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Isolation and Classification of Lipoxygenase from the Rind of Pistacio Khinjuke and Investigation of its Affinity Toward Certain Inhibitors in Mice: in vivo and in vitro

Ameera A. AL-JURAISI
University of Mosul

Omar Y. AL-ABBASY
University of Mosul

Abstract: This study includes purification of lipoxygenase from the rind of pistacio khinjuke by applying ammonium sulfate precipitation (70%), dialysis, ion exchange chromatography, and the slab electrophoresis technique. One isoenzyme has been obtained with a special activity of 1230x10-4 unit/mg protein and a molecular weight of 101997 dalton. The partially purified rind lipoxygenase has been characterized and inhibited by quercitine rutinoside and melatonine. The type of inhibition seemed to be uncompetitive and the Ki values were 1.77 and 1.62 mM, respectively. Due to the importance of lipoxygenase for the animal, this study also deals with the determination of its affinity toward quercitine rutinoside and melatonine in the serum of normal and diabetic male mice. The best doses for lipoxygenase inhibition were 523.3 and 8 Mg/Kg body weight of quercitine rutinoside and melatonine, respectively. Treatment of animals with these doses led to a significant decrease in lipoxygenase activity, glucose level, and total cholesterol/high density lipoprotein cholesterol ratio in animal serum. Therefore, these natural inhibitors seem to serve as a drug for reducing the complications of some diseases (e.g. diabetes mellitus) which are affected by this enzyme.

Keywords: Lipoxygenase, Melatonine, Quercitine rutinoside, *Pistachio khinjuke*

Introduction

In the progress of science and technology, plants became part of the interests of WHO and researchers, in turn, started working on isolating the important plant components in order to use them in the proper form. One of these plants is Pistacio Khinjuke, which is an oily plant abundant in Turkey, Iran, Afganistan, Syria, Palestine, and Iraq (Al-Juwari, 2009). Due to its pleasant taste and its special chemical components, this plant is used for different purposes. It contains terpens and phenolic compounds (Pirbalonti and Aghaee, 2011), in addition to lipids, proteins, vitamins, antioxidants, and minerals (Agar et al, 1995). Its oil contains a high percentage of essential fatty acids such as linoleic acid and linolenic acid (Safari and Alizadeh, 2007). One of the enzymes that contribute to metabolizing these fatty is lipoxygenase, LOX (Linoleate: oxygen oxidoreductase, EC 1.13.11.12), which stimulates the selective-oxygen entrance in the unsaturated bond of the fatty acid producing hydrooeroxide (Iranshahi et al, 2009; Silva et al, 2001). In addition to its wide spreading in the plant kingdom, LOX is also widely spread in the animal kingdom, including humans (Al-Sultan et al, 2004). Inhibition of LOX in humans decreases the complications of diseases such as diabetes mellitus caused by hydroperoxides.

This study deals with the isolation and purification of LOX from the rind of Pistacio Khinjuke by applying different separation methods, in addition to studying its affinity toward quercitine rutinoside and melatonine as natural inhibitors, in vivo and in vitro, using normal and diabetic male mice.

Materials and Methods

Materials

The Plant Used

In this study, the fruit of Pistacio Khinjuke was used. It was obtained from the local market of Duhok City in November, the time of its ripeness and reaping. The rind was used after its being peeled from the plant fruit.

The Animals Used

Ninety males of albino mice were used in this study. Their weight range was 30-35 gm. They were supplied with water and fodder and they were exposed to the same environmental conditions of natural light and temperature (25^{o} C).

Methods

Preparation of Rind-Crude Extract

Distilled water was added to 45 gm of the rind (5:1 v:w). The mixture was ground for 10 mins, then frozen by liquid nitrogen, and left to dissolve at room temperature. The dissolved mixture was filtered through several layers of gauze, and then centrifuged for 15 mins at 4000xg (Muhaisin et al, 2008). The clear filtrate was then collected and used as a crude extract in the subsequent tests.

Evaluation of Protein Concentration

The protein concentration in the crude extract of the rind was evaluated according to the modified Lawry method (Scharcterle and Pollack, 1973). A calibration curve of absorbance (at 650 nm) of different concentrations (ranging between 0-240 Mg/ml) of Bovine-serum albomin was used for evaluation (Fig. 1).

