight, percentage increase in life span, mean survival time, tumor volume, packed cell volume, and viable and non-viable tumor cell count. The normal animals, those who received saline alone, did not show any significant changes in all the parameters studied. The EAC-bearing mice showed increased body weight due to the increased ascites volumes by actively proliferating peritoneal cells, whereas the bodyweight was decreased in treated groups. In the EAC control group, the mean survival time was 16.0 days, whereas, it increased significantly in all treated groups. A better effect was observed in medium dose treatment with an increase in the life span by 99.5%, when compared to the carcinoma control group. Increased life span and decreased

Table 1. Effect of fish venom on body weight changes and survival time

Parameters	Normal + Saline	EAC control (10^6 cells/ml)	FV (LD) + EAC	FV (MD) + EAC	FV (HD + EAC)	5-FU + EAC
Body weight/g	22 ± 2.0	40.20 ± 0.3	30.50 ± 0.4	24.6 ± 0.3[a]	29.4 ± 0.02	25.2 ± 0.02
Mean survival time/d	—	16.00 ± 0.1	23.2 ± 0.1[a]	35.9 ± 0.19[a]	30.40 ± 0.2[a]	39.5 ± 0.3[a]
Increase life span %	—	—	34.12	99.5	68.75	119.49
Tumor volume/ml	—	4.5 ± 0.07	3.37 ± 0.03[a]	0.8 ± 0.01[a]	2.41 ± 0.01[a]	—
Packed cell volume/ml	—	2.11 ± 0.84	1.4 ± 0.04[a]	0.23 ± 0.01[a]	1.2 ± 0.01[a]	—
Viable tumor cell count/10^{10} cells L^{-1}	—	12.30 ± 0.07	0.32 ± 0.01[a]	0.68 ± 0.04[a]	0.84 ± 0.06[a]	—
Non-viable tumor cell count/10^{10} cells L^{-1}	—	0.89 ± 0.05	1.62 ± 0.06[a]	—	1.57 ± 0.05[a]	—

(Values are means ± SD, $n = 6$, [a]$p < 0.01$ vs EAC control group, Body weight of normal mice 22 ± 2 g).

Table 2. Changes in the levels of TBARS, hydroperoxides, and GSH content in liver of EAC and FV treated mice

S.No	Groups	TBARS mmol/100 g	Hydroperoxides mmol/100 g	GSH mmol/100 g
1.	Normal	0.94 ± 0.02*	0.75 ± 0.01*	3.35 ± 0.09*
2.	EAC control	1.82 ± 0.01	1.95 ± 0.02	1.20 ± 0.01
3.	EAC + FV (LD)	1.20 ± 0.01	1.25 ± 0.01	1.90 ± 0.01
4.	EAC + FV (MD)	0.90 ± 0.01*	0.89 ± 0.02*	2.40 ± 0.01*
5.	EAC + FV (HD)	1.28 ± 0.01	1.30 ± 0.01	1.98 ± 0.01
6.	EAC + 5-FU	0.99 ± 0.02*	0.85 ± 0.01*	3.65 ± 0.09*

(Values are means ± SD, $n = 6$, $^{*}p < 0.001$ vs EAC control group).

Table 3. Showing the changes in the activities of antioxidant enzymes in liver of EAC-bearing and FV treated mice

S.No	Groups	SOD[A] U/mg protein	CAT[B] U/mg protein	GST[C] U/mg protein	GPx[D] U/mg protein
1.	Normal	4.49 ± 0.35[a]	26.4 ± 0.07[a]	19.2 ± 0.18[a]	7.77 ± 0.72[a]
2.	EAC control	2.09 ± 0.26	10.8 ± 0.07	8.24 ± .0.06	4.32 ± 0.01
3.	EAC + FV (LD)	3.15 ± 0.01	12.5 ± 0.03	10.53 ± 0.01	5.45 ± 0.05
4.	EAC + FV (MD)	3.90 ± 0.03[a]	19.5 ± 0.01[a]	15.9 ± .009[a]	6.7 ± 0.05[a]
5.	EAC + FV (HD)	2.98 ± 0.01	13.0 ± 0.04	11.35 ± 0.07	5.78 ± 0.04
6.	EAC + 5-FU	3.96 ± 0.35[a]	20.4 ± 0.07[a]	16.2 ± 0.18[a]	6.97 ± 0.72[a]

(Values are means ± SD, $n = 6$, $^{a}p < 0.001$ vs EAC control group).
[A] Amount of enzyme required to give 50% inhibition of NBT reduction.
[B] Micromoles of H_2O_2 utilized per min.
[C] Micromoles of CDNB conjugated with GSH/min.
[D] Micromoles of GSH utilized/min/mg protein.

bodyweight resulted in the decreased tumor volume, packed cell volume, and viable tumor cell count in a dose dependent manner.

