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Effect of *Echis carinatus* snake venom and its purified fractions F_{11} and F_{11A} on the evolution of Ehrlich ascites tumor (EAT) cells

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Abstract

The present study was undertaken to investigate the effects of *Echis carinatus* crude venom and some of its isolated toxic fractions on Ehrlich ascites tumor (EAT) cells transplanted into the peritoneal cavity of the white female mice as an experimental model of fast growing tumor. The fractionation of the crude venom by gel filtration on sephadex G-75, yielded four fractions (F_1, F_{11}, F_{111} and F_{1V}) while, the refractionation of these fractions by ion exchange chromatography using diethylamino ethyl cellulose (DEAE-cellulose) gave nine subfractions (F_{1A}, F_{1B}, F_{11A}, F_{11B}, F_{11C}, F_{111A} , F_{111B}, F_{1VA} and F_{1VB}).All these separated subfractions were pure when tested by a third chromatographic step using SDS-PAGE and scanned with densitometer scan. Some *in vitro* biological properties of the crude venom and its isolated fractions were studied. It was found that F_{11} showed the highest value of protein content, followed by F_{111} and F_{11A}. On the other hand, it was found that no interrelationship between phospholipase enzyme activity (PLA_2) and the protein contents of the crude venom and its isolated fractions. Toxicity studies were conducted on adult female Swiss albino mice to determine the approximate acute median lethal dose (LD_{50}) of the crude venom and its purified fractions. LD_{50} of crude venom, F_{11} and F_{111} were found to be 1.92, 2.35 and 2.75 µg/g b.w. In addition, F_1 and F_{1V} were non-lethal, while after ion exchange chromatography, the only toxic fraction was F_{11A} (LD_{50} = 1.85 µg/g b.w.). The inhibitory effect of crude venom, F_{11} and F_{11A} on tumor growth was studied by treatment of EAT bearing mice with two intraperitoneul injections of the sublethal doses (20 and 2% LD_{50}) of crude venom and its purified fractions F_{11} and F_{11A}. The growth inhibitory effect of 20 and 2%LD_{50} of the crude venom, F_{11}, and F_{11A} was confirmed by a remarkable decrease of the viable tumor cells. This was accompanied by a significant increase in the number of dead cells, and increased percentage of survivals. On the other hand, the animals inoculated by EAT cells showed a significant increase in polymorphonuclear (PMN) and mononuclear (MN) leukocyte cells influx into the peritoneal fluid.

It could be concluded that *Echis carinatus* crude venom and its isolated toxic fractions F_{11} and F_{11A} showed a significant antitumor activity against EAT cells transplanted in the intraperitoneal cavity of white female albino mice.

Introduction

Many experimental studies have been carried out using snake venoms for the treatment of animal tumors. However, we have seen many controversies on this subject. Some authors have reported that tumor treatment with specific snake venoms fractions has an important toxic effect on tumor cells, while others have reported no antitumor effects after inoculation with snake venom (Da Silva *et al.,* 1996). The literature contains a layout of evidence regarding

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effect of snake venoms on the inhibition of tumor cell proliferation in animals (Martikianen *et al.,* 1993; Lipps, 1994; Stevens-Teruss *et al.,* 1996; Juhl *et al*., 1997 and Costa *et al.,*1998).

Preliminary and essential experiment for evaluation of the validity of antitumor agent depends on its efficiency in elongation of life (patients or experimental) and /or improvement in their general health. Also, it has been known that criteria used for evaluating the tumor-inhibitory effect of a compound include; inhibition of tumor growth, histologic changes (decrease in mitotic figures and amount of necrosis) and survival time of the host (Fahim *et al.,* 1988).

Most attempts have been carried out to use snake venoms in the therapy of many painful diseases (Ibragimov and Rosulova, 1987; Fernandez-Gomez *et al.,* 1994 and Mollman *et al.,* 1997). With the development of several techniques, various attempts have been tried to recognize a suitable venom or venom fraction to be used for the treatment of many types of malignant tumors (Cohen and Quisted, 1998; Costa *et al.,* 1998; Lipps, 1999 and De Carvalho *et al.,* 2001).

The exact mechanisms that cause tumor regression in experimental animals after treatment with crude venom and/or fraction of this venom still are unknown. According to Lipps, (1999) certain fractions isolated from snake venoms revealed a direct cytolytic activity on tumor cells. Many researchers have demonstrated also that snake venoms may induce apoptosis in both normal and tumor cells (Torii *et al.,* 1997 and Abe *et al*., 1998). In most of these works, the proposed apoptosis induction mechanism was H2O2 production induced by the venom enzyme L- amino oxidase. This enzyme was purified from Crotalus adamanteus venom and its structure showed a high degree of homology with murine interleukin-4 (Abe *et al*., 1998). On the other hand, many studies have shown that leukocytes may effectively participate in venom-induced tumor cell elimination (Gallagher *et al*., 1990 ; Smyth *et al*., 1990 and Da Silva *et al*., 1997).

The present study was designed to investigate the effect of the viper *Echis carinatus* crude venom and its isolated purified fractions F11 and F11A on the growth of Ehrlich ascite tumor (EAT) cells and the possible mechanism of action.

Material and Methods:

Animals

Adult female albino mice weighing from 20-30 grams were purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Cairo). The animals were maintained for one-week acclima - tization period on commercial standard pellet diet and tap water *ad libitum.*

Venom

Echis carinatus venom was milked from snakes kept in the Serpentarium at the laboratory of physiology, Department of Zoology, Faculty of science, Ain Shams University. The venom was lyophilized and stored desiccated at 4ºC in the dark.

Fractionation of the crude venom- Gel filtration:

The crude venom was dialyzed against distilled water for 48 hr at 4ºC and lyophilized. Lyophilized venom was fractionated by the method of Laurent and Killander (1964), using a 1.7 X 80 cm column of sephadex G-75 (super fine) otained from Pharmacia fine chemicals (Upssala, Sweden) and

0.03M phosphate buffer, pH 7.4 as eluent. The procedure was carried out at 4ºC and 3ml fractions were collected in test tubes. The total protein content of each tube was determined by absorbance measurement at 280nm (A_{280}). Appropriate fractions were pooled, then dialyzed against distilled water, and lyophilized.

Refractionation by DEAE-cellulose:

Lyophilized fractions were refractionated by the method of Sugihara *et al*. (1983) using Diethylamino ethyl cellulose, anion exchanger (DEAE-cellulose) purchased from Pharmacia fine chemicals (Upssala, Sweden) and ammonium acetate buffer of increasing moralities at pH 7,column dimension 1.7 X 30 cm at a flow rate 35ml/hr.

SDS-PAGE – Gel electrophoresis:

Purity of the isolated repurified fractions was tested using slab gel electrophoresis (SDS-PAGE) according to the method of Ornstein (1964). Gels were scanned using Gelman digital computing densitometer at 700nm, 20 cm/min and 0.5 volt.

Phospholipase A_2 (PLA_2) activity and the total protein contents:

Crude venom and each of the lyophilized fractions were assayed for PLA_2 activity according to the method of Augustyn and Elliot (1969). The total protein content was determined according to the method of Lowry *et al*.(1951).

Determination of the LD_{50}:

The LD_{50} of crude venom and its purified fractions was carried out according to Meier and Theakston (1986).

Ehrlich ascites tumor (EAT) cells:

The first inoculate of EAT cells was kindly provided by the National Cancer Institute, Cairo University, Egypt. EAT cells were thereafter propagated in our laboratory weekly i.p injection of 0.3ml of 1:5 saline solution of freshly drawn ascites fluid from a donor mouse bearing 6-8 days old ascites tumor, into 3 mice with a mean weight of 24±2g. Each inoculm contained approximately $12X10^6$ cells. Transplantation was carried out using sterile disposable syringes under aseptic conditions (Fujimoto *et al*., 1976).

Antitumor effect of the crude venom and its isolated fractions F_{11} and F_{11A}

A total number of 160 female mice were involved in the present study. Twenty of them were injected with saline (0.1 ml, i.p) and served as cotrol group (G_1). The remaining 140 female mice were inoculated (i.p) with 6 X 10^6 EAT cells/ animal. Implantation of cells/ animal was performed under complete aseptic conditions and in the next day the animals were randomly divided into seven equal groups (n=20); G_2-G_8.

G_1- Saline control
G_2 -Tumor control (EAT).
G_3 - EAT+ 20% LD_{50} crude venom.
G_4 -EAT+ 2% LD_{50} crude venom.
G_5 - EAT+ 20% LD_{50} F_{11}.
G_6 - EAT+ 2% LD_{50} F_{11}.
G_7 - EAT+ 20% LD_{50} F_{11A}.
G_8 - EAT+ 2% LD_{50} F_{11A}.

The crude venom and the fractions (F_{11} and F_{11A}) were injected intraperitoneally 24hr and 5 days after inoculation by EAT cells. Each group of animals were subdivided into two equal subgroups (n=10) to reach a total number of 16 subgroups.

The first 8 subgroups of animals were used for determination of mortality rate and survival time throughout the experimental period (36) days.

The remaining 8- subgroups of animals (n=10) were sacrificed at the end of the experimental period (14 days), and the ascitic fluid was obtained from each animal. Individual ascitic

fluids were collected into centrifuge tubes chilled in an ice bath. EAT cells were first separated from the ascites fluid by centrifugation at 1500 r.p.m. for two minutes , and the supernatant was discarded. The packed cells were washed twice with ice-cold saline until become completely blood free. The backed cells were then resuspended in a fixed volume of saline for counting. For this purpose, 0.2 ml 0f 0.32% trypane blue (Sigma,USA) were mixed with 0.2 ml of EAT cells, left for 3 minutes then placed on standard hemocyto-meter. The viable (non-stained) and the dead tumor cells/ml were counted according to the method of (Liman Mc *et al.,* 1957). Differential counts for PMN and MN leukocyte cells were performed on fixed and stained cell suspensions (0.5 % crystal violet dissolved in 30% acetic acid) using standard hemocytometer.

Results*:*

Fig. (1) shows the profile of fractionation of the crude *Echis carinatus* venom on sephadex G-75 column (1.7 X 80cm, phosphate buffer 0.03M,pH 7.4). The protein was resolved into four main fractions F_1, F_{11}, F_{111} and F_{1V}. Each fraction was refractionated on DEAE-cellulose, using ammonium acetate buffer 1M, pH7 column (1.7 X 30 cm) at a flow rate 35 ml/hr (Fig. 2,3). It was found that, F_1 yielded two types of protein F_{1A} and F_{1B} (Fig.2). F_{11} gave 3 peaks F_{11A}, F_{11B} and F_{11C}, while, F_{111} and F_{1V} yielded two peaks for each F_{111A} , F_{111B}, F_{1VA} and F_{1VB} respectively (Fig. 3).All of these separated fractions were pure when using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig.4, 5).

Table (1) shows the results of the protein contents, PLA_2 activity and LD_{50} for crude venom and its purified fractions. It was found that, the protein content for the crude venom was 166 ug /ml and for isolated and purified fractions were 20.85, 57, 15.5, 14.1, 6.7, 55, 7.6, 5.8, 25, 8.6, 8.75 and 3.8 ug /ml for F_1, F_{11}, F_{111}, F_{1V}, F_{1A}, F_{1B}, F_{11A}, F_{11B}, F_{11C}, F_{111A}, F_{111B}, F_{1VA} and F_{1VB} respectively. The level of PLA_2 activity for the crude venom was 525ug hydrolyzed lecithin / hr/ mg protein, while, it was found that PLA_2 levels in the isolated and purified fractions were 3.65, 8.7, 1.1, 7.65, 9.1, 20.5, 1.2, 15.2, 14.5, 3.78, 22.8, 10.9 and 29.5 ug hydrolyzed lecithin/hr/ mg protein for F_1, F_{11}, F_{111}, F_{1V}, F_{1A}, F_{1B}, F_{11A}, F_{11B}, F_{11C}, F_{111A}, F_{111B}, F_{1VA} and F_{1VB} respectively.

On the other hand, LD_{50} for crude venom F_{11} and F_{111} was found to be 1.92, 2.35 and 2.75 ug/g b.w. respectively. In addition, after ion exchange chromatography step, the only toxic fraction was F_{11A} and its LD_{50} was found to be 1.86 ug/g b.w. All other separated fractions were non lethal up to 10 ug/g b.w., these results illustrated that, there was no direct relationship between toxicity and PLA_2 activity either in crude venom or in its purified fractions.

Fig.(6) illustrate the percentage of survival of animals inoculated with EAT cells and those inoculated and treated with *Echis carinatus* venom and its fractions (F_{11} and F_{11A}). It was found that animals inoculated with EAT cells and treated with the crude venom and its fractions have increased survival time and decreased mortality when compared with the untreated animals.