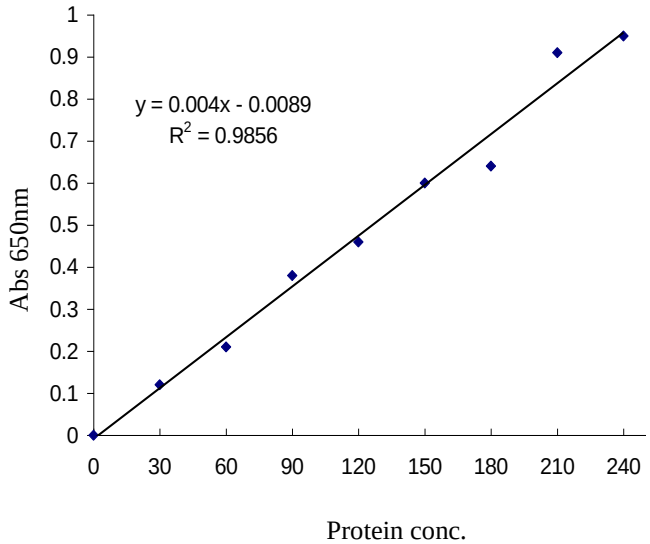

Figure 1. Calibration curve for protein conc.-determination

Determination of LOX Activity

The activity of LOX was determined according to Shastry and Rao (1975). This method depends on the oxidation of linoleic acid (as a substrate of LOX) producing conjugated diens. The increase in absorbance was followed at 234 nm for 5 mins, using 1800 UV Spectrophotometer. The molare absorption (Ɛ) of conjugated diens is 25000 M^{-1}, Cm^{-1}

$$\text{Activity} = \frac{\Delta A_{234} \times 10^6}{0.1\ ml \times \varepsilon}$$

Purification of LOX

Several techniques were used for the purification of LOX from the rind of Pistacio Khinjuke. These techniques are:

Precipitation by Ammonium Sulphate

The ammonium sulphate was added gradually to the crude extract of the rind with continual magnetic stirring for 1 hour at 4^{o} C. The saturation of ammonium sulphate ranged between 0% and 60%. Twenty-four hours later, the solution was centrifuged for 15 mins at 4000xg to separate the precipitate which then was dissolved in the phosphate-buffer solution at pH 6.8 (Muhaisin et al, 2008). The protein content and the LOX activity were determined. The remaining solution was used in the next step of purification.

Dialysis

Using a cellophane tube, with M.wt cut of 20KD, the solution produced from the previous step was dialysed against 20 mM phosphate-buffer solution pH 6.8 at 4^{o} C for about 12 h. The protein content and the LOX activity of the produced extract were determined. The remaining solution was used in the next step of purification.

Ion-Exchange Chromatography

The sample (10 ml) of the extract produced from the dialysis was let to pass through the ion exchange column previously prepared. The resin used was Diethyl amino ethane-cellulose and the phosphate-buffer solution NaH_2PO_4-Na_2HPO_4 (20mM) at pH 6.8 was used for elution. The buffer solution passing through the column during the separation had gradient concentrations ranging from 20 to 200 mM. The eluted fractions were collected every 5 mins using an alternating push pump. The velocity of filtration was 60 ml/h. Each collected sample was concentrated by lyophilizing after the determination of protein thconcentration and LOX activity.

Determination of LOX-Molecular Weight

The LOX-molecular weight was determined by using slap-electrophoresis which was applied on the concentrated protein extract obtained from the previous separation step using Sodium Dodecyl Sulphate (SDS) gel as an Anionic detergent that surrounds the charged groups of the proteins leading to separating the proteins according to their molecular weights (Robyt and White, 2001). A standard curve was adopted to evaluate the molecular weight of LOX.

Determination of Carbohydrate Content in LOX

The Molish test was applied on the LOX obtained after ion exchange chromatography to identify the presence of carbohydrate content in LOX structure by using glucose solutions as a standard (Robyt and White, 2001).

Characterization of LOX

For the characterization of LOX, the optimum pH and temperature were determined, in addition to evaluating the effect of substrate (linoleic acid) concentration on LOX activity.

Inhibition of LOX

Quercitine rutinoside and Melatonine were examined as natural inhibitors for LOX. In addition, different concentrations of the substrate were used in order to determine the type of inhibition (Befani et al, 2001).

Testing the Type of LOX Inhibition

In order to test the type of LOX inhibition, the activity was pursued using different concentrations of the substrate (linoleic acid) ranging from 0.01 to 0.8 mM with 150 MM of quercitine rutinoside and 10 MM of melatonine. After 30 minutes of incubation of the solutions (0.2 ml of the LOX extract of the rind with 0.1 ml of the inhibitor and substrate solutions), the activity of LOX was determined (Befani et al. 2001).

Animal Experiments

Definition of Inhibitors' Effective Dose

In order to define the effect of inhibitors' dose on LOX, several groups of animals of 5 mice each were used. The animal groups were orally treated once a day with 0.5 ml of saline solution containing quercitine rutinoside with concentrations 0.0, 130.8, 261.6, 392.5, 523.3 and 785 microgram/kg body weight, and melatonine with the concentrations 0.0, 4, 8, 12, 16, and 20 microgram/kg body weight (for the second half of the animal groups). After 5 days of treatment, the activity of LOX in the blood which was withdrawn by orbital sinus puncture was evaluated in order to determine the effective dose of inhibitors.

