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تاثير مستخلص الانثوسيانين لقشور الباذنجان في فعالية انزيم الليبيز المنقى من دم المصابين بالسكري النوع الأول

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Effect of *Solanum Melongena* peel anthocyanin extracts in Lipase Activity which purified from TI-diabetes patients

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الملخص:

الليبيز من الانزيمات المهمة التي تعمل على تنظيم عملية التمثيل الغذائي للدهون الثلاثية في الدم والعضلات والأنسجة الدهنية. نقي انزيم الليبيز من دم مرضى السكري من النوع الأول باستخدام مجموعة من التقنيات، الترسيب بكبريتات الأمونيوم، والفرز الغشائي، وكروماتوغرافيا التبادل الأيوني الموجب بواسطة المبادل الايوني CM-cellulose. وجد أن الفعالية النوعية هي 0.0117 و0.0252 و0.1035 وحدة/ملغم بروتين على التوالي، ووجد أن عدد مرات التنقية هي 1.88 و4.06 و16.96 على التوالي. وجد ان الوزن الجزيئي 76.5 كيلو دالتون. تناول البحث أيضًا الظروف المثلى للإنزيم، تأثير تركيز الإنزيم ودرجة الحرارة والرقم الهيدروجيني والركيزة. وجد أن الظروف المثلى هي 100 ميكرولتر من الإنزيم عند 37 درجة مئوية و7.2 و1.2 على التوالي. تم عزل مستخلصات الأنثوسيانين من قشور الباذنجان بمزيج من المذيبات (حمض الهيدروكلوريك، الميثانول، الكلوروفورم) وتم تشخيصها وتقديرها بواسطة كروماتوغرافيا السائل عالي الأداء، وكانت الكمية الكلية ومركبات الأنثوسيانين السيانيدين الدلفينيدين المالفيدين الفاوانيا 439.25 64.35، 215.15، 79.9، 79.85 مايكروغرام/كغم من قشر الباذنجان على التوالي. أظهرت المستخلصات تثبيطًا تنافسيًا لفعالية اللايبيز عند تركيز 20 و40 جزء في المليون.

الكلمات الدالة: الأنثوسيانين، ملف الدهون في الأحماض الدهنية، مؤشر تصلب الشرايين، داء السكري.

Abstract

Lipase is a crucial enzyme regulating triglyceride metabolism in the blood muscle and adipose tissue. lipase was purified from the blood of the patients with type 1 diabetes using a range of techniques

including precipitation with ammonium sulfate, dialysis, and ion exchange chromatography by CM-cellulose. The specific activity was found to be 0.0117, 0.0252, and 0.1035 U/mg protein, respectively, purification fold was found to be 1.88, 4.06, and 16.96, respectively. Molecular weight was 76.5 KD. The research also examined the optimal conditions for the enzyme, including the effect of enzyme concentration, temperature, pH, and substrate. The optimal conditions were found to be 100 µl of enzyme at 37°C, 7.2, and 1.2, respectively. Anthocyanin extracts were isolated from eggplant peel by a mixture of solvents (HCL, Methanol, chloroform) identification and estimation of these compounds by HPLC, total and anthocyanin compounds Cyanidin Delphinidin Malvidin Peonidin 439.25 64.35, 215.15, 79.9, 79.85 µg/kg of eggplant peer respectively. The extracts exhibited competitive inhibition of LPL activity at concentrations of 20 and 40 ppm.
Keywords: Anthocyanin, fatty acids lipid profile, atherogenic index, diabetes mellitus.