Table (2) and Fig. (7 a,b) demonstrated the effect of *Echis carinatus* crude venom and its purified fractions (F_{11} and F_{11A} ;20 and 2% LD_{50}) on the viable and dead EAT

cells count *in vivo* (10^6 cell /ml). It was found that the growth inhibitory effect of 20% LD_{50} of the crude venom, F_{11} and F_{11A} was confirmed by a significant ($P<0.001$) decrease of the viable tumor cells by -51.87, -61.9 and –69.04% respectively. This was accompanied by a significant increase in the number of dead cells by 760.7, 411.9 and 388.1% respectively. On the other hand, at a dose level of 2% LD_{50} there was a significant ($P<0.001$) decrease in the viable tumor cells by -42.84, -29.39 and –63.75% and by a significant increase in the number of dead cells by 0.95, 345.5and 206.7% for crude, F_{11} and F_{11A} respectively. These results showed that the crude venom and F_{11A} had the superior effect in the treatment of tumor cell growth.

Table (3) and Fig. (8 a,b) show the changes in leukocyte cell count (PMN and MN) in animals inoculated with EAT cells and those inoculated with EAT cells and treated with crude venom and its purified fractions (20 and 2% LD_{50}) in comparison with saline control animals. It was found that the influx of both PMN and MN leukocytes was significantly increased in animals inoculated with EAT cells. On the other hand, in EAT cell bearing animals treated with crude venom and its fractions the influx of leukocytes was decreased but it was still significantly higher than that of saline control group.

Table (1): Protein content, phospholipase A_2 (PLA_2) activity and approximate lethal dose (LD_{50}) in crude *Echis carinatus* snake venom and its isolated and purified fractions.

Parameters **Crude venom** & its fractions	Protein content **(µg/ml)**	**PLA_2 (µg/hr/mg protein)**	**LD_{50} (µg/gm)**
Crude venom	166	525	1.92 µg/gm
F_1	20	3.65	Non toxic
F_{11}	85	8.7	2.35 µg/gm
F_{111}	57	1.1	2.75 µg/gm
F_{1V}	15.5	7.65	Non toxic
F_{1A}	14.1	9.1	Non toxic
F_{1B}	6.7	20.5	Non toxic
F_{11A}	55	1.2	1.86 µg/gm
F_{11B}	7.6	15.2	Non toxic
F_{11C}	5.8	14.5	Non toxic
F_{111A}	25	3.78	Non toxic
F_{111B}	8.6	22.8	Non toxic
F_{1VA}	8.75	10.9	Non toxic
F_{1VB}	3.8	29.5	Non toxic

Table (2): Effect of *Echis carinatus* crude venom and its purified fractions F_{11} and F_{11A} (20 and 2% LD_{50} on the viable and dead Ehrlich ascites tumor (EAT) cells count in vivo (10^6 cell/ml).

Groups Parameters	Saline G_1	Tumored G_2	Tumored and venom treated					
			Crude venom		F_{11}		F_{11A}	
			20%LD_{50} G_3	2%LD_{50} G_4	20%LD_{50} G_5	2%LD_{50} G_6	20%LD_{50} G_7	2%LD_{50} G_8
Viable cells $\bar{X} \pm$ SE. % change Sig.	0	162±2.3	78.2±3.9 -51.87 P<0.001	92.88±5.8 -42.84 P<0.001	61±3.6 -61.9 P<0.001	114.6±5.8 -29.3 P<0.001	50.3±4.1 -69.04 P<0.001	58.9±4.3 -63.75 P<0.001
Dead cells $\bar{X} \pm$ SE. % change Sig.	0	4.2±0.71	36.1±3.3 760.7 P<0.001	7.95±0.4 89.3 P<0.001	21.5±3.2 411.9 P<0.001	18.7±2.9 345.5 P<0.001	20.5±3.5 388.1 P<0.001	12.8±1.6 206.7 P<0.001

P<0.001 = very highly significant.

Table (3): Polymorphonuclear (PMN) and mononuclear (MN) leukocyte cells count in peritoneal fluid (10^6 cell/ml) of EAT cells of tumored and tumored treated animals.

Groups Parameters	Saline G_1	Tumored G_2	Tumored and venom treated					
			Crude venom		F_{11}		F_{11A}	
			20%LD_{50} G_3	2%$LD_{50}G_4$	20%LD_{50} G_5	2%LD_{50} G_6	20%$LD_{50}G_7$	2%LD_{50} G_8
PMN $\bar{X} \pm$ SE. Sig.	0.6±0.18	2.85±0.03 P<0.001	1.81±0.12 P<0.001	1.79±0.04 *P<0.001*	1.96±0.02 2 P<0.001	1.94±0.03 P<0.001	1.72±0.06 P<0.001	1.63±0.028 *P<0.05*
MN $\bar{X} \pm$ SE. Sig.	2.9±1.9	6.6±0.27 P<0.001	5.25±0.28 P<0.001	4.26±0.19 P<0.001	5.18±0.28 P<0.001	4.91±0.24 P<0.001	3.94±0.36 P<0.001	3.59±0.20 P<0.05

P<0.05 = significant. ***P<0.001 = very highly significant.***

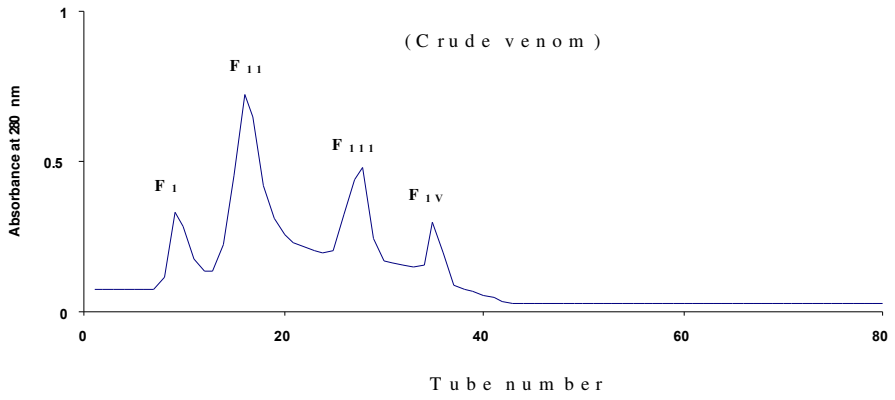


Fig. (1): Chromatographic separation of *E. carinatus* crude venom on sephadex G-75 using phosphate buffer (0.03M, pH 7.4).