Induced Diabetes in Mice

Diabetes in mice was induced by injecting alloxan intraperitoneally (180 mg/kg body weight dissolved in tmessaline solution) after starvation for 24 hours (Miura et al, 1995). After 2 hours of injection, the animals were given glucose solution (5%) to avoid their death as a result of an acute decrease in glucose. One day later, the animals were allowed to take what they need of water and forage. The afflication with diabetes was checked by daily examination of the presence of glucose in the animals' urine using the relevant test-strip.

Treatment of the Animals with the Effective Dose of Quericitine Rutinoside and Melatonine

The animals were divided into 6 groups of 5 animals each. The groups are:
Group 1: normal animals not treated with inhibitors,
Group 2: normal animals treated with the effective dose of quercitine rutinoside,
Group 3: normal animals treated with the effective dose of melatonine,
Group 4: animals with induced diabetes, not treated with inhibitors.
Group 5: animals with induced diabetes treated with the effective dose of quercitine rutinoside, and
Group 6: animals with induced diabetes treated with the effective dose of melatonine.

Five days later, the LOX activity in the animal serum was determined, in addition to enzymatically determining the levels of glucose, total cholesterol, and cholesterol of high-density lipoprotein.

Results and Discussion

As previously indicated, several techniques were used for the separation and purification of LOX from the rind of Pistacio Khinjuke, depending on the difference in the molecular weight and charge and the affinity of the proteins in the aqueous extract of the tissue.

As shown in Table 1, the specific activity of LOX in the crude was 68×10^{-4} U/mg of protein. After ammonium sulphate precipitation, the specific activity increased to 120×10^{-4} U/mg of protein, or more specifically, it was multiplied by 1.76 times and the amount of recovery of total activity was 6.58% compared with the total activity of LOX in the crude extract.

Table 1. Purification steps of LOX

Purification steps	Total voulume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Multiplication No. of purification	Recovery activity %
Crude extract	200	4448	30.6	68×10^{-4}	-	%100
Amonum sulfate prespitation *	48	167.52	2.016	120×10^{-4}	1.76	6.58
Dialysis	50	102	1.7	166×10^{-4}	2.44	5.55
Ion exchange	100	15.5	1.92	1230×10^{-4}	18.08	6.27

U: means amount of LOX which oxidizes one □m of linoleic acid in one unit

After dialysis, the specific activity increased to 166 x 10^{-4}. This means that the dialysis resulted in an increase in the specific activity by 2.44 times and the amount of recovery of total activity was 5.55% compared with the total of LOX in the crude extract.

Elution of the extract produced for the dialysis showed a clear peak at an elution volume of 90-185 ml, using DEAE- cellulose as an anionin exchanger (Fig. 2) with the specific activity 1230 x 10^{-4} U/mg of protein as shown in Table 1 above. This means that the purification was multiplied by 18.08 times and the percentage of recovery was 6.27%. This result agrees with that arrives at by Casey (1998) and Muhaisin et al (2008) who state that oily plants, herbs, and cereals contain a considerable amount of LOX.

John et al (1981) precipitated LOX from rice bran-crude extract by 20-60% ammonium sulphate saturation, then purified it by ion exchange chromatography. They found that its specific activity was 99.5 x 10^{-2} U/mg of protein. This reflects less activity compared with what was found in our study. That is, LOX activity in oily plants is higher than that in cereals.

