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Isolation and Characterization of Xanthine Oxidase from Tissues of Benign and Malignant Colon Tumors Patients

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ABSTRACT

The present research included the determination of xanthine oxidase XO in tissues of benign and malignant colon tumor patients.

Six samples were collected from patients in surgery unit in Al-Zahrawi hospital in Nineveh Governorate, three of them were benign and the others were malignant colon tumors.

The results showed that there are (62%) increase in the specific activity of the enzyme in malignant than benign.

The research also included an isolation and partial purification of XO using gel filtration chromatography from benign and malignant colon tumor tissues. The number of purification folds was (12) fold from benign and (17) folds from malignant.

The molecular weight of XO was determined using gel filtration on sephadex G-100 and it was found to be (199000 ± 2000 , 191000 ± 2000) dalton from benign and malignant colon tumor respectively.

The results also showed that the enzyme gave a maximum activity at (1.5×10^{-4} M) of xanthine as a substrate and phosphate buffer at pH (8.5), temperature of (40/°C) and incubation time (6 minutes). Using linweaver-Burk plot, the K_m was (2.5×10^{-4}M) and V_{max} was (7.1×10^{-5}) U/ml.

The research also shows an inhibition effect of some metal ions like Cu^{+2}, Hg^{+2}, Ag^{+2} (but not by Co^{+2}) and some chemical compounds like N-acetyl cysteine, 1-phenl-3-pyrazolidinone on XO activity.

Keywords: Colon cancer, Xanthine oxidase.

عزل ودراسة خواص أنزيم الزانثين اوكسديز من أنسجة مرضى أورام القولون الحميدة والخبيثة

الملخص

تضمن البحث تقدير فعالية إنزيم الزانثين اوكسديز في أنسجة مرضى أورام القولون الحميدة والخبيثة إذ جمعت العينات من وحدة الجراحة في مستشفى الزهراوي في محافظة نينوى وكان عددها ست عينات ثلاثة منها لمرضى ذوات ورم خبيث والباقية لمرضى ذوات ورم حميد.

أشارت النتائج إلى حدوث ارتفاع معنوي بنسبة (62 %) في الفعالية النوعية للإنزيم في الورم الخبيث مقارنة مع الحميد كما تضمن البحث عزل وتنقية الإنزيم باستخدام تقنية الترشيح الهلامي وكان عدد مرات التنقية (12) مرة للحميد و(17) مرة للخبيث كما تم تقدير الوزن الجزيئي للإنزيم وكان بحدود (199000 ± 2000 ، 191000 ± 2000) دالتون للإنزيم من الورم الحميد والخبيث على التوالي.

كما تضمن البحث تحديد الظروف المثلى لعمل الإنزيم من الورم الخبيث وأظهرت النتائج أن أعلى فعالية للإنزيم كان عند تركيز (1.5×10^{-4}) مولاري باستخدام الزانثين كمادة أساس وعند استخدام محلول الفوسفيت المنظم عند أس هيدروجيني (8.5) عند درجة حرارة (40/°م) وزمن تحضين (6) دقائق. وباستخدام رسم لينويفر-بروك كانت قيمة k_m (2.5×10^{-4}) مولاري. والسرعة القصوى كانت (7.1×10^{-5}) وحدة/ ملي لتر.

كما تم دراسة التأثير التثبيطي لبعض المركبات والمعادن على فعالية الإنزيم وكانت Cu^{+2}، Hg^{+2}، Ag^{+2}، N-acetylcystine ، 1-phenyl 3-pyrazolidinone، أما Co^{+2} أظهر تأثيراً تنشيطياً.

الكلمات الدالة: سرطان القولون، زانثين اوكسيديز.

INTRODUCTION

Colon cancer of colorectal carcinoma (CRC) is one of the most prevalent malignancies of the gastrointestinal tract in developed countries. Each year ≈ 1 million new cases of CRC are diagnosed world wide, where half a million patients die from this disease every year (Weitz *et al.*, 2005); (Birrl *et al.*, 2006); (Jemal *et al.*, 2009).

Xanthine oxidase (XO EC 1.1.3.32) is a form of the house keeping enzyme xanthine oxido-reductase (XOR) which is a component of the innate immune system. The enzyme involved in multiple features of innate immunity and it is central to the evolution and function of this defense system (Vorbach *et al.*, 2003).

The enzyme is a highly versatile flavoprotien enzyme ubiquitous among species (from bacteria to human and with in the various tissues of mammals (Broges *et al.*, 2002).

