Anti- Lipase Activity of Some Plant Extracts on The Effectiveness of Lipase Enzyme Isolated and Purified From *Pseudomonas aeruginosa*

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Abstract

The *Pseudomonas aeruginosa* strain grows optimally in the temperature of 40°C and pH 7.The lipase was extracted and purified by precipitation with 45% saturation of ammonium sulfate, dialysis and filtration chromatography by Sephadex G-150, two peaks of protein appeared in last step, the lipase activity was observed in second peak. The purified lipase has specific activity 3.67 unit/mg with protein concentration 0.27 mg/ml. Analysis of purified lipase was done by HPLC, the individual peak was identified by comparing the retention time of peak with standard. *Pseudomonas aeruginosa* lipase was treated with different concentration of plant extract to determine their effect on the production and activity of this enzyme. The methanolic extract varied in their effect *Quercus infectoria* reduced the effectiveness of the enzyme activity more than *Trigoella foenum graecum*.

Key words: Anti-lipase, Plant extract, *Ps. aeruginosa* , Purification

Introduction

Lipases are industrially important enzymes and are produced by a variety of microorganisms **(1).**Lipase (Triacylglycerol acylhydrolases E.C. 3.1.1.3) is widely distributed in animals, plants and microorganism, is a soluble enzyme that catalyzes the hydrolysis of ester bonds in water insoluble, lipid substrates. Lipases thus comprise a subclass of the esterase. **(2)** Lipase hydrolyses triglycerides to fatty acid and glycerol and under certain condition, catalyses the several reaction forming glycerides from glycerols and fatty acids. (**3)**

$$\text{Triacyl glycerol} + H_2O \xrightarrow{\text{Lipase}} \text{n Diacyl glycerol} + \text{fatty acid anion}$$

Lipases are ubiquitous in nature and are produced by several sources **(4).**Most commercially useful lipases are of microbial origin..Lipase producing microorganisms include bacteria, fungi, yeasts, and actinomyces .Microbial lipases have gained special industrial attention due to their stability, selectivity, and broad substrate specificity **(6)** .Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer **(7)**. Lipases form an integral part of the industries ranging from food, dairy, pharmaceuticals, agrochemical and detergents to oleo-chemicals, tea industries, cosmetics, leather and in several bioremediation processes. Because of the vast applications, newer microbes are to be screened for production of lipases having desirable properties. The understanding of structure function relationships will enable researchers to tailor new lipases for biotechnological applications **(8).**A variety of lipases are produced from both Gram-positive and Gram-negative bacteria. Greater part of bacterial lipases comes from Gram-negative bacteria and the most important Gram-negative genus is Pseudomonas which contains at least seven lipase producing species, that are *P. aeruginosa*, *P. alcaligenes*, *P. fragi*, *P. glumae*, *P. cepacia*, *P. fluorescens* and *P. putida* **(9)**.*Quercus infectoria* Oliv (Family Fagaceae), the main constituents found in the galls of *Q.infectoria* are tannin (50-70%) that have the ability to precipitate proteins and small amount of free gallic acid and ellagic acid **(10).**Fenugreek (Trigonella foenum graecum) is an annual herb that belongs to the family Leguminosae., Saponins **(11)**, flavonoids **(12),** galactomannans **(13)**, were isolated from fenugreek seeds in previous phytochemical research studies. In the present study, lipase from *Pseudomonase aeruginosa* has been isolated, partially purified and characteristic with respect to its stability at different temperature and pH. As well as the study of the inhibitory effectiveness of plant extracts against lipase enzyme.

Material and Method

Collection of producer microorganism

The bacterial strain *Pseudomonas sp* used in this study was isolated from food. The isolates were identified on the basis of various morphological and biochemical characteristics. Lipolytic bacteria were typically detected and screened through the appearance of clearing zones

by using a selective medium (**14**), which containing Tween 80 or olive. The diameter ratio of clear zone and colony was measured.

Optimization of lipase production

Mineral media ($NH_4H_2PO_4$ 0.1g, KCl 0.02g, $MgSO_4$ 0.02g, Yeast extract 0.3g, Olive oil 5ml, D/W 100mL, pH = 7.2) was used for time course optimization. 1mL of 24hr old *Pseudomonas* culture was inoculated in 100mL of mineral media and incubated on rotary shaker adjusted at 30°C and 120 rpm. After every 24hrs and lipase activity was determined.

