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Estimating Lipoxygenase and Gamma-glutamyl Transferase Activities in Sera of Colon Cancer Patients with Partial Purification of Lipoxygenase

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Abstract:

Colon cancer is an abnormal growth of cells that occurs in the large intestine. Sometimes growth remains restricted for a relatively long time before it becomes a malignant tumor and then spreads through the intestinal wall to the lymph nodes and other parts of the body. The study aims to estimate the effectiveness and partial purification of lipoxygenase (LOX) enzyme and measure gamma-glutamyle transferase (GGT) activity in serum patients of colon cancer in Baghdad. The study included (80) case male patients with colon cancer with (50) samples of apparently healthy males (control) as comparison group. The result displayed a noteworthy increase in lipoxygenase effectiveness (805.0±517.23 IU/L) in serum of patients with colon cancer compared with control (114.6±49.77 IU/L). The enzyme was purified by the precipitation of the serum protein using (40% $(NH4)_2SO4$) then removing the remaining salts by dialysis. The column of gel (sephadex G.100) was used to separate the enzyme from another protein, in this step a single peak was obtained. The effective part of lipoxygenase at yield (71.42%) and folds (11.033). The ion exchange chromatography (DEAE–CeA50) was used to isolate LOX isoenzyme, two bands (LOX1 and LOX2) were acquired with different degree of purity (16.372) and (*12.16*) folds respectively. The result displayed a noteworthy increase in the (GGT) activity in patients (58.69±16.94IU/L) (probability P≤ 0.000) compared with control (12.79±5.68 IU/L). The increase in activity of LOX can be used as a tumor marker to detect the colon cancer disease.

Key words: Colon cancer, Gamma-glutamyl transferase(GGT), Lipoxygenase (LOX).

Introduction:

Colorectal cancer is the fourth most common type of cancer in the world and the second most common cause of death, with (145.600) new case cancer diagnosis and (51.020) case deaths estimate in 2019(1).

Although colorectal cancer is treatable (especially by surgical removal), it leads to death if not detected early. It is an abnormal growth of cells that occurs in the large intestine, sometimes the growth remains restricted for a relatively long period before becoming a malignant tumor, then it spreads through the bowel wall to the lymph glands and other parts of the body (2,3).

Usually colon cancer begins with non-cancerous growth called (polyp), that later promotes on internal lining of the colon to grow slowly for 10-20 years (4).

The factors such as ulcerative colitis , age, genetics ,smoking , diet and obesity are found to increase the risk of developing colon cancer(5).

The metabolism of fat in the human body, especially the pathway of arachidonic acid, plays a major role in chronic inflammation and colon cancer(6).

Phospholipase A2 enzymes (PLA2) stimulate the formation of free fatty acids such as arachidonic acid from phosphate-lipid related membranes, these enzymes have been shown to be involved in cancer formation in laboratory mouse models (7,8).

The families of lipoxygenase and cycloxygenase are the most important enzymes in the arachidonic acid metabolic pathway (9), which are found in high concentrations in many tumors including rectal cancer, breast cancer, lung cancer, brain cancer, skin cancer and prostate cancer (10-15). Leukotrienes C4 are a metabolite of polyunsaturated fatty acids that are metabolized by lipoxygenase, where the GGT enzyme enters in the metabolism pathway of Leukotrienes C4(16).

The aim of this study is to estimate the effectiveness and partial purification of lipoxygenase and estimate GGT enzyme in colon cancer patients.

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Materials and Methods:

Collection of Samples:

Blood samples of colon cancer patients (40-80 years) were obtained from the Teaching Oncology Hospital at the City of Medicine and the National Center for Oncology - Baghdad for the period (18-2-2018 to 28-2-2019). The samples were 80 samples of blood. Approval of the Ministry of Health, Book No. (4237) dated 4/2/2018.

A total of 50 blood samples were collected from apparently healthy individuals as a control group (40-80 years). The samples were collected by drawing blood from the vein (5mL) using a syringe and placing the blood in a gel tube. The tubes were placed in the centrifuge at 1252g for 10 minutes to obtain serum. The serum was kept by eppendorf tube in deep-freeze at -20 ° C until testing.