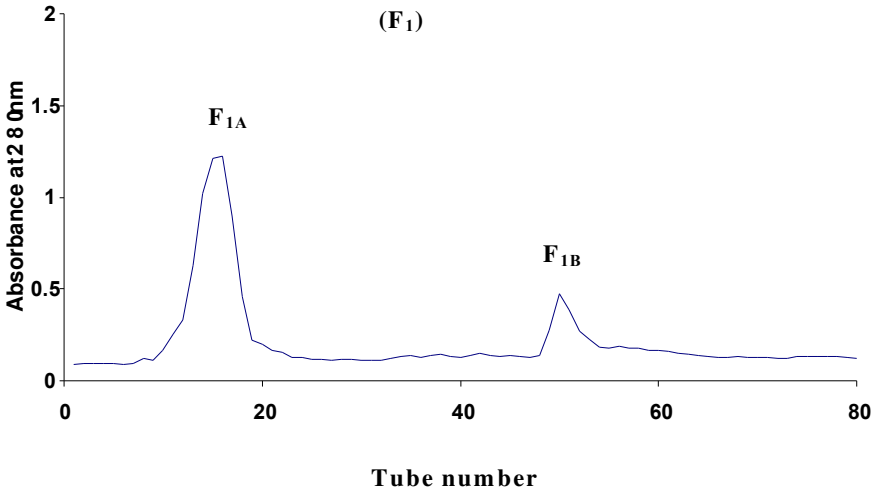


Fig. (2): Refractionation of F_1 on DEAE- cellulose using ammonium acetate buffer 1M, pH 7 (column dimensions 1.7X30cm) at flow rate 35ml/hr.

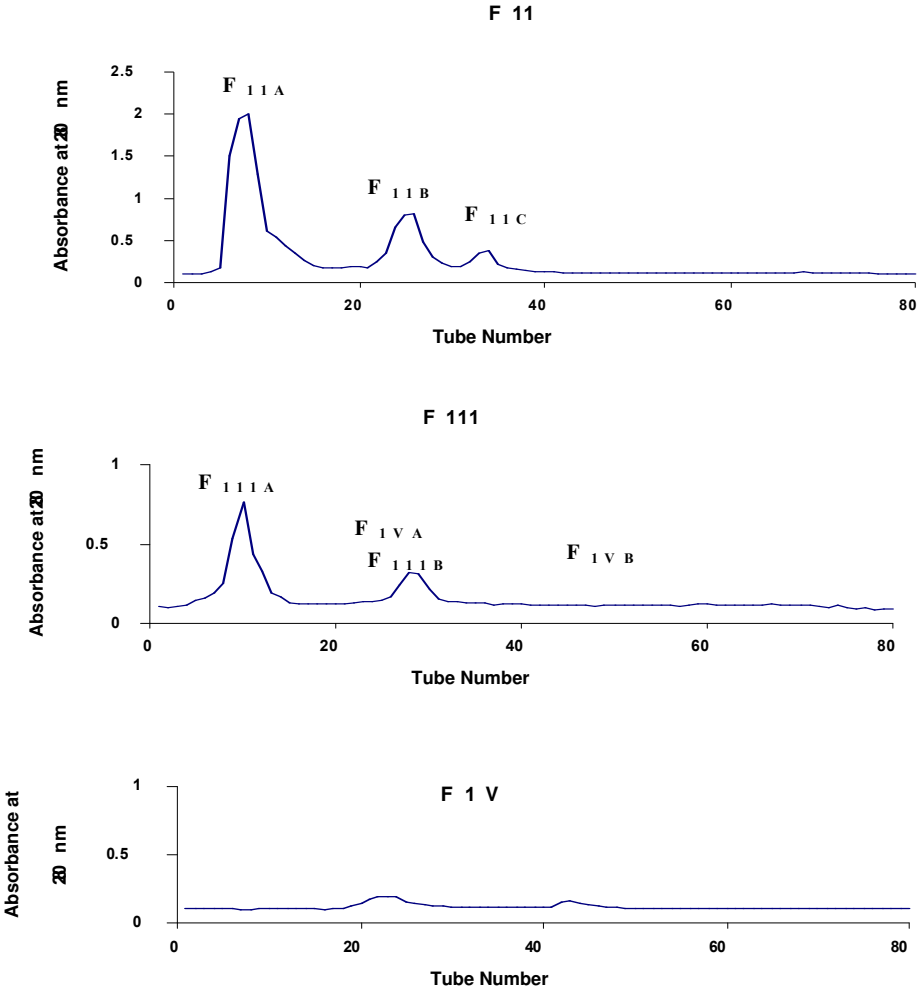


Fig. (3): Refractionation of $F_{11,}$ F_{111} and F_{1V} on DEAE-cellulose using ammonium acetate buffer 1M, pH7 (column dimensions 1.7X30cm) at a flow rate 35 ml/hr.