Lipase activity

Lipase activity was determined using olive oil hydrolysis, 10% Olive oil emulsion in 2% gum acacia was used as substrate. Reaction mixture composed of 0.5mL substrate emulsion, 0.4mL 0.1M Tris-HCl buffer (pH-7.2), and 0.1mL lipase solution. In blank lipase solution was replaced with equal amount of distilled water. Reaction was carried out at 30°C for 30min. Reaction was stopped by adding 20mL acetone and ethanol (1:1). Liberated fatty acids were titrated with 0.05N NaOH using phenophthalene indicator. Amount of NaOH required to achieve end point (colorless to pink) was recorded.

Determination of pH and Temperature optimum

The optimum temperature and pH of crud lipase was assayed for its activity at different temperatures (20-60° C) and different pH (3-10) **(14).**

Purification of lipase

Lipase purification was carried out at 4°C. The culture medium was centrifuged at 10000 rpm for 20 min to obtain crude enzyme, then the supernatant fluid was subjected to precipitation with ammonium sulphate to 45% saturation and stirred for 2 h.. Lipase activity was determined in the precipitate after collected and dissolved in buffer pH 7.2 and dialyzed against same buffer. The enzyme mixture was loaded on Sephadex G-150 column (1.8 X 65 cm) equilibrated with 100 mM Tris buffer, pH 7.5 The enzyme was then eluted with the same buffer with a flow rate of 1 ml/min. Fractions of 3 ml were collected. The active fractions that contained lipase enzyme were pooled and assessed for protein content. The resulting enzyme was utilized for the characterization of the extracellular lipase. The protein content at each stage of enzyme purification was determined according to the Lowery method.

Analytical RP-HPLC of Purified Lipase

Analysis of purified lipase was done by HPLC under following conditions: Column (C18) (250mm x 4.6mm) 5µ, Mobile phase: 0.01 M Potassium di hydrogen phosphate (20%), acetonitrile(20%),methanol (40%) orthophosphoric acid (20%). flow rate: 1.0ml/min. Protein was detected at 280 nm.

Preparation of methanolic extract

Plant parts were air dried at room temperature. After drying, the plant material was powdered and extracted by soxhlet apparatus (Quick fit (England) using methanol as solvent. The extract was filtered using Whatmann filter paper No.1 and concentrated at 40°C in a rotator vacuum evaporator.

Anti-lipase activity of crude methanolic extracts

The inhibitory activity of methanolic extract against lipase was tested against lipase extracted from the *pseudomonas aeruginosa.* Lipase inhibitory activity of different concentrations of methanolic extract for each of the *Quercus infectoria* and *Trigoella foenum graecum* was tested by mixing 100µl of each concentration of methanol extract, 8ml of oil emulsion and 1ml of lipase followed by incubation of 30 minutes. The reaction was stopped by adding 1.5ml of a mixture solution containing acetone and 95% ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02M NaOH using phenolphthalein as an indicator **(15)** Percentage inhibition of lipase activity was calculated using the formula:

Lipase inhibition = A - B / A X 100

Where:

A: lipase activity

B: activity of lipase when incubated with ethanol extract.

Statistical analysis

Data obtained in the study were statistically analyzed using Analysis of Variance (ANOVA) and means were separated using Fisher's Least Significant Difference (LSD) at both 1 and 5% levels of significance **(16).**

Result and Discussion

Optimization of lipase production

The effect of incubation time has resulted to 72 hours as optimum incubation time for the growth of the organism and the activity of lipase at this point of time was found to be 0.41 U/ml. Further incubation was found to be negatively affecting the yield (Table 1). Similar results were obtained by (**17**).

Table 1: Time course of lipase production

Lipase activity (U / ml)	Incubation Time (In Hours)
0.13	24
0.25	48
0.41	72
0.22	96

Effect of pH on lipase production

Media pH is one of the important physical parameter which can influence on bacterial growth as well as production. Many studies have been reported the importance of culture pH. Benattouche and Abbouni Reported that the *Pseudomonas aeruginosa* was able to grow in the pH range from 6 to 8 and produced maximum lipase (38.5 U/ml) at pH 7**(18)**. Sujatha and Dhandayuthapani also reported that maximum lipase activity from *Pseudomonas aeruginosa* KDP (19.32 U/ml) was obtained at pH 7.0 **(19).** Similarly, in the present study maximum lipase production (0.56 U/ml) was obtained at pH 7 Fig (1).

