Inhibition of Partially Purified Xanthine Oxidase Activity from renal failure patients by Theophylline and Metronidazole

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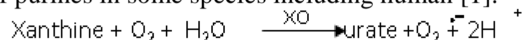
Abstract
Xanthine oxidase (XO) was purified from sera of patients with renal failure by using ammonium sulfate and dialysis with a specific activities 2.1×10^{-3} and 5.6×10^{-3} unit/mg protein respectively. By using anion exchange chromatography techniques, One protein peak of XO activity was obtained with specific activity 78×10^{-3}unit/mg protein and with purification fold of 134.02 compared to crude enzyme. One isoenzyme was obtained having a molecular weight of 255344 Dalton approximately.
Inhibition of purified XO was studied by deferent concentrations of Theophylline and Metronidazole drugs. The maximal inhibitory effect was exhibited with 70 and 80µM Theophylline and 16 and 18 mM metronidazole. The inhibition type of XO by Theophylline was competitive. Vmax value was 0.0416 unit/ml, and Km value without inhibitor was 0.833 mM, however K'_m values were 2 and 2.85 mM, and inhibition constant K_i values were 29.57 and 23.67 mM respectively with 70 and 80µM of Theophylline.
The inhibition type of XO by Metronidazole was non competitive. Km value 0.833 mM and Vmax value without inhibitor was 0.0416 unit/ml. However, Vmax values were 0.027 and 0.023 unit/ml and inhibition constant Ki values were 17 and 19mM by using 16 and 18 mM of Metronidazole respectively.

Key words: Xanthine Oxidase, Theophylline, Free radicals, Imidazoline.

Introduction
Xanthine oxidase (XO)(E.C.1.1.3.22) catalyzes hypoxanthine to xanthine, and can further xanthine to uric acid, and play an important role in the catabolism of purines in some species including human [1].



The enzyme is highly versatile flavoprotein enzyme ubiquitous among species from bacteria to human and with in the various tissues of mammals [2]. It is a key in degredation of DNA and RNA [3] and it is believed to be one source of reactive oxygen species in the failing heart [4].
This enzyme is involved in free radicals production associated with exercise in patients with chronic obstructive pulmonary diseases (COPD) [5]. XO an enzyme responsible for superoxide production,was change in nitrotyrosine formation in COPD subjects and significantly elevated compared with healthy subjects [6]. XO also play an important role in cellular oxidative status, detoxification of aldehydes, various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases and chronic heart failure[7,8].
[9] showed that XO is increased in plasma and liver of diabetic rats and the inhibition of XO should prevent oxidative stress in diabetes. Allopurinol administration of COPD subjects significantly decrease XO activity [6]. Treatment with allopurinol decreases oxidative stress in type 1 diabetes patients, glutathion oxidation and the increase in lipoperoxidation are prevented [9].
XO is partially purified from sera of patients with gout and inhibited by allopurinol and caffeine as a prodrug [10]. XO activity is elevated in early asymptomatic stages of cardiac dysfunction and the chronic inhibition of XO by allopurinol improved contractile function [4]. Recent data have suggested that chronic inhibition of XO improves survival and cardiac function in postischemic cardiomyopathy [11,12].
The aim of research is inhibition of XO activity by a one of drug which naturally existing compounds in many herbs and plants for using to treat or prevent many diseases.

Materials and Methods
Collection of blood samples: Human blood was collected from males patients with renal failure. Venous blood samples were drawn by a range 5-10 milliliter for each sample.
Blood serum isolation: The serum is isolated by putting tubes in a water bath at 37oC for 10 min, and centrifuged at 13000xg by refrigerated centrifuge for 10 min. The supernatant was taken to conserve in freezing [13].
Determination of protein: Buiret method was used to determine total protein concentration [14].
XO assay: Xanthine oxidase activity was determined by using the method of [15]. This method involved enzymatic oxidation of xanthine which is followed spectrophotometrically by measuring uric acid formation at 293 nm. A unit is defined forming one micromole of uric per minute at 25 oC. the molar absorbarcy of uric acid is 1.22x 104 cm-1.
Purification of XO:
Precipitation of enzyme: Enzyme was precipitated by addition 0- 45% gradually from ammonium sulfate in a cold bath and left over night at 4 oC, then centrifuged at 13000xg.
Dialysis: Serum was dialyzed for about 15hrs. against 50 mM of phosphate buffer solution of pH=7.5 at 4 oC with 5 changes.
Fractionation on DEAE-Cellulose column: The dialyzed enzyme solution was applied on DEAE-Cellulose anion exchanger column 2.5×40cm, which has been equilibrated with gradient phosphate buffer

50-250 mM of pH=7.5. Fractions of 5 ml volume were collected. Flow rate was 60 ml/hr.