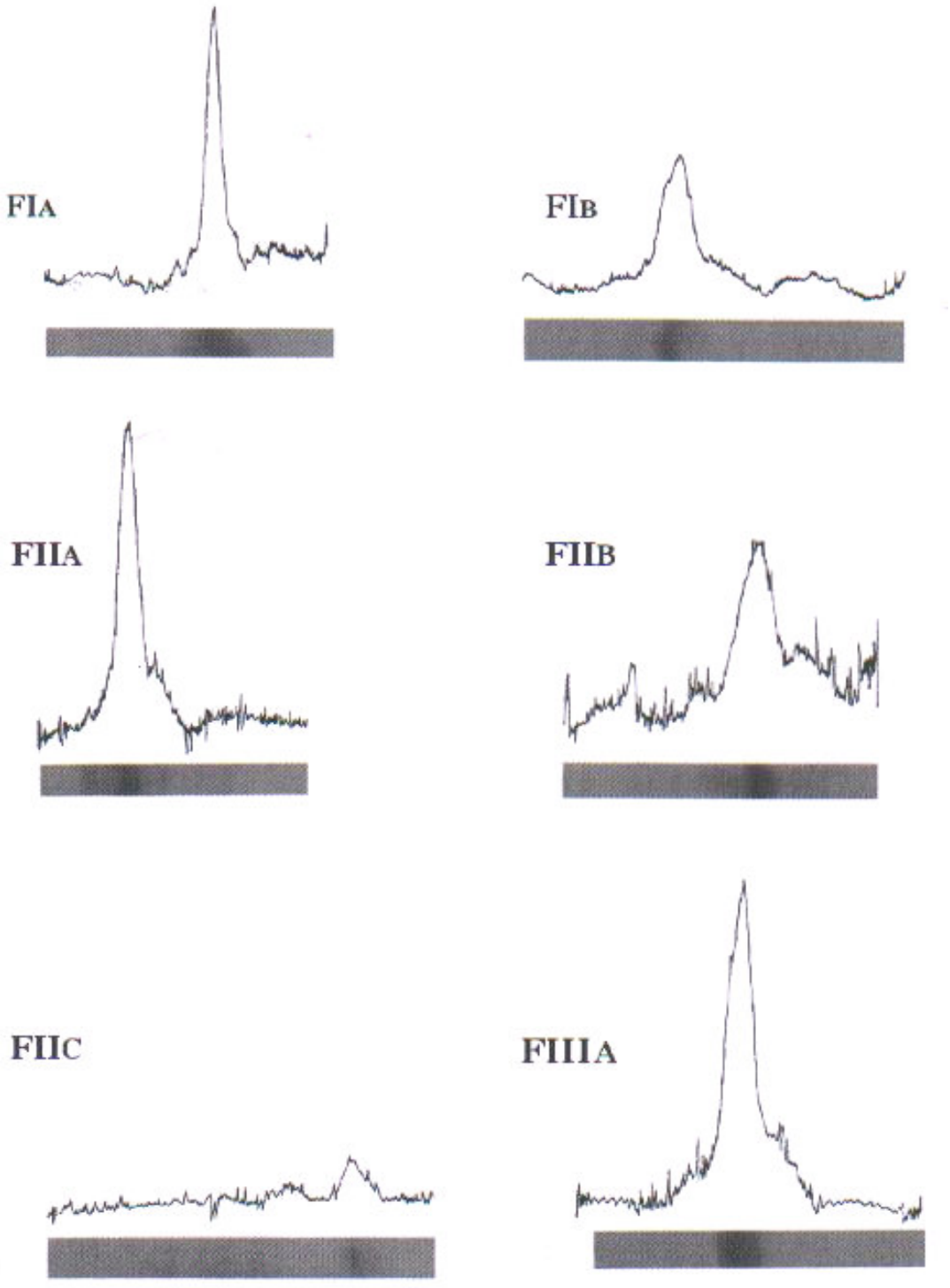


Fig.(4): Densitometric scan of FIA, FIB, FIIA,FIIB, FIIC and FIIIA fractions of ***Echis carinatus*** snake venom separated by SDS-PAGE electrophoresis.

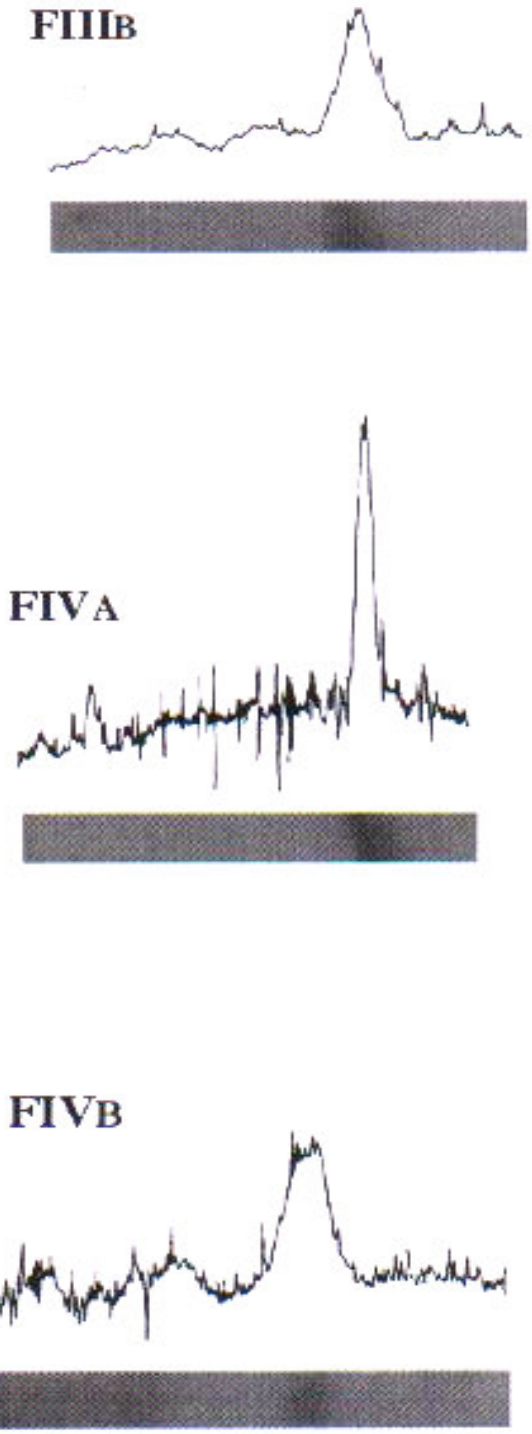


Fig.(5): Densitometric scan of FIIIB, FIVA, and FIVB, fractions of ***Echis carinatus*** snake venom separated by SDS-PAGE electrophoresis.

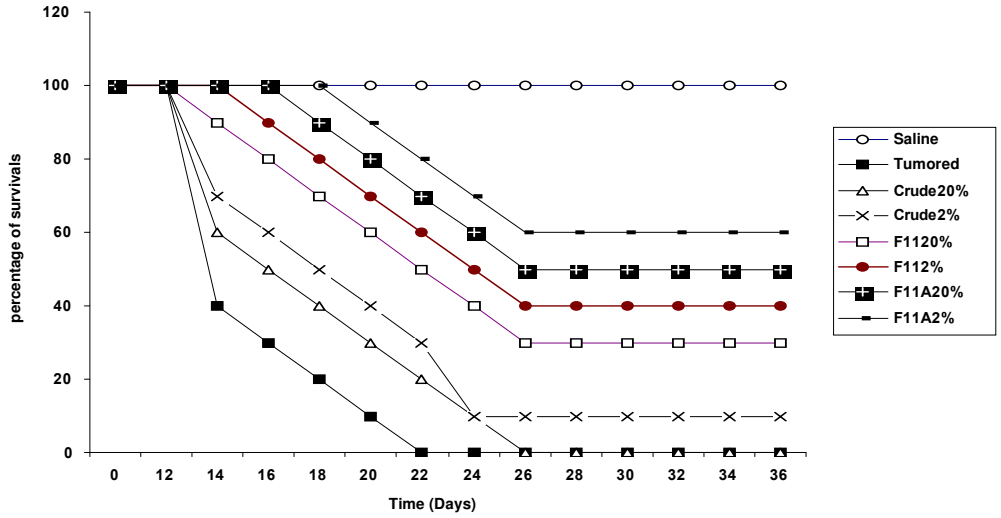


Fig. (6): Survival percentages of animals inoculated with EAT cells and treated with *Echis carinatus* crude venom and its purified fractions F_{11} and F_{11A}.