It plays an important role in the development of colon cancer (Hashimoto *et al.*, 2005) and in generation of free radical in diabetes (Desco *et al.*, 2002). It is a key enzyme in

degradation of DNA, RNA (Linder *et al.,* 2009) and it is believed to be source of reactive oxygen species in the failing heart (Jennifer *et al.,* 2005).

In the last decade much interest has been focused on the possible role of xanthine oxidase in initiating and modulating the immune and metabolic response to infection (Harrison, 2002).

There is overwhelming acceptance that xanthine oxidase serum levels are significantly increase in various pathological states like hepatitis, inflammation, ischemia, reperfusion carcinogenesis and aging. The ROS generated in the enzymatic process are involved in oxidative damage (Broges *et al.,* 2002) also it was found that xanthine oxidase activity was elevated in chronic obstructive pulmonary diseases (COPD) compared with healthy subjects (Ichinose *et al.,* 2003).

The aim of this research is to determine the XO activity which would be a marker for colon cancer infection and finding an inhibitor to this enzyme which might be useful in drug industry for colon cancer.

MATERIALS AND METHODS

Sample Collection:

Tissue samples (6) were collected immediately after operation from patients in surgery unit in Al-Zahrawi hospital in Nineveh Governorate, (3) patients are benign and the others are malignant.

Tissue Homogenate Preparation:

The samples were cut off, rinsed with normal saline from each tissue. A weight of (1.0) gm was taken and cut into small pieces and homogenized by hand using phosphate buffer (0.1M, pH 8.2). The ratio of (1 : 10 w/v) in an ice bath then the homogenate was sonicated for four cycles of (15) seconds with (15) seconds interval in between. Then homogenate was filtered through double layer of sterile gauze and centrifuged at (1000 xg) for (30) minutes in a cooling centrifuge. The supernatant was separated and divided in a liquid and kept frozen at (-20) for protein and enzyme activity measurement (Tietz, 1999).

Determination of Enzyme Activity:

Xantine oxidase activity was determined using the method of (Ackermann and Brill, 1974).

This method involved enzymatic oxidation of xanthine which is followed spectrophotometerically by measuring uric acid formation at (293 nm):

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{XO} \text{Urate} + H_2O$$

Protein Estimation:

The method used for protein determination in tissues homogenates was the modified lowery (Schacterle and Pollack, 1973) where bovine serum albumin was used as a standard protein.

Isolation and Purification of the Enzyme from Colon Tumors:

The enzyme was purified from the benign and malignant colon tumors patients by:

1- Protein Precipitation by Ammonium Sulphate:

Total protein from tissues extract was isolated by precipitation using ammonium sulfate with (75%) saturation (Robyt and White, 1987).

The supernet was separated by cooling centrifugation at (10000 xg) for 30 minutes, protein concentration was estimated and XO activity was measured.

2- Dialysis:

The supernet (6 ml)and (10 ml) from malgnanit and benign colon tumer of supernet respectively were dialyzed overnight at (4ºC) using sodium bicarbonate buffer. The final volume was measured and the protein concentration and xanthine oxidase activity were determined.

3- Gel Filtration Chromatography:

Gel filtration was used to purify the enzyme and for molecular weight determination. (Robyte and White, 1987).

A- The column used in this technique has a dimension of (2 × 87) cm containing Sephadex G-100 to (85 cm) height. The proteinous sample (2.0) ml which was prepared in section (2) after dialysis was applied to column, the fractions were collected at flow rate of 70 ml/h. using a phosphate buffer as eluent.

The proteins compound in each fraction collected were detected by following the absorbance at 280 nm using UV/visible spectrophotometer then the fractions were collected and assayed for XO activity.

B- Determination of Aproximate Molecular Weight by Gel Filtration:

The aproximate molecular weight of xanthine oxidase from malignant and benign colon tumor was estimated using sephadex (G-100) and calibrated with known molecular weight proteins.

The optimum condition temperature, time of incubation, pH, concentration of enzyme, and substrate were determined and the effect of some metal ions and chemical compounds as inhibitors were performed.

RESULTS AND DISCUSSION

The result showed that the specific activity of XO in malignant colon tumor is higher than benign and the increment was about (62%). This result was in agreement with previous studies which showed a significantly higher XO levels in human brain tumors (Alabachi, 2006). Also the same result was found in patients with glioma cells (Griquer *et al.*, 2006) and other disease like (COPD) (Ichinose *et al.*, 2003).