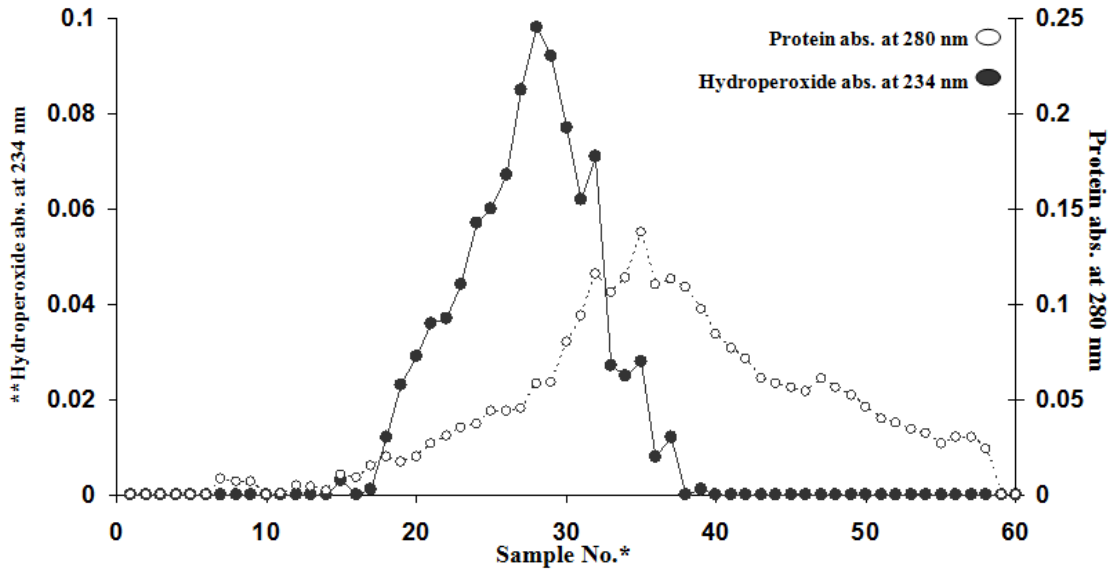

Fig. 2: Elution pattern of LOX purification

*** each sample collected through 5 min.**
**** Hydroperoxide is a product of linoleic acid oxidation by LOX**

LOX- Carbohydrate Content

By applying the Molish test, LOX was identified as a glycoprotein and the amount of carbohydrate content was 10.28%. This result agrees with that found by Al-Araji for the LOX purified from soybean, 10.3 and from peanuts, 13% (Muhaisin et al, 2008).

LOX Characterization

The present study deals with the characterization of the LOX appearing in the peak of the eluted extract of ion exchange chromatography. It was found that LOX optimum pH was 8.5 using borate- HCl buffer solution (Fig. 3). The pH affects the three-dimensional structure of the enzyme which controls its activity (McKee and McKee, 2001). Each enzyme has its own optimum pH. It is worth-mentioning that the optimum pH Found experimentally is not necessarily identical to the physiological amount (Christenson et al, 2001).

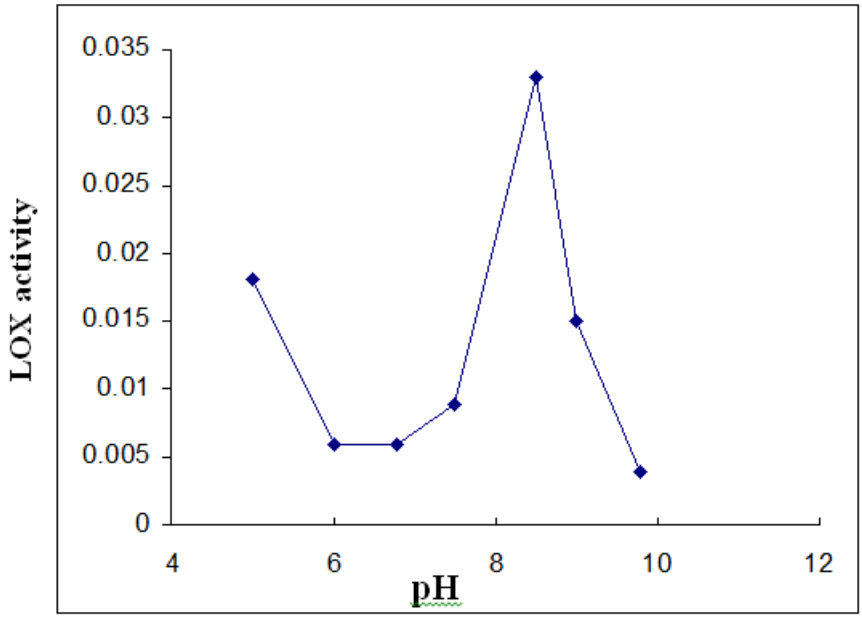

Fig.3: Effect fo pH on LOX activity

Fig. 4 shows that the LOX- optimum temperature was 25° C, which agrees with that of LOX isolated from tomato (Yilmaz, 2001) but not with that isolated from ice-bran or from peanuts, 30° C and 40° C, respectivey (John et al, 1981; Al-Sultan et al, 2004).

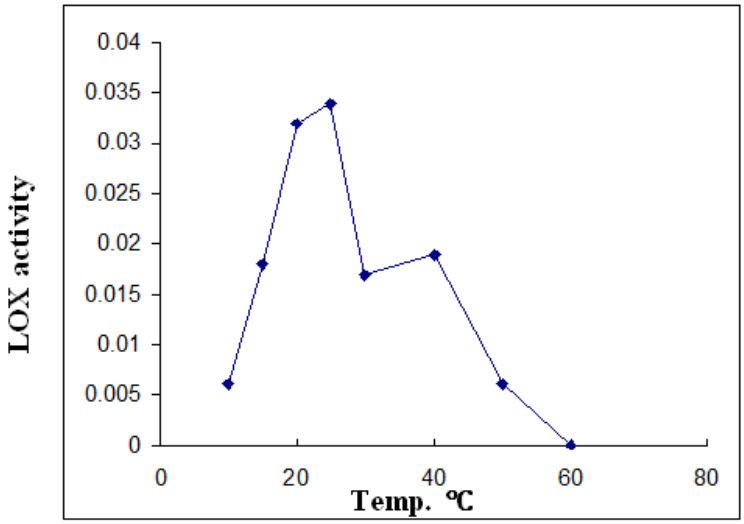

Fig.4: Effect of temp. on LOX activity

The effect of substrate (linoleic acid) concentration on LOX activity was determined using different concentrations ranging between 0.0 and 0.8 mM. The amounts of Km and Vmax were found to be 0.166 mM and 0.0277 U/ml/min, respectively (Fig. 5). The value of Km was lower than 1 mM, which means that LOX has high affinity to bound with linoleic acid as a substrate (Fayyih, 2000).

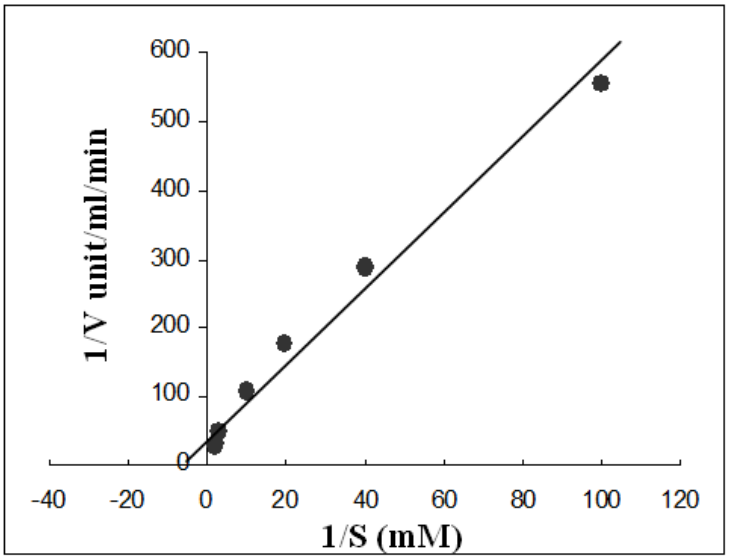

Fig.5: Effect of substrate concentration on LOX activity applying Linewaver-Burk plot

Comparing these results with those arrived at in previous studies, it was found that the amounts of LOX- Km and Vmax depended on the substrate used and the source of LOX , whether it be rice bran, raw rice, tomato, sunflower, peanuts, or others (Shastry and Rao, 1975; John et al, 1981; Boyes et al, 1992; Malekian et al, 2000).

Evaluation of LOX Molecular Weight

The molecular weight of LOX present in the isolated peak of ion exchange chromatography was evaluated by applying SDS- PAGE electrophoresis. As shown in Fig. 6, there is a single band for LOX, which indicates that the Lox isolated from the rind of Pistacio Khinjuke has one isoenzyme, and its M.Wt is 101997 Dalton. The molecular weight of LOX differs according to the source from which it is isolated. Boyington et al (1993) found that the M.Wt of the LOX isolated from peanuts was 100680 Dalton, but Al-Sultan et al (2004) and Ding et al (1996) found it to be 104000 and 105000 Dalton, respectively, with one enzyme.

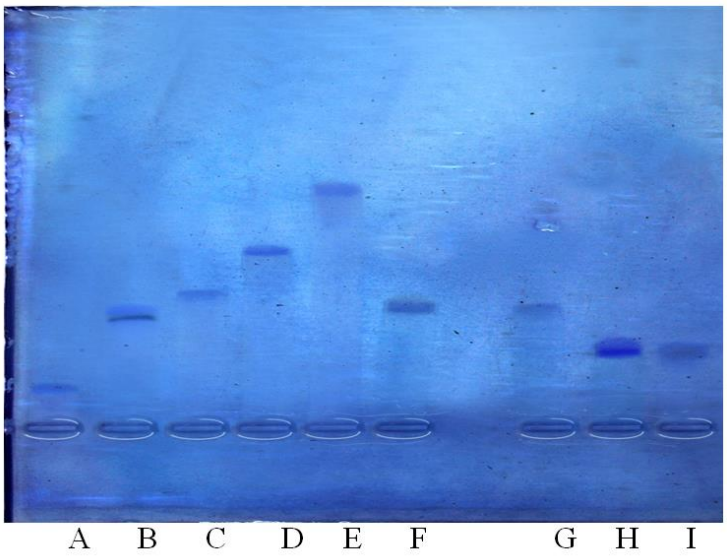

Fig. 6: Picture of electrophoresis of LOX using SDS-PAGE

A: urase, B: bovine albumin, C: peroxidase, D: trypsine, E: lysozom, F: rind lipase, G: karael lipase, H: rind LOX and I: karnel LOX

Hurt and Axelord (1977) stated that the LOX isolated from Phaseolus Vulgaris had two isoenzymes, each consisting of a single polypeptide chain with M.Wt ranging between 100000 and 106000 Dalton. In addition, Axelord et al (1972) isolated four LOX- isoenzymes from soybean consisting of a single polypeptide chain with relatively similar M.Wt (about 100000 Dalton).