Biochemical findings. Tables 2 and 3 depict the effect of treatment with FV on lipid peroxidation as evidenced by the formation of TBARS and hydroperoxides in liver. The lipid peroxidation indices were significantly increased in the liver of EAC-bearing mice when compared to the normal ones. Treatment with FV not only decreases the tumor burden, but also the oxidative stress on liver and a better effect was observed with the medium dose treatment of the venom. Regarding the antioxidant status, the tumor implant have decreased the activities of enzymes like SOD, CAT, GPX, GST, and GSH levels in the liver. Again treatment with various doses of FV was found to restore the above levels to near normal.

Histological findings. Figure 1a shows the hepatocytes of normal liver with portal trait. The liver tissue of EAC (Fig. 1b) bearing mice shows the presence of carcinoma cell clumps in between the liver tissue, that are anaplastic in nature. The photomicrographs of FV treated liver appear to be normal without any tumor cell invasion (Figs. 1c–f).

Immunostaining of PCNA. Figures 2 and 3 depict the effect of lionfish venom on PCNA expression in hepatocytes of EAC-bearing mice and are compared with the expression in normal mice. In EAC-bearing mice the mean expression of PCNA (84%) was significantly higher than that of normal hepatocytes (43%). Administration of lionfish venom decreased the expression of PCNA in all the three doses tested and a significant decrease was observed with the medium dose treatment of lionfish venom (51%).

The present study was carried out to evaluate the antitumor and hepatoprotective efficacy of the fish venom—*P. volitans*. A reliable criteria on for assessing the potential of any anticancer agent is the prolongation of life span of the animals.[22] A decrease in tumor volume and viable tumor cell count observed by us can be considered as an important marker of reduced tumor burden and enhanced the life span of EAC-bearing mice. Andreani et al.[23] have suggested that an increase in the life span of ascites bearing animals by 25% is considered as an indicative of significant drug activity. This suggests that the cytotoxic activity of fish venom on the EAC cells might be due to mechanisms other than direct cytolytic effect.

In this context several authors have reported the anticancer potential of snake venom and exact mechanism of them are not still understood.[8] Lipps[24] has suggested that venom acts directly on the tumor cells and causes their lysis. But, Markland[25] has suggested that venom act indirectly by destroying the microenvironment produced by the tumor cells. In our earlier work we have demonstrated that fish venom induces apoptosis and causes DNA fragmentation by activation of caspase in EAC cells.[26]

Cell proliferation, a characteristic feature of cancer, is inversely proportional to lipid peroxidation and cancer itself imparts an oxidative stress on the host organism. This is illustrated by the rapid elevation of lipid peroxidation products and impaired antioxidant status of animals xenografted with various tumors.[27] Several studies have demonstrated that tumor-bearing animals can experience a systemic change of antioxidant enzymes in organs distant from the tumor.[10] A study by Zatrowski and Nathan[28] suggested that tumor cells produce substantial