The higher activity of XO is due to the function of the enzyme which catalyzes the breakdown of nucleotides to form uric acid, which contributes to the antioxidant capacity of the blood (Griquer *et al.*, 2006).

The purification steps of XO enzyme from benign and malignant colon tumor are showed in Tables (1 and 2) which indicate that the purification folds of enzyme were (12) and (17) from benign and malignant respectively.

Table 1: Partial purification of XO from benign colon tumor tissues.

Steps	Volume (ml)	Total protein (mg)	Total activity (U/L)*	Specific activity (U/mg)**	Purification folds	Yield %
Crude homogenate	30	45	72.11	1.6	1	100
High speed supernatant	28	31.36	66.16	2.11	1	92
After precipitation	10	8.1	54.59	6.74	4	76
Dialysis	8	4.51	41.61	9.22	6	58
Gel filtration sephadex (G-100)	24.5	1.35	24.84	18.4	12	34

* Enzyme unit: the amount of enzyme to consume one μ mole of substrate per minute.
** specific activity of enzyme: number of enzyme units per milligram of protein.

Table 2: Partial purification of XO from malignant colon tumor tissues.

Steps	Volume (ml)	Total protein (mg)	Total activity (U/L)*	Specific activity (U/mg) **	Purification folds	Yields %
Crude homogenate	30	69.3	124.74	1.8	1	100
High speed supernatant	27	49.41	111.47	2.3	1	89
After precipitation	6	9.36	89.21	9.53	5	72
Dialysis	5	6.6	78.21	11.84	7	63
Gel filtration sephadex (G-100)	21	1.8	53.46	29.7	17	43

***** Enzyme unit: the amount of enzyme to consume one μ mole of substrate per minute.
** specific activity of enzyme: number of enzyme units per milligram of protein.

Gel filtration chromatography

This technique was used to isolate and purify XO from benign and malignant colon tumor . The results showed that there are three proteinous peaks detected at 280 nm. The XO activity was found in the first peak at elution volume of (90.6) ml and (95.3) ml from benign and malignant colon tumor as shown in Fig. (1-2) respectively.

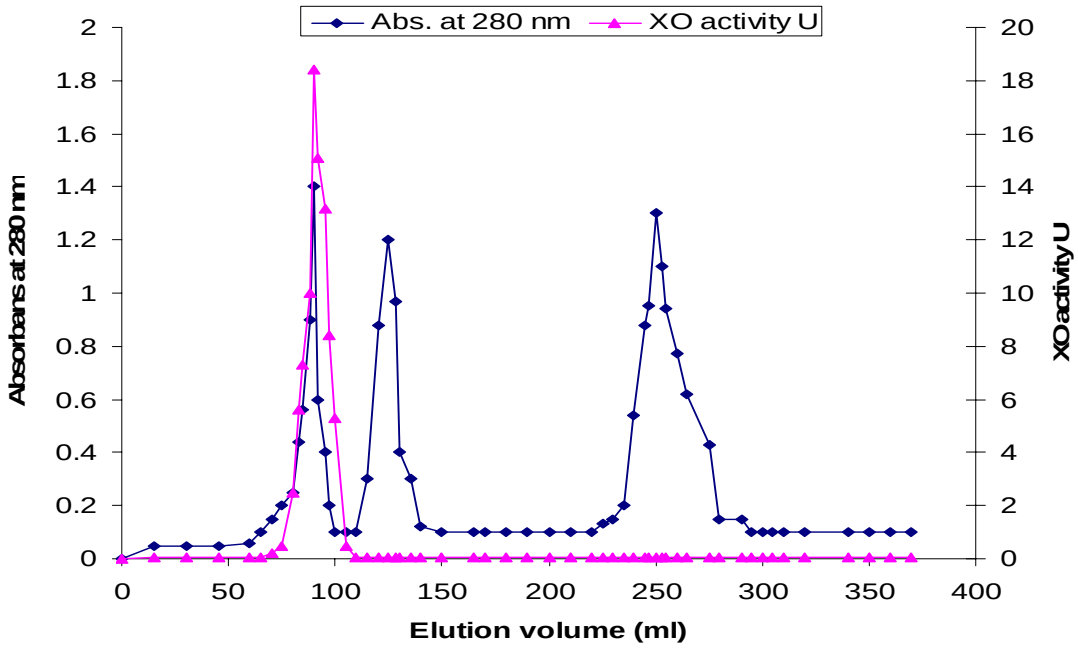

Fig. 1: Partial purification of XO from benign colon tumor tissues using sephadex G-100

