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Inhibition and Characterization of Polyamine Oxidase (Pao) Which Purified from Atherosclerosis Patients

Al-Lehebe, Nashwan.I; Al-Abbasy, Omar Y; Al-Flayeh. Israa S.

University of Mosul, College of Education for Pure Science, Department of Chemistry

Correspondingauthor
e-mail: nashwan78ibrahem@uomosul.edu.iq
e-mail:chem.omar1978@uomosul.edu.iq
e-mail:israasahil80@uomosul.edu.iq

Abstract: Plants are an important source of drugs, and their use in medicine has a long history. *Terminalia bellirica* Roxb. is a plant that is widely used to treat a variety of ailments, including cardiovascular disease and diabetes. The purpose of this study is to examine the inhibitory effect of petroleum ether and ethyl acetate extracts of *T. bellirica* seeds on purified polyamine oxidase from atherosclerosis patients. Polyamine oxidases play an essential role in maintaining the polyamine balance in vertebrates to ensure the viability of cellular life.Polyamine oxidases oxidize free and acetylated polyamine compounds as substrate. The effectiveness varies according to the tissue present, and the products of the enzymatic reaction depend on the source of the enzyme.
The research included partially purification of Polyamine oxidases from red blood cells hemolysate atherosclerotic patients, using ammonium sulfate sedimentation, dialysis, ion-exchange chromatography, and finally Sodium dodecyl sulfate-electrophoresis migration. Two isoenzymes of polyamine oxidase (I, II) activitieswere obtained with specific activities of (0.148 and 0.163 $x10^{-3}$) Unit\mg protein and molecular weights of 101.9 and 94.2 kilodaltons respectively.Optimum conditions enzyme activity was obtained at 125 microliters of the enzyme, phosphate buffer solution at pH 7.2, temperature 37°C, incubation time 10 min., 125mM of spermine as substrate concentration, and with potassium ion as an activator. A high specificity toward the polyamine, spermine compared to mono and di-amine compounds. The existence of flavine adenine dinucleotide was detected for all isoenzymes. Polyamine oxidase (II) activity was non-competitively inhibited by ethyl acetate and petroleum ether *T. bellerica* extracts. The Michaelis-Menten constantvalue was unchanged at 0.016mM for control and with two extracts. And the maximum velocity values without extracts were 0.024 U/ml, However, ethyl acetate and petroleum ether extracts were 0.014 and 0.011 U/ml respectively.

Keywords*: Oxidative stress, Artery disease, Polyamine. Terminalia bellerica*

INTRODUCTION

Plants play an important role in the development of new therapeutics. *Terminalia bellirica* Roxb corresponds to the Combretaceae family and is found in India's deciduous forests. It is used in prescriptions for diarrhea, cough, bronchitis, and allergic eruptions. In their chemical composition, the seeds of this plant contain a large number of tannins in addition to flavones, coumarin, saponin, alkaloid, terpenoid, and some other antioxidant compounds such as ellagic acids and gallic [1, 2].The discovery and characterization of several enzymes involved in heart disease have resulted in a large number of potential drug targets for metabolic disorders [3]. Atherosclerosis is a known advanced arterial disease that contributes to the emergence of cardiac stroke disease and the loss of the most major causes of death in developed nations. Atherosclerosis tends to result mostly from initial injury caused by mechanical and environmental factors to the artery endothelium, triggering an inflammatory response in the walls of the vessel [4]. It is particularly prevalent in the populations of developed

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countries and is increasingly burdensome in developing countries [5].It is increasingly recognized that in the initiation and development of atherosclerotic plaque, both lipoprotein retention and inflammatory cellular components are intricately related. Furthermore, inflammatory cells such as monocytes and, subsequently, foam cells have also been directly linked to atherosclerotic disease progression [6].