The Effect of Inhibitors on LOX Activity

The inhibition effect of quercitine rutinoside and melatonine with different concentrations ranged between 15 and 150 and 2-10 MM, respectively, and LOX activity was tested. As shown in Tables 7 and 8, the gighest inhibition was 47.61% and 50% of LOX activity at 75 and 4 MM of quercitine rutinoside and melatonine, respectively. These concentrations represent the Inhibitor Concentration 50, IC 50.

Table 2. Inhibiton of LOX by quercitine rutinoside

Inhibitor conc. Mm	LOX activity U/ml	Inhibition effect %
0	0.0336	-
15	0.0312	7.14
25	0.0256	23.8
50	0.0336	0
75	0.0204	39.2
100	0.0216	35.7
125	0.0204	39.2
150	0.018	46.4

Table 3. Inhibition of LOX by melatonine

Inhibitor conc. Mm	LOX activity U/ml	Inhibition effect %
0	0.024	-
2	0.018	25
4	0.0146	39.16
6	0.0168	30
8	0.0204	15
10	0.0102	57.5

LOXs are sensitive to antioxidants which lead to inhibiting the formation of lipid hydroperoxides (Kumara Swamy and Satish, 2008). Flavonoids (especially quercitine rutinoside) are important antioxidants. Their inhibition capability is due to their affinity for hydrogen bonding with oroteins, which affects the biological activity of such proteins (Birari and Bhutani, 2007).

Melatonine is one of the antioxidants and it belongs to alkaloids. Rackavo et al (2007) stated that the inhibition effect of alkaloids isolated from Mahonia Aquifolium on LOX activity, i.e. its antioxidant effect, seems to protect the cells from peroxidation (Chen et al, 2003).

The Type of LOX Inhibition

As shown in Figs 7 and 8 (applying the Lineweaver-Burk equation), the type of inhibition of LOX by quercitine rutinoside and melatonine seems to be uncompetitive. In the case of using quercitine rutinoside, the value of Km decreased from 0.0588 mM to 0.0357 mM, and the value of Ki mounted to 1.77 mM (Fig.7).

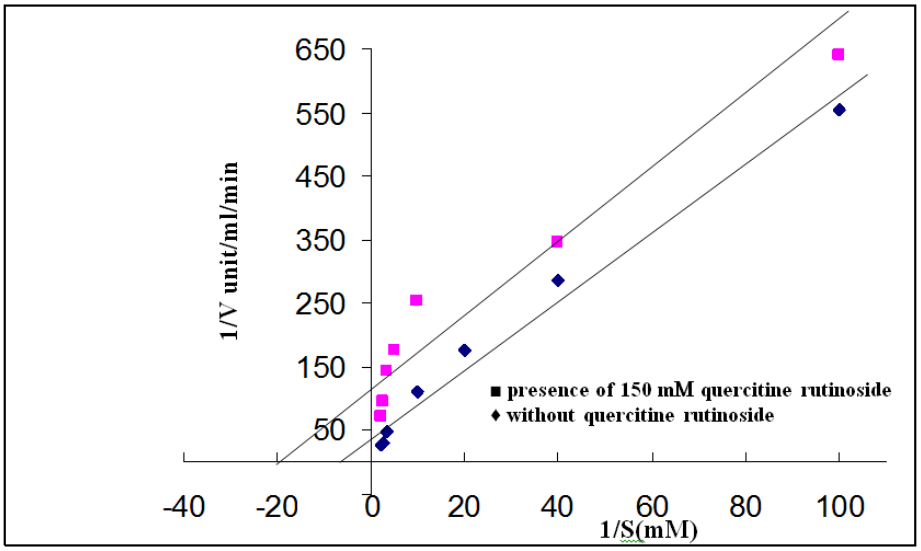

Fig.7: Effect of quercitine rutinoside on LOX activity

When melatonine was used, the value of Km decreased from 0.0769 mM to 0.0476 mM, and the value of Ki mounted to 1.62 mM (Fig. 8).

