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Knowledge to Wisdom

ISOLATION AND PARTIAL PURIFICATION OF PHOSPHOLIPASE FROM VENOM OF*ECHIS CARINATUS* AND STUDY OF ITS EFFECT ON LIPID PROFILE OF HYPERCHOLESTEROLEMIA MALE RATS

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ABSTRACT

The current study included isolation and purification of phospholipase from *Echis caraintus* venom that obtained from Said Dakhell after drying of venom, the purification steps were performed on crude enzyme comprise ion exchange chromatograph use sephadex G-25, after that gel filtration chromatography by use carboxy methyl. Nine groups of male rats (*Rattus norvegicus*) used one group (negative group) and two groups of all for induced high cholesterol diet for and two groups for injected by purified enzyme, and other groups for prevention and induced high cholesterol and injected by purified enzyme. The results showed a significant increase in the body weight of male rats in groups which induced high cholesterol fed and a significant decrease in the body weight of male rats in groups treated with phospholipase.

The results were recorded a significant increase in concentrations TCH, TG, VLDL, LDL and Atherogenid Index in groups treated with cholesterol and a significant decreasing in groups injected with phospholipase, While HDL concentration showed a significant decreased in groups treated with cholesterol and a significant increasing in groups injected with phospholipase.

KEYWORDS: Phospholipase, *Echis Carinatus*, Lipid Profile

INTRODUCTION

Echis carinatus is a venomous viper species found in parts of the Middle East, central Asia, and especially the Indian subcontinent. It is the smallest of the big four dangerous snakes of India as found in Sri Lanka, Bangladesh, Pakistan, Afghanistan, Oman, United Arab Emirates, Iran and south of Iraq. The snake is found in place faraway about15 kilometers southeast of the city of Nasiriya in Thi-qar in an area called Said Dakhell so called Said Dakhell snake as well as to the large presence in this region **(Joger, 1984; Whitaker, 1990; McDiarmid et al., 1999; Afrasiab et al., 2012)**. Snake venom is modified saliva to a large extent, consists in a set of glands called endocrine toxicity which secretes a poison which to base fangs modified for injection, as instilled in the body of the prey and thus enters the poison to the body of the prey and acts on the paralyzed **(Bauchot and Roland, 1994)**. The glands that secrete the zootoxins are a modification of the parotid salivary gland found in other vertebrates and they are usually lying on each side of the head below and behind the eye and encapsulated in a muscular sheath, the glands have large alveoli in which the synthesized venom is stored before being conveyed by a duct to the base of channeled or tubular fangs through which it is ejected.Venoms contain more than 20 different compounds, mostly proteins and polypeptides **(Halliday and Tim, 2002; Bottrall *et al*., 2011)**.

The term phospholipase is refer to the group heterogeneous of enzymes have the ability to hydrolysis of one or more of the ester linkage in compounds fats phospholipids which serve as a substance of this enzyme and each enzyme is able to break the ester bond, so this enzyme divided into several main types PLA, PLB, PLC, PLD **(Ghannoum, 2000)**.

The major lipids are fatty acids, Triglycerides (TG), cholesterol (free and esterified cholesterol) and phospholipids (PL). They are important in maintaining the structure of cell membrane (cholesterol and phospholipids), steroid hormone synthesis (cholesterol), and energy metabolism (TG and fatty acid) **(Liu, 2002)**. The term lipid has been loosely defined as any of a group of organic compounds that are insoluble in water but soluble in organic solvents **(Smith, 2000)**. Because lipids are water-insoluble molecules, they cannot be transported in aqueous solutions, such as plasma. For that reason, lipids are transported in plasma as macromolecular complexes known as lipoproteins **(Bauer, 2004; Johnson, 2005)**. Plasma lipoproteins are typically classified into five major subclasses on the basis of their densities HDL, LDL, IDL, VLD and Chylomicrons (CM) **(Betteridge, 2000)**.

MATERIALS AND METHODS

Collection of Snakes

*Echiscarinatus*was obtained from Said Dakhell and put it in a glass cage the dimensions (50 * 50)cm after that collected several samples of snake venom andthe venom was collected and preserved in glass dishes in the freezer directly, then drying the venom sample by freeze dryer device in the Food Industries Department. Agriculture College, Basra University.