1. INTRODUCTION

Diabetes mellitus (DM), which is brought on by deficient in insulin production, inefficient insulin utilization, or both, is the result of abnormalities of the metabolism (Aronson, Goldenberg, Boras, Skovgaard, & Bajaj, 2018; Newman et al., 2022). (DM) is defined by immune-mediated diabetes in childhood (Type 1 diabetes), insulin resistance (Type 2 diabetes), hyperglycemia during pregnancy or other acute and chronic hyperglycemia, difficulties resulting from the environment, infections, genetics, or medications, or abnormalities affecting the islets of Langerhans beta cells (Baynes, 2015; Nair, 2007). Since type 2 diabetes mellitus (T2DM) is a serious public health concern or threat in the twenty-first century, no nation is safe from the diabetes invasion these days (Zimmet, 2003). The estimated global prevalence of type 2 diabetes is expected to increase to 6.4% by 2030 from the percentage of 4.6% in 2000. In the last ten years, there has been a significant rise in the prevalence of diabetes because of factors such as increasing average age in the community, inherited predispositions, insufficient nutrition, a sedentary lifestyle, and rising obesity rates in tandem with development (Abbas & El-Yassin, 2022). Pancreatic lipase is the primary enzyme involved in the breakdown of dietary fat, the reduction of fat accumulation in adipose tissue, and the regulation of weight gain. These benefits are shown in individuals with diabetes who are overweight or obese. Pancreatic lipase hydrolyzes and breaks down triacylglycerol, which accounts for approximately 90–95% of the fats that are consumed. In the past, lipase inhibitors were thought to be a viable treatment for obesity if they could prevent the early transfer of triglycerides from the intestinal lumen. Orlistat is a potent inhibitor of pancreatic, gastric, and carboxylic ester lipase. It is a hydrolyzed form of the lipase inhibitor lipstatin, which is produced from Streptomyces toxitricini.

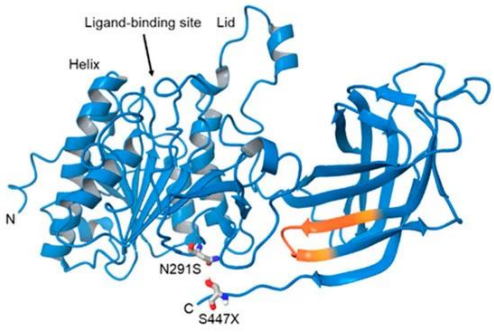


Figure 1: Tertiary structure of lipase (Chen, Xie, Jin, & Huang, 2014)

Treating obesity, which is a significant risk factor for type 2 diabetes (Savych & Marchyshyn, 2021) Research has shown a substantial correlation between the level of C peptide and pancreatic enzyme function.(Fonseca, Berger, Beckett, & Dandona, 1985) Trypsin is the first enzyme to be lost in the sequence of enzyme loss in the exocrine pancreatic damage associated with human diabetes, whereas lipase is the final one to be lost (Junglee et al., 1982). Foods high in anthocyanins are members of the polyphenol family and are primarily found in dark fruits and vegetables (think blueberries, cranberries, and eggplant) and red cabbage and radishes. Many research investigations have demonstrated the important role that these substances play in preventing or lessening type 2 diabetes (T2D) complications. It was discovered in experiments using isolated omental adipocytes and rodents. It has been observed that

anthocyanin compounds influence the transmission of GLUT4 (insulin-regulated glucose transporter), which in turn affects the activation of PPARγ (peroxisome proliferator-activated receptor γ) in skeletal muscle and adipose tissue. They also increase the secretion of adiponectin and leptin. These effects are responsible for controlling the body's metabolism of carbohydrates. These substances additionally lessen inflammation in the body. (Różańska & Regulska-Ilow, 2018).
The eggplant fruit is distinguished by its high concentration of antioxidant-rich phenolic compounds. (Cao, Sofic, & Prior, 1996). Nasunin is assumed to be the primary component in the peel of Japanese eggplant types, and the anthocyanins found in eggplant peel are mostly derived from delphinidin. Nasunin was first isolated in 1933 by Kuroda Wada who found residues of delphinidin, glucose, and coumaric acid at which time the structure was named delphinidin-3-diglucoside acylated with coumaric acid (Noda, Kneyuki, Igarashi, Mori, & Packer, 2000).

Anthocyanin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

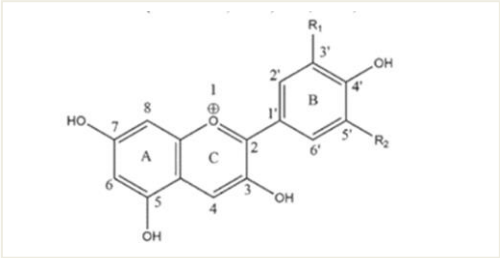


Figure 2: The basic structure of anthocyanidins (Zhao et al., 2018)

2 Methodology

2.1 Eggplant taxonomy (Kavitha, Revathi, & Kingsley, 2008)

Name	Brinjal
Kingdom	Plantae
Class	Magnoliopsida
Subclass	Asteridae
Order	Solanales
Family	Solanaceae
Genus	Solanum
Species	melongena

2.1.1 *Plant protocol preparation*

Eggplant vegetables were collected from the local market in Iraq/Baghdad. A peeling machine was used to separate the rinds from the pulp, and by using a Magic Bullet 600-watt blender was used to crush them with distilled water 1:1. Freeze and thaw for three times, followed by 30 minutes of ultrasonic treatment at 7°C, about 500 g of rinds were used to make a fresh puree. The puree was dried in an air oven at 40°C, and the next step involved grinding the resulting powder for extraction.

2.1.2 *Anthocyanin extraction*

Neff and Chory(Neff & Chory, 1998) suggested using methanol and chloroform to extract anthocyanins. Consequently, for the next step, samples were thoroughly mixed with 15 mL of methanol, 10 mL of water, 0.15 mL of HCL, and 25 mL of chloroform. The mixture was then incubated at 4 °C for 24 hours on a shaker in the dark, and the mixture was centrifuged for 15 minutes at 4 °C and 7000 rpm. The absorbance of the supernatant at 530 and 657 nm was measured.

2.1.3 *Analysis of anthocyanin*:

HPLC model SYKAM (Germany), column C18 – ODS (25 cm * 4.6 mm), and the detector UV-Vis at = 520 nm were used to investigate and estimation of anthocyanin compounds. The mobile phase 95 / 5 (v/v) mixture of water (pH 7.0) and (2 %) formic acid, and the flow rate at 0.8 mL/min (Shim, Kim, Seo, Park, & Ha, 2014).

2.1.4 ***lipase (LP) purification***

Blood sample collection: Blood samples were collected by drawing (5 ml) of venous blood using a disposable syringe, placing it in gel tubes, letting it clot at room temperature, and centrifuging at 3000 rpm for 10 minutes. The serum was obtained using a micropipette, placed in clean, sterilized tubes, and stored frozen at (-20°C) until the biochemical parameters under investigation were analyzed.

Protein Determination: Total protein was estimated by the modified Lowry method (Waterborg, 2009).The standard curve for protein was prepared by taking different concentrations from bovine serum albumin ranging between (0-500)µg/ml. 1 ml of the reagent alkaline copper sulfate was added to 1 ml of a sample, the tubes were mixed and left for (10) minutes at room temperature, and then (4) ml of phenol reagent was added with quick mixing. The tubes were placed in a water bath at (55) °C for 5 minutes, left to cool down and read the absorbance at 650 nm.

LPL assay: LPL assay by using the para-nitrophenyl acetate as a substrate and monitoring the release of p-nitro phenol as a product at 420nm.

Precipitation: The enzyme was precipitated using ammonium sulfate salt at a saturation rate of 60% in an ice bath for 24 hours, the protein precipitate of the enzyme was obtained using cryogenic centrifugation at 10,000 rpm.

Dialysis: Dissolve the precipitate with a minimum amount of distilled water and place it in a dialysis bag with a separation limit of 10,000 kilodaltons, and place the bag in an ice bath for six hours(Meana et al., 2016).

Ion Exchange Chromatography: The dialysate LPL was transferred to a cation exchanger, CM-cellulose column (2.5x40 cm), followed by 15 mM phosphate buffer pH 7.2. The elution was collected at a flow rate of 1ml/min. The protein was spectrophotometrically monitored at 280 nm of all fractions. The fraction that has PAO activity was pooled and lyophilized.

SDS-PAGE electrophoresis: The molecular weight of LPL was determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sharma et al., 2021). Protein bands were visualized by staining with Coomassie brilliant blue R250. Molecular weight markers proteins.

2.1.5. ***Optimal conditions of LPL activity***

Enzyme volume: the optimal concentration of enzymes was studied by adding different volumes (22-100 µl) of LPL to the reaction mixture.

pH range: The effect of pH on the LPL activity was studied with Na-Na-phosphate buffer solutions at pH= 6.7, 7.1, 7.3, 7.5, 7.7, 7.9, 8.1, and 8.3.

Effect of Temperature on PAO: The effect of temperature on LPL activity was determined by incubating LPL at different temperatures of 10 to 70°C.

Substrate influence: The effect of the substrate was studied using increasing concentrations of para-nitrophenyl acetate at a concentration between 0.1 and 0.3 mM. The kinetics variables of LPL Km and Vmax values of the enzyme were calculated from the Lineweaver-Burk plot.

2.1.6 ***Inhibition of LPL*****:** The final test in this study was to illustrate the effect of anthocyanin extract by adding 10 -100 ppm to the enzyme mixture, the more inhibition effect was chosen to study the inhibition type. (Befani, Grippa, Saso, Turini, & Mondovi, 2001).