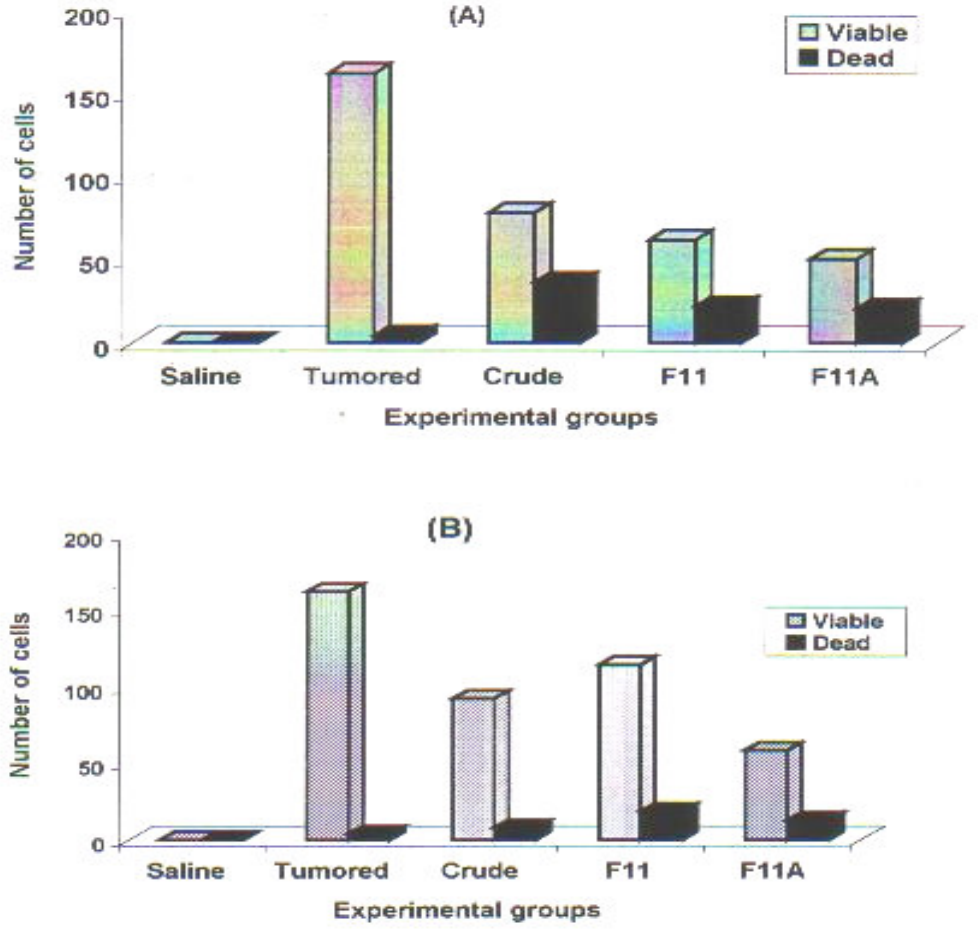
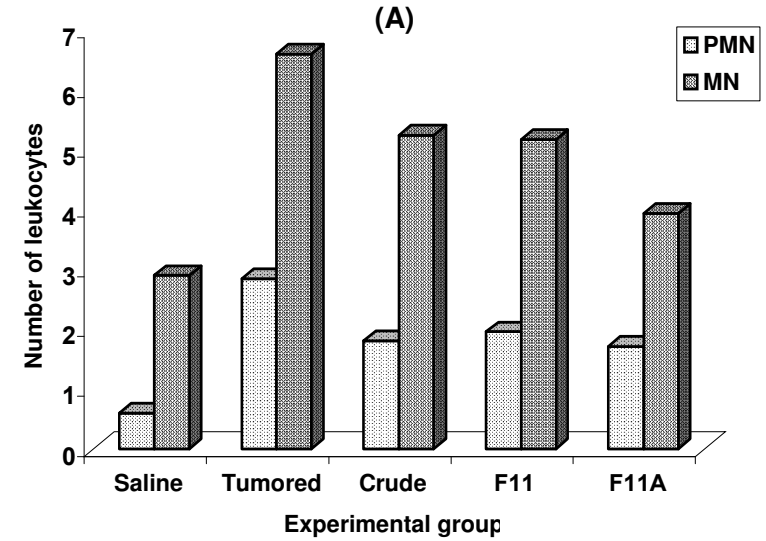


Fig.(7 A,B): The number of viable and dead cells (N x10^6 cells/ml) in the peritoneal cavity of the animals on the 14th day of tumor evolution.(A=20% LD_{50}& B=2%LD_{50}))



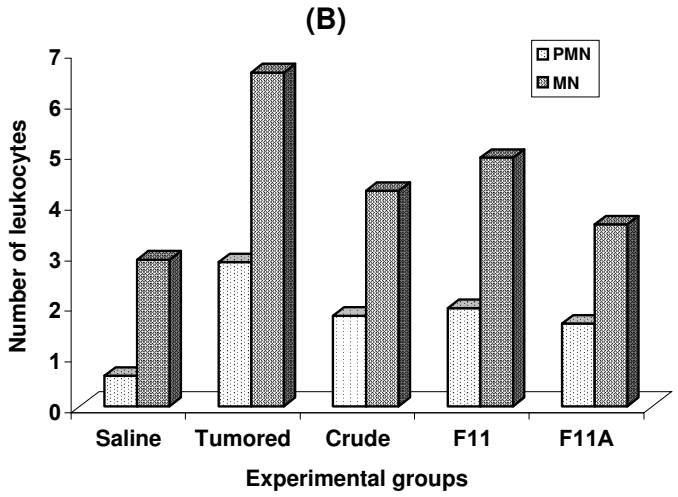


Fig.(8 A,B): The number of PMN and MN cells (N x10^6 cells/ml) in the peritoneal cavity of the animals on the 14th day of tumor evolution.(A=20% LD_{50}&B=2%LD_{50}))

Discussion

Several studies have been reported in an attempt to find a specific venom or venom fraction to inhibit the growth and destroy different types of cancer (Chim-Matyas and Ovida, 1987 and Calteneo *et al*., 1993). These studies

used *in vivo* (active bearing tumor animals or induced tumor formation) and *in vitro* techniques. On the other hand, several authors referred to the

mitogenic effects of snakes and scorpion venom and venom fractions (Abu sinna *et al*., 1992; Lipps, 1994, 1999; Cohen and Quisted, 1998; Schmitmeier *et al.,* 2000; de Carvalheo *et al.,* 2001and Ritter and Markland, 2001).