Determination of molecular weight

The fractions 75-160 showing XO activity were pooled together and lyopholized and subjected to the Sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE, was carried out using the method of Laemmli [16] on a Tris-HCl buffer 1M, pH 8.9, 30% polyacrylamide containing 10% SDS. For electrophoresis in the presence of SDS, samples and control were boiled for 3 min in the sample buffer, which contained 5% 2-mercapto ethanol and 1% bromophenol lue. Protein bands were visualized by staining with Coomassie brilliant blue R250. Electrophoresis was performed horizontal slab mini-gel apparatus [Labnet, USA] at 1000 V for 5-6 h.

A reference proteins were insulin, pepsin, Trypsin, Egg albumin , BSA and Urase (Fig.1).

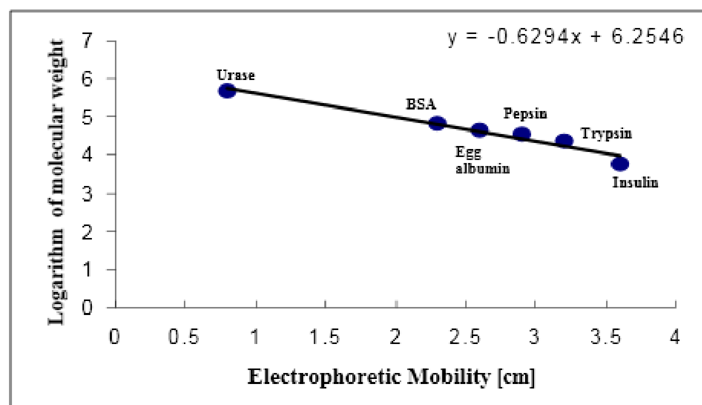


Fig. 1: Standard curve for SDS-PAGE determination of protein molecular weight

Inhibition of XO: Xanthine oxidase activity was inhibited by addition 0.1 ml of inhibitor to 0.2 ml of enzyme and incubated for 10 min at 25 °C [17]. The concentrations of substrate, xanthine were 0.2-1.5 mM.

Results and Discussion

Table [1] shows purification steps of XO. The specific activity of crud enzyme was 0.582×10^{-3} unit/mg protein, after ammonium sulphate and dialysis became 2.1×10^{-3} and 5.6×10^{-3} unit/mg protein respectively. Figure 2 explains the elution profile of purified XO by anion exchange chromatography. We obtained a one peak at elution volume 75-160 ml with specific activity 78×10^{-3} unit / mg protein and with purification fold of 134.02 compared to crude enzyme .

Table 1: XO purification steps from patients with renal failure

Purification steps	Volume [ml]	Protein concentration [mg/ml]	Total protein [mg]	Activity [U/ml]	Total activity U*	Specific activity $\times10^{-3}$[U/mg]	Yield %	Purification fold
Crude	**13**	**51.5**	**669.5**	**0.03**	**0.39**	**0.582**	**100**	**-**
Ammonium sulphate	**14**	**33.2**	**464.8**	**0.07**	**0.98**	**2.1**	**251.28**	**3.6**
Dialysis	**13**	**21.4**	**278.2**	**0.120**	**1.56**	**5.6**	**400**	**9.62**
Ion exchange	**91**	**0.41**	**37.31**	**0.032**	**2.912**	**78**	**746.66**	**134.02**

