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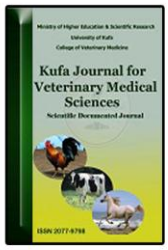

Toxicopathological and biochