KINETIC STUDIES ON EOSINOPHIL PEROXIDASE ENZYME ISOLATED FROM HEMOLYSATED BLOOD CELLS OF INDUCED ATHEROSCLEROTIC MICE AND ITS INHIBITION WITH SOME ISOFLAVONE COMPOUNDS

Nahida Saieed Al- Chalabi[a] and Eman Saieed Al-Choka[a]

Keywords: Atherosclerosis, inhibitors, eosinophil peroxidase enzyme, isoflavone compounds

This study includes an isolation, partial purification and study of the kinetic properties of Eosinophil peroxidase (EPO) enzyme from hemolysate blood cells in induced atherosclerosis and normal mice, then comparison between them. Isolation of two protein peaks I and II were carried out using ion exchange chromatography (DEAE cellulose), specific activity for these two peaks I and II for EPO enzyme for normal and induced atherosclerosis were (0.018, 0.02) and (0.126, 0.031). unit mg $^{-1}$ of protein respectively, peak I has a high specific activity for EPO enzyme which was isolated from induced atherosclerosis mice. The optimum condition of EPO for the peak I separated from hemolysate blood cells in induced atherosclerosis mice showed an optimum reaction incubation time at 21 minutes, pH of sodium acetate buffer 6, temperature at 30 °C, volume of enzyme 500 µL and the substrate concentration was about, 200 µM. When we used Line Weaver-Burk plot, the maximum velocity (Vmax) and Michale's - Menten constant (Km) were found to be 0.045 unit ml^{-1} and 400 µM L^{-1} respectively, all cationic metal ions such as Mg^{+2}, Na^{+}, Ca^{+2}, K^{+}, Mn^{+2}, Hg^{+2} (10 mM) shows a catalytic effect on the activity of EPO enzyme but maximum that for Hg^{+2} ions. Also at this study shows an inhibition effect of some isoflavone compounds such as genistein, daidzein, biochanin A and formononetin on the activity of this enzyme. The results indicate different relative inhibition in enzyme activity, for these compounds, according to Line Weaver- Burk plot genistein compound shows a competitive inhibition whereas daidzein, biochanin A and formononetin shows non competitive inhibition for EPO enzyme..

* Corresponding Authors
E-Mail: nahida_alchalabi@yahoo.com
[a] Department. of chemistry, College of Education, University of Mosul, Mosul, Iraq

Introduction

Atherosclerosis disease is one of cardiovascular diseases word wise in the world, which is characterized by a thicking wall of large, medium and small volumes of arteries. Mechanism appearance of the lesion of atherosclerosis represented by oxidation of low density lipoprotein (LDL-C), swallowing the oxidized LDL-C particles by phagocytic cells and formation of foam cells with adherence and penetration of monocyte cells for the wall of the blood vessel then the formation of cytokines of vascular smooth muscle cells .[1]

Atherosclerosis consider to be a disease mediated inflammation which is occur by a complex interactions with leukocytes, plates and vascular wall of the cells, this inflammatory interaction reinforced by liberation of atherosclerosis plaques, [2] because of EPO enzyme present plenty in Eosinophil leukocyte , [3] Which is a smaller constituent present in phagocytic leukocyte circles in blood under normal conditions, which where can be much larger in blood for different disorders of inflammation.[4] Thus the level of EPO enzyme was increased at different disorders of inflammation, one of them is vascular inflammation . The substrates for EPO to be (Br^{-}, Cl^{-}, I^{-}) in addition pseudo SCN^{-} which is substitute for halides as a substrate in the reaction of EPO using hydrogen peroxide.[5]

In addition to halides there is another substrate which was done is ortho methoxy phenol (guaiacol) in the reaction which is dependent on hydrogen peroxide.[6] It is found that bromide and thiocyanate which are a main substrates of EPO in vivo among another halides [7].

The aim of present study was to purified EPO enzyme from the Hemolysate blood cells in mice induced atherosclerosis and study the inhibition of it by some isoflavone compounds

Materials and methods

Animals used

Female albino mice were used for this study which was provided from the two Colleges of Education and Medicine for the University of Mosul , with approximate weight about 30 - 35 g , placed in a special cage provided for this purpose and provided with water and special animal feed continuously.

Induction of Atherosclerosis

The animal was divided to two groups each containing 20 mouse. Control group leaved takes animal feed and water *ad Libitum* for 60 days. Groups exposed to atherosclerosis which gives hydrogen peroxide 1 % with a drinking water for 60 days.

After the end of 60 days, these animals anesthesized with diethylether for some seconds then blood was pulled from the sinus puncture using special capillary tubes, put the blood in a clean, dry and sterilized plan tube. Serum of blood was isolated after clotting and centrifugation at 1008*g* for 10 minutes, then the sample of serum was conserved in a freeze at (-18 °C). The preparation of hemolysate cells of the blood takes place according to the method,[8] which was prepared by taking the isolated blood cells after serum was isolated, washed three times with a solution 0.9% NaCl with using a refrigerated centrifuge at 4 °C with 1008*g* for 5 minutes.

The precipitate wase taken which included blood cells after washing and hydrolyzed it with the addition twice their volume with distilled water. The tubes which contains hemolysate cells of the blood were put in a freeze at (-18 °C), then separation of the filterate of hemolysate cells was carried out blood after centrifugation using cold centrifuge at 4 °C with a velocity 1008xg for 30 minutes. After centrifugation the solution of hemolysate cells of the blood were taken for using the following experiments directly in order to isolate EPO enzyme from it.

Protein estimation was carried out for blood cells hemolysate (BCH) according to modified Lawry method ,[9] using a standard curve for bovine serum albumin for the determination of protein.

The animals after finishing the pull of their bloods, sacrified and explanation of it and then aortic artery was taken, placed in neutral formarline 10 % as a fixer for 72 hours.

Surely atherosclerosis was occurred, histological sections were carried for the aorta and then staining it by a dye hematoxylin and Eiosene [10] and the method of AL− Haj [11] was followed for colouring of these tissues sections.

Estimation the activity of EPO activity in hemolysate blood cells

Estimation of EPO activity was carried out according to Desser *et al*., method, [12] this method depends on following of an increasing for concentration of products at a wave length 470 nm.

The following equation demonstrate the action of EPO enzyme.

$$4\text{guaiacol} + \xrightarrow{EPO} \text{Tetraguaiacol} + 4H_2O_2$$

Reagents and procedures

Reagents used were the following: 1. Buffer solution of sodium phosphate (100 mM , pH 7). 2. Guaiacol solution (100 μM). 3. Hydrogen peroxide solution (100 μM).

Table 1. explains all substances added in each tube (sample and blank).

	Sample	Blank
Hemolysate of blood cells	250 μL	
Distilled water		250 μL
Phosphate buffer	250 μL	250 μL
Guaiacol	250 μL	250 μL
Hydrogen peroxide	250 μL	250 μL

Calculations

The EPO activity (φ) was expressed as:

$$\varphi = \frac{1000\Delta A V_t}{E_0 V_s d}$$

where

ΔA= the difference in absorbance at on minute .

V_t= Total volume

E_0= molar extinction coefficient (26600 L mol^{-1})

d= path length (1 cm).

V_s= sample volume

Partial purification of EPO enzyme from hemolysate cells of the blood

Partial purification of EPO enzyme of hemolysate cells of the blood for both induced atherosclerosis and normal mice were carried out after estimated of the activity of this enzyme using the following steps:

Dialysis

10 ml of hemolysate cells of the blood was dialyzed overnight at 4 °C using sodium phosphate buffer (100 mM , pH 7). The final volume was measured , the protein concentration was estimated then EPO activity was also determined.

Ion- exchange chromatography

DEAE - cellulose was used as anion exchanger for partial purification of enzyme. (9 ml) of the sample was applied to a column (2.5 × 40 cm) containing DEAE - cellulose to (37 cm)height. Elution was carried out at a flow rate 1.2 ml min^{-1} (72 ml h^{-1}) using sodium phosphate (100 mM, pH 7) at 4 °C as eluent. The volume of fractions produced were collected manually then the concentration of protein in each tubes were applied according to modified Lawry method. After plotting a diagram for absorbance against fractions number of the elution volume indicate peaks for the proteins of EPO, collection each fractions due to each peak , lyophilized until obtained a dry substance as a powder of EPO enzyme which is used in the following experiments .

Factors affecting EPO activity

To determine the optimal conditions of EPO enzyme several experiments had been designed including enzyme volume, reaction time, temperature, substrate concentration, pH and effect of some metal ions on peak I of EPO enzyme activity.

Results and Discussion

The cross sections for aortic artery for adult female mice (control) shows a three layers for the wall represent internal, middle and external layers empty from foam cell (Fig.1), whereas the cross sections for aortic artery for female mice treated with H_2O_2 (1 %) in drinking water showed a change in the tissue indicating a disease was occurred representing a thickness in the lining wall of the aorta with proliferation of vascular smooth muscle cells with the presence of foam cells (Fig. 2). This is an indication for lesion in the lining of the blood vessel due to oxidative stress induced with H_2O_2 that contribute to produce lipid peroxides which act to change permeability of membrane causing lesion internal during contribution of the oxidation of LDL particles and then produce atherosclerosis. [13]

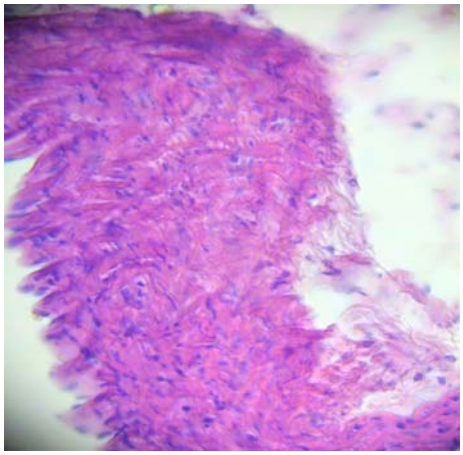

Figure 1. A three layers (internal, middle and external) in the aortic artery wall represent for normal female mice and empty from foam cells.