Measuring the LOX Activity in Blood Serum:

The method of measuring the activity of the LOX enzyme (liu,1998) (17) is based on stimulating the oxygen reaction with the unsaturated fatty acids containing (cis, cis -1.4-pentadiene). It consists of a sequential system of double bonds that increase absorption at a wavelength of 234nm where the absorption intensity is directly proportional to the concentration of the enzyme (18). The unit of enzyme is defined as the amount of enzyme that changes in absorbance by 0.001 / sec at wavelength (234nm) under ideal conditions.

Estimation Protein Concentration:

The biuret method was used to estimate the concentration of the protein in the samples(19).

Separation and Purification of LOX from Serum Patients of Colon Cancer:

LOX is purified using the following steps:

Precipitation by Ammonium Sulphate:

The serum proteins were deposed by adding (0.9) gm of ammonium sulphate (0-40%) to 4ml of serum for patients with colon cancer, which was gradually added in ice bath with magnetic stirrer (15 minutes) until all the ammonium sulphate has been dissolved. Then the solution was placed in the centrifuge for 15 minutes and at a speed of 17608g to separate the precipitation from the leachate, the precipitate was dissolved with the least amount of the buffer solution (Buffer phosphate pH 7(0.001M)). Then, the enzyme activity and protein concentration were measured.

Dialysis:

The process of dialysis for the dissolved protein was done to remove the ammonium sulphate residues that was used to precipitate the proteins, using a dialysis bag. The dissolved protein was added into the bag and immersed in the buffer solution (Buffer phosphate (0.001M) pH 7). This process was carried out for 24 hours, with the solution being changed periodically. This step of purification was done at 4 ° C to maintain the activity of the enzyme. The activity and protein concentration of the enzyme were measured after the end of the process.

Gel Filtration

The gel filtration technique is based on the difference in molecular weights. This step was used to purify the LOX enzyme from proteins and associated salts. The filter column of the Sephadex G.100 was used.

- A column separating diameter (2cm) and length (70cm) with a filter at the end of which prevents the granulation of the resin outside was used , the process of casting the column was performed by using resin solution and pouring the resin solution on the walls slowly and homogeneously so as not to form air bubbles that impede the separation process, the column was then washed with a quantity of buffer solution (Buffer phosphate(0.001M) pH 7), and the flow velocity was set at (1mL / min).
- Four mL of product in dialysis step were added slowly and gradually over the resin surface and on the column walls and left for 5 minutes to soak into the resin.
- The gel filtration process was initiated using 250mL of the buffer solution (Buffer phosphate(0.001M) pH 7). The extracts were extracted from the gel filtration column at a size of 5 mL per part.
- The activity and the protein concentration of the lox enzyme were evaluated.

Ion Exchange Chromatography:

This technique was used to purify the isoenzyme of the LOX .

- DEAE-Cellulose (A50) was prepared by dissolving 20 gm of DAEA-Cellulose A50 in 250 mL of Buffer phosphate pH (7), leaving the solution suspended for 24 hours and at 4 ° C. The solution was switched several times from time to time to remove the soft minutes from the suspended solution until the pH reaches 7.
- NaCl solution (1 M): was prepared by dissolving 5.85gm of sodium chloride in 100 mL of (Buffer phosphate (0.001M) pH 7) solution. Other solution were obtained with

graduated concentrations of NaCl (0.1,0.25,0.5,0.75 M).

- A glass column diameter (3cm) and length (30cm) contains a filter at the end which prevents the resin granules from leaking out of it was used, the process of casting the column was performed by using resin solution with pouring the resin solution on the walls slowly and homogeneously so as not to form air bubbles that impede the process of ion exchange, then The column was washed with 250mL of the buffer solution (Buffer phosphate (0.001M) pH 7) and the flow time and velocity were set at 1mL / min.
- Three ml of protein from the gel filtration step were added slowly on the column walls and left to soak into the column. The separation process was initiated using (500mL) of the buffer solution containing NaCl (25,50,75,100 mM) progressive concentrations and the elute parts (3 mL) were collected for each part. Then the activity of the LOX and the protein concentration was evaluated.

Measuring GGT Activity in Blood Serum:

The Szasz method (20) was used to measure the effectiveness of the GGT enzyme, and the reaction equation is shown as follows:

$$L-GAMMA-Glutamul-3-carbooxy-4-natroanilid+Glaycylglycin \xrightarrow{GGT} 5-Ammino-2-nitro-benzoat+L-GAMMA-Glutamul-Glycylglycin$$

The activity of the enzyme is directly proportional to the formation of 5-amino-2-nitro-benzoate at a wavelength of 405nm.

