

استخلاص وتوصيف انزيم اريل استيريز من لب وقشره ثمره

القشطه

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الخلاصة:

تضمن البحث استخلاص وتوصيف انزيم اريل استيريز من المستخلص المائي لللب وقشره ثمره القشطه باستخدام التقنيات الحيوية المختلفة. اظهرت النتائج ان اللب يحتوي على اعلى فعالية للأنزيم من القشرة كما وظهرت نتائج تقنيه الترشيح الهلامي على عمود الفصل الحاوي على Sephadex G-75 احتواء محلول الراسب البروتيني الناتج من الترسيب بالأسيتون للمستخلص المائي لللب ثمره القشطه على ثلاث حزم بروتينية متباينة الفعالية لأنزيم اريل استيريز, الفعالية للحزمة A ١٦٥٣ والحزمة B ٢٣١٠,٩ لكن الحزمة C كانت قليلة جدا, وقد اظهرت الحزمة الاولى والثانية فعالية نوعيه ٢٣١٠٧,١٨٣٦٦ IU/ml/mg وبنقاوة على التوالي ٢٦,٥٣١, ١٢,٥٨ مره اما الحزمة الثالثة فتحتوي على اقل فعالية نوعيه للأنزيم وقد تم تقدير الوزن الجزيئي للنظير الأنزيمي الاول والثاني وكان مقدارها ١٢٩,٩٢,٨٩,٨٦ كيلو دالتون على التوالي باستخدام تقنيه الترشيح الهلامي ثم حددت الظروف المثلى لعمل الانزيم المنقى جزئيا, وبينت النتائج ان الانزيم يعمل في المحلول المنظم (Tris-HCl) وبتراكيز ٩ mM واس هيدروجيني امثل (٧,٢) ودرجه حراره مثلى (C ٣٧ °) و زمن حضان مقداره 25 دقيقه وباستخدام منحنى لاينير-بيرك وجد ان السرعة القصوى التي يعمل بها الانزيم تساوي 211U/ml وان قيمه ثابت ميكالس- منتن تساوي (2.8mM)



الكلمات الدالة : أرييل أستريز , اننونا موريكاتا . الترشيح الهلامي , الظروف المثلى .

Extraction and characterization of Arylesterase enzyme from (peel and pulp) of *Annona muricata* fruit.

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Abstract

The extraction of arylesterase from the aqueous extract of (peel and pulp) of *Annona muricata* fruit was conducted using different biochemical techniques. It was shown that, max activity was obtained in the pulp than in peel, and by using gel filtration chromatography on sephadex G-75 for the pulp part, the solution of the proteinous precipitate produced by acetone precipitation, contained three proteinous peaks. The activity for peak A (1653) and peak B(2310.9) but the third peak C was very low. while maximum specific activity was obtained in the second peak (B) which showed (23107), (18366) IU/ml /mg for (A) and very low for the third one (C), and (12.58),(26.531) folds of purification for B and A peaks respectively .Furthermore, the comparative molecular weight of the partially purified isoenzymes arylesterase





(peaks B and A) using gel filtration were found to be (92,129), (86,890) Dalton respectively.

The optimum conditions of arylesterase were determined as maximum activity was obtained using (9 mM) Tris – HCl as a buffer at pH (7.2), with incubation temperature (37°C), incubation time (25min) and (4mM) of phenyl acetate as a substrate. Using Linweaver–Burk plot, it was found that maximum velocity (Vmax) and Michaela’s constant (Km) had the values of (211UI/ml) and (2.8 mM) respectively.