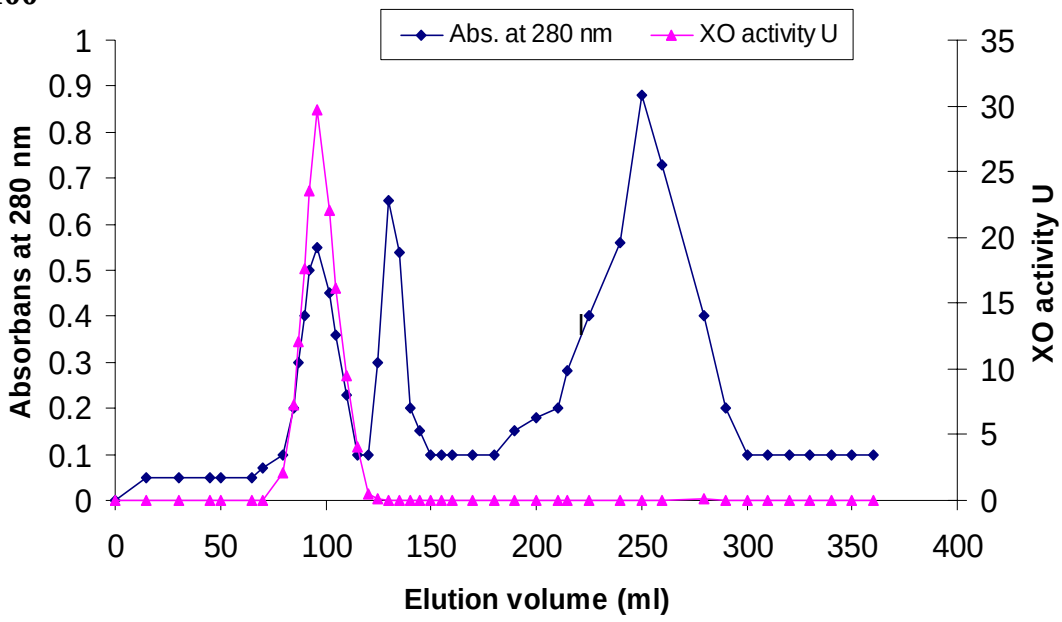

Fig. 2: Partial purification of XO from malignant colon tumor tissues using sephadex G-100

Also the molecular weight was estimated for XO from benign and malignant colon tumor.

Using sephadex G-100 column (2×87) cm calibrated with known molecular weight proteins which were listed in Table (3) then from the plot of logarithmic molecular weight of each material indicated in the table versus their elution volumes gave a straight line as illustrated in Fig. (3) and results showed that the molecular weight of XO was about (199000 ± 2000 and 191000 ± 2000) dalton from benign and malignant colon tumors and this result is comfortable with other previous studies like (Pacher *et al.*, 2006).

Table 3: Elution volumes of known molecular weight materials on sephadex G-100.

Material	**Molecular weight (Dalton)**	**Elution volume (ml)**
Blue dextran	2000000	82
Glucose oxidase	186000	100
BSA	67000	140
α- amylase	58000	166
Papein	23000	240
Tryptophan	204	280

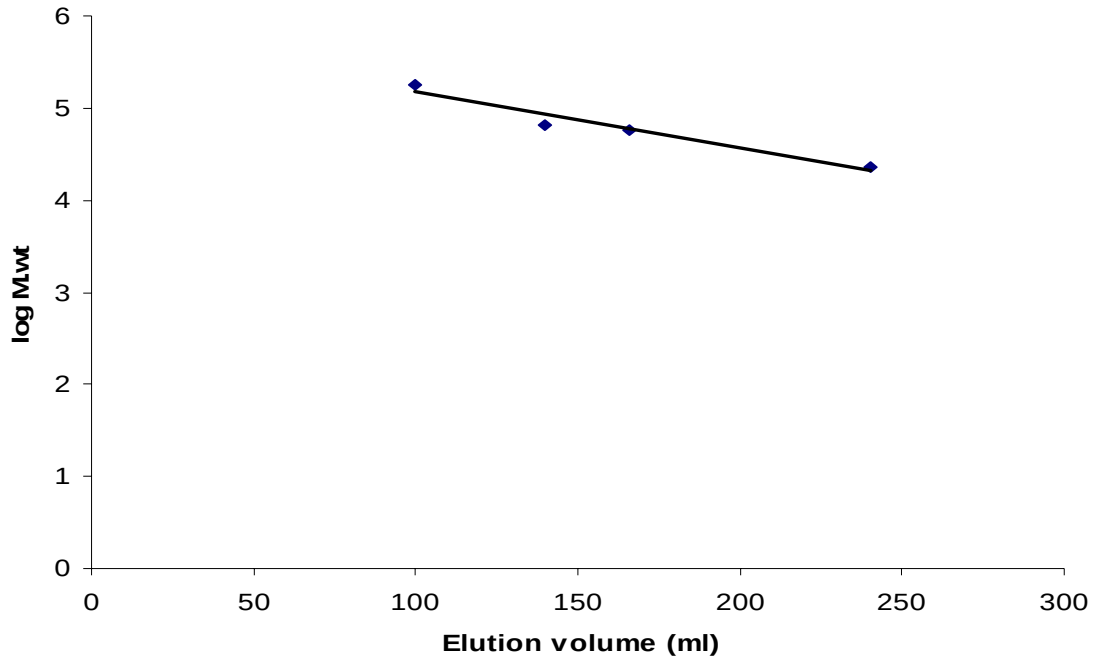

Fig. 3: Plot the logarithm molecular weight of known proteins versus elution volume on Sephadex G-100.

Optimum Condition of XO

The activity of XO in malignant colon tumor tissues has been detected using different concentrations of enzyme, temperatures, pH, times, substrate concentrations in order to evaluate the optimum conditions of the enzyme activity.

1- Effect of enzyme concentration on the enzyme activity:

The activity of XO was measured in the presence of different concentrations of the enzyme and the results showed that maximum activity of the enzyme was obtained using (10μg/ml) as shown in Fig. (4). The activity is essentially the measurement of the initial velocity under conditions that make it the maximum velocity. The activity should be proportional to the amount of enzyme added. That is, if two or three times as much enzyme is added the activity should be two or three times as great (Robyt and White, 1987).

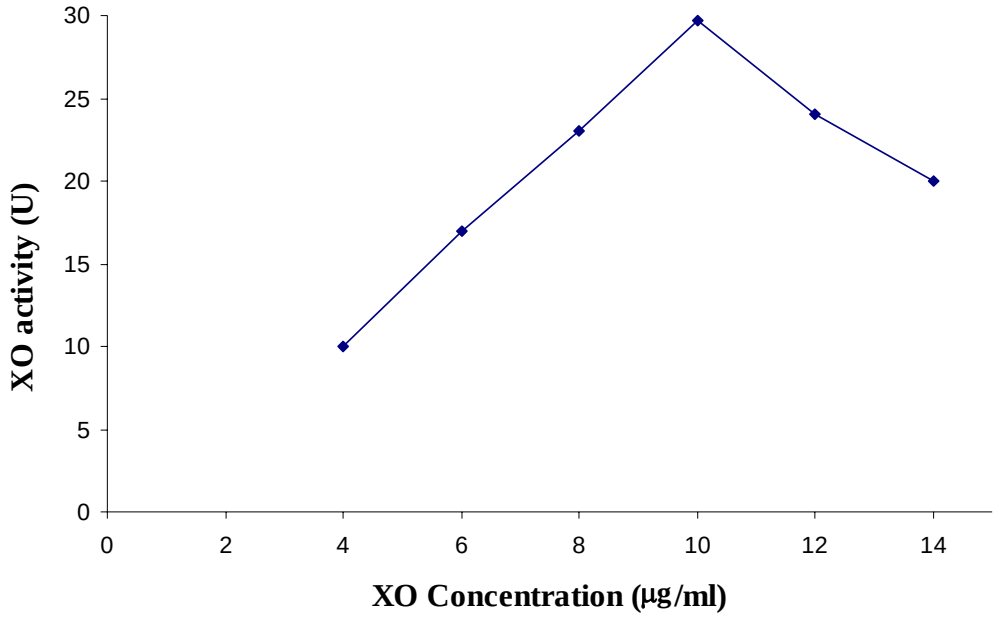

Fig. 4: Effect of enzyme concentration on XO activity.

2- Effect of incubation time on XO activity

The activity of the enzyme was measured in different incubation times. The results indicate that the maximum activity was obtained after 6 minutes as shown in Fig. (5). The activity of an enzyme is the amount of reaction that a certain amount of enzyme will produced in a specific period of time. The activity is determined by measuring the amount of product produced or the amount of substrate consumed per unit of time under high concentrations or saturating conditions of substrate. (Robyt and White, 1987).