Extraction and Purification of the Phospholipase Enzyme

Phospholipase enzyme was extracted and purified according to the method **(Dale and Smith, 1971)**.

- **PhosphateBuffer Solution:**

  Attended a phosphate buffer solution with the concentration of 0.02 Mof mixing K_2HPO_4 and KH_2PO_4 with the pH =7.2. Venom crude freeze-dried cooling-sized 25 mg dissolve in 10 mL of phosphate buffer (0.02 M and pH 7.2).

- **Ion Exchange Chromatography by Use SephadexG-25**:

  Attended Ion exchanger according method **(Whitaker, 1972)**

- **Gel filtration chromatography (Carboxy Methyl Cellulose)**:

  Attended the gel according to the company's instructions processed pharmacia fine chemicals

Phospholipase Assay

Assay the activity of the enzyme by titration of fatty acids produced by phospholipase enzyme with sodium

Determination of Protein Concentration

To determine the concentration of protein in the enzyme solutions, based on the curve of bovine serum albumin standard (BSA), use the method of **(Bradford, 1976)**

Preparing of Food (Cholesterol Diet)

5% of high cholesterol diet prepared from 50 g of cholesterol dissolved in 200 ml of olive oil and heated in a water bath, after soluble of cholesterol in the oil added to 1 kg of feed, and cut into small pieces fit with the size of the

holes in the lid iron to boxes, to facilitate the process taken up by rats **(Cook *et al.*, 1950)**.

Experimental Animals

The 54 adult male rats (*Rattusnorvegicus*) weighting (160-200) g of (10-12) weeks old were used in the present study. Animals were housed in the animal house of Biology department, Sciences College, Thi-Qar University, Iraq. Animals were housed in plastic cages and metallic clasp covers with wooden chips. During the experimental period six animals were kept in each cage and they were housed under standard laboratory conditions 12h light: 12h dark photoperiod (LD) at 20ºc - 25ºc. Also, the animals supply with water and food (*ad libitum*) during the experiment.

Experimental Design

The experiment was contained fifty four of male rats, they were divided routinely into nine groups (every group contained six animals) as follow:-

- **The First Group (control):** (negative) the animals injected intrapritional (I.P.) 0.1mL of normal saline (0.9% sodium chloride) daily for 21 days.
- **The Second Group:** The animals treated daily with high cholesterol diet for 15 days.
- **The Third Group:** The animals treated daily with high cholesterol diet for 15 daysthen with the normal fed (do not content high cholesterol) for one week.
- **The Fourth Group:** The animals injected (I.P.) 0.05mL of the purified phospholipase of second purification stage daily for 7 days.
- **The Fifth Group:** The animals injected (I.P.) 0.1mL of the purified protease of second purification stage daily for 7 days.
- **The Sixth Group:** The animals treated daily high cholesterol diet and injected (I.P.) 0.05mL of the purified phospholipase of second purification stage for 15 days.
- **The Seventh Group:** The animals treated high cholesterol diet and injected (I.P.) 0.1mL of the purified phospholipase of second purification stage for 21 days.
- **The Eighth Group:**The animalsinjected (I.P.) 0.05mL of the purified phospholipase of second purification stage after treated with high cholesterol diet for 21 days.
- **The Ninth Group:** The animalsinjected (I.P.) 0.1mL of the purified phospholipase of second purification stage after treated with high cholesterol diet for 21 days.

After 24 hours of last inject, the animals numbed by diethyl ether the blood was drawn from each animal of experimental groups by heart puncture method. 2ml of blood were put into clean tubes, which were contained anticoagulation for purpose the measurement of blood parameters. The remaining blood was put in the tubes without EDTA and centrifugation at 3000 rpm for 15 min for obtained serum in order to determine the biochemical tests, and analyzed for the levels Serum lipid profile.

Body Weight

The body weight of male rats was measured in the first day and the last day of the experiment by using balance (Animals balance Stanton 461, (Germany)).