3 Results and discussion:

3.1 ***Anthocyanins study***:

The anthocyanin compounds found in the extracts with amounts indicate that cyanidin had a higher concentration than the other compounds than malvidin as shown in Table 1

Table 1: the concertation of anthocyanidin compounds in eggplant peel

Anthocyanidin compounds	Peonidin	Cyanidin	Malvidin	Delphinidin	Total
µg of aqueous extracts	19.8	66.2	24.6	21.8	132.4
µ g /kg eggplant peel	64.35	215.15	79.9	79.85	439.25

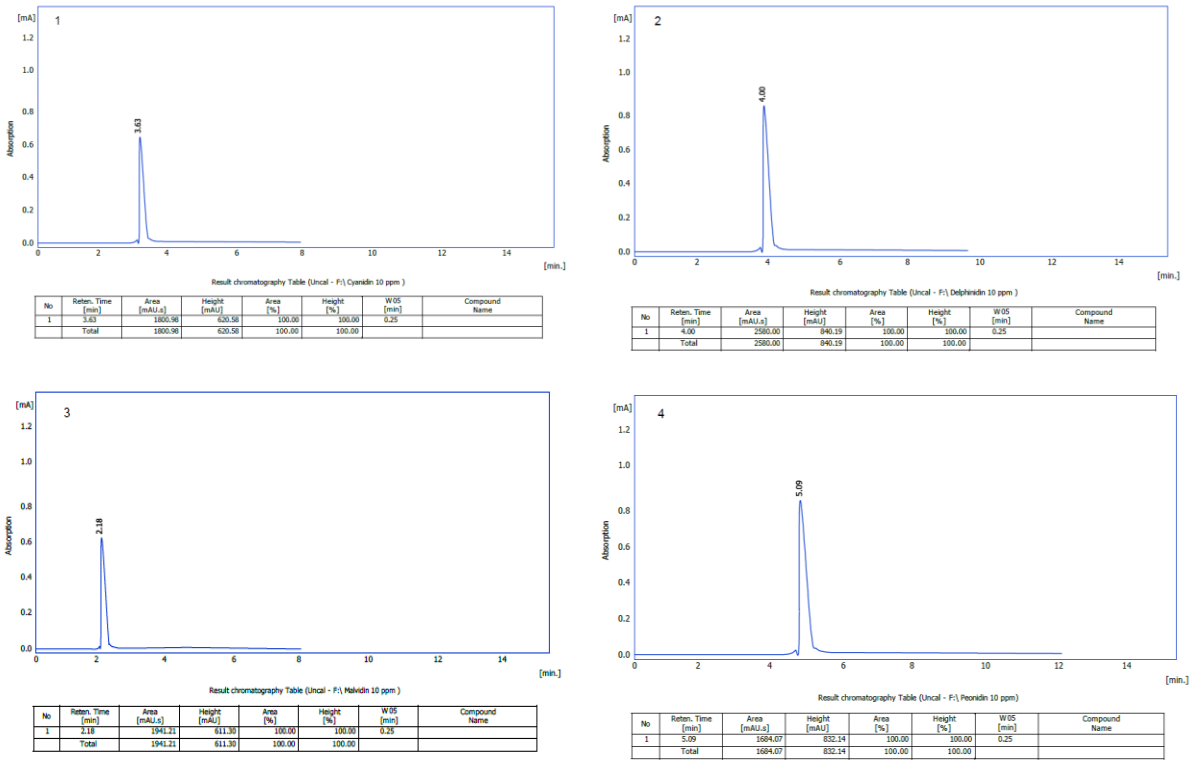
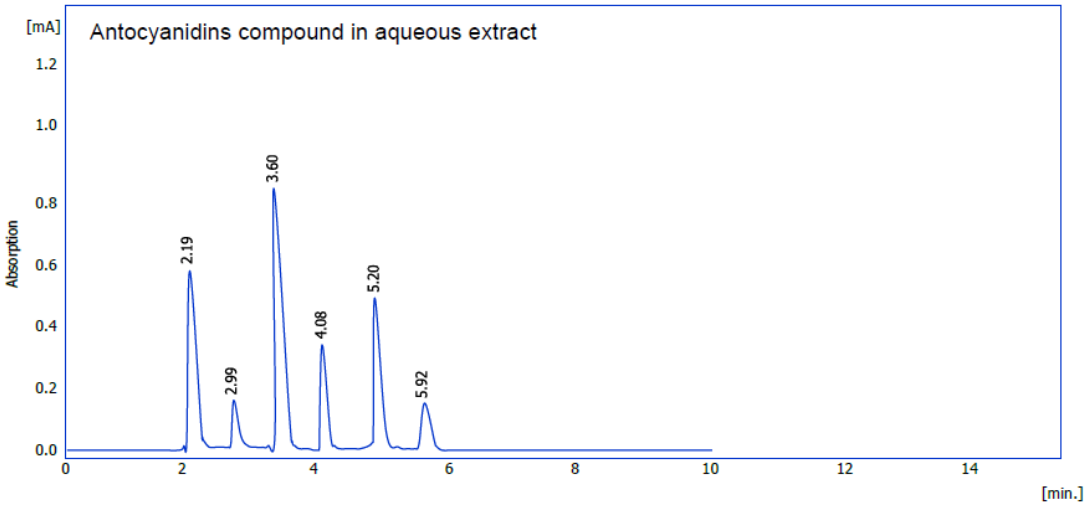