***U: A unit is defined forming one micromole of uric per minute at [25] °C**

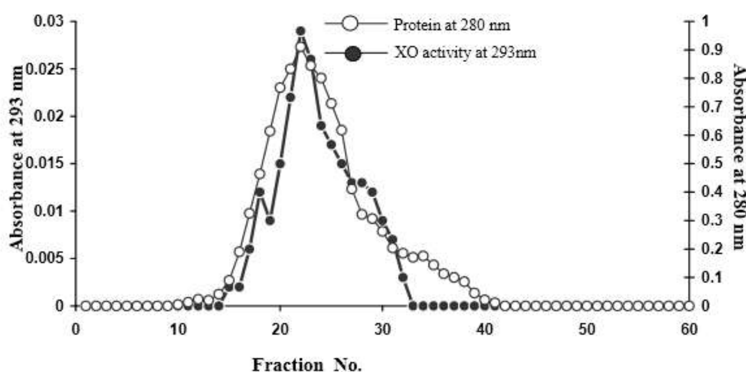


Figure 2: Purification of XO by DEAE-cellulose chromatography

Xanthine oxidase was partially purified from sera of patients with gout, one protein peak of XO activity was obtained with specific activity 0.149 unit/mg protein and with purification fold of 496.66 compared to crude enzyme [10]. XO is isolated and partially purified by gel filteration chromatography from benign and malignant colon tumor tissue and the number of purirification fold was 12 and 17 fold respectively [18].

Ammonium sulfate fractionation and ion exchange chromatography on DEAE- cellulose column resulted in 10 fold increase in enzyme activity from normal and malarial infected mice [19]. From rat liver, XO was purified by anion exchange chromatography on a Q-Sepharose column resulted in a highly purified 1200-fold , with a specific activity of 3.64 U/mg and with a 20% yield [20].

Sodium dodecyl sulfate electrophoresis: On sodium dodecyl sulfate electrophoresis, The purified enzyme showed a single band on SDS-PAGE (Fig. 3 , lane 6). The molecular weight of the enzyme was estimated to be 255344 Da. The enzyme is large, having molecular weight of 270KD and has 2 flavin molecules (bound as FAD) and 2 molybdenum atoms as cofactors and are the active sites of the enzyme [21].

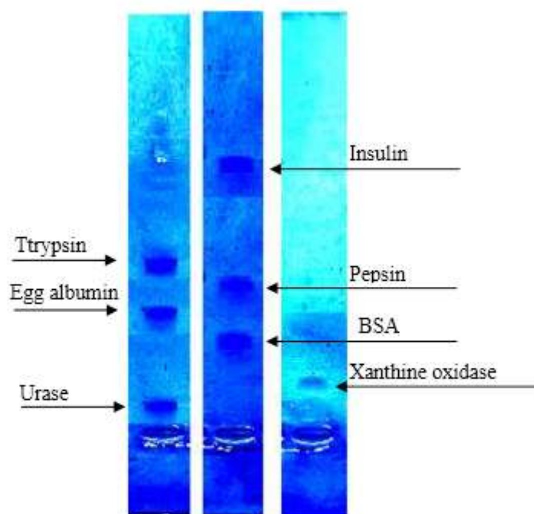


Figure 3 : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of Xanthine oxidase. Mol. wt. markers:1. Urase 480 KD , 2. BSA 67 KD , 3. Egg albumin 45 KD, 4. Pepsin 36 KD, 5. Ttrypsin 23 KD and 6. Insulin 5.734 KD , Lane 7 partially purified Xanthine oxidase approximately 258 KD.

The purified enzyme from human cytosol was shown as a single band of 300 KD on polyacrylamide gel electrophoresis[22]. Purified xanthine oxidase from mouse liver after induction with bacterial lipopolysaccharide has an apparent molecular weight of 300,000 in its native state and it is suggested to be constituted of two identical subunits of 150,000 each [23]. The purified enzyme from rat liver gave single bands of approximately 300 kDa on a polyacrylamide gel electrophoresis [20]. The native XO enzyme in *Arthrobacter sp*. should be a dimer with 280 kDa approximately of a protein composed with two different peptides. The optimal pH and temperature of this enzyme were determined at about pH 7 and 50 ◦C [24].

Inhibition of XO activity :

XO play an important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases and chronic heart failure[7].The inhibition of XO is very important because the oxidation of xanthine to uric acid which plays a crucial role in gout [25].

Table 2 shows the percentage inhibition of XO activity by Theophylline and metronidazole with an explained concentrations:

Table 2:Inhibition of XO activity by Theophylline and Metronidazole

Theophylline [µM]	% inhibition	**Metronidazole** [mM]	% inhibition
0	0	**0**	0
10	7.14	2	14.28
20	14.28	4	18.57
30	24.28	6	24.28
40	31.42	8	30
50	40	10	35.71
60	44.28	12	42.85
70	48.57	14	44.28
80	52.85	16	47.14
90	62.85	18	51.42
100	44.28	20	42.85

The best inhibition of XO activity was 48.57% and 52.85% with concentration 70 and 80 µM by Theophylline respectively. However 47.14% and 51.42% with concentration 16 and 18 mM by Metronidazole respectively.