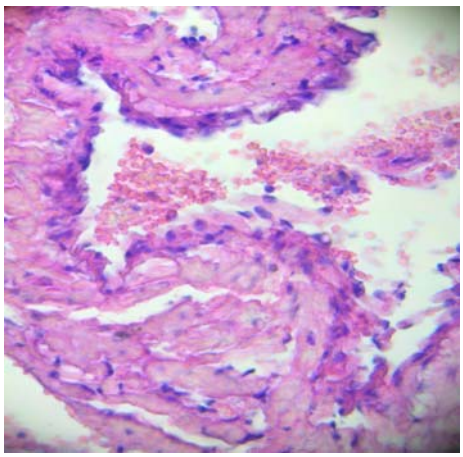

Figure 2. Thickness in the aortic lining wall (a) with proliferation of vascular smooth muscle cells (b) presence of foam cells (c).

Partial purification of EPO enzyme from hemolysate cells of the blood

Purification by dialysis

Results in Table 2 shows specific activity of EPO enzyme in hemolysate cells of the blood for normal mice was after dialysis process 0.016 unit mg $^{-1}$ protein i.e. increase 2.66 folds, 215.68 percent recovery compared to total activity of crude enzyme 100 % . The specific activity of EPO enzyme in hemolysate cells of the blood for atherosclerosis mice group was 0.037 unit mg $^{-1}$ protein after dialysis process, i.e. increased 2.46 folds before dialysis 0.015 unit mg $^{-1}$ protein and 202.5 % recovery compared to total activity of crude enzyme 100 % as indicated in (table 2).

Purification by ion- exchange chromatography

Results indicated in (Fig .3) presence of two peaks which have activity of EPO enzyme in hemolysate cells of the blood for normal mice as indicated in (Table 1). Peak I was appeared at elution volume 78 -132 ml with a specific activity 0.018 unit mg $^{-1}$ protein , i. e. increased 3 folds compared to specific activity of crude enzyme which have recovery percent about 58.23 %. Whereas peak II at elution volume 144- 186 ml, specific activity 0.02 unit mg $^{-1}$protein , i. e. more 3.33 folds than specific activity of crude enzyme which have recovery percent was 74.11 % compared to 100 % specific activity of crude enzyme (Table 2).

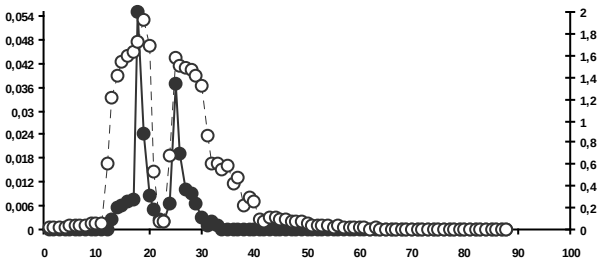

Figure 3. Recovery sample from purified enzyme EPO from hemolysate cells of the blood for normal mice by anion exchange chromatography using exchanger DEAE − cellulose packed separation column with dimensions (2.5 × 40 cm) at rate of flow of 1.2 ml min $^{-1}$ (72 ml h^{-1}).

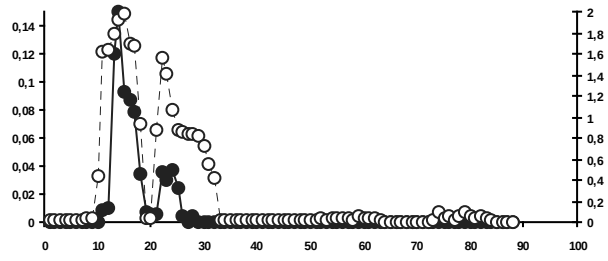

Figure 4. Recovery sample from purified enzyme EPO from hemolysate cells of the blood for induced atherosclerosis mice by anion exchange chromatography using exchanger DEAE − cellulose packed separation column with dimensions (2.5 × 40 cm) at rate of flow of 1.2 ml min $^{-1}$ (72 ml h^{-1}).

Table 2. Purification steps for EPO enzyme in hemolysate cells of the blood for normal mice.

Purification steps		Total volume, ml	Total protein, mg	*Activity (unit ml^{-1})	Activity in enzymatic unit		Purification folds	Reco-very,%
					Total	Specific		
Crude enzyme		10	169.37	0.102	1.02	0.006	1	100
Dialysis		9.2	132.25	0.24	2.2	0.016	2.66	9.2
Ion-exchange chromatography	Peak I	54	31.87	0.011	0.594	0.018	3	58.23
	Peak II	42	37.06	0.018	0.756	0.02	3.33	74.11

*Enzymatic unit (*U*) indicate to the amount of enzyme that oxidize one micromole of substrate in one minute.

Table 3. Purification steps for EPO in hemolysate cells of the blood for induced atherosclerosis mice.

Purification steps		Total volume, ml	Total protein, mg	*Activity, (unit ml^{-1})	Activity in enzymatic unit		Purification folds	Reco-very, %
					Total	Specific		
Crud enzyme		10	153.75	0.24	2.4	0.015	1	100
Dialysis		9	128	0.54	4.86	0.037	2.46	202.5
Ion-exchange chromatography	Peak I	48	25.02	0.066	3.16	0.126	8.4	131.66
	Peak II	30	23.77	0.025	0.75	0.031	2.06	31.25

*Enzymatic unit (*U*) indicate the amount of enzyme which oxidize one micromole of substrate in one minute.

The results in Fig. 4 was indicated two peaks present which also have an activity of EPO enzyme in hemolysate cells of the blood for induced atherosclerosis mice as shown in Table 3. Peak I appeared for the activity of EPO enzyme at elution volume 66-114 mL, its specific activity 0.126 unit mg^{-1}protein, i. e. increased about 8.4 folds over specific activity of crude enzyme which have 131.66 % recovery compared to total activity of crud enzyme . But peak II shows elution volume 132 -162 mL, its specific activity 0.031 unit mg^{-1} protein, i-e greater about 2.06 folds than for crude enzyme and 31.25 % recovery (Table 3).

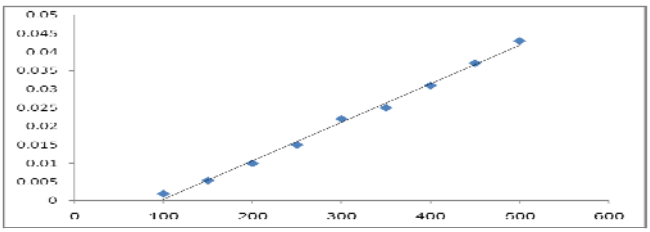

Figure 5. Effect of the enzyme extract volume on EPO enzyme activity

Study of the kinetic properties for EPO enzyme after partial purification of it from hemolysate cell of the blood in induced atherosclerosis mice.

To determine the optimal conditions of EPO enzyme in hemolysate cells of the blood in induced atherosclerosis mice. The following experiments had been designed including.

Effect of enzyme volume on EPO activity

The measurement for the activity of EPO enzyme in the presence of different volumes of the enzyme which was purified partially from hemolysate cells of the blood for induced atherosclerosis mice for peak I about 100- 500 µl . Fig. 5 shows the relationship between enzyme activity and volumes of enzyme with a constant other factors. It was shown that the rate of enzyme reaction increases with increasing the volume of enzyme reaches maximum at 500 µl. This results was in agreement with, [14] which shows the rate of enzymatic reaction increases with increase the volume of enzyme if substrate is sufficient with constant other factors.

Effect of buffer solutions on enzyme activity

The study of buffer solutions effect on the rate of enzymatic reaction of purified EPO isozyme I using different buffer solutions at a concentration 0.1M as follows:

1.Tris- HCl buffer	$C_4H_{11}NO_3$-HCl buffer.
2. Trisodium Citrate buffer	$C_6H_8O_7$-$C_6H_5O_7Na_3$ buffer.
3. Sodium bicarbonate buffer	Na_2HCO_3-Na_2CO_3 buffer.
4. Phosphate buffer	Na_2HPO_4-KH_2PO_4 buffer.
5. Phosphate buffer	Na_2HPO_4-NaH_2PO_4 buffer.
6. Sodium acetate	CH_3COONa.

The preferable enzyme activity were obtained for isozyme I which was purified from enzyme EPO was with sodium acetate buffer as indicated in Fig 6. This result is similar to the results obtained for purified enzyme EPO from uterus rat females.[15]

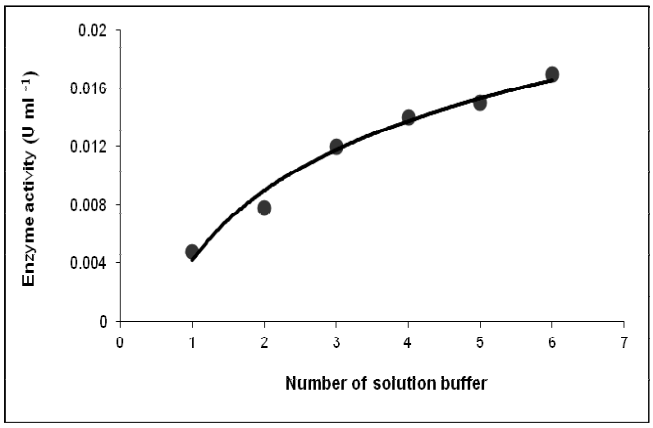

Figure 6. Effect of buffers solution on EPO enzyme activity

Effect of pH on EPO enzyme activity

The influence of pH upon the EPO activity was investigated using the sodium phosphate buffer solution with different pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8) as shown in Fig. 7. We show that the EPO activity of peak I was maximum at 6 , thus in all further experiment incubation were carried out at this point. Other studies showed that optimum pH for EPO purified from uterus of female rat was 5.6 using the same buffer.[15]