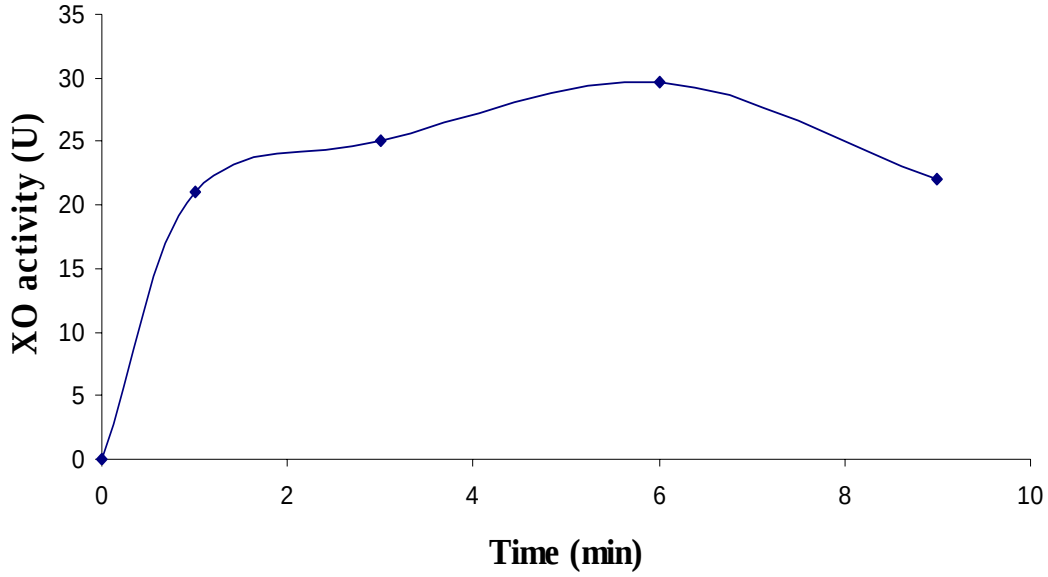

Fig. 5: Effect of incubation time on XO activity.

3- Effect of pH on XO activity

The effect of pH on XO activity was investigated at different pH values (4.5-9.5) as shown in Fig. (6). The results showed that maximum activity of XO was at pH 8.5. This result was in agreement with others (Carro *et al.*, 2009); (Rashidi *et al.*, 2009). Thus, all further experiments incubation was carried out at this point ,since the velocity of enzyme-catalyzed reactions depends on pH value. Enzymes have pH optima and often give bell-shaped curves of velocity versus pH, although other shapes have been observed. The pH optimum for different enzymes varies depending on the nature of the catalytic groups. (Robyt and White, 1987).