Determination of Serum Lipid Profile

The reagents were supplied by Biolabo (France), and Serum total cholesterol was measured according to **(Allan and Dawson, 1979)**, and Serum TG was measured according to **(Tietz *et al.*, 1999)**. While serum HDL was measured according to **(Lopes-Virella, 1977)**, and measurement of LDL and VLDL according to **(Friedwald *et al.*, 1972)**. LDL, VLDL and atherogenic indexwas measured as follows:

LDL(mmol/L) = Total cholesterol – (HDL + VLDL)

VLDL(mmol/L) = serum TG /2.2

Atherogenic Index = LDL / HDL

Statistical Analysis

Statistical analysis was done using the software SPSS version 17.0, the results were expressed as mean ± standard deviations (mean ± SD). One way ANOVA was used to compare parameters in different studied groups. P-values ($P < 0.01$) were considered statistically significant.

RESULTS AND DISCUSSIONS

Effect of Extracted Phospholipase from Venom of *Echis Carinatus* on Body Weight

The result in the table (1) showed a significant increase ($P < 0.01$) in the body weight of(the second and the third) groups as compared with the first group(control group). At these times, there was a significant reduction ($P < 0.01$) in body weight of (the fourth and the fifth) groups as compared with the first group, with greater decrement ($P < 0.01$) in the body weight of the fourth and fifth groups than this with the eighth and ninth groups. Also there was a significant reduction ($p < 0.01$) in the sixth, seventh, eighth and ninth groups as compared with the first group. On the other hand, non-significant differences can be observed between (the sixth and the seventh) groups. These results are similar to the result of Onyeike *et al.* (2012), they reported that there was a significant increase ($p < 0.01$), in the weight of the hypercholesterolemia rats after two weeks of feeding. These results showed the significant decrease in body weight of the male rats laboratory treatment phospholipase extracted from the *Echis Carinatus* the decrement in body weight as a result of a decrease in food consumption on the one hand, or may be the result of a defect in metabolism and absorption of necessary for the life of the object **(Marchlewics *et al.*, 2006)**. Leidich *et al.* (1998) reported to the influence of the phospholipase on the digestive system layer of oral epithelial of laboratory rats and thus lose the ability to food.

Table 1: Changes in the Body Weight in the All Studied Groups (N=6)

Groups	Increase in Body Weight(Gm) Mean ± S.D
The first	34.5 ± 17.67^{c}
The second	96.16 ± 13.04^{a}
The third	60.16 ± 30.20^{b}
The fourth	11.17 ± 8.70^{de}
The fifth	10.83 ± 13.37^{de}
The sixth	3.50 ± 2.58^{e}
The seventh	2.33 ± 1.86^{e}
The eighth	29.66 ± 10.32^{d}
The ninth	18.83 ± 11.68^{cde}
L.S.D	22.16

Effects of Purified Enzyme on Serum Lipid Profile

Serum Total Cholesterol (TC) Concentration

The results presented in table (2) showed a significant increase (P < 0.01) in the serum concentration of TC in (the second and the third)groups as compared with the first group while there was non-significant deferent can be observed between (the second and the third) groups. At these times, there was a significant reduction (P < 0.01) in the serum concentration of TC in (the sixth, the seventh, the eighth and the ninth)groups as compared with the second group, with greater decrement (P < 0.01) in TC concentration in 0.1 mL phospholipase extract treated ninth group than this with 0.05 mL treated the third group than this with extract treated the eighth group. On the other hand, non-significant (P < 0.01) differences can be observed between (the eighth and the ninth) groups compared to the first group.

Also, there was non-significant (P < 0.01) differences can be observed between (the fourth and the fifth) groups compared to the first group, there was a significant decrease (P < 0.01) in (the sixth and seventh)groups compared to (the second and the third) groups. The increase in the serum concentration of TC in the second group was due to rats were treated high cholesterol diet similar to the result of Onyeike *et al*. (2012). On the other hand, the reduction in TC concentration resulting from a defect in absorption from the small intestine and to produce by liver because the liver plays a critical role in the production of cholesterol through bile secretion (**Reinner *et al*., 1989**). Also, the decline in cholesterol level back to the effect of the phospholipase in raising the level of HDL and reduce the level of LDL associated with cholesterol, has active role in reducing the level of cholesterol in the blood and that because high active biological in removing cholesterol from tissues, then transported to the liver which acts to undermine it and put in the bile so it act to protect the body from the risk of exposure to disease of the coronary arteries (**Jacobs *et al*., 1990**).