Figure 3:

Illustrate the chromatogram of HPLC of the standard anthocyanin compounds (1-delphinidin, 2-malvidin, 3-peonidin, 4-cyanidin, 10ppm)

The benefits of these compounds include protection against colorectal cancer. (do Nascimento & da Fonseca Machado, 2023) allergies (Merecz-Sadowska et al., 2023) and cardiovascular disease, (Mohammadi et al., 2024)enhancement of microcirculation, prevention of peripheral capillary fragility, prevention of diabetes, improvement of vision, and anti-allergic, anti-inflammatory, antiviral, antiproliferative, anti-mutagenic, anti-microbial, and anti-carcinogenic properties (Dhalaria et al., 2020).

Figure 4: Illustrate the chromatogram of HPLC of the anthocyanidin compounds found and detected in the *solanum melongena* aqueous extract



Result chromatography Table (Uncal - F:\ Aqueous sample)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.19	177854.08	590.25	26.00	26.00	0.20	
2	2.99	25414.15	180.99	9.00	9.00	0.10	
3	3.60	233952.40	860.22	27.00	27.00	0.30	
4	4.08	90652.14	362.81	11.00	11.00	0.15	
5	5.20	102632.00	492.10	16.00	16.00	0.20	
6	5.92	29654.11	178.99	11.00	11.00	0.10	
	Total	660158.79	2665.41	100.00	100.00		

3.2 LP-

purification

Even though lipoproteins are hydrolyzed by LPL at the EC surface in the vascular lumen, these cells do not produce. In the heart, LPL is synthesized in cardiomyocytes before its transfer to the vascular lumen (Pirahanchi, Anoruo, & Sharma, 2023).

The LPL was purified from the sera of patients with type 1 diabetes patients by using the salt of ammonium sulfate precipitation, dialysis, ion exchange chromatography, and polyacrylamide SDS-electrophoresis. The method of precipitation was used as a first step in purifying the enzyme. The results in Table (2) indicated that the specific activity of the LPL enzyme was 0.0117 enzyme units/mg of protein, meaning that it increased by 1.88 times after the precipitation process. The recovery of the LPL activity was 92.48 compared to the total activity of the enzyme in the crude extract.

A dialysis technique was applied in the next step in purifying the enzyme. It is a method of excluding the ammonium sulfate as well as peptides, amino acids, ions, and small molecular compounds. The results in Table (2) indicated that the specific activity of the LPL is 0.0252 U/mg of protein, meaning that it has increased by 4.06 times and the amount of recovery l, of the total activity of LPL was 208.89 compared to the total activity in the crude extract.

The solution that yielded from the dialysis was applied in cationic ion exchanger CM cellulose (figure 5).