Effect of Temperature on lipase production

The enzyme activities were observed at 20, 30, 40 and 50°C. The optimum Temperature was observed at 40°C for maximum production Fig (2). The optimal Temperature for lipase production by *Peudomonass xinjiangensis* CFS14 was 40°C **(20).** Tembhurkar *et. al* also reported the enzyme from *Psedomonas spp* worked optimally at slightly higher Temperature; 50°C,at which lipase activity was 0.357 $\mu M.min^{-1}.mg^{-1}$.**(17)**.

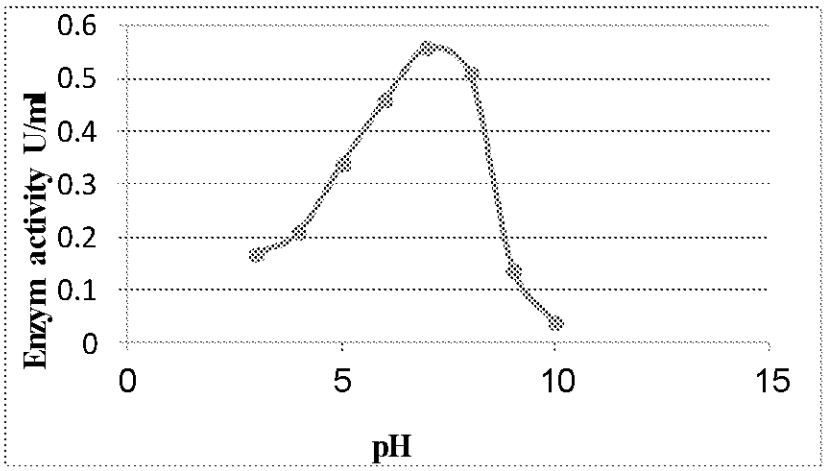

Fig (1): Effect of different pH on lipase production by *Ps. Aeruginosa*

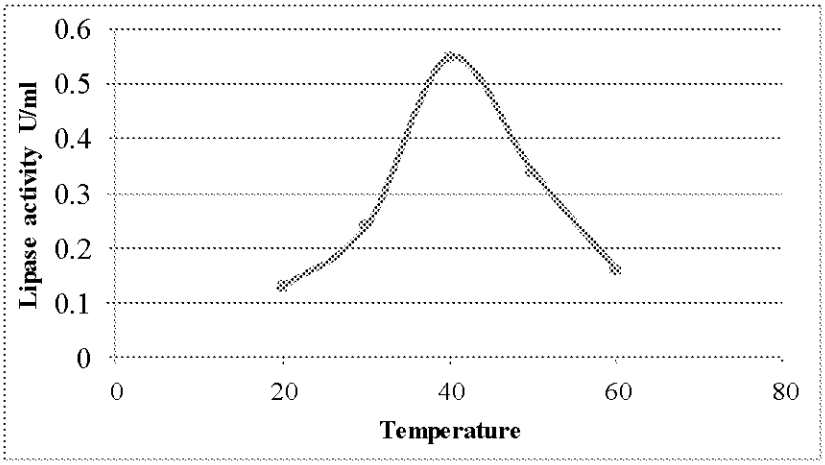

Fig (2): Effect of different temperature on lipase production by *Ps. aeruginosa*

Determination of protein

Protein concentration was determined by the method of Lowry **(21)** Using Bovine Serum Albumin as standard (Figure 3).