Theophylline (1,3-dimetylxanthine) is a drug uses for bronchodilator as well as occurs as a natural product in the tea [27], besides polyphenolic compounds and catechin. Metronidazole, a drug containing imidazoline moieties. It using for anti-inflammation which may be happened via free

radicals production. The majority of XO inhibitors are heterocyclic aromatic compounds and aldehydes or alcohols that mimic purine and aldehyde substrates of XO [21].
Quercetin - oxathiolanone as well as quercetin inhibited xanthine oxidase. Approximately 0.05 µM quercetin - oxathiolanone inhibited the activity by 50%, whereas 50% inhibition by quercetin was observed at approximately 0.4 µM [28].
The inhibition type of XO by Theophylline appeared to be competitive as indicated by Lineweaver-burk plot Figure 4. The results show that Km value without inhibitors was 0.833 mM. However $K'm$ value were 2 and 2.85 mM with 70 and 80µM of Theophylline respectively. Vmax value remained stable 0.0416 unit/ml. This inhibition of XO by Theophylline is due to its structure analogue to that of substrate, xanthine.
In other hand, the inhibition type of XO by using allopurinol and caffeine was competitive with a Km value 40 mM and Vmax value 0.135 unit/ml [10]. The inhibition of XO by 2,4-dihydroxybenzophenon and 2,3,4-trihydroxybenzophenon is produced a competitive reversible type [29]. Lin[30] reported the inhibition of xanthine oxidase by apigenin and quercetin was competitive.

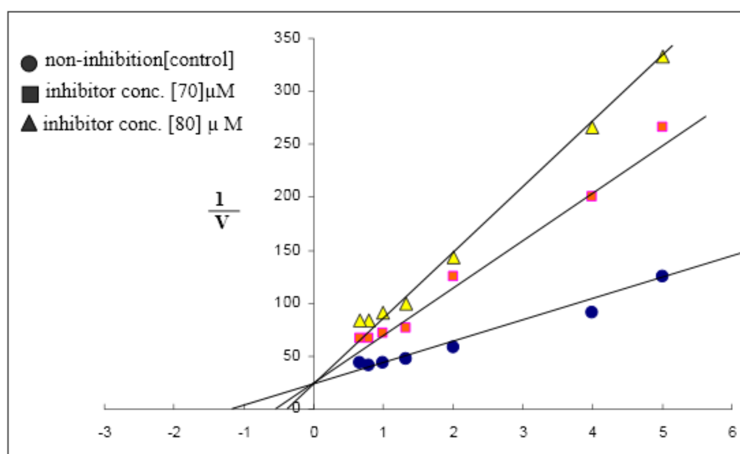


Fig. 4: Lineweaver-Burk plot shows inhibition type on XO activity by Theophylline

The inhibition type of XO by Metronidazole appeared to be non competitive as indicated by Lineweaver-burk plot Figure 5. There is no change in Km value 0.833 mM. Vmax value without inhibitor was 0.0416 unit/ml. However, Vmax values were 0.027 and 0.023 unit/ml by using 16 and 18 mM respectively.
Therefore, the inhibition of XO occurs either with interaction of purine binding sites, like allopurinol [31], or interaction of FAD cofactor binding sites, like benzimidazole [32]. The results of Lineweaver-Burk plot can be interpreted as Caulerpenyne inhibits xanthine oxidase, and was observed non-competitive inhibition [33]. Oxypurinol (1,2- Dihydropyrazolo (4,3-e) pyrimidine - 4,6-dione) is an efficient tight binding inhibitor of XO and exerts non-competitive type inhibition[1]. The root extracts and phytochemicals of *Tephrosia purpurea* which contain of significant amount of polyphenols and flavonoids inhibited bovine milk XO and revealed noncompetitive mode of inhibition [34].

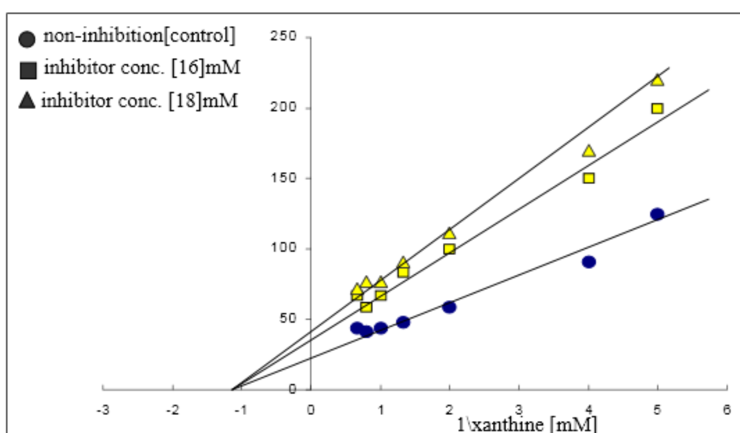


Fig. 5: Lineweaver-Burk plot shows inhibition type on XO activity by Metronidazole

Accordingly, the inhibition constant Ki values calculated and appeared to be 29.57 and 23.67 mM with Theophylline and 17 and 19mM with metronidazole respectively. From these results, metronidazole has an inhibitory effect best than Theophylline on XO activity

Reactive nitrogen species (RNS) cause airway inflammation via oxidative stress and lipid peroxidation[35]. Cell damage caused by free radicals appears to be a major contribute to aging and to generative diseases of aging such as cancer, cardiovascular diseases, cataracts, immune system decline and brain dysfunction [36].

It was hypothesised that inhibition of XO might decrease RNS production in COPD airways through the suppression of superoxide anion production [6].

These results suggest the continuous intake of herbs and plants containing theophyille such as green and black tea or some containing imidazoline ring may be inhibited XO activity which plays an important role in many diseases and suppress free radicals production such as superoxide anion, then prevent or treat many diseases.

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تثبيط فعالية أنزيم الزانثين اوكسيديز المنقى جزئيا من مرضى الفشل الكلوي بواسطة عقاري الثايوفلين والميترونيدازول

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الملخص

نقي أنزيم زانثين اوكسيديز (XO) من مصل المرضى المصابين بالفشل الكلوي باستخدام الترسيب بكبريتات الامونيوم والفرز الغشائي وكانت الفعالية النوعية للانزيم 2.1×10^{-3} و5.6×10^{-3} وحدة أنزيمية/ملغم بروتين على التوالي. بأستخدام تقنية كروماتوغرافيا التبادل الايوني السالب، وتم الحصول على قمة بروتينية واحدة تحوي فعالية نوعية لانزيم XO مقدارها 78×10^{-3} وحدة أنزيمية/ملغم بروتين، وبنقاوة بلغت 134.02 مرة مقارنة بالأنزيم الخام. تم التوصل إلى أن للإنزيم متماثلا واحدا بوزن جزيئي 255344 دالتون تقريبا.

درس تثبيط فعالية أنزيم XO باستخدام تراكيز مختلفة لعقاري الثايوفلين والميترونيدازول، وظهر اعلى تأثير تثبيطي باستخدام 70 و80 مايكرومولار من الثايوفلين و16 و18 ملي مولار من الميترونيدازول.

تبين أن نوع تثبيط فعالية انزيم XO مع الثايوفلين كانت من النوع التنافسي. وجد ان قيمة Vmax ثابتة وهي 0.0416 وحدة أنزيمية/مللتر، وان قيمة K_m بدون مثبط 0.833 ملي مولار، وكانت قيمتا $K'm$ 2 و2.85 ملي مولار وقيمتا ثابت التثبيط Ki 29.57 و23.67 ملي مولار باستخدام 70 و 80 مايكرومولار من الثايوفلين على التوالي.

كما تبين أن نوع تثبيط فعالية انزيم XO مع الميترونيدازول كانت من النوع غير التنافسي. حيث وجد ان قيمة Km ثابتة وهي 0.833 ملي مولار، وان قيمة Vmax بدون المثبط 0.0416 وحدة أنزيمية/مللتر، في حين اصبحت 0.027 و0.023 وحدة أنزيمية/مللتر وقيمتا ثابت التثبيط Ki 17 و 19 ملي مولار باستخدام 16 و 18 ملي مولار من الميترونيدازول على التوالي.