Serum Triglycerides (TG) Concentration

The results presented in table (2) showed a significant increase (P < 0.01) in the serum concentration of TG in (the second and the third)groups as compared with the first group and significant increase (P < 0.01) in the serum concentration of TG in the third group compared with the second group. At this time, there was non-significant differences can be observed between (the fourth and the fifth) groups compared to the first group. At these times, there was a significant reduction (P < 0.01) in the serum concentration of TG in (the sixth, the seventh, the eighth and the ninth) groups as compared with the second group. On the other hand, non-significant differences can be observed between (the fourth, the sixth, the seventh, the eighth and the ninth) groups compared to the first group. These results are similar to the result of Onyeike *et al*. (2012). This result due to the effect of the enzyme in the level of hormones because the level of triglyceride will be down in hyperthyroidism or because to increasing in Lipoprotein lipase (LPL) **(Abrams *et al*., 1981; Alterihy *et al*., 2012)**. The triglyceride level is low related to a decrease in weight may occur due to malabsorption or anemia or lack the level of digestive enzymes and some vitamins in the body, such as vitamin B_{12} by affecting the transfer of amino acids through the intestine wall, so effecting on the formation of digestive enzymes important in the process of absorption, as well as the hypertriglyceridemia may be caused by other factors, such as liver disease **(Moll, 2010)**.

High Density Lipoprotein (HDL)

The results presented in table (2) showed a significant decrease (P < 0.01) in the serum concentration of HDL in (the second and the third) groups as compared with the first group but non-significant differences can be observed between (the second and the third) groups. At these times, there was a significant increase (P < 0.01) in the serum concentration of HDL in groups (the fourth, the fifth and the sixth) as compared with (the second and the third) groups. On the other hand, non-significant can be observed between (the fourth, the fifth, the sixth, seventh, the eighth and the ninth) groupscompared

to the first group. These results are similar to the result of Onyeike *et al.* (2012). The treatment of male rats for laboratory with phospholipase enzyme cause a rise in the level of moral HDL compared with the control group, and is due to many reasons, including the low level of cholesterol or the low level of LDL, VLDL, or to increase Lecithin Cholesterol Acyl Transferase (LCAT) **(Glueck *et al.*, 1986; Tan *et al.*, 1991)**. This change in HDL level may be due to the enzyme that activates (LCAT), which works to integrate the free cholesterol with high density lipoproteins and therefore lead to raising the level of HDL blood **(Ghule *et al.*, 2006)**.

Low Density Lipoprotein (LDL)

The results presented in table (2) showed a significant increase ($P < 0.01$) in the serum concentration of LDL in (the second and the third) groups as compared with the first group while non-significant differences can be observed between (the second and the third) groups. At these times, there was a significant reduction ($P < 0.01$) in the serum concentration of LDL in (the fourth and the fifth)groups as compared with the first group, with greater decrement ($P<0.01$) in LDL concentration in (0.1mL) purified enzyme treated the ninth group than this with (0.05mL) purified enzyme treated the eighth group. On the other hand, non-significant differences can be observed between(the fifth, the sixth and the seventh) groups compared to the first group. This result of groups which treated with phospholipase enzyme come from lower the proportion of triglyceride and thus lead to lower cholesterol levels, as well as that the large proportion of cholesterol be portable on (LDL-c) lead to inhibition the absorption of cholesterol by gastrointestinal tract and removal the large amount from LDL-c of blood by stimulating the receptorLDL-c and finally increase the activity of the enzyme that destroyed the cholesterol by increasing the metabolic enzymes **(Korotaeva *et al.*, 2009)**.

Very Low Density Lipoprotein (VLDL)

The results presented in table (2) showed a significant increase ($P < 0.01$) in the serum concentration of VLDL in (the second and the third)groups as compared with the first group and a significant increase ($P<0.01$) in the serum concentration ofVLDLin the third group compared with the second group. At these times, there was a significant reduction ($P < 0.01$) in the serum concentration of VLDL in (the fourth, the fifth, the sixth, the seventh, the eighth and the ninth)groups as compared with the second group. On the other hand, non-significant differences can be observed between (the fourth, the fifth, the sixth, the seventh, the eighth and the ninth)groupscompared to the first group. The increase of VLDL levels in (the second and the third) groups that reported in this study was compatible with finding of Onyeike *et al*., (2012). The present study indicated that treatment of male rats vitro purified enzyme led to obtain a significant decrease in the VLDL in the blood whereby carries 90% of the triglyceride on the chylomicron (lipoproteins which transport of triglyceride in blood from small intestine to fatty tissue) and 10% carry on the VLDL, usually exposed TG to catabolism and break down of compound to produce to ATP which used when happen deficiency in carbohydrates because purified enzyme have catabolism and increasing metabolism processes and decreasing in TG in blood which lead to decreasing in VLDL because of obtain on a large amount of TG, also, may be the decreasing back to decreasing in VLDL (**Howell *et al.*, 1998**).