Key words: Arylesterase, *Annona muricata*. gel filtrations , optimum conditions

Introduction

Arylesterase (EC 3.1.1.2) is a carboxylic ester hydrolase catalyzing the hydrolysis of phenolic esters such as phenyl acetate to phenol and acetate. It occurs in microorganisms [1],[2] plants [3], [4] and other living organisms including humans [5],[6],[7]. This enzyme catalyses the hydrolysis of toxic metabolites (e.g. paraoxon) and so it is called paraoxonase., and it is involved in the degradation of plastics and hydrolysis of organophosphates [8], phenylacetate, estrogenester, The health benefits of green tea have been extensively studied in the past few decades. Nowadays, tea is considered as a source of dietary constituents endowed with biological and pharmacological activities with potential benefits to human health Results of the current study revealed that green tea extract reversed the elevation of liver enzymes, lipid peroxidation and Advanced oxidation protein products in addition to attenuating the reduction of SOD,CoQ10 and paraoxonase (arylesterase) levels [9][10].





Arylesterase hydrolyses hydrogen peroxide which is produced by arterial wall cells in patients with atherosclerosis [11]. Also it presents In the serum ,liver,brain etc.cells of human and rats [12],[13] .It is extracted from green tea, grape. The extract from *Annona muricata* induced necrosis of pancreatic cancer (PC) cells by inhibiting cellular metabolism. The expression of molecules related to hypoxia and glycolysis in PC cells (i.e. HIF-1 α , NF- κ B, GLUT1, GLUT4, HKII, and LDHA) were down-regulated in the presence of the extract. In vitro functional assays further confirmed the inhibition of tumorigenic properties of PC cells. Overall, the compounds present in the whole extract inhibited multiple signaling pathways that regulate metabolism, cell cycle, survival, and metastatic properties in PC cells [14],[15]. and Ginger. Graviola is indigenous to most of the warmest tropical are as All parts of the graviola tree are used in natural medicine in the tropics, including the bark, leaves, roots, fruit, and fruit seeds [16]. (*A. muricata*) contains a variety of components which attribute to the various biological activities. The roots and bark can be of aid for diabetes, but can also be used as a sedative. The purpose of the present review is to highlight the various traditional uses, phytochemistry and pharmacological reports on Guyabano. Sourop contain many enzymes, polyphenol oxidase was obtained from ripe sourop by using 0.2M phosphate buffer (PH 7.5) [17].

Annona has a long, rich history of use in herbal medicine, antispasmodic, sedative, and nervine for heart conditions, coughs, flu,





difficult childbirth, asthma, hypertension, parasites and annona: a novel promising natural-derived drug that inhibits tumorigenicity and metastasis of pancreatic cancer cells in vitro and in vivo through altering cell metabolism[18]. Inhibit of annonaceous acetogenins on class II 3-hydroxy-3-methylglutaryl coenzyme A reductase from Streptococcus pneumonia, have an antibacterial effect (*in vitro*) of Moringa oleifera and Annona muricata against Gram positive and Gram negative bacteria [19][20].

The aim of this research is to extract and characterize arylesterase enzyme from medicinal plant((peel and the pulp parts) of the common names of the fruit(Graviola, soursop, Brazilian paw paw,Annona muricata.) due to its medical , biological applications.

2-Materials and Methods

2.1 Raw Material

Annona muricata (Custard apple) fruits (the Indian source) were taken from locally market in Erbil, it was restored at 25°C.

2.2 Extraction of Enzyme

Arylesterase extracted from (peel and pulp) of the Annona Muricata by the following method 120g of the pulp and 16 g of the peel





are used separately, were mixed with 400 and 70 ml of distilled water using a blender respectively for 10 min. at 4°C in water bath. Extraction was performed using high speed refrigerated centrifuge for 15min. at 4000xg speed and 4°C temp, (this experiment was carried in duplicate).

2.3 Enzyme activity assay

Arylesterase was assayed by using phenyl acetate as substrate, measured spectrophotometrically at 270 nm in an automated shimadzu UV-1601, UV visible spectrophotometer. Buffer substrate was (50 mM Tris-HCl buffer, pH 8.0 containing 2 mM Calcium (1.5ml)). Phenyl acetate in Isopropyl alcohol was added such that the Phenyl acetate concentration was 2mM and the isopropyl alcohol concentration was less than one percent. to 2.99ml of buffered substrate 10µl of the sample from different fractions, were add mixed together as a test against the blank which included all above solution except enzyme which used with 5µL of distilled water instead. One unit of arylesterase activity represents the amount of enzyme catalyzed hydrolysis of (1µmol.) phenyl estate in 1 min (table1).