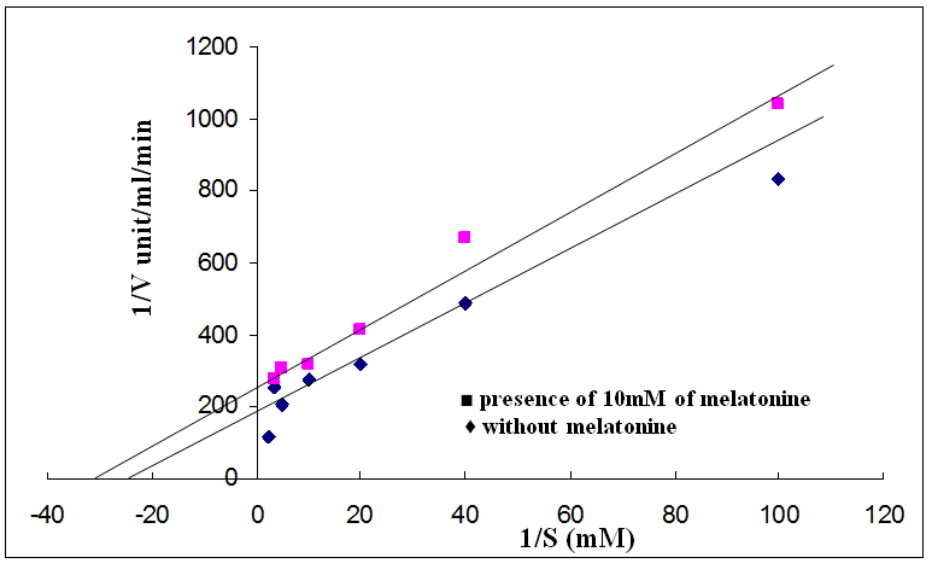

Fig.8: Effect of melatonine on LOX activity

Since the value of Ki in the case of using melatonine was lower than that in the case of using quercitine rutinoside, the inhibition effect of melatonine on LOX activity was higher than that of quercitine rutinoside.

Animal Tests

Determination of the Effective Dose for inhibition

Tables 9 and 10 show the most effective doses of quercitine rutinoside and melatonine for inhibiting LOX in the serum of normal albino male mice were 523.3 and 8 Mg/Kg body weight, respectively.

Table 4. The effective dose of quercitine rutinoside for inhibiting LOX *IN VIVO*

Quercitine Rutinoside Dose □g/Kg Body Weight	0.0	130.8	261.6	392.5	523.3	785
LOX Activity U/ml	6.51±0.11 e	4.37±0.3 d	3.89±0.11 c	2.97±0.1 a	2.7±0.11 a	3.31±0.09 b

Different letters indicate significant difference (P<0.05).

Table 5. The effective dose of melatonine for inhibiting LOX, IN VIVO

Melatonine Dose □g/Kg Body Weight	0.0	**4**	**8**	**12**	**16**	**20**
LOX Activity U/ml	5.8±0.11 **d**	3.21±0.13 **c**	2.11±0.16 **a**	3.25±0.33 **c**	2.59±0.16 **b**	3.09±0.09 **b**

Different letters indicate significant difference (P<0.05).

The Effect of LOX Inhibition on Biochemical Parameters

Tables 6 and 7 below show the effect of the IC50 dose of inhibitors on the level of glucose (Glu), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and LOX activity in normal and diabetes-induced male mice, respecdtively.

Table 6. Effect of LOX-inhibitors on the level of biochemical parameters in mice

Groups	**Glucose conc. mM/l**	**Chol. Conc. mM/l**	**HDL-C mM/l**	**LOX activity U/ml**
	Avarege ± Standard deviation			
Normal Mice	**c 6.66±0.46**	**A 2.42±0.11**	**A 0.85±0.05**	**B 6.42±0.78**
Normal mice treated with 523.3 mg quevcitine rutinexide /kg body weight	**b 4.13±0.12**	**A 2.39±0.22**	**B 1.15± 0.07**	**A 2.30±0.26**
Normal mice treated with 8 mg metatonie /kg body weight	**A 2.19±0.13**	**A 2.21±0.19**	**C 1.5±0.16**	**A 2.43±0.43**

The vertically different letters mean a significant difference at a probability level p≤0.05

Table 7. Effect of LOX-inhibitors in the level of biochemical parameters in diabetic mice

Groups	**Glucose conc. mM/l**	**Chol. Conc. mM/l**	**HDL-C mM/l**	**LOX activity U/ml**
	Avarege ± Standard deviation			
Normal Mice	**c 6.15±0.69**	**Ab 2.90±0.17**	**b 0.9±0.05**	**B 6.26±1.05**
Diabetic mice not treated with inhibitors	**d 11.25±0. 31**	**C 3.84±0.25**	**a 0.57±0.05**	**C 20.20±1.79**
Diabetic mice treated with 523.3 □g quercitine rutinoside /kg body weight	**b 4.85±0.20**	**B 3.28±0.19**	**c 1.55±0.1**	**A 2.71±0.41**
Diabetic mice treated with 8 □g melatonice /kg body weight	**a 4.02±0.04**	**A 2.69±0.18**	**c 1.68±0.17**	**A 1.91±0.43**