Polyamine oxidase (PAOs) (EC:1.5.3.11) is a monomeric enzyme that belongs to oxidoreductase.PAOs are a flavoprotein enzyme family but FAD is bound non-covalently to the protein. PAO has an important function in bioprocess, it is available in most vertebrates and invertebrates tissues especially in mitochondria [7]. Also, it is found in mammals plants, bacteria, and fungi [8]. This enzyme was studied in different tissues of humans, rats, and rabbits [9]. PAOs induce catalysis the oxidation of free and acetylation terminal amine group in polyamines compounds producing H_2O_2 and NH_4^+[10]. Polyamines (PAs) are small, linear, or occasionally branched polycations derived from amino acids, and found in almost all eukaryotic cells [11]. PAs are essential for cell proliferation, differentiation, and they have functioned as anti-inflammatories, antioxidants,and free radical scavengers.They are involved in several physiological processes such as DNA stabilization and regulation of gene expression [12, 13]. Polyamine compounds can interfere with diseases related to cardiac tissues, heart failure, [14, 15] as well as atherosclerosis [16, 17]**.** Al-fayadh (2020) [18] indicated that there was an insignificant increase in the level of activity of the enzyme polyamine oxidase in the serum of epilepsy patients compared with the level of its concentration in the serum of the control group. After using many biochemical techniques for purification, a single peak of the enzyme was detected in patients with chronic kidney disease [19]. Also, PAO was purified from red blood cells of diabetic females patients, and the enzyme activity was observed elevated markedly in patients than of normal [20].

This project aims to purify PAO from atherosclerosis patients, and then study the inhibitory effect of *Terminalia bellerica extracts* on its activity as a natural inhibitor.

MATERIALS AND METHODS

Collection of blood samples: The study included six volunteers of male atherosclerosis patients from Ibn-Sina hospital, Mosul city, Iraq. Five milliliters were collected in a tube containing ethylenediamine tetraacetate, centrifuged with 15000g for 10 minutes at 4C to obtained plasma and red blood cells (RBC).

Preparation of red blood cells hemolysate (RBCH): RBCs were washed three times with 0.9% NaCl solution and centrifuged with 15000g for 10 minutes at 4℃. the supernatant contained RBCH [21].

Collection and preparation of Plant: *T. bellirica* seeds were obtained from Iraqi medicinal herbal marketplaces. The plant's scientific name has been verified. The seeds were air-dried before being ground into a fine powder. For 24 hours, 200 ml of ethyl acetate and petroleum ether were used to extract the powdered material (20g). The suspensions were filtered and air-dried before being stored at -20°C until needed. Just before the enzyme assay, a solution of 10 mg of each extract in 100mM phosphate buffer was prepared.

PAO assay: PAO activity was monitored spectrophotometrically in an automated Shimadzu UV- 1800 UV Spectrophotometer by a modified method of (Dahel *et al.,* 2001)[22] using spermine as substrate. The decrease in absorbance at 410nm due to the reduction of potassium ferricyanide as electron acceptor was followed. One unit of PAO activity was defined as that amount of enzyme which oxidized 1 micromole of spermine per min.

Protein Determination: Total protein was estimated by the modified Lowry method [23]. The standard curve for protein was prepared by taking different concentrations from bovine serum albumin

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ranging between (0-500) micrograms/ml. One ml of alkaline copper sulfate reagent was added to 1 ml of a test sample. After good mixing, the tubes were left for (10) minutes at room temperature, then (4) ml of phenol reagent was added with quickly good mixing. The tubes were placed in a water bath at (55) °C for 5 minutes, left to cool down and read the absorbance 650 nm.

Purification of PAO:

Step I: Dialysis: Ten milliliters of RBCH was dialyzed for 24 hrs. using phosphate buffer, pH 7. A magnetic stirrer was used to stir the solution at 4 ° C overnight. The buffer was adjusted every 6 hours [24].

Step II: Ion Exchange Chromatography: The dialysate PAO was transferred to a column (2.5x40 cm) contains anion exchanger, DEAE-cellulose, followed by 15 mM phosphate buffer, pH 7.2. Elution was performed at a flow rate of 1.3 ml/min. The fractions were collected and Protein was spectrophotometrically monitored at 280 nm. PAO activity was determined, pooled, and lyophilized.

SDS-PAGE electrophoresis: The molecular mass of PAO isoenzymes produced after ion exchange was determined by using polyacrylamide of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) [25]. Protein bands were visualized by staining with Coomassie brilliant blue R250. Molecular weight markers contained Urase (480 kDa), Bovine Serum Albumin (67 kDa), Peroxidase (40 kDa) Trypsin (23.8 kDa), and Cytochrome Oxidase (13 kDa).

Optimum conditions of PAOisoenzymes activity:
Enzyme volume: To study the optimal concentration of enzymes, PAO isoenzymes were added into the reaction mixture of different volumes (25-150 µl).
Buffers type: The enzymatic reaction mixture was incubated with the following buffers systems at pH 7.2 Sodium acetate, Citric acid, Tris-HCl, Na-Na-phosphate, Na-K- phosphate.

pH range: The effect of pH on the enzyme was determined with Na-Na-phosphate buffer solutions pH 6, 6.4, 6.8, 7.2, 7.6, and 8.