Protein estimation was carried out according to Lowry's method to estimate serum protein concentration as well as protein in purified fractions [21]

2.4 Partial Purification of Arylesterase

2.4.1. Precipitation





The enzyme was precipitated using (60:40) ratio of acetone and distilled water (v/v) .The addition of the acetone was slowly with stirring at 4 °C for 60 min., The solution was left for 24 hours in the refrigerator and the precipitate was isolated using high speed refrigerated centrifuge for 15 min., 6000xg speed at 4°C. The activity of the enzyme and the protein contents were estimated before and after precipitation, the sample was dried using Lyophilization and stored at - 20 °C.

2.4.2 Dialysis

The active fraction enzyme sample from the precipitation step was dialyzed against (0.01 M, potassium phosphate, buffer pH 7.2) for 24 hours at 5°C and the enzyme activity and the protein contents were estimated by using (dialysis bag across a 30 kDa. rate dialysis membrane)

2.5 Gel Filtration Chromotography

Preparation of the gel column and the fractionation procedures were determined as previously mentioned by (Ammar,1975). For this purpose, column (2x100cm) and sephadex G-75 were used. A tow ml of the enzyme sample after dialysis was loaded carefully to the top of the column and allowed to pass through the gel and eluted with distilled water with flow rate (53 ml/hr). Fraction of two ml was collected by fraction collector at 5°C. The protein content in all fractions were monitored spectrophotometrically at 280 nm figure(1). Active fraction were pooled assayed for total enzyme activity and protein.





2.6 Molecular weight determination

The molecular weight of peak as source of arylesterase was determined by the elution volume from sephadex G–75 column (2×100cm) calibrated with known molecular proteins weight that as listed in table (2,3). Then from the plot of logarithmic molecular weight of each material in dictated in show figure (2) versus the elution volumes giving a straight line.

2.7 Characterization of Arylesterase.

To develop assay conditions where arylesterase shows a maximum activity, a series of experiments were performed these included changing the enzyme concentration, the incubation time, the incubation temperature, buffer concentration, the pH of the conditions and the substrate concentration.

3. Results and Discussion.

The activity of Arylesterase from the pulp part of the fruit was shown in Table (1).

Table 1. Arylesterase activity (U) extracted from raw pulp and peel.

Parts of Fruits	Activity U
Pulp	988.6
Peel	465.6



3.1 Extraction and partial purification of Arylesterase

The aqueous extracted solution of the peel part of *Annona muricata* was added to the column using gel filtration chromatography on sephadex G-75. The solution of the proteinous precipitate produced by acetone precipitation contained three proteinous peaks. The activity for peak A (1653)U/mg and peak B(2310.9)U/mg but the third peak C was very low, maximum specific activity was obtained in the second isoenzyme peak B which showed (23107)U/mg and (18366)U/mg for isoenzyme peak A and very low for peak C and 12.58, 26.531 folds of purification for B and A iso-enzymes respectively(Fig1) .