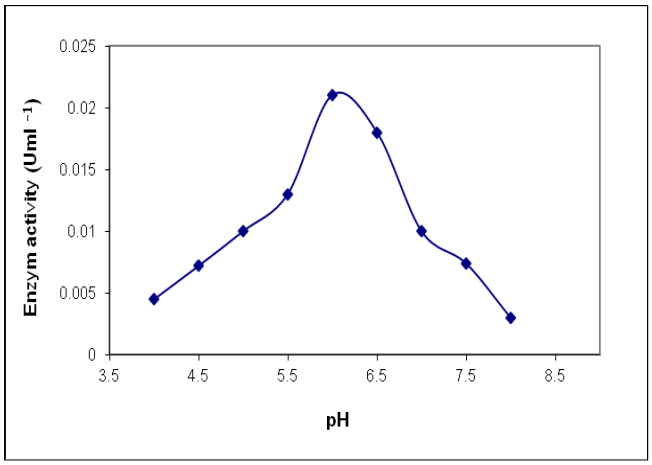

Figure 7. Effect of pH on EPO enzyme activity

Effect of time on enzyme activity

Maximum EPO activity observed after 21 minute of the reaction as shown in Fig. 8.

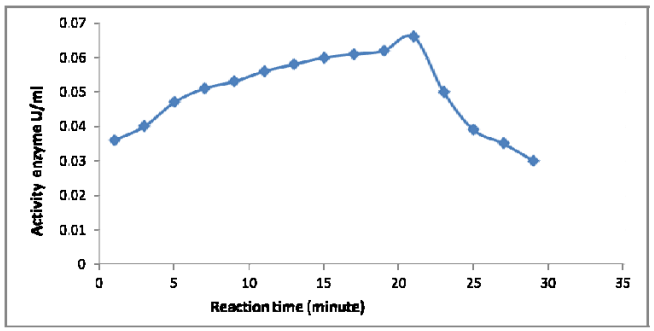

Figure 8. Effect of reaction time on EPO enzyme activity

Effect of Temperature on enzyme activity

It seems that by increasing temperature lead to increase EPO activity, the maximum activity of the enzyme showed at 30 °C followed by decreasing in enzyme activity as shown in Fig. 9 so that 30 °C used as optimal temperature to estimate EPO activity in the following experiments, Other studies [15] showed that the higher activity of EPO which was purified from uterus rat femals was at 37 °C. Also the results of isozyme I of EPO from hemolyzate cells of the blood showed a lost of enzyme activity at 60 °C for 10 minutes, this results indicated that EPO enzyme is unstable at high temperature more than that of optimum temperature .

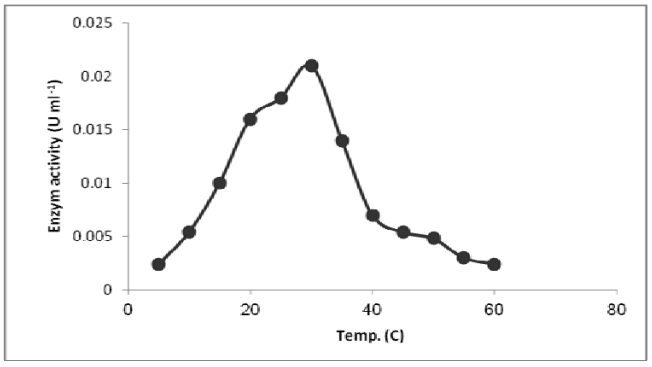

Figure 9. Effect of Temperature on EPO enzyme activity

Effect of some metal ions on enzyme activity

Effect of different cations Hg^{+2}, Mn^{+2}, K^{+}, Ca^{+2}, Na^{+}, Mg^{+2} were studied on the rate of enzymatic reaction of EPO at optimum conditions which was indicated before. The results shows all the positive ions for chloride salts of these metals which was added to a reaction mixture have catalytic effect on the activity of enzyme and the higher catalytic effect appeared when mercury ion was added, this increasing may be due to stabilization of the transition state by metal ions of the reaction between enzyme and its substrate .[16]

Table 3. Optimum conditions to evaluate peak I EPO activity partially purified from hemolysate cells of the blood for induced atherosclerosis mice.

Enzyme volume, µL	Type of solution buffer	pH	Reaction time, min	Temperature, °C	Metal ions	Substrate conc., µM
500	sodium acetate	6	21	30	Hg^{+2}	200

Table 4. Optimum inhibitory concentration of GE and DA on EPO enzyme activity purified from hemolysate cells of the blood in induced atherosclerosis mice.

GE concentration, μM	Activity, (U ml $^{-1}$) ×100	Percentage for inhibition %	DA concentration, μM	Activity (U ml $^{-1}$) ×100	Percentage for inhibition, %
Control without inhibitor	1.26	0	Control without inhibitor	1.14	0%
1	0.66	47.6	1	0.84	26.31
2	0.42	66.6	2	0.72	36.8
3	0.72	42.8	3	0.075	73.68
4	0.84	33.3	4	0.36	68.4
5	0.54	57.1	5	0.78	31.5
6	0.48	61.9	6	0.54	52.6

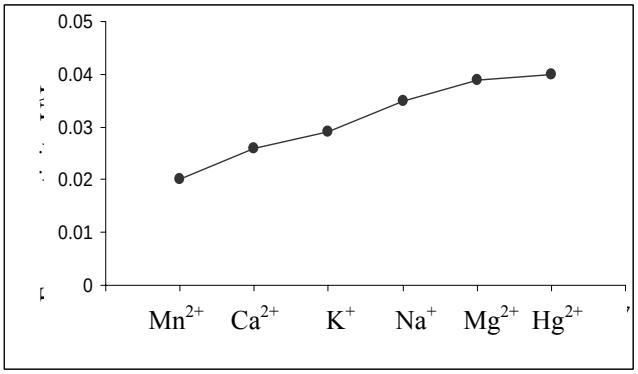

Figure 10. Effect of cation ions on EPO enzyme activity

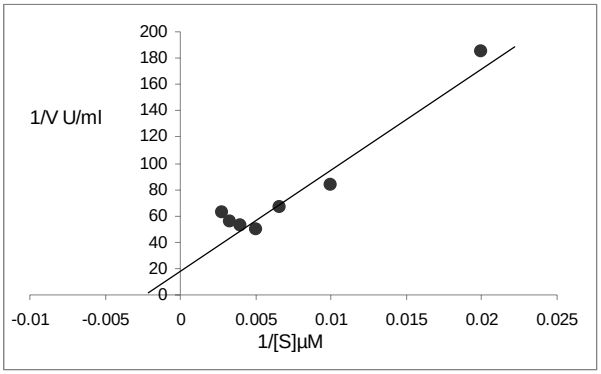

Figure 11. Effect of substrate concentration on EPO enzyme activity

Effect of substrate concentration on EPO enzyme activity

The activity of enzyme was measured in the presence of different concentrations of guaiacol as a substrate. It was found that the maximum activity obtained by using 200 μM of guaiacol.

Line Weaver-Burk plot as shown in Fig. 11. The maximum velocity (V_{max}) and Michaele's − Menten constant (K_m) were found to be 0.045 U ml $^{-1}$ and 400 μM respectively.

Other studies found K_m value for EPO enzyme separated from guinea pig bone marrow equal to 4 mM using guaiacol as substrate .[6]

From all the above experiments we can conclude that optimum conditions for peak I EPO enzyme isolated and partially purified from hemolysate cells of the blood for induced atherosclerotic mice as shown in (Table 3).

Inhibition Effect of isoflavone compounds on peak I for EPO enzyme

It was observed from(Table 4 and 5) that activity of peak I EPO enzyme purified from hemolysate cells of the blood in induced atherosclerosis femal mice decreased when using different concentration of isoflavone compound (GE, DA, BI, FO).

Table 5. Optimum inhibitory concentration of BI and FO on EPO enzyme activity purified from hemolysate cells of the blood in induced atherosclerosis mice.

BI concentration, μM	Activity (U ml $^{-1}$) ×100	Percentage for inhibition, %	FO concentration, μM	Activity (U ml $^{-1}$) ×100	Percentage for inhibition, %
Control without inhibitor	1.08	0	Control without inhibitor	1.14	0
1	0.54	50	1	0.24	78.94
2	0.9	16.6	2	0.18	84.21
3	0.66	38.8	3	0.3	73.68
4	0.42	61.1	4	0.18	84.21
5	0.96	11.1	5	0.42	63.15
6	1.02	5.5	6	0.36	68.42

It was found, that the concentration 2, 3, 4 µM for GE, DA, BI respectively posses more inhibition than other concentrations used in this experiment, whereas FO posses an inhibition concentration at 2 and 4 µM with a higher percent of inhibition 84.21 % than that produced by other isoflavones DA, GE, BI, these results of inhibition was indicated in (table 5). This enzyme inhibition may be due to the action of BI to inhibit phosphodiesterase enzyme which convert cAMP to AMP, therefore BI act to increase the level of cAMP which is decrease the total number of inflammation cells (Eosinophil and Basophil cells) lead to decreasing the level of EPO present in inflammatory Eosinophil cells [17]. Since other isoflavone compounds such as DA, FO have a similar structure to BI (fig .12), may be act similarly for inhibition for EPO enzyme as indicated by BI compound, also the inhibition of these compounds may be have a similar structure to estrogen hormone, where estrogen decreased the total number of inflammation cells. consequently this isoflavone compounds may be binding with estrogen receptors .[18]

Also the results obtained for GE isoflavone compound posses a competitive inhibition toward EPO enzyme.

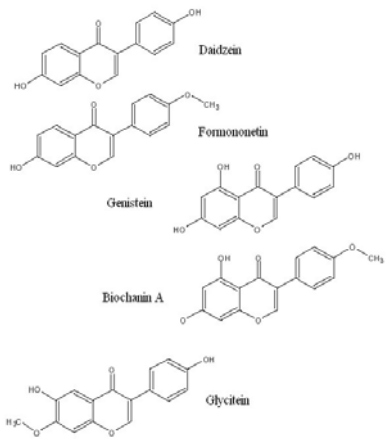

Figure 12. Chemical structure of different isoflavone compounds.

Type of inhibition for EPO enzyme by isoflavone compounds

The type inhibition of peak I EOP purified from hemolysate cells of the blood for induced atherosclerosis femal mice was carried out for isoflavone compounds (FO, BI, DA, GE) by measurement of the activity of enzyme with the presence of optimum concentration of inhibitor used as indicated in (Table 6).

The results showed a competitive inhibition was indicated for EPO enzyme with GE isoflavone compound according to Line Weaver- Burk plot (Fig. 13), whereas the inhibition were non competitive for DA, BI, FO isoflavone compounds according to Line Weaver- Burk plot also (figs. 14,15,16). The values of inhibition constant with these compounds GE, DA, BI, FO were 1.44, 4, 5, 3 µM respectively as indicated in Table 6.

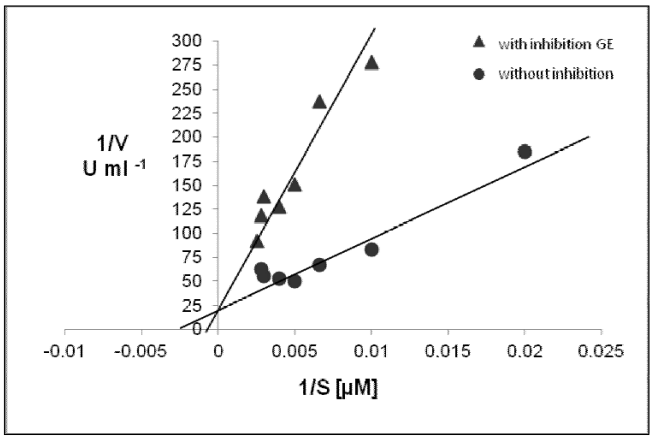

Figure 13. Inhibition enzyme activity from EPO purified of hemolysate cells of the blood for induced atherosclerosis mice by isoflavone compound GE.

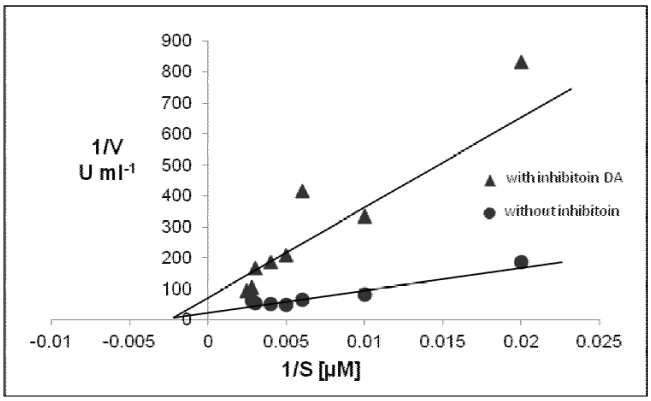

Figure 14. Inhibition enzyme activity from EPO purified of hemolysate cells of the blood for induced atherosclerosis mice by isoflavone compound by DA .

Table 6. Type of inhibition for EPO purified from hemolysate cells of the blood for induced atherosclerosis mice .

Optimum concentration (µM)	K_m (µM) without inhibitor	K'_m (µM) with inhibitor	V_{max} (unit ml^{-1}) without inhibitor	V'_{max} (unit ml^{-1}) with inhibitor	K_i (µM)	Kind of inhibition
(GE)2	400	833	0.045	0.045	1.44	Competitive
(DA)3	400	400	0.045	0.014	4	Non competitive
(BI)4	400	400	0.045	0.022	5	Non competitive
(FO)2	400	400	0.045	0.01	3	Non competitive

K_m - Michael's-Menten constant, K'_m - appearance Michael's-Menten constant, V_{max} - maximum velocity, V'_{max} - appearance maximum velocity, K_i - inhibition constant.