Effect of Temperature onPAO: The temperature effect on PAO activity was determined by incubating PAO and spermine for 10 minutes at different temperatures of 0 to 60°C.

Incubation time: A partially purified isoenzymes were incubated at various periods between (0-16 min).

Metal ions: The purified PAO was pre-incubated with 10 mM of different metal ions Na^{+1}, K^{+},$Mg^{+2,}$ andCa^{2+} for 30 min.

Substrate Specificity: Substrate specificity was studied by using 100 mM spermine, cadaverine, hexylamine, and butylamine.

Kinetic Parameters: The kinetics parameters of PAO were estimated using spermine as substrate at various concentrations (25-150mM). *K*m and *V*max values of enzyme were calculated Lineweaver-Burk plot

Inhibition of PAO (II): The activity of PAO was inhibited by the addition of 0.2 ml of either ethyl acetate or petroleum ether extracts to 0.1ml of enzyme and incubated for 30 min at the optimum temperature (Beffani, 2001). Spermine concentration was prepared between (25-175 mM).

FAD Identification: The coenzyme FAD was detected by 8800 UV/VIS Spectrophotometer

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(Shimadzu), between (200-600) nm.

RESULTS AND DISCUSSION:

PAO Purification

Polyamine oxidase causes an increase in the level of cellular oxidative stress, causing the generation of active aldehyde classes such as acrolein and also generates hydrogen peroxide. Figure (1) explains the elution profile of partially purified PAO by ion-exchange chromatography. We obtained two peaks at elution volumes (136-175) and (247- 286) ml with specific activity (0.148×10^{-3}) and (0.163×10^{-3}) unit/mg protein respectively table 1. The presence of more than one characteristic peak for the enzyme indicates that the enzyme has more than a stereoscopic confirmation, this is what Cervelli et al. (2001) [26] have shown.

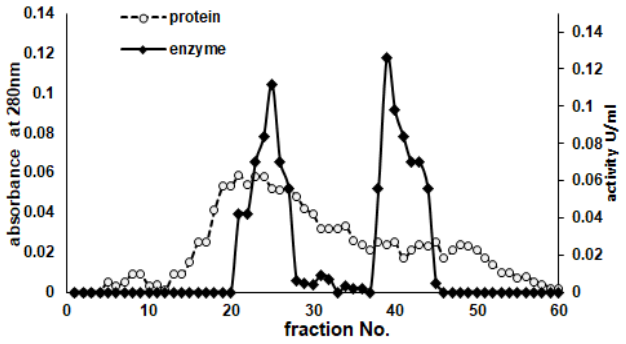

Figure (1) explains the elution profile of partially purified PAO by ion-exchange chromatography.

These results according to the PAO purification from the serum of people withnon-insulin-dependent diabetes, two distinct peaks were obtained [27]. The enzyme was purified from red blood cells of women with type1 diabetes, which was also found in three isoenzymes [20]. While Al-Jubouri (2002) [28] found that there are four distinct peaks of red blood cells for people with schizophrenia using the same purification steps.On the other hand, PAO was isolated by DEAE-cellulose chromatography from the cow brain and only one peak was gained with a purification fold of 17.71 [29].

Determination of Molecular Weights of PAO Isoenzymes

When determining the molecular weights of isoenzymes by electrophoresis using SDS-PAGE, each isoenzyme has shown to have a single protein peak. This indicates that both isoenzymes consist of a single protein unit. After comparing the electrophoretic mobility of isoenzymes for the purified PAO enzyme after the electrophoresis process the molecular weights of (101.9 and 94.2)kDa are found respectively by standard curve (Fig. 2) of the relationship between the molecular weight logarithm of the standard proteins

Table1:PAO purification steps from atherosclerosis patients

Purification steps	Volume (ml)	Total protein (mg)	Activity (U/ml)	Total activity U*	Specific activity $\times10^{-3}$ (U/mg)	Yield %	Purification fold

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Crude		10	572.77	1.807	18.07	0.0315	100	1
Dialysis		9.4	487.27	3.097	29.11	0.059	161.09	1.87
Ion-exchange DEAE-cellulose	Peak I	47	62.832	0.203	9.338	0.148	51.67	4.69
	Peak II	47	46.48	0.162	7.614	0.163	42.13	5.17

and the distance these proteins made towards the positive electrode as shown in(Fig. 3).