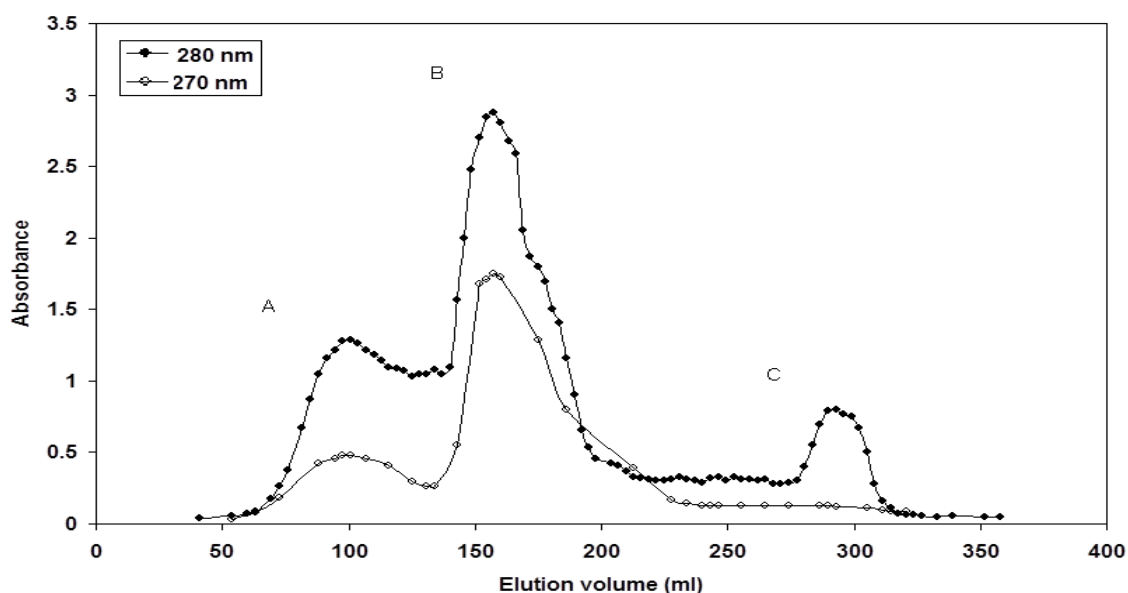


Fig.1. Gel filtration chromatography for Arylesterase from the peel of *Annona muricata* fruit on sephadex G-75 (2x100 cm) eluted with distilled water with flow rate 53ml/h.

* (280 nm for protein and 270 nm for Arylesterase activity)

Table 2.Purification of first and second protein bundle arylesterase in the pulp of *Annona muricata* fruit.

Purification steps		Protein concentration mg/ml	Activity U	Specific activity U/mg	Yield %	fold of purifications
Extracted of raw substance		3.9	988.6	253.48	100	1
aqueous extracted solution		3.5	880.31	251.51	89.04	0.99
Acetone precipitation(40:60)		2.4	1661	692.08	188.68	2.751
gel filtration chromatography on Sephadex G-75	First protein peak	0.091	1653	18366	99.51	26.531
	Second protein peak	0.10	2310.7	23107	139.78	1.258

3.2 Molecular weight determination.

The molecular weight of unknown compound separated by the column chromatography could be determined from the standard curve. The comparative molecular weight of peak as a source of arylesterase enzyme is approximrely equal to peak A 86.895 Da, peak B 92.129 Da



showed in table (3) and figure (2) this result is highly agrees with [14][15][24]

Table(3) standards used for the determination of the partially purified Arylesterase enzyme

Elution volume (ml)	Molecular weight (Da)	Substances
99	2,000,000	Blue dextran
197	58,000	α -amylase
215	45,000	Egg albumin
240	36,000	Pepsin
303	5,750	Insulin
116	87,890	First protein bundle
163	92,129	Second protein bundle