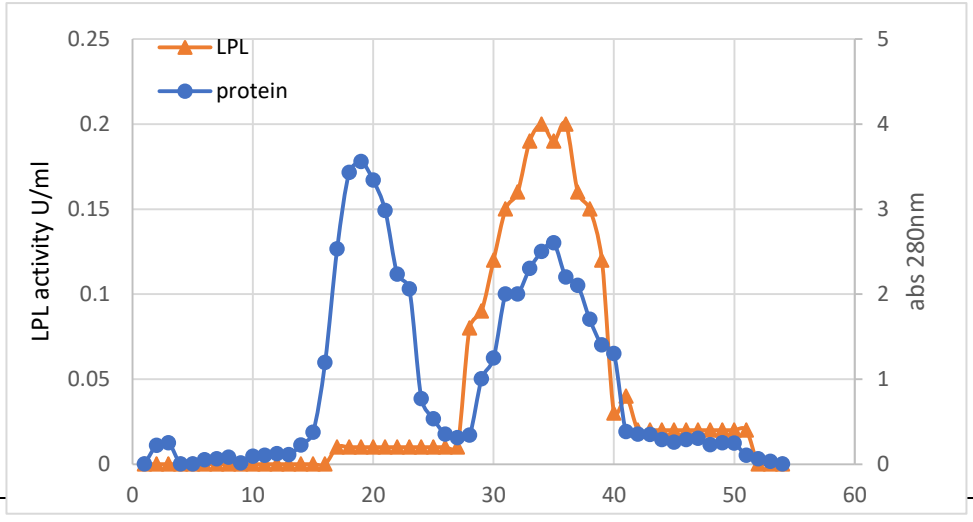


figure 5: Recovery model obtained from purifying the lipase enzyme from the blood serum of patients with type 1 diabetes using a cation exchanger at a flow rate of 60 ml/hour CM-cellulose

Two proteinous peaks and one LPL enzyme peak were obtained from CM-cellulose ion exchange chromatography. The LPL peak appeared at a profile volume (53ml) that has a specific activity of 0.1035 U/mg of protein with several purification folds of approximately 16.96, and a recovery of 1194.78 times.

Table 2: Purification steps LPL which purified from Type I diabetes mellitus

Purification steps	**Vol. ml**	**Protein mg/ml**	**Total protein mg**	**Activity u/ml**	**Total activity u/ml**	**Specific activity u/mg protein**	**Purification steps**	**% yield**
Crud	8	26.28	201.24	0.163	1.304	0.0062	1	100
Precipitation	6	17.06	102.63	0.201	1.206	0.0117	1.8	92.84
Dialysis	6	8.98	107.76	0.227	2.724	0.0252	4.06	208.89
CM-cellulose	53	2.84	150.52	0.294	15.58	0.1035	16.96	1194.78

When the molecular weights were determined of the LPL peak using SDS-PAGE, comparing some standard protein compounds which was illustrated in figure (6) below

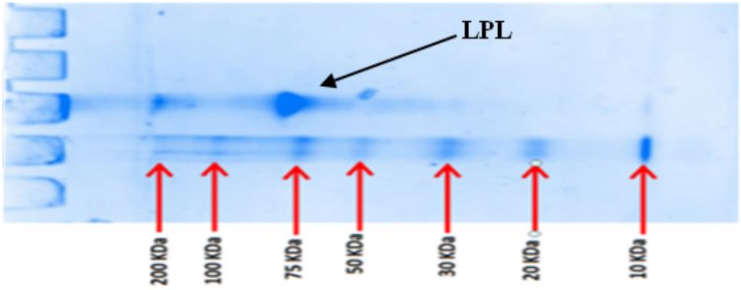


Figure 6: The migrated distance of LPL and standard proteins by SDS-PAGE

it has been shown the molecular weight is approximately 76.5KD by plotting the Mr. of versus the rf of the standard protein compounds Figure (7)

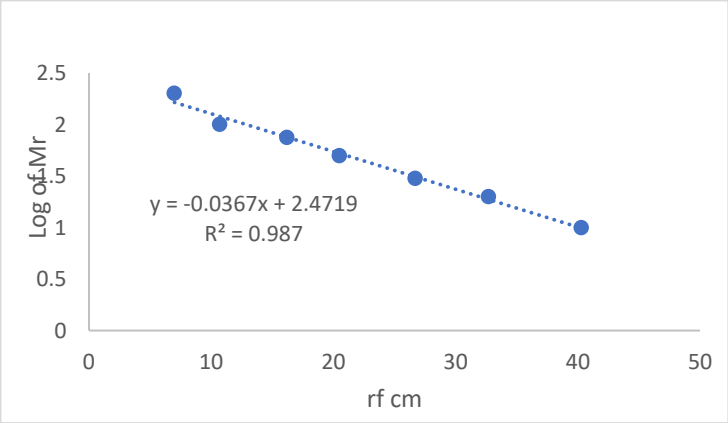


Figure 7: Standard curve to determine LPL Molecular weight by SDS-PAGE

3.2.1 *The Kinetic Study of LPL*

The effect of the concentration of purified LPL has been studied by using different volumes of enzymes ranging between 10-100 µl. The results indicate that a volume increase of up to 100 µ led to an increase in the speed of the enzymatic reaction for the purified Figure (8).