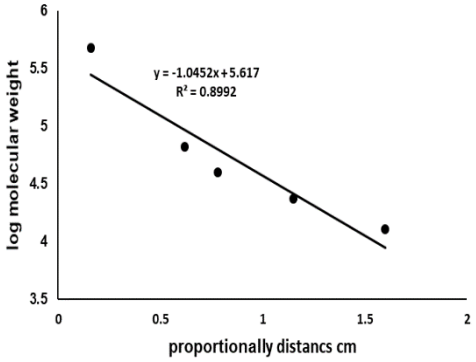
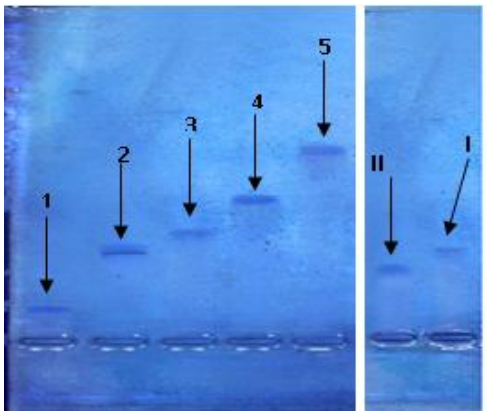


Figure 2. Standard curve to determine PAOMolecular weight isoenzymes by SDS-PAGE



1-urase, 2-BSA, 3-peroxidase, 4-trypsine, 5-cytochrome oxidase I and II SMO isoenzymes which purified from atherosclerosis RBCH

Figure 3. Shows the migrated distance of isoenzymes with standard proteins

The molecular weights of the three isoenzymes of PAO that purified from the healthy women's blood were found to 74.5, 72.5, and 69.3 kDa respectively, which were lower than the molecularweights of the isoenzyme which was purified from type I and II diabetes, higher than 100 kDa. After purifying of PAO from non-treatment diabetic females RBC three peakswere obtainedwith M.wet (106, 104, and 130.7) kDa respectively[30]. A single peak of the enzyme was detected in patients with chronic kidney disease with a molecular weight of56 kDa using SDS-electrophoresis [19].

Kinetic study of PAO enzyme:

The effect of the concentration of purified PAO isoenzymes has been studied by using different volumes of isoenzymes ranging between 25-150 microliters. The results indicate that a volume increase of up to 125 microliters led to an increase in the speed of the enzymatic reaction for the purified isoenzymes Figure (4).

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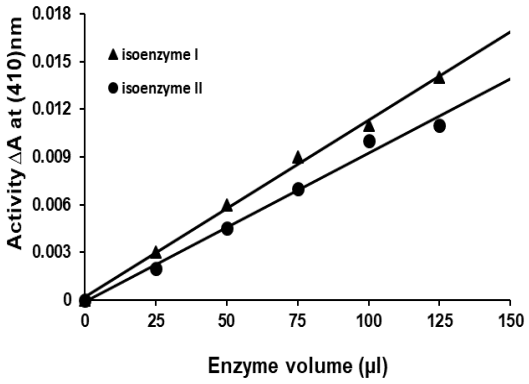

Figure 4. Effect of isoenzyme volume(µl) on activity

The results in Figure (5) showed that the highest activity of enzymatic isoenzymes can be obtained by using the Na-Na-phosphate buffer solution after stabilizing the acidic function at pH 7.2. When usingNa-Na-phosphate buffer between (6-8 pH) the results indicated that the best activity of both isoenzymes was pH=7.2 (Figure 6).

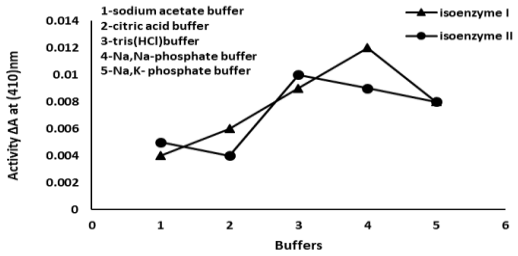

Figure 5. Effect the buffer solutions on enzyme activity

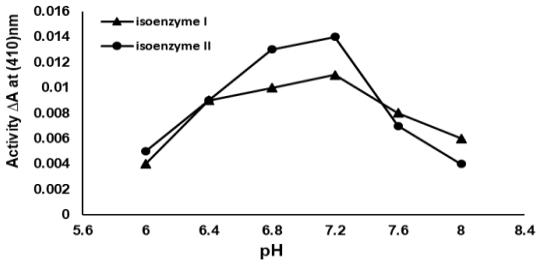

Figure 6. Effect of pH on enzyme activity

Figure 7 shows the temperature effect on activity isoenzymes. As is clear the optimum temperature of the enzyme was 37ºC. After incubation of the enzyme for (10) minutes at different temperatures ranged between (0-60)ºC, the activity was decreased until the enzyme lost its activity at 60°C.