Statical Analysis:

Statistical analysis was carried out using SPSS (version 16). Graphs were drawn using the Excel (2010), where ANOVA, arithmetic mean and standard deviation were used. The minimum probability factor (p 0.05%) was statistically significant .

Results and Discussion:

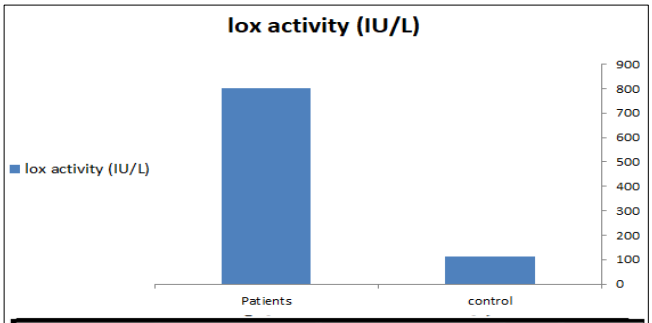
The study included (80) males with colon cancer. The study also included (50) samples of healthy (control) males, as comparison groups, and the range the age for patients and healthy between (40-80) years.

Measurement of LOX Activity in Blood Serum:

The activity of LOX in patients with colon cancer and control was measured using the method of liu (1998).

The results of the study included the statistical values of colon cancer patients and the biochemical variables measured in serum patients and control group.

The results showed that there was an increase in the activity of LOX in the blood serum of patients with colon cancer. A statistical comparison between the effectiveness of LOX in patients' and control showed a significant excess in enzyme effectiveness in patients with probability (P≤0.000) compared with control, as shown in Fig. (1).



Parameter	Control Mean±S.D N=50	Patients Mean±S.D N=80	T test	P value
LOX (IU/L)	114.6± 49.77	805± 517.23	5.94	0.000

Figure 1. The effectiveness of LOX in sera of control and patient

Overall, the results indicated an increase in the activity of LOX in the serum of colon cancer patients, previous scientific literature did not indicate that the enzyme's activity was measured from the serum of colon cancer patients, but indicated an increase in the activity of the enzyme in human colon cancer cell lines(21, 22, 23), this high effectiveness was reported to be highly correlated with reproduction of cancer cells ,angiogenesis ,and ,resistance to apoptosis(24, 25).

Also the increase in enzyme activity is due to the increase in the digestion of unsaturated fatty acids and the release of Eicosanoid compounds that promote the growth of cancerous tumors (26).

Separation and Purification of LOX from Serum Patients of Colon Cancer:

LOX was separated and purified in several steps as shown in the Table(1).

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Table 1. Separation and purification of the Lox enzyme from serum patients of colon cancer

Step	Elute (ml)	Activity (IU/L)	Total activity (IU)	Protein con. (g/L)	Total protein (g)	Specific activity (IU/g)	Purification (fold)	Yield %
Crude	6	420	2.52	78.3	0.4698	5.363	1	100
Ammonium Sulphate (0-40)	5	480	2.4	24	0.12	20	3.729	95.23
Dialysis	4	540	2.16	13.6	0.0544	39.705	7.403	85.71
Gel filtration sephadex G100	5	360	1.8	6.093	0.0365	59.17	11.033	71.42
Ion exchange DEAE-C A50 Isoenzyme- II	3	180	0.54	2.05	0.00615	87.804	16.372	28.57
Isoenzyme- I	3	120	0.36	1.84	0.00552	65.217	12.16	21.42

The first step was precipitating and separating the enzyme from blood serum by using ammonium sulphate salt at a concentration (0-40)%. In the second step, the dialysis was performed to obtain a degree of purity and desalting . In the third step size-exclusion chromatography technique was used to purify the Lox from the proteins and other salts associated with the enzyme. The filtration column of the sephadex G-100 resin was used in this step, a single peak was obtained at yield (71.42) % and (11.033) times of purification as shown in Fig. (2).

In the final ion exchange chromatography technique step was used to separate the LOX isoenzyme that based on the difference in charge. DEAE-Cellulose A50 resin was used, two isoenzyme were obtained with varying degrees of purity at a yield (28.57)%, (21.42)%, respectively and times of purification (16.372), (12.16) as shown in Fig. (3).