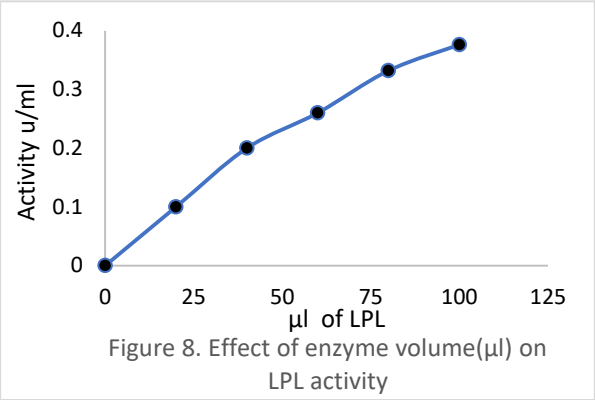


Figure 8. Effect of enzyme volume(µl) on LPL activity

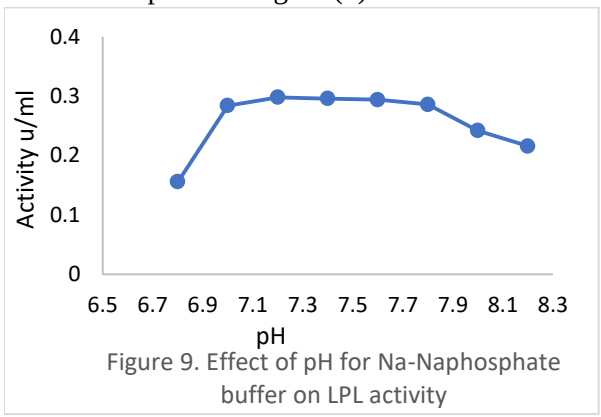


Figure 9. Effect of pH for Na-Naphosphate buffer on LPL activity

Figure (9) shows the activity of the enzymatic reaction using the sodium phosphate buffer solution at pH 7.2.

This result is consistent with what the researcher found for the enzyme purified from palm pollen (Okunwaye, Obibuzor, & Okogbenin, 2015).

Where it is found about 8.7 for LPL purified from human post-heparin plasma (HAYASHI, TAJIMA, & YAMAMOTO, 1986).

While the optimum temperature of LPL activity was found at 37°C Figure 10

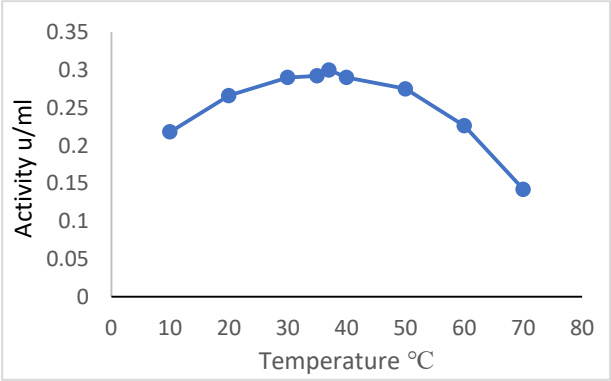


Figure 10. Temperature effect on LPL activity

When studying the influence of the substrate on LPL activity the result in Figure 11,12 illustrates that LPL follows the Michael-Menton equation and from Lineweaver plot the amount of the kinetic parameters Vmax=1.2 u/ml and Km = 8.33 mM.