In this study, *Echis carinatus* crude venom was fractionated on sephadex G-75 column (1.7X80cm) eluted with phosphate buffer pH 7.4. The protein was resolved into four peaks. Further purification of the isolated fractions, on anion exchanger (DEA-cellulose) yielded nine fractions.

The present study revealed that *Echis carinatus* crude venom and its purified fractions (F_{11} and F_{11A}) showed a phospholipase A_2 (PLA_2) activity and were associated with an obvious inhibition of tumor growth in EAT cell inoculated animals. This suppression of tumor growth was accompanied by increased percentage of survivals. The PLA_2 activity encount - ered in the present study was previously demonstrated in *Echis carinatus* venom and its fractions by Fletcher et al. (1980). In addition, Kemparaju et al. (1994) separated three types of PLA_2 enzymes (neutral, acidic and basic) from *Echis carinatus* venom by using gel filtration on sephadex G-50 column.

The tumor suppression induced by *Echis carinatus* and its purified fractions may be attributed to the presence of PLA_2, which is highly destructive to cell membrane via conversion of lecithin to lysolecithin (Chaim-Matyas et al., 1991). This possibility was suggested by Tu (1973) who postulated that tumor inhibition may be partially attributed to the cytopathic effect of PLA_2 contained in *Naja nigricollis* crude venom and its fractions. Moreover, other studies demonstrated a potent cytotoxic activity to PLA_2 present in cytotoxins isolated from different sources, such as pancreatic, bee venom, scorpion as well as snake venom (Chaim-Matyas et al., 1991, 1995). In contrast, other studies reported that venom PLA_2 has no necessarily tumoricidal activity (Martikainen et al., 1993).

The antitumor effect of the crude *Echis carinatus* venom and its purified fractions may be explained by the fact that snake venoms are a complex mixture of many different types of toxins. These large toxins are composed of two polypeptide chains (A and B) linked by disulfide bonds. Chain A is toxic for the process of protein synthesis in the cell, while chain B binds to the cell surface. These cytotoxins may be combined with lipoproteins in the plasma membrane of susceptible cells, causing cell shrinkage (Chaim-Matyas and Ovadia, 1987). In addition, this protein apparently has the specificity to among the lipoproteins involved in the activity of Na^+- K^+ ATPase in different cells. This process may affect the plasma membrane without disrupting its integrity and also may induce permeability changes for ions such as calcium and sodium. As a consequence, there might be a calcium influx, which is known to affect mitochondria. This cytotoxic activity in the mitochondria of malignant cells may decrease ATP production which in turn may be followed by an increase in lysosomal activity leading to vaculization and alterations of the endoplasmic reticulum which assumed a microsomal-like appearance two hours following intoxication (Oron *et al.,* 1992).

The exact mechanisms that cause tumor regression in experimental animals after treatment with crude venom and or any fraction of this

venom still are unknown. Certain fractions isolated from snake venoms revealed a direct cytolytic activity on tumor cells. The cancer cell inhibitors Atroporin (AT) and Kaotree (KT) were isolated by fractionating *Crotalus atrox* and *Naja Kaouthia* snake venom respectively by high-pressure liquid chromatography (Lipps, 1999). The homogenous preparation of AT and KT showed killing effects on various types of human (breast, colon, liver, ovary etc.) and animal cancer cells while having no effect on normal cells of mouse kidney, liver and spleen.

A four-step chromatography was developed to purify contortrostatin (CN), a snake venom protein inhibitor of platelet aggregation and a powerful antagonist of tumor growth, angiogenesis and dissemination, from the complex mixture of proteins in southern copperhead snake venom *Agkistrodon contortix contortix* (Swenson *et al*., 2000 and Bolger *et al*., 2001). CN is a member of the disintegrin family of protein, which are characterized by the presence of an amino acid sequence (Arg-Gly-Asp) that serves as the binding glycoproteins, which are found on the surface of normal and malignant cells (Schmitmeier *et al*., 2000; Swenson *et al*., 2000; Markland *et al.,* 2001 and Ritter and Markland, 2001).

De Carvelho *et al.* (2001), isolated and characterized a lectin (polyvalent carbohydrate binding protein of non-immune origin)from the venom of the snake *Bothrops jararacussu.* This lectin (BjcuL) has been shown to bind to lactose moieties and induce agglutin - ation of erythrocytes. The authors postulated that, the lectin may serve as an interesting tool for combating tumor progression by inhibiting tumor cell and endothelial cell growth.

Many studies demonstrated also that snake venoms may induce apoptosis in both normal and tumor cells (Torii et al., 1997; Abe et al., 1998 and Tan et al., 2000). In most of these works, H2O2 production induced by the venom enzyme L- amino acid oxidase may be responsible for the induction of apoptosis. This enzyme was purified from the venoms of *Crotalus addam - anteus, Bothros jararaca* and *Cerastes ceracres* (Tan and Pannduri, 1991 and Pessatti et al., 1995 and Zaki et al., 1995), and its strcture showed a high homology with murine interleukin-4 (Raibekas et al., 1998). As *Echis carinatus* belongs to the same family (viperidae) which is characterized by the presence of L- amino acid oxidase, the possible antitumor action of this enzyme on EAT cells in the present study can not be excluded. However, venom biodistribution in the body should also be considered for apoptosis, as it was demonstrated that the venom should have enough contact time with tumor cells to induce apoptosis (Tanigawa *et al.*, 1994).