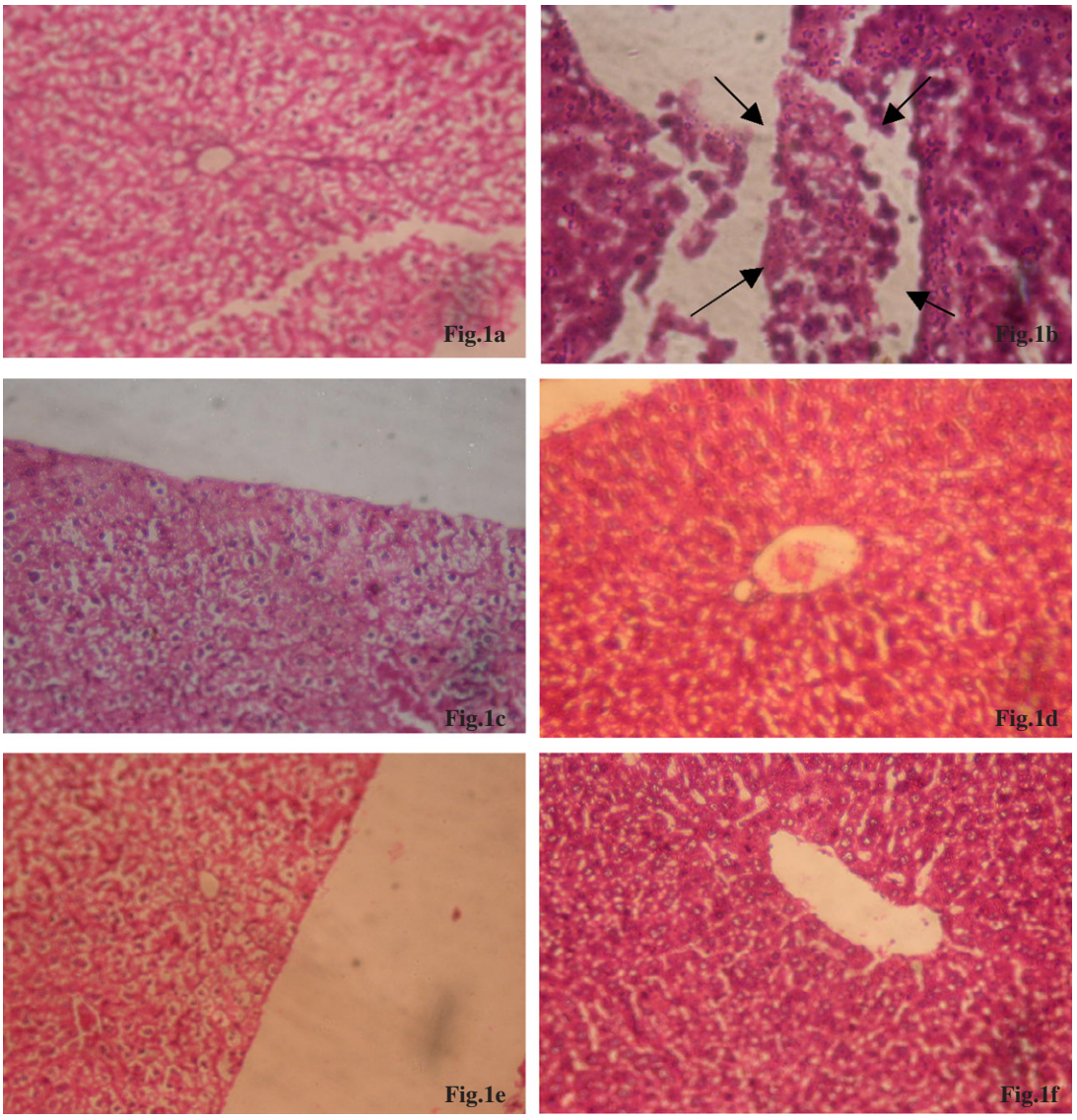

Figure 1. Showing the histological changes of EAC xenografted and FV treated mice liver. (a) 20× H&E: showing hepatocytes of normal liver. (b) 20× H&E: showing tumor cell clumps in between the liver tissue that are anaplastic in nature (→). (c–e) 2 0 X H&E: showing normal hepatocytes of FV treated mice without any tumor mass. (f) 20× H&E: showing normal hepatocytes of 5-FU treated mice without any tumor mass.