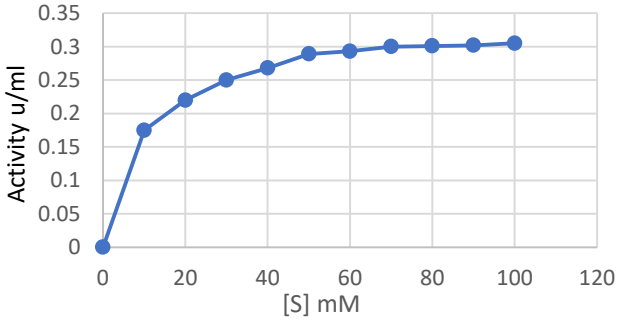


Figure 11. Michael-Menton plot of LPLactivity

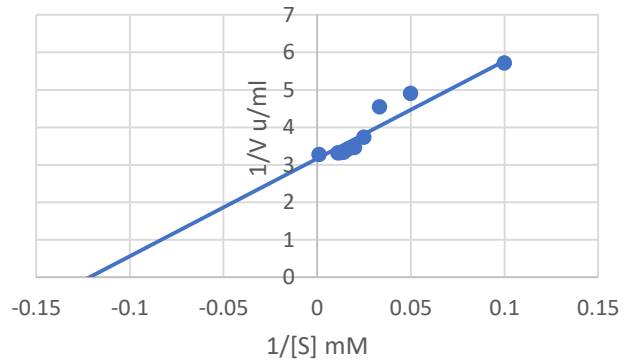


Figure 12. Lineweaver plot of LPL activity

3.2.2 *Study effect of Anthocyanin extract on LPL activity*

The result in Table 4 illustrates that two concentrations appear more than others that inhibit LPL activity 20 and 40 ppm

Table 4: Inhibition percentage of LPL activity by different concentrations of Anthocyanin extracts

Anthocyanin con. ppm	LPL activity u/l	Inhibition percentage
Control	0.294	0
10	0.232	21.08
20	0.226	23.12
30	0.242	17.68
40	0.228	22.44
50	0.258	12.24
60	0.260	11.56
70	0.268	8.84
80	0.270	8.16
90	0.274	6.8
100	0.280	4.7

Figures 13, and 14 Lineweaver plot show the two concentrations of 20 and 40 ppm of anthocyanin extracts have the same inhibition type competitive inhibition. The kinetic parameters of the two 20 and 40 ppm inhibitors are illustrated in Table 5

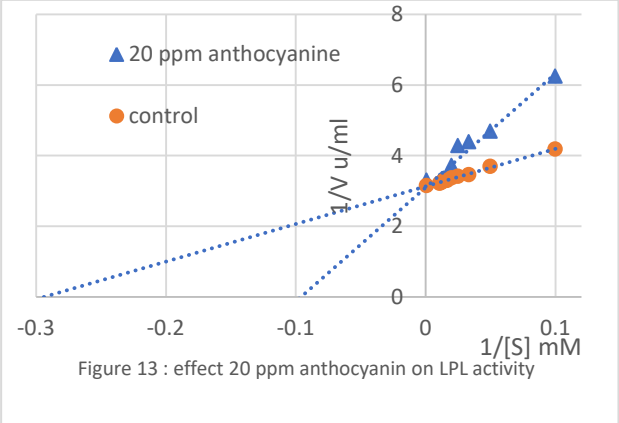


Figure 13 : effect 20 ppm anthocyanin on LPL activity

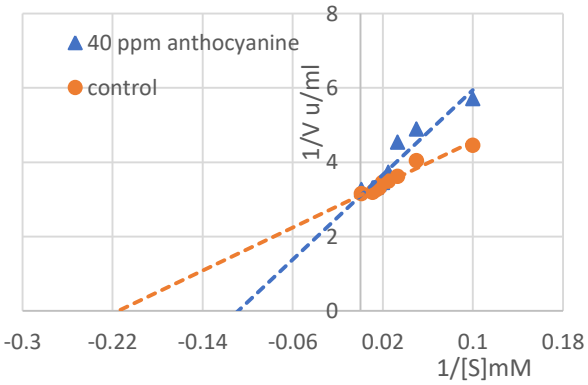


Figure 14 : effect 40 ppm anthocyanin on LPL activity

Table 5: The kinetic parameters of LPL inhibition

Conc.of anthocyanin	Km (mM) of control	Km (mM) with inhibitor	Vmax(u/ml) of control	Vmax(u/ml) of with inhibitor	Inhibition type
20 ppm	5	9.09	0.322	0.322	competitive
40 ppm	3.75	11.11	0.322	0.322	competitive

These results Compatible with the (Wei et al., 2011) The result showed that the Cyanidin-3-O-β-glucoside decreased the LPL activity in mice by given with food, this effect may be because of the ability of anthocyanin compounds that have some active hydroxyl group to interact with enzyme molecules by any position that Which causes a connection disability with a substrate.

4 CONCLUSION

The study showed that the anthocyanin compounds are good bioavailable concentrations in eggplant peels. Also found that they can inhibit the LPL enzyme at several concentrations, while the best concentrations were 20 and 40 ppm, and the type of inhibition is competitive.

5 ACKNOWLEDGMENT

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