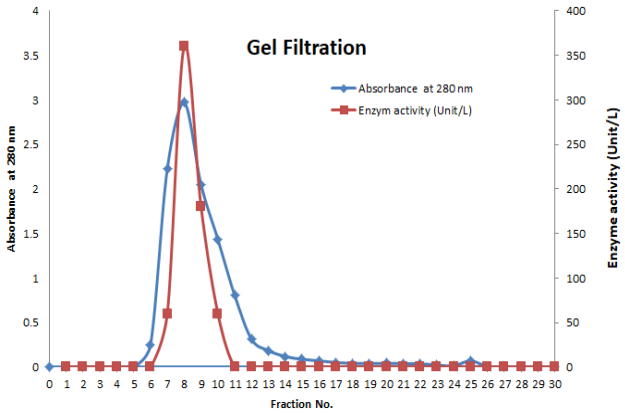


Figure 2. Activity and absorbance at 280nm for the fraction of Gel filtration step of Sephadex G-100 resin.

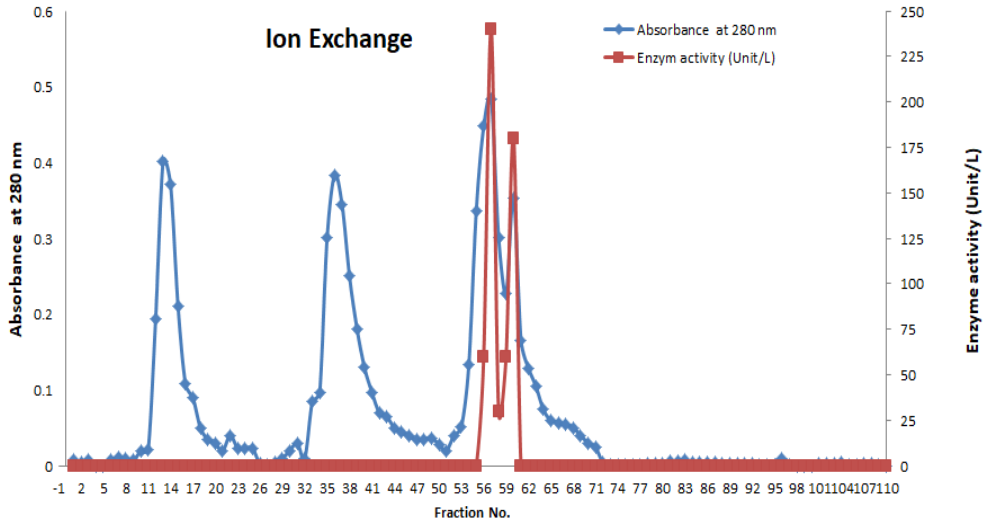
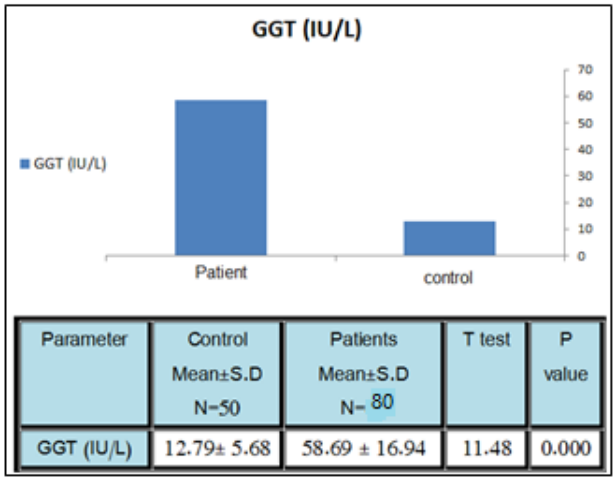


Figure 3. Activity and absorbance at 280nm for the fraction of ion exchange step by using DEAE Cellulose A-50 resin.

It has been noted in previous scientific literature that LOX was purified from various sources purified from the serum of male patients with cardiovascular disease (27) ,purified from serum in men with asthma (28) and it was also purified from the serum of women with breast cancer (29). Previous scientific literature has also indicated that the enzyme was purified from the colon cancer cell line(30) but did not indicate that the enzyme was purified from blood serum of colon cancer patients. Also the scientific literature indicated that the enzyme was purified from various other sources, including soybeans, where the number of times of purification (7.7 times) at yield (41%)(31). The enzyme was also purified from Human Placental at yield (21.84%)(32).