The present study also showed that EAT cell inoculation was associated with a marked increase in the peritoneal influx of both PMN and MN leukocyte cells which may play an important role in inhibition of tumor growth. This finding is in line with the result of Gallagher et al.(1990) and Smyth et al.(1990) who showed that mononuclear leukocytes may effectively participate in tumor cell elimination. In addition, Da Silva (1997) found that, after treatment with *Crotalus durissus terificus* venom, macrophages showed an increase in size together with the presence of large cytoplasmic vacuoles, suggesting high cellular activity.

The interaction of snake venom and animal cells may cause the release of certain chemical modulators of the inflammatory response (Fecchio et al., 1990). Snake venoms might have direct

and / or indirect action on tumor cells by stimulating the host cells, mainly macrophages. Such stimulation might induce production and release of several cytokines such as TNF-α, IL-1, IL-6 and IL-8 (Barraviera et al., 1995 and Da Silva, 1997). Some of these cytokines have direct cytotoxic effect on tumor cells while others act on the other cells such as natural killer cells (NK) and cytotoxic T lymphocytes to activate them. In addition, these cytokines might stimulate production of C-reactive protein and complement factor C3 that would act as opsonins on tumor cells in the liver (Da Silva, 1997).

Venom can also act on the endothelial cells promoting the release of IL-6 and IL-8 (Lomont et al., 1993 and Barraviera et al., 1995). IL-8 act as a chemotatic factor for leukocytes, mainly for neutrophils. Neutrophils have the properties of chemotaxis, adherence to immune complexes and phagocytosis. The activated PMN can lead to the elimination of tumor cells via oxidative and non oxidative mechanisms (Barraviera et al., 1995). In addition, polymorphonuclear leukocytes can be activated by TNF-α released by the mononuclear phagocytes. Thus, venom could also act as an indirect inducer of neutrophils via the release of TNF-α.

On the other hand, it was reported that malignant cells would produce a microenvironment around them, using substances of the host itself that protected them against defensive responses of the immune system. This microenvironment would be composed of fibrin deposits from the nearly blood vessels.Crotalase, an enzyme present in viper venoms including *Echis carinatus* venom, was found to apparently destroy the microenvironment produ -ced by the tumor cells. Crotalase attaks directly fibrinogen found in abnormal microclots leading to the formation of soluble fibrin monomers that are rapidly removed by secondary activa - tion of the fibrinolytic system (Markland, 1986). Thus, one of the possible explanations for the inhibition of tumor growth encountered in the pres - ent study is that the presence of crotal - ase enzyme, will unmask the tumor cells and consequently will facilitate the action of various cytotoxic agents.

It could be concluded that *Echis carinatus* venom and its purified fractions have an antitumor effect in EAT cells inoculated animals. PLA2 and/or various immunological defensive mechanisms may induce this effect, however, other mechanisms can not be excluded.

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تأثير سم الحية ايكس كاريناتيس و مفصولاته F_{11} و F_{11A} علي نمو خلايا ايرليش السرطانية

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اجريت هذه الدراسة لإلقاء الضوء علي تأثير السم الخام للإيكس كاريناتيس وبعض من مفصولاته ذات السمية علي نمو خلايا ايرليش السرطانية المنقولة الي التجويف البريتوني في اناث الفئران البيضاء كنموذج لحيوان تجريبي .

تم فصل السم الخام عن طريق الفصل الكروماتوجرافي باستخدام عامود سيفادكس G-75 وتنتج هذا اربع مفصولات هي F_1,F_2,F_3,F_4 بينما نتج عن اعادة فصل هذه المفصولات باستخدام الترشيح التبادلي الأيوني تسعة مفصولات هي$F_{1A},F_{1B},F_{11A}, F_{11B}, F_{11C}, F_{111A}, F_{111B},F_{1VA}, F_{IVB}$

تلا ذلك اختبار درجة النقاوة لهذه المفصولات باستخدام الفصل الجيلاتيني الكهربي (الهجرة الكهربية) وقد ثبتت نقاوة جميعها بواسطة مسح شرائح الجل الناتجة لكل مفصول بمقياس الكثافة اللونية . وقد أجريت بعض الدراسات البيلوجية (خارج الزجاج) للسم الخام ومفصولاته وقد أظهرت النتائج أن المفصول F_{11} يحتوي علي أعلي نسبة بروتين يليه في ذلك F_{11A},F_{111} ومن ناحية اخري بينت النتائج أن السم الخام ومفصولاته تحتوي علي انزيم الفوسفوليز A_2 وقد وجد انه ليس هناك علاقة بين نشاط انزيم الفوسفوليز A_2 والمحتوي البروتيني لكل من السم الخام ومفصولاته . و عند تحديد الجرعة نصف المميتة للسم الخام ومفصولاته أظهرت النتائج أن السم الخام ومفصولاته F_{11A},F_{11}كانت الجرعة نصف المميتة ميكروجرام/ جرام من وزن الجسم علي الترتيب . بينما بعد اعادة التنقية باستخدام التبادل الأيوني كانت جميع المفصولت غير سامة حتي الجرعة10 ميكروجرام /جرام من وزن الجسم ماعدا الفصول فكانت الجرعة نصف المميتة ميكروجرام من وزن الجسم . وقد أوضحت الدراسة أيضا ان معالجة الفئران الحاملة لخلايا إيرليش السرطانية بحقنتين في التجويف البريتوني لكل من الجرعتين (20% و2%) من الجرعات نصف المميتة لكل من السم الخام ومفصولاته F_{11},F_{11A} أدي هذا الي نقص معنوي في عدد الخلايا السرطانية الحية بعد 14 يوما من المعالجة مصحوبا بزيادة معنوية في عدد الخلايا الميتة وإطالة زمن الإعاشة للفئران المعالجة .

وقد أظهرت الفئران الحاملة لخلايا ايرليش السرطانية أن هناك زيادة معنوية في عدد الخلايا الدموية البيضاء عديدات الأنوية واحادية النواة عند مقارنتها بالمجموعة الضابطة وبعد المعالجة بالسم الخام ومفصولاته F_{11},F_{11A} فقد استمرت هذه الزيادة المعنوية في عدد خلايا الدم مقارنة ايضا بالمجموعة الضابطة .

مما سبق يمكن استنتاج ان السم الخام ومفصولاته F_{11},F_{11A} لها تأثير ايجابي مضاد لنمو الخلايا السرطانية المنقولة عن طريق الحقن البريتوني للفئران البيضاء و الذي ربما يعزي الي وجود انزيم القوسفوليبيز A_2 بالإضافة الي نشاط جهاز المناعة من خلال خلايا الدم البيضاء .