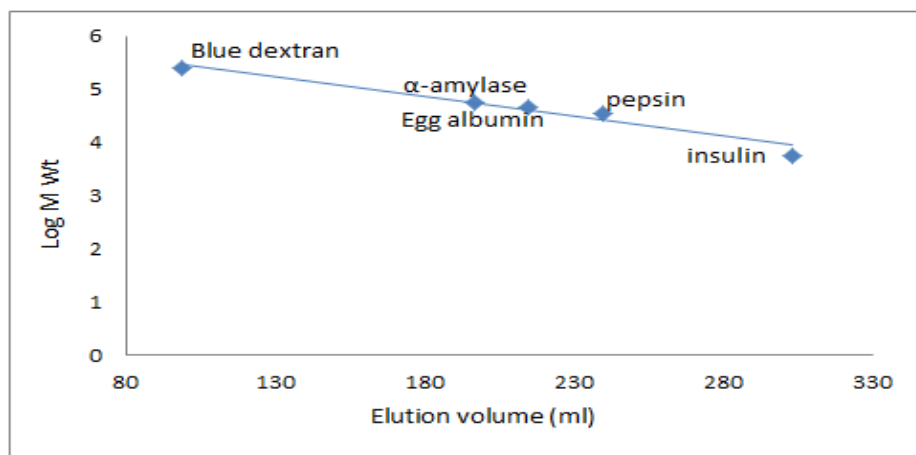
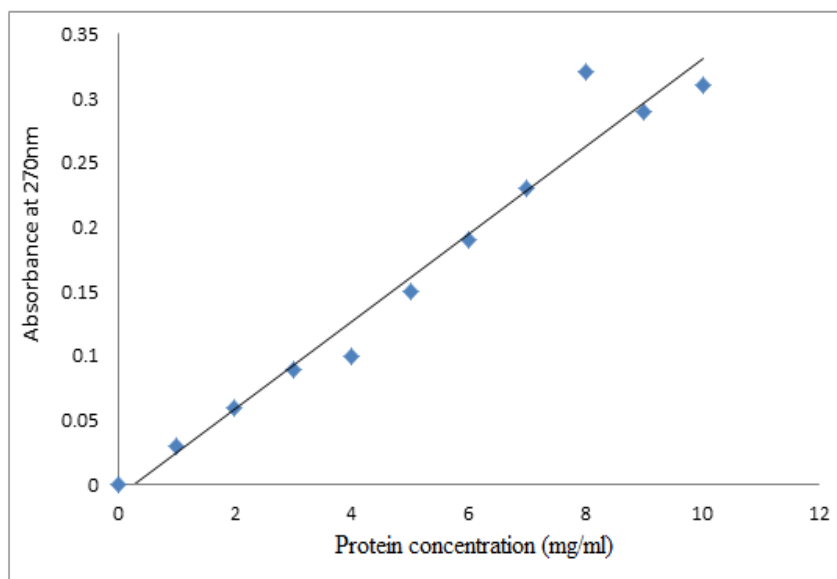


Fig 2. The molecular weight standard curve

3.3 Characterization of Arylesterase

3.3.1 Effect of enzyme concentration on arylesterase activity

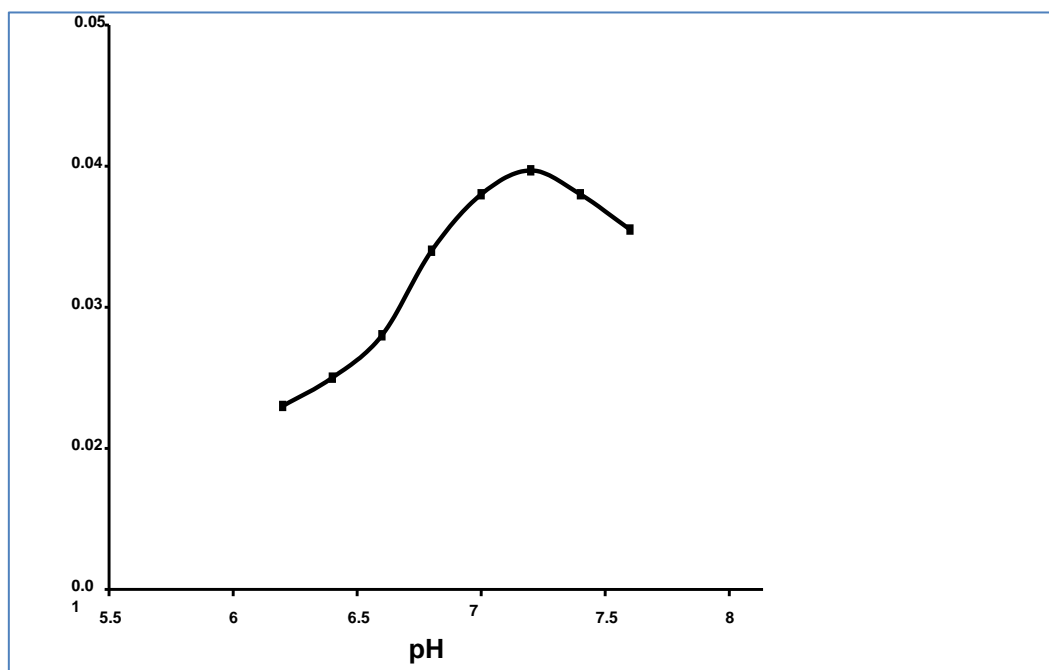
It is important to establish that the activity varies linearly with enzyme concentration the activity of enzyme was measured in the presence of different concentrations of partially purified enzyme (1-10) $\mu\text{g/ml}$ as shown in figure (3). The results enzyme and the optimum enzyme concentration was 8 $\mu\text{g/ml}$ indicated that the enzyme activity increased linearly with increasing the concentration of the protein as a source of the enzyme in study use 8 $\mu\text{g/ml}$.