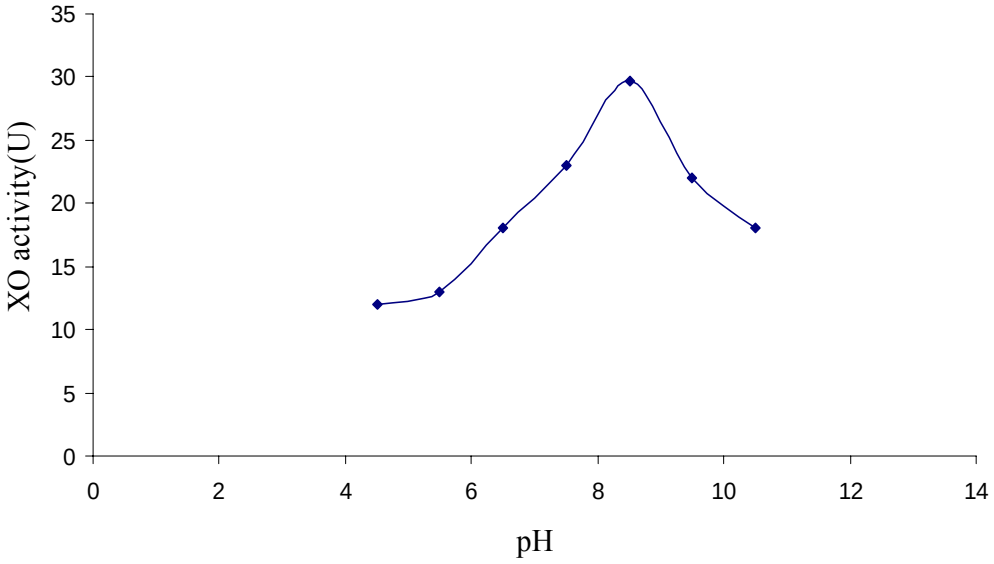

Fig. 6: Effect of pH on XO activity

4- Effect of temperature on XO activity

The activity of XO was assayed at range of different temperatures (20-60°C) as shown in Fig. (7). The results indicated that maximum activity was at (40°C) then decreased, This result was comfortable with other studies (Mithico *et al.*, 1999); (Carro *et al.*, 2009). The velocity of an enzyme catalyzed reaction increases with an increase in the temperature. The velocity approximately double for every 10°C rise in temperature. In contrast with ordinary chemical reactions, enzymatic reactions, have optimum temperatures and then rapidly decrease with further temperature increase. The loss of activity at the higher temperatures is due to the thermal conformational changes of the enzyme (Robyt and White, 1987).

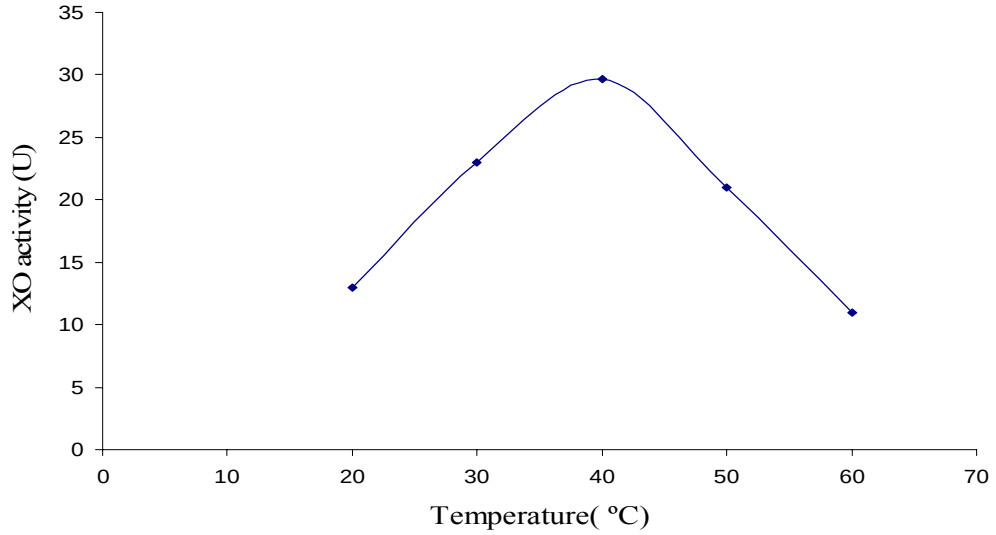

Fig. 7: Effect of temperature on XO activity

5-Effect of Substrate Concentration on XO activity:-

To determine the effect of substrate concentration on XO activity a series of experiments were performed at different concentration of xanthine (0.5-2.5) × 10^{-4} M as a substrate as shown in Fig. (8).

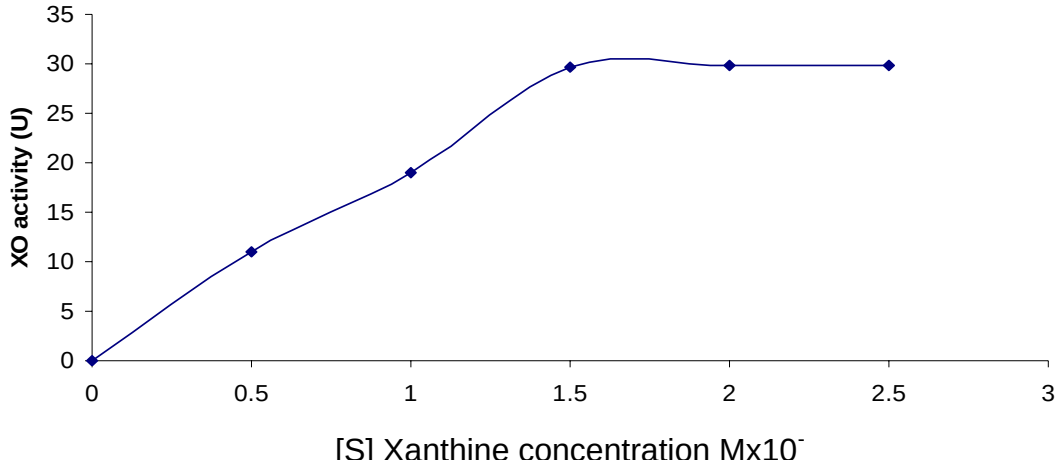

Fig. 8: Effect of substrate concentration on XO.