Table 2: Changes in Lipid Profile in All Studies Groups (N=6)

Groups	TC (mmol/L) Mean±S.D	TG (mmol/L) Mean±S.D	HDL (mmol/L) Mean± S.D	LDL (mmol/L) Mean±S.D	VLDL (mmol/L) Mean±S.D
The first	2.79 ± 0.29^{b}	1.28 ± 0.20^{cd}	0.93 ± 0.14^{ab}	1.28 ± 0.60^{bc}	0.58 ± 0.09^{c}
The second	4.88 ± 0.71^{a}	3.54 ± 0.45^{b}	0.77 ± 0.09^{bc}	2.50 ± 0.66^{a}	1.61 ± 0.20^{b}
The third	5.08 ± 0.67^{a}	3.99 ± 0.40^{a}	0.76 ± 0.09^{c}	2.51 ± 0.44^{a}	1.81 ± 0.18^{a}
The fourth	2.34 ± 0.39^{b}	1.17 ± 0.20^{d}	1.10 ± 0.27^{a}	0.77 ± 0.39^{c}	0.53 ± 0.09^{c}
The fifth	2.29 ± 0.17^{b}	1.15 ± 0.27^{cd}	1.03 ± 0.08^{a}	0.74 ± 0.40^{bc}	0.52 ± 0.02^{c}

Table 2: Contd.,

The sixth	2.89 ±0.55[b]	1.14±0.05[d]	0.97±0.08[ab]	1.40±0.27[bc]	0.52 ±0.08[c]
The seventh	2.87±0.22[bd]	1.65±0.10[cd]	0.93±0.07[ab]	1.20±0.30[bc]	0.75±0.05[c]
The eighth	3.14± 0.52[b]	1.38±0.26[cd]	0.98±0.14[a]	1.50±0.61[d]	0.63±0.12[c]
The ninth	2.29± 0.35[b]	1.35±0.23[cd]	0.99±0.15[a]	0.60±0.41[c]	0.61±0.11[c]
L.S.D	1.01	0.42	0.21	0.74	0.18

Atherogenic Index Levels

The results presented in table (3) showed a significant increasing ($P < 0.01$) in the Atherogenic Index levels in (the second and the third)groups as compared with the first group. Also, non-significant differences can be observed between (the second and the third)groups. At these times, there was a significant reduction ($P < 0.01$) in the Atherogenic Index levels in (the sixth, the seventh, the eighth and the ninth)groups as compared with groups (the second and the third), with greater decrement ($P < 0.01$)in Atherogenic Index levels concentration in the ninth group compared with groups (the second and the third) and there was a significant reduction ($P < 0.01$) in the Atherogenic Index levels in the sixth group compared with groups (the second and the third).

On the other hand, non-significant differences can be observed between groups (the fourth, the fifth, the seventh, the eighth and the ninth) compared to the first group. These result was due to the high level with low level which leads to decreasing in the Atherogenic Index levels.

Table 3: The Atherogenic Index Levelsin the All Studied Groups (N = 6)

Groups	Atherogenic Index(Mmol/L) Mean ± S.D
The first	1.40 ± 0.49[bc]
The second	3.18 ± 0.57[a]
The third	3.29 ± 0.66[a]
The fourth	0.71 ± 0.46[bc]
The fifth	0.67 ± 0.49[bc]
The sixth	1.16 ± 0.56[c]
The seventh	1.22 ± 0.38[bc]
The eighth	1.67 ± 0.88[bc]
The ninth	0.70 ± 0.52[bc]
L.S.D	0.88

Justifications of Research

Objective: According to the wide-spread snake with saw-scaled viper (said Dakheel Snake) and with view to what caused horror and death by its toxic effects, the present study aimed to extract and purify the phospholipase enzyme out of *Echis carinatus sochureki* (Said Dakheel Snake) venom, and study its effects in blood parameters in male rats.

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