Fig(3) Effect of enzyme concentration on arylesterase activity

3.3.2 Effect of pH on arylesterase activity.

The influence of pH upon the activity of arylesterase was investigated using buffer solution with pH values (6 , 6.5 , 6.8 , 7 , 7.2 , 7.5), The optimum pH was found to be 7.2 for partially purified Arylesterase from *Annona muricata* (Fig.4)

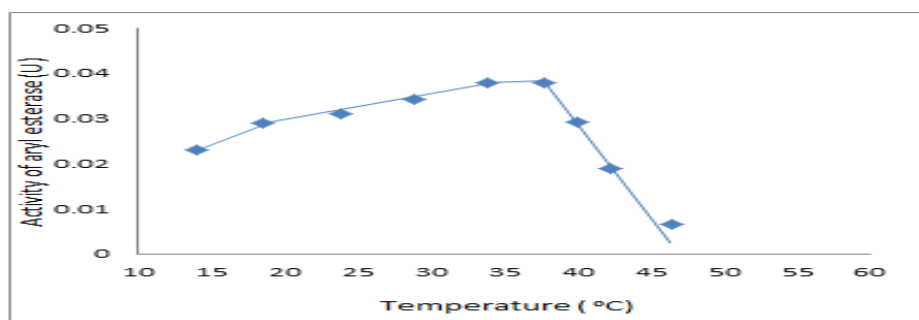


Fig(4) Effect of pH on arylesterase activity

3.3.3 Effect of Temperature on arylesterase activity .

The role of enzyme–catalyzed reactions, like most chemical reactions, increases with temperature. The initial reaction rate will rise with temperature until it becomes impossible to. In practice, most enzymes are completely in activated above (80 °C) [25]. In this study used (0,5,10,15,20,25,30,37,40,45) °C, it has been found that as the temperature increased, there was a concave up increase in the enzyme activity until it reached a maximum value at a temperature of (37°C) and the acivity was dropped to less than 37% at 70 °C as shown in figure (5).

Fig.
(5)
Effect of



Temperature on arylesterase activity

3.3.4 Effect of incubation time on partially purified enzyme.

To determine the stability of arylesterase activity under assay conditions, a series of experiments were performed at different time intervals. The result indicated that maximum enzyme activity was obtained after (25min) figure (6).