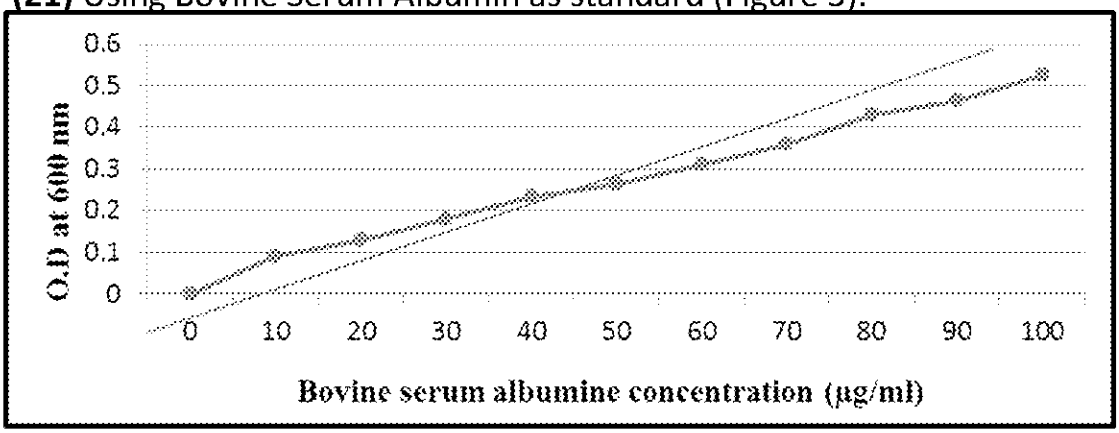

Fig (3): Standard curve of protein

The specific activity for enzyme was determined as following:-

$$\text{Specific activity (Unit /mg protein)} = \frac{\text{Enzymatic activity (Unit/ml)}}{\text{Protein concentration (mg/m)}}$$

Partial purification of lipase:

Ps. aeruginosa lipase was purified by simple steps which included precipitation with 45%, saturation of ammonium sulfate and gel filtration chromatography by Sephadex G-150 (Pharmcia fine chemical), the eluted fractions of the last step contained two protein peaks Fig. (4). The two

peak (fractionation tubes 19-20) showed lipase activity about (0.81،0.99) unit/ml and specific activity 3.67 unit/mg and the purification fold was 5.206 times with 30.938 % recovery was obtained after the single step purification on gel filtration chromatography As in table (2). The result was approximately compatible with other studies that deal with lipase of *Ps. aeruginosa* , such as ammonium sulphate 40% saturation(**14**). Analysis of purified lipase was done by HPLC, the individual peak was identified by comparing the retention time of peak with standard Fig (5).Fig (6) Represented the HPLC(Shimadzu Corporation,Kyoto,Japan) chromatogram of Gel filtration with Sephadex G -150. The purified lipase showed three peaks with retention time (4.323, 4.920 and 5.069) min the peak with retention times of 4.323min represent the presence of lipase with concentration 0.035 ppm.

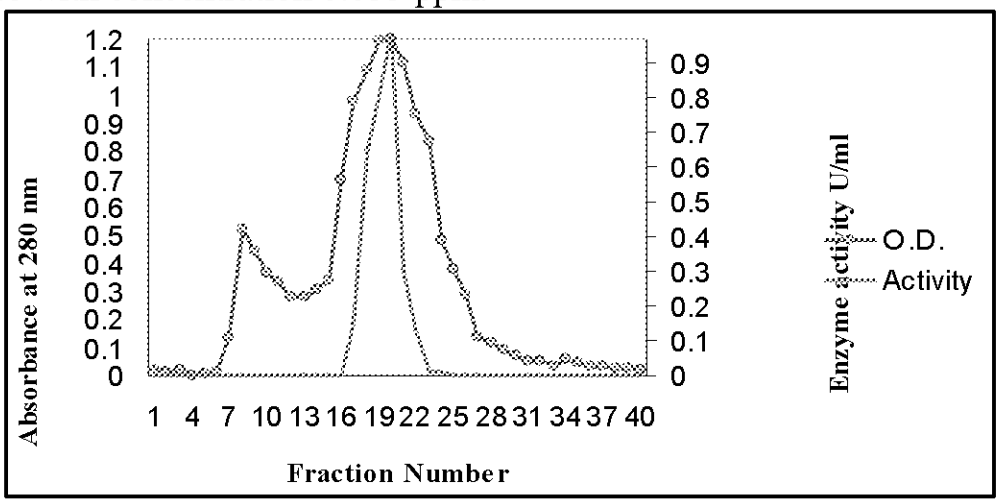

Figure (4): Gel filtration for partial purification of lipase from *Ps .aeruginosa* by Sephadex G -150 column