The vertically different letters mean a significant difference at a probability level p≤0.05

Inducing diabetes by the dose 180 mg alloxan/kg body weight led to a significant increase in Glu. Level (P< 0.05) because alloxan destroys the beta cells of the islets of Langerhans due to the increase in free radicals

leading to decreasing insulin secretion and then increasing blood Glu. This means that diabetes was induced (Saravanan and Pari, 2995). In addition, induced diabetes significantly increased TC, which may be attributed to the increase in the level of free fatty acids due to the random analysis of triglycerides to provide a source of energy instead of Glu. The accumulation of free fatty acids activates the regulatory enzyme of cholesterol synthesis, β-hydroxy-β-methyl glutaryl-CoA, HMG-CoA, reductase (De-Man et al, 1996). On the other hand, induced diabetes led to decreasing HDL-C, which may be attributed to the fact that diabetes mellitus is accompanied by a defect in lipid metabolism since the transition of CE and the cholesterol ester transfer-protein from HDL to VLDL is increased. Therefore, HDL becomes compact with triglycerides, leading to easy destruction and then loss of apo AI, which is an important component of HDL, with urine. A decrease in apo AI leads to a decrease in HDL and its cholesterol (Pikto-Pictkewic et al, 2005).

Diabetes induction by alloxan led to a significant increase in LOX because a high level of Glu. promotes the increase in 12/15 LOX in several types of cells such as vascular smooth muscle cells and pancreatic β-cells, leading to increasing hydroxyl-eicosatetraenoate, HETE, produced according to LOX action. When the animals with induced diabetes are injected with insulin, the levels of LOX and the compounds produced as a result of its action decrease (Imig, 2006; Natarajan and Nadler, 2004). The increase in the level of LOX is considered to be an indication of affication with diabetes, leading to accumulation of HETE, and retardation of blood-flow in the kidney, retardation of the glomerular filtration rate and finally damaging them (Imig, 2006).

Treatment of the diabetic animals with quercitine rutinoside and melatonin reflected a noticeable effect on the studied parameters. They significantly decreased Glu. level ($P< 0.05$), which may be due to activating glycolysis and glyconeogenesis (Shibib et al, 1993; Sharkar et al, 1996).

The level of TC significantly decreased (Table 7 above) because these inhibitors decrease Glu. level, which reflects its contribution to energy production and decreasing the production of fatty acids, then inhibiting HMG-CoA reductase, and the regulatory enzyme of cholesterol synthesis (Chang et al, 2004; De-Man et al, 1996). In addition, the flavonoids may play a role in the transformation of cholesterol to its derivatives , leading to decreasing its level in the blood (Panda, 2004).

Table 7 also shows that HDL-C significantly increased ($P <0.05$), which may attributed to the fact that the flavonoids and alkaloids increase the synthesis of HDL in the liver and intestine cells (Murray et al, 2009).

Treatment of the normal and diabetic animals with quercitine rutinoside and melatonine affected the activity of LOX in the mice blood (Tables 6 and 7 above). It significantly decreased because these compounds are antioxidants and their indolic and phenolic groups act as H-donor, leading to restricting LOX activity (Moosman and Behl, 2000).

Inhibition of LOX is an important means of overcoming many chronic diseases because LOX, the leucotriens, and HETE, which are the compounds produced as a result of its action, are directly related to diabetes (Imig, 2006), cardio-vascular diseases (Hou et al, 2004), some types of cancer (Murphy and Gljon, 2007; Janken et al, 2006), and other diseases.

Scribner et al (2000) stated that Masaprocol (as an indicator) decreased LOX activity and the compounds produced from its action (12 and 15-HETE), which negatively affect the function of the kidney. In addition, this inhibitor decreased the levels of tryglycerides and free fatty acids and improved the sensitivity of the cells of diabetic patients to insulin. Cinnamyl-3,4-dihydroxy cyano cinnamate and Baiclein are LOX- inhibitors used for the treatment of diabetes mellitus (Dellipizzi et al, 2000; Nozawa et al, 1990).

There is no doubt that future studies will deal with searching for active and safe inhibitors for LOX to over-come the complications of diabetes mellitus, especially its effect on the kidney function (Imig, 2006).

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Author Information

Ameera A. Al-Juraisi
University of Mosul
Mosul / Iraq
Contact e mail: ameeraaziz2000@yahoo.com

Omar Y. Al-Abbasy
University of Mosul
Mosul / Iraq