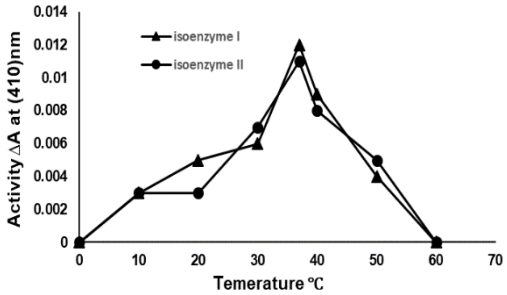

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Figure 7. Effect of temperature on enzyme activity

The results explain of enzymatic activity have also exhibited that the best incubation time has been 10 minutes (Figure 8), which had maximum activity.

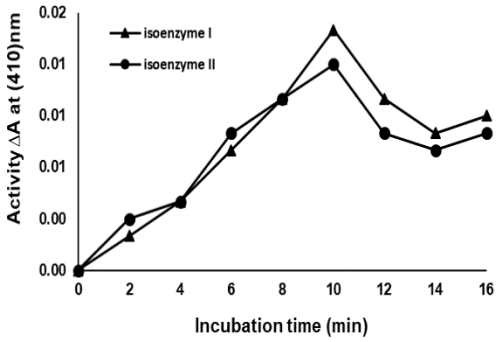

Figure 8. Effect of Incubation time on enzyme activity

The effect of cation on the rate of the enzymatic reaction was indicated that potassium ions had the highest effect of all isomers (Figure 9).

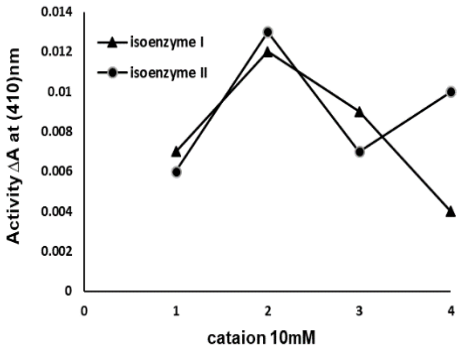

Figure 9. Effect of Positive ion on enzyme activity

Finally, the effect of spermine concentration between (25-150)mM on isoenzymes activity showed that the highest efficacy was achieved by using 125 mM (Figure 10).

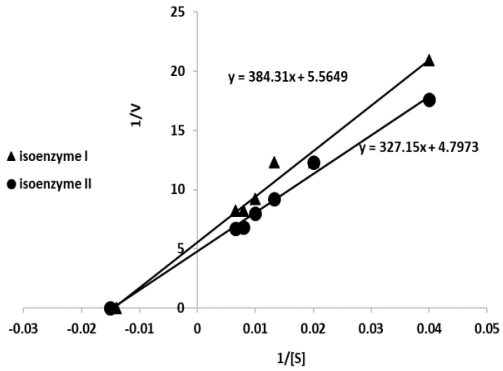

Figure 10. Effect of spermine on enzyme activity

The kinetic variables of the reaction were shown in Table (2).

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Table 2: kinetic parameters of PAO isoenzyme by spermine as substrate

PAO isoenzymes	Km(mM)	Vmax (unit/ml)	Best concentration [S]mM
I	69.06	0.18	125
II	68.2	0.20	125

Specificity of PAO:
Table 3 shows that the spermine substrate has better specificity than cadaverine, hexylamine, and finally butylamine at 100 mM concentration.