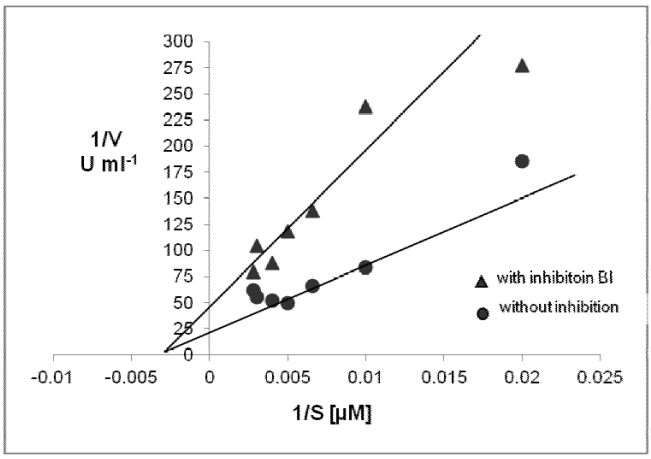

Figure 15. Inhibition enzyme activity from EPO purified of hemolysate cells of the blood for induced atherosclerotic mice by isoflavone compound BI.

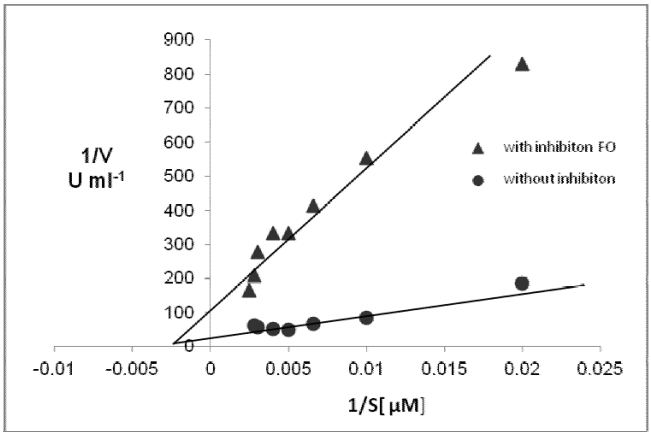

Figure 16. Inhibition enzyme activity from EPO purified of hemolysate cells of the blood in induced atherosclerotic mice by isoflavone compound FO.

REFERENCES

[1]Singh, A. K., Singh M ., Gupta, A. K. and Singh, P. K., *Uniq. J. Ayurved. Herbal Med..*, **2014,** *2(1),* 1-5.

[2]Schuett, H., Luchtefeld M., Grothusen, C., Grote, K. and Bernhard, S., *Thromb. Haemost*., **2009**, *102*, 215-222.

[3]Olsen, L. R ., Syse, K., Little, C. and Christensen, B. T., *Biochem . J.,***1985 ,** *229* ,779-784.

[4]Henderon, P .J., Byun, J ., Williams, V .M., Mccormick, L. M., Parks, C. W. and Ridnour, A .L., *Proc. Natl. Acad. Sci. USA,* **2001**, *98 (4),*1631 – 1636.

[5]Mccormick, M. L., Roeder, T. L., Railsback, M. A. and Britigan, B. E., *J. Biol. Chem.,* **1994,** *269(45)*, 27914-27919.

[6]Keyhani, E., Zarei, M. A. and Lashgarblooki-Livani, T., *FEBS Letters.,* **1999,** *452,* 233 236.

[7]Vandelen, C. J. and Kettle, A. J., *Biochem. J.,* **2001**, *358,* 233-239.

[8]Rapoport, S. M., Schewe, T., Wiesner, R., Halangk, W. and Ludwig, P. *Eur. J. Biochem.,* **1979,** *99,* 545-561.

[9]Schacterle, G. R. and Pollack, J. K., *Biochem*., **1979,** *51,* 654-655 .

[10]Culling, C. F. A., Allison, R. T. and Barr, W. T., *Cellular Pathology Technique*. 4th Ed. Butterworth and Co. Ltd, **1985,** 641.

[11]Al-Haj, H. A. *Preparations microscopic optical theory and applications.* House march for publication and distribution, and printing the first edition, Amman Jordan, **2010.**

[12]Desser, R. K., Himmelhoch, S. R., Evans, W. H., Januska, M., Mage, M. and Shelton, E., *Arch. Biochem. Biophys.,* **1972,** *148,* 452.

[13]Devasagayam, T., Tilak, J. C., Boloor, K. K., Sane, S. K., Ghaskadbi, S. S. and Lele, R., *J. Assoc. Physicians India,* **2004 ,** *52,* 794-802.

[14]Ahmed, Y. and Hilali, L. A., *Biochemistry, Part I*, Dar Ibn Al Atheer for printing and publishing, conductor/Iraq, **2010,** 278.

[15]Olsen, R. L. and Little, C., *Eur. J. Biochem.,* **1979,** *101*, 333-339.

[16]Murray, R. K., Bender, D. A., Botham, K. M., Rodwell, V. W., and Weil, P. A., *Harpers' illustrated biochemistry*". **2009**, 28th. Ed., Mc Graw Hill company N.Y.

[17]Ko,W., Lin, L., Shen, H., Lai, C., Chen, C. and Shih, C., *Evidence-Based Compl. Altern. Med.,* Article ID *635058*, **2011,** pp 1-13.

[18]Shore, L. S. and Lyttle, R., *Pharm. Struct. Activity Relations.,* **1986,** 253-256.

Received: 04.06.2014.
Accepted: 29.09.2014.