Table (2): Purification of *Pseudomonas aeruginosa lipase*

Purification Steps	Volume (ml)	Enzyme activity (U/ml)	protein Concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Fold purification
Crude enzyme	**80**	**0.12**	**0.17**	**0.705**	**9.6**	**100**	**1**
Precipitation with 45% saturation of $(NH_4)SO_4$ after dialysis	**7**	**2.92**	**4**	**0.73**	**20.44**	**212.917**	**1.035**
Gel filtration with Sephadex G -150 column	**3**	**0.99**	**0.27**	**3.67**	**2.97**	**30.938**	**5.206**

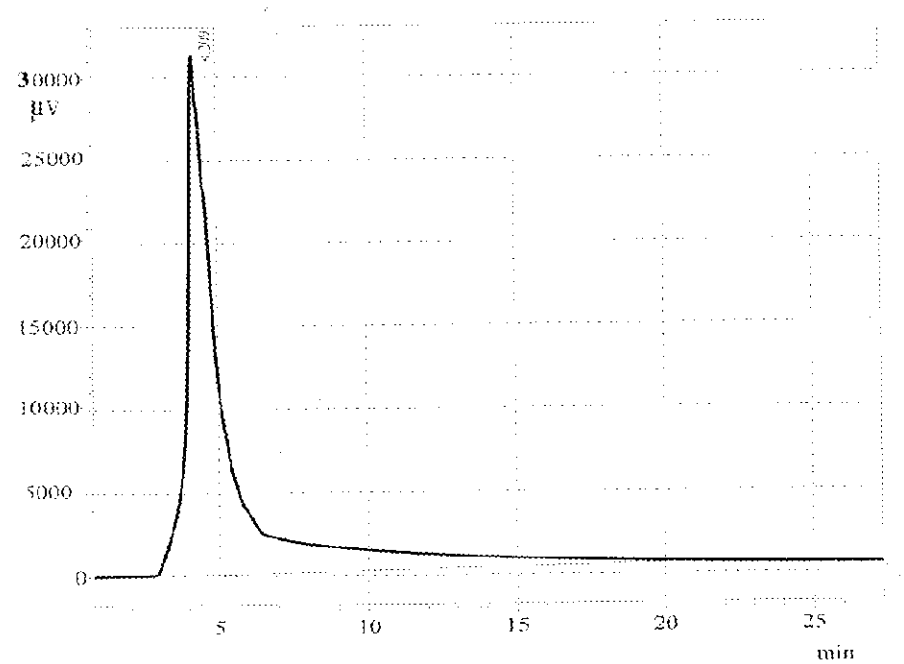

Fig (5): HPLC chromatogram of standard lipase

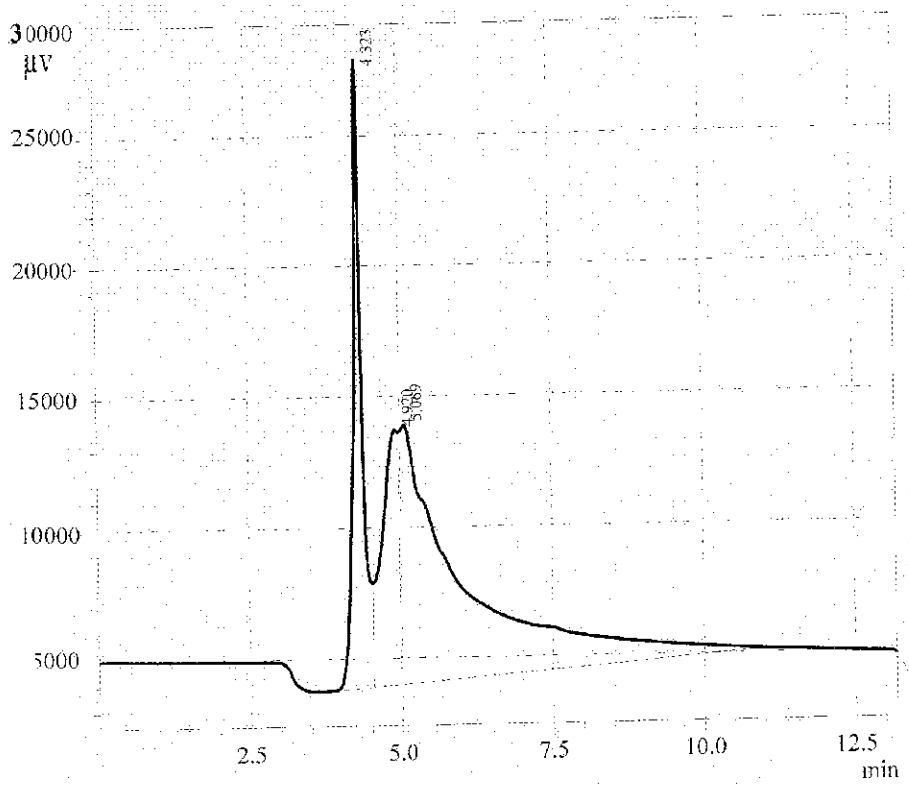

Fig (6): HPLC chromatogram of gel filtration chromatography by Sephadex G-150 of purified lipase

Lipase inhibitory activity of methanolic extract for each of the *Quercus infectoria* and *Trigoella foenum graecum*

*Ps.*aeruginosa lipase was treated with different concentration of plant extract to determine their effect on the production and activity of this enzyme. The methanolic extract varied in their effect *Quercus infectoria* reduced the effectiveness of the enzyme activity more than *Trigoella foenum graecum* Where the specific gravity about (0.36, 1.58) U/mg respectively in the concentration of 20 mg/ml Table (3). In this study we demonstrated that galls of *Quercus infectoria*, has strong anti lipase activity (82.8) % while *Trigoella foenum* graecum has a weak inhibitor activity (49.5) % at concentration 20 mg/ml Table (4).The galls of *Quercus infectoria* is a rich source of tannins which are polyphenolic compounds. Polyphenols from plants have an affinity for proteins, primarily through hydrophobic, as well as hydrogen bonding and therefore exhibit inhibitory activity for enzymes, because of aggregation