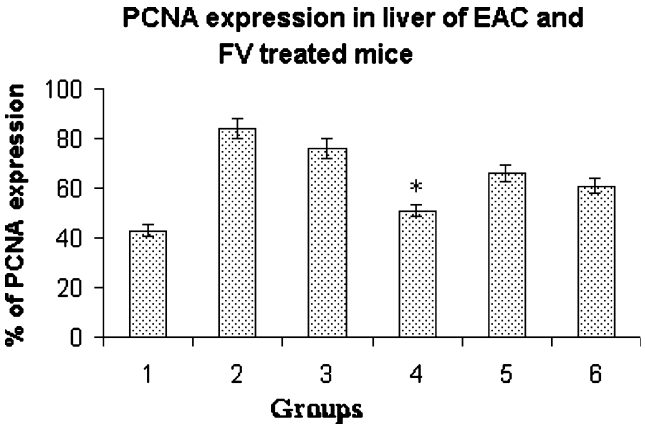

Figure 2. PCNA expression in liver of EAC and FV treated mice.

amount of hydrogen peroxide (H_2O_2), which may be released into circulation for being transported to the liver for detoxification.[29] Furthermore, growing tumors sequester essential antioxidants from host tissues and meet their demand.[30] Therefore, the observed elevated levels of TBARS and hydroperoxides in liver of EAC-bearing mice may be due to the excessive generation of H_2O_2 (by peritoneal cells) that has been transferred to liver for detoxification along with the sequestration of antioxidants by tumor cells.[31] Whereas, treatment with FV effectively decreased the levels of TBARS and hydroperoxides depicting their protective role on the liver by decreasing the tumor burden.

The increased levels of lipid peroxidation in liver of tumor-bearing animals could attribute to the observed decrease in GSH levels, GPx and GST activities. GST and GPX are biotransformation enzymes involved in the detoxification of xenobiotics, carcinogens, free radicals, and peroxides by conjugating these toxic substances with GSH, ultimately protecting the cells and organs from oxidative stress.[29] Treatment with different doses