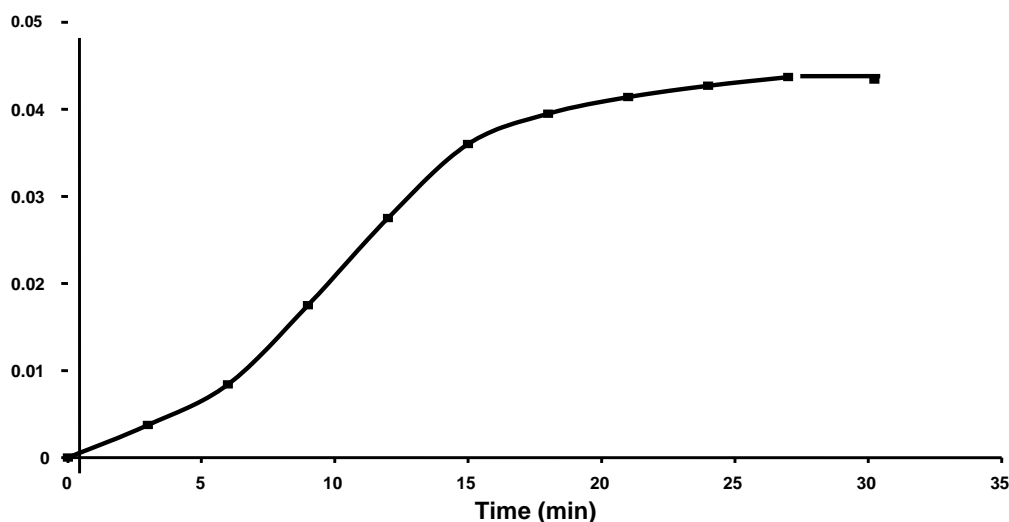


Fig.(6) effect of incubation time on enzyme activity

3.3.5 Effect of substrate concentration on the enzyme activity.

For many enzymes, the rate of catalysis (U) varies with the substrate concentration. The rate of catalysis (U) is defined as the number of moles of product formed per second at a fixed concentration of enzyme; (U) is almost linearly proportional to [S] when [S] is small. At high [S] (U) is nearly independent of [S] [26]

To determine the effect of substrate concentration on the enzyme activity, a series experiments were performed where the concentration of substrate was varied figure(7) Assays were conducted as described previously using phenylestate in concentration (1.5–7) mM. maximum activity was obtained with (2.8mM) of phenylestate further, increase in the concentration of the substrate did not alter the activity of the enzyme

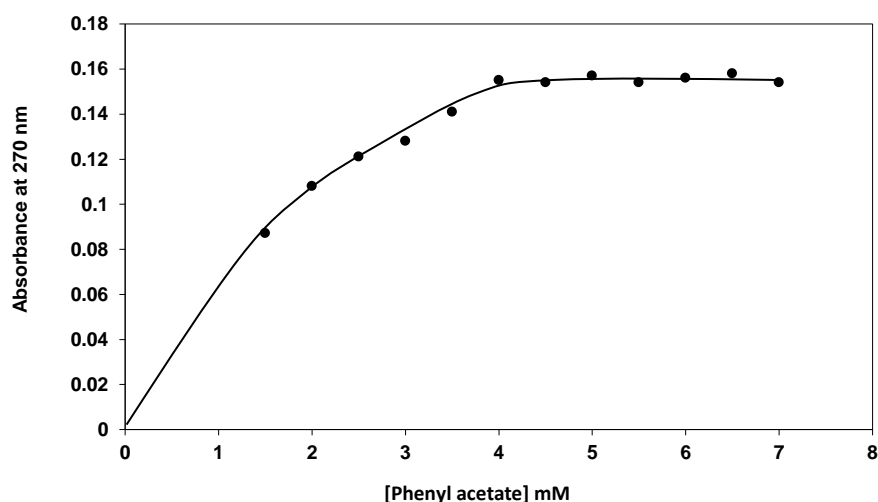


Fig 7. Effect of substrate concentration in arylesterase activity

The michaelis – Menten constant (K_m) of the enzyme was determined from figure (7) and found to be (4) mM as similar result was obtained using a linreciprocal of the substrate concentration. A linear relationship was obtained figure (8) giving a K_m value of (2.8) mM and V_{max} (211)U/ml. the values were similar to K_m values of arylesterase in root of Ginger (Zingiber) , $K_m=3.26\mu\text{mol}$ and $V_{max}=209.3 \text{ U/ml}$ [27].

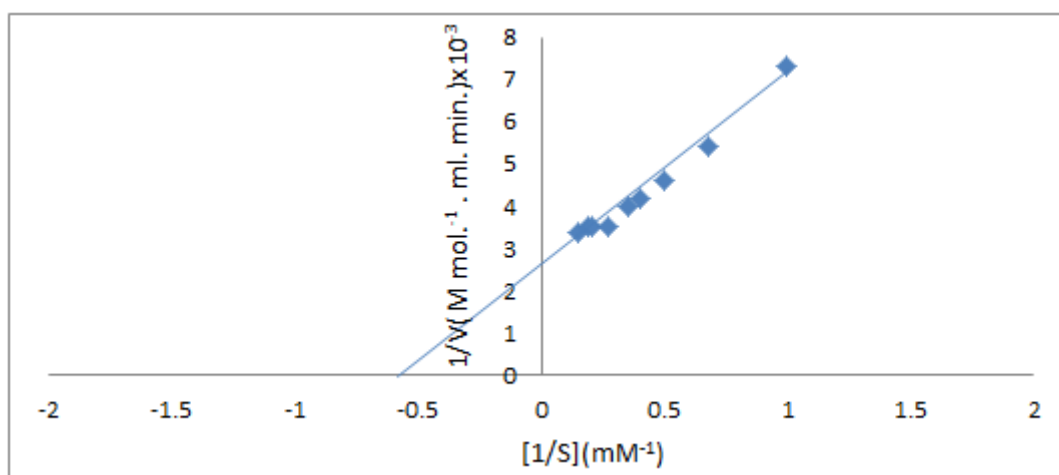


Fig.(6) Linweaver- Burk plot of purified arylesterase

Natural products, especially those derived from plants, have been used to help mankind sustain its health since the dawn of medicine. Over the past century, the phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated



significant scientific interest in the biological activities of these substances [28]. Despite these studies, a restricted range of plant species has experienced detailed scientific inspection, and our knowledge is comparatively insufficient concerning their potential role in nature. Hence, the attainment of a reasonable perception of natural products necessitates comprehensive investigations on the biological activities of these plants and their key phytochemicals [29]. In a pharmaceutical landscape, plants with a long history of use in ethno medicine are a rich source of active phytoconstituents that provide medicinal or health benefits against various ailments and diseases. One such plant with extensive traditional use is *Annona muricata*. In this review, we describe the botany, distribution and ethnomedicinal uses of this plant, and we summarize the phytochemistry, biological activities and possible mechanisms of *A. muricata* bioactivities.

The methanolic extracts of *A. muricata* leaves were investigated to observe the microanatomical changes in the pancreatic islet cells of streptozotocin induced Diabetic Wistar rats. Three groups viz control, untreated diabetic group, and *A. muricata*-treated diabetic group consisted of ten adult rats each [17].

Food derived antioxidants have a strong potential for long term use as chemo-preventive agents in disease states involving oxidative stress such as tea which is a food source rich in phenolic compounds that has many important functions including antioxidant, antimutagenic &





antiinflammatory activities, Paraoxonases (PONs) are a family of three enzymes termed PON1, arylesterase and PON3, the best known among the paraoxonases is PON1 which degrades oxidized phospholipids in low-density lipoproteins (LDL) and HDL and, as such, plays a role in the organism's antioxidant system Alterations in circulating PON1 levels are associated with a variety of diseases involving oxidative stress , The liver plays a key role in the synthesis of PON1 and chronic liver diseases are associated with increased oxidative stress and lipid peroxidation, also a reduction of serum PON1 Coenzyme Q10 (CoQ10) is an essential component of oxidative phosphorylation at mitochondrial level, and also functions to stabilize cell membranes as well as acting as a potent antioxidant antihypertensive, anti-atherogenic, neuro-protective & bioenergetic. Indeed, it affects the function of all cells in the body, making it essential for the health of all tissues and organs. From this point of view, we aimed to evaluate the protective role of green tea against experimental hepatitis with emphasis on CoQ10 and paraoxenase status [9].

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