Measurement of GGT Activity in Blood Serum:

The results of the statistical analysis also showed a higher activity of GGT in colon cancer patients compared to control as shown in Fig.(4).



Parameter	Control Mean±S.D N=50	Patients Mean±S.D N= 80	T test	P value
GGT (IU/L)	12.79± 5.68	58.69 ± 16.94	11.48	0.000

Figure 4. The activity of GGT in sera of patients and control groups.

Previous scientific literature has indicated a high GGT activity in serum colon cancer patients (33, 34). The reason for the high activity of GGT is due to that the GGT is involved in generating free radicals and peroxidation of unsaturated fatty acids, which are involved in various tumorigenesis (35, 36).

Conclusion:

1- There is an increase in the activity of LOX enzyme in patients compared to the healthy group. This increase in enzyme activity in patients can be used as a tumor marker to detect the presence of colon cancer with other tumor markers.

2 -There was a significant increase in the activity of the enzyme GGT in patients with colon cancer compared to the healthy group.

3- Two isoenzymes of LOX were obtained using ion exchange chromatography.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in Tikrit University.

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تقدير فعالية انزيم اللايبوكسيجنيز وانزيم كاما-كلوتامايل ترانسفيريز في امصال دم المرضى المصابين بسرطان القولون مع تنقية جزئية لانزيم اللايبوكسيجنيز

مسلم عباس عبد العادلي **نادية احمد صالح الجبوري**

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الخلاصة:

سرطان القولون هو النمو غير الطبيعي للخلايا الذي يحدث في الامعاء الغليظة، احيانا يبقى النمو مقيدا لفترة طويلة نسبيا قبل ان يتحول الى ورم خبيث لينتشر بعدها من خلال جدار الامعاء الى الغدد اللمفاوية واجزاء اخرى من الجسم. هدفت الدراسة الى قياس فعالية وتنقية جزئية لانزيم اللايبوكسيجنيز وتقدير فعالية انزيم (*GGT*) في امصال دم المرضى المصابين بسرطان القولون في مدينة الطب-بغداد. اشتملت الدراسة على (80) عينة لاشخاص مصابين بسرطان القولون من الذكور، وكذلك اشتملت الدراسة على (50) عينة اشخاص اصحاء(سيطرة) من الذكور كمجموعة مقارنة. بينت النتائج وجود ارتفاع في فعالية انزيم اللايبوكسيجنيز *(805.0±517.23 IU/L)* عند مرضى سرطان القولون مقارنة مع المجموعة الضابطة *(114.6±49.77 IU/L)*. تمت تنقية الانزيم من مصل المرضى بواسطة الخطوات التالية: الترسيب بكبريتات الامونيوم (40%) بعدها استخدم الفرز الغشائي لازالة المتبقي من الملح، التنقية تمت باستخدام عمود تقنية الترشيح الهلامي (*sephadex G-100*) حيث امكن الحصول في هذه الخطوة على قمة واحدة، كانت نسبة الاسترداد في هذه الخطوة (71.42%) وبعدد مرات تنقية (11.033) مرة. تم فصل المتناظرات لانزيم اللايبوكسيجنيز باستخدام تقنية كروموتوغرافيا التبادل الايوني (*DEAE – Cellulose-A50*) ، امكن الحصول في هذه الخطوة على حزمتين (*LOX1 and LOX2*) وبدرجات نقاوة مختلفة (16.372) و (12.16) مرة على التوالي. اظهرت النتائج ايضا ارتفاع في فعالية انزيم كاما كلوتامايل ترانسفيريز (*GGT*) في امصال دم المرضى *(58.69±16.94IU/L)* وبمستوى احتمالية (*P≤ 0.000*) مقارنة مع مجموعة السيطرة *(12.79±5.68 IU/L)*. ان الارتفاع في فعالية انزيم اللايبوكسيجنيز يمكن ان يستخدم كمعلم سرطاني للكشف عن سرطان القولون.

الكلمات المفتاحية: سرطان القولون، غاماغلوتامايل ترانسيفيريز (*GGT*), لايبوكسيجنيز(*LOX*).