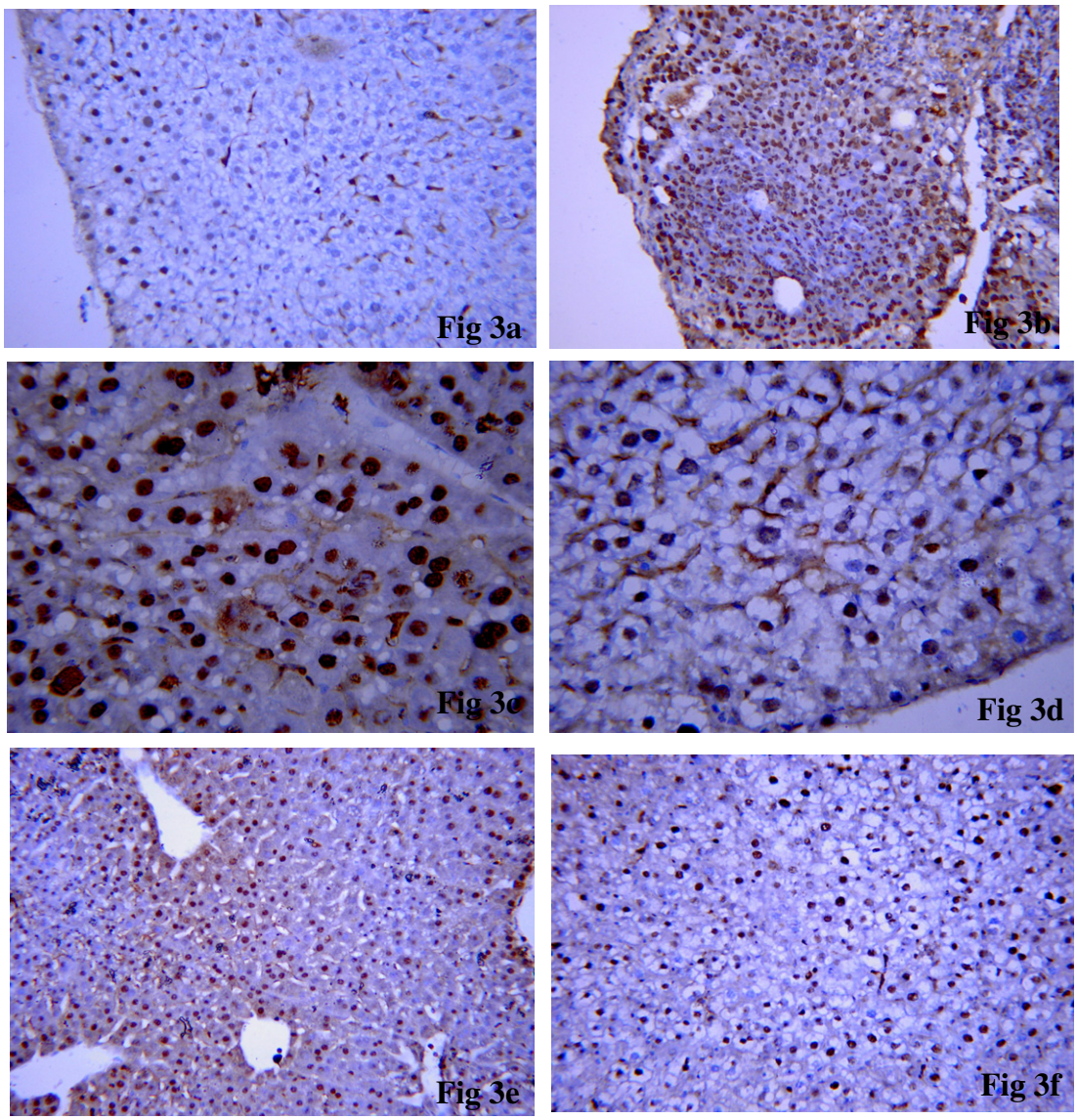

Figure 3. Illustrating the PCNA expression pattern in liver of EAC-bearing and FV treated mice (20×). (a) PCNA expression in normal liver. (b) Overexpression of PCNA in EAC-bearing mice liver. (c) Decreased expression of PCNA in venom (LD) treated mice. (d) Significantly decreased expression of PCNA in venom (MD) treated mice. (e) Decreased expression of PCNA in venom (HD) treated mice. (f) Decreased expression of PCNA in 5-FU treated mice liver.

of FV was observed to decrease the tumor burden, hence decrease the oxidative stress, which in turn results in the restored activities of GST, GPx, and GSH levels.

SOD and CAT are considered as the primary antioxidant enzymes, since they are involved in the direct elimination of active oxygen species.[32] CAT depression both in activity and immunoreactivity in liver is a well-established phenomenon accompanying tumor burden.[33] Decreased SOD activities were also seen in liver, spleen, kidney, lungs, and leg muscle in EAC-bearing mice.[34] Our results are in agreement with the above findings and treatment with FV effectively reduced the oxidative stress in EAC-bearing mice and thereby restored the activities of enzymic antioxidants. In this context Ramanaiah and Venkaiah[9] has reported that, scorpion venom has the SOD activity whose action is inhibited by specific antivenom. Apart from this, in the past few years several peptides have been reported to exert different mechanisms of action in free radical mediated oxidative sequences by radical scavenging and metal ion chelation.[35]

The biochemical findings are supported by the histopathological study, where the liver of EAC-bearing mice shows the presence of anaplastic tumor cells in between the liver tissue, which were absent in all treatment groups. In all the above studied parameters, the medium dose treatment of lionfish venom than that of other two low and high doses respectively, this may be correlated with the findings of Abu-sinna et al.[7] who has suggested a dose dependent effect of snake venom on EAC cells wherein, the low dose may not be sufficient and high dose might have produced any other effects, respectively. Hence the medium dose of lionfish venom is thought to be of optimum to treat the cancer cells.

The observed results are further confirmed by the immunohistogical staining of PCNA in EAC-bearing and venom treated mice. PCNA, an auxiliary protein of the DNA polymerase δ, is a proliferation-associated marker expressed in proliferating cells. Its maximal expression is attained during the late G1- and S-phase period of the cell cycle.[36] PCNA has been used in different neoplasm to measure the growth fraction of the tumors in relation to clinical behavior, expression, size of tumor, and distant metastases are indicators of an aggressive biological behavior. PCNA is a cell cycle-related antigen and its expression progressively increases incrementally as the tissue progresses from normal through premalignant stages to carcinoma.[37,38] The observed result, increased expression of PCNA in EAC-bearing mice, and which decreases upon treatment with lionfish venom are in line with previous studies with various anticancer and antimetastatic compounds.[39] Even though several studies have been carried out on the PCNA expression, we for the first time report that the PCNA expression decreases on treatment with lionfish venom. Thus, the present study clearly demonstrates the antitumor; hepatoprotective, and antimetastatic effects of FV on EAC-bearing mice and further studies are warranted for better understanding of the exact role of lionfish venom.

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