Table 3.Isoenzymes specificity towered differentamines

PAO Isoenzyme I			PAO Isoenzyme II	
Amine compounds	Enzyme activity	Relative activity %	Enzyme activity	Relative activity %
Spermine 100Mm	0.121	100	0.135	100
Cadaverine 100Mm	0.081	66.66	0.108	79.98
Hexylamine 100mM	0.062	51.23	0.054	39.88
Butylamine 100mM	0.031	25.61	0.04	29.54

Inhibition of PAO enzyme:
It has been found that both ethyl acetate and petroleum etherof*Terminalia bellirica*extracts have an inhibitory effect on the activity of PAO. The IC50 which were used for investigating the inhibition model for PAOII 0.23 and 0.30 mg/ml respectively.

The plotting of Lineweaver-Burk (Fig. 11 and 12) shows that the inhibition mode was noncompetitive by using both extracts. It has been observed that the value of Km was stable (64.1mM), while the value of Vmax was 0.023 with the absence of extracts but it was 0.014 and 0.011 U/ml with ethylacetate and petroleum ether extracts respectively. This is unlike to (Al-Abbasy, 2010)[27] studywhose found a competitive inhibition of PAO by vitamin E and metformin which partially purified from plasma type II diabetes. Inhibition of the purified PAO enzyme from red blood cells for women with type I diabetes with thiourea compounds prepared from the semicarbazide and thiosemicarbazide compounds has been also competitive [30].

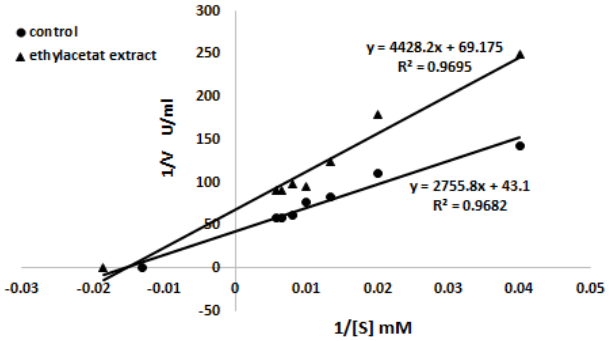

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Figure 11. Lineweaver-Burke plot of PA (II) Inhibition by ethyl acetate*T. bellerica*extract

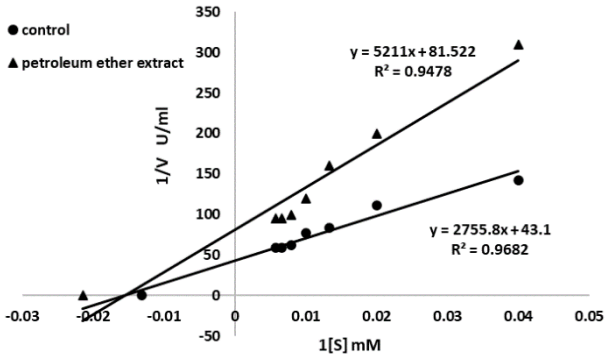

Figure 12: Lineweaver-Burke plot of PAO (II) Inhibition type by petroleum ether *T. bellerica*extract

Angiogenic activity of *T. bellerica* leaf ethanolic extract was demonstrated in mice using an in vivo sponge implantation assay. The extract also resulted in a significant increase in hemoglobin content. These findings revealed that *T. bellerica* ethanolic leaf extract has a high angiogenic potential [31].In all antioxidant assays, the ethyl acetate fraction outperformed the crude acetone extract. The antioxidant potential was compared to that of the well-known antioxidant butylated hydroxyl toluene, and the total phenolic and flavonoid content in crude extract and fractions was correlated [32].*T. bellerica* has been informed to own effective antioxidant action. It contains a lot of tannins, which are a type of antioxidant. Tannins are polyphenolic compounds that are found in plants. Plant polyphenols bind to proteins in a variety of ways, including hydrophobic and hydrogen bonding as a result of the aggregation of enzyme proteins, they have inhibitory activity for enzymes [33].

Coenzyme :

The results of the UV-Visible spectrum showed that there have been two distinct peaks λmax between 360-415 due to FAD absorption. Previous studies have indicated that PAO is accompanied by FAD and is called Flavo-enzyme. There are distinct absorption peaks at the approximate wavelength of 360-450 indicating the presence of FAD [34].The presence of Flavin adenine dinucleotide (FAD) as a cofactor has been detected using UV. Technology and showed two bands at wavelengths of (325-430)nm [35].

CONCLUSION:

In this studyPAO which present in atherosclerosis patients was purified and characterized. The inhibition of purified PAO activity byethyl acetate and petroleum ether *T. bellerica* extracts was observed non-competitively. FAD existing with both PAO peaks as a coenzyme.

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