of enzyme proteins (**22**. Tannins are commonly defined as water–soluble polyphenolic compounds ranging in molecular weight from 500 to 3000 Daltons that have the ability to precipitate proteins **(10)**.

As well as contain *Quercus infectoria* on quercetin **(23)** which could play an important role against lipase producing microorganisms **(24).** In addition to containing *Quercus infectoria* on alkaloids which are inhibitors of the enzyme lipase **(25)**.

Table (3): Specific gravity of the lipase enzyme in different concentrations of plant extract.

Rang of Specific activity (U/mg)± Standard error		
Concentration mg/ml	*Quercus infectoria*	*Trigoella foenum graecum*
0	b3.67±0.02	a3.67±0.02
1.25	b3.07±0.02	a3.41±0.01
2.5	b2.63±0.01	a2.93±0.02
5	b2.15±0.03	a2.48±0.04
10	b0.41±0.02	a2.15±0.02
20	b0.63±0.02	a1.58±0.02

- The different numbers refer to significant differences.
- Probability (P≤0.01).
- The result means to five replicate.

	Inhibition %	
Concentration (mg/ml)	*Quercus infectoria*	*Trigoella foenum graecum*
1.25	16.1	7
2.5	28.3	20.2
5	41.4	32.3
10	61.6	41.4
20	82.8	49.5

Table (3): Anti-lipase activity for eachof *Quercus infectoria* and *Trigoella foenum graecum* against lipase.

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التأثيرالمضاد لبعض المستخلصات النباتية تجاه فعالية أنزيم اللايبيز المعزول والمنقى من بكتريا *Pseudomonas aeruginosa*

الخلاصة

تنمو بكتريا *Pseudomonas aeruginosa* بشكل مثالي بدرجة حرارة 40 م واس هيدروجيني 7 . أستخلص الأنزيم من هذه العزلة وتم تنقيته بأستخدام عملية الترسيب بكبريتات الامونيوم بنسبه تشبع 45% والتنقية بأستخدام عمود الهلام Sephadex G-150 حيث ظهرت قمتين بروتينية وتم قياس الفعالية النوعية للأنزيم بعد التنقية حيث وجد أنها تساوي 3.67 وحدة /ملغم وبتركيز بروتين 0.27 %ملغم/مل .قد تم تحليل أنزيم اللايبيز بأستخدام تقنية وبأستخدام تقنية الكروماتوكرافي السائل عالي الأداء(HPLC) .كما أستخدمت تراكيز مختلفة من المستخلصات النباتية لدراسة تأثيرها على فعالية انزيم اللايبيز.