The activity was increased with increasing the substrate concentration till reached a limiting value of substrate concentration of (1.5×10^{-4}M). Further increase in substrate concentration did not produce any significant change in the activity and follow Michealis Minten kinetics. The Linweaver-Burk plot was shown in Fig. (9). The maximum velocity (V_{max}) and Michaelis Minten constant (k_m) were found to be (7.1×10^{-5} U/ml) and (2.5×10^{-4} M) respectively as shown in Fig. (9). This result was in agreement with other studies (Mithico *et al.*, 1999).

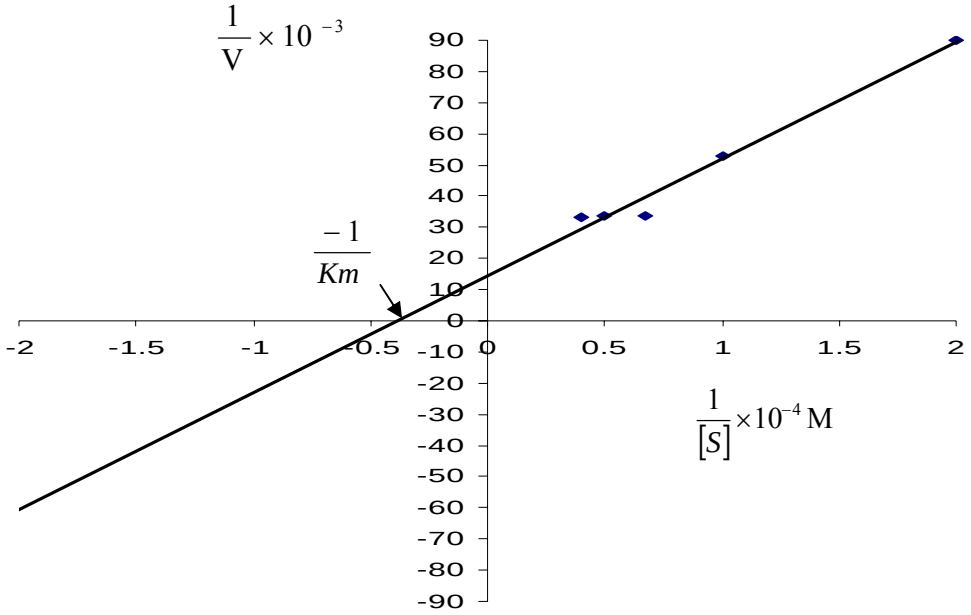

Fig. 9: Linweaver-Burk plot for XO activity in Malignant colon tumor

Table 5:The optimum conditions for XO.

Condition of XO	Concentration of XO µg	Time min	pH	Temperature °C	Concentration of substrate M	Activity U/ml
Optimum	10	6	8.2	40	1.5×10^{-4}	33.2
The assay method	8	5	8.5	25	1×10^{-4}	25.8

6-Effect of some metal ions and some compound on XO Activity

XO activity was measured in the presence of some metal ions like Cu^{+2}, $Hg^{+2}$and Ag^{+2}, which showed an inhibitory effect on XO. This results was comfortable with other studies (Mandal *et al*,2000). The metal ions have strong affinity towards XO. The interaction of these metal ions with XO significantly effects the kinetic parameters of the oxidative half reaction and this may be the main cause for the inhibition of XO activity by the metal ions (Mandal *et al.*, 2000).

On the 0ther hand, Co^{+2} was activated XO because it increased xanthine oxidase XO-derived reactive oxygen species (ROS) (Griquer *et al.*, 2006). Also the effect of N-acetylcysteine, was investigated and it seemed to have an inhibitory effect. This result was in agreement with other studies (Yamakawa *et al*., 2000).

Also the effect of 1-phenyl-3-pyrazolidinone had been studied and showed an inhibitory effect on XO activity. Other research had been studied other compound similar to its structure but not this compound. So, this compound might be useful in drug industry for colon cancer.

Table 6: Effect of some metal ions and compound on XO activity.

Compound	**XO activity (U)**	**% inhibition**
Non	29.7	100
Cu^{+2}	11.2	38
Ag^{+2}	10.6	36
Hg^{+2}	8.3	28
N-acetylcysteine	6.1	21
1-phenyl-3-pyrazolidinone	5.4	18
Co^{+2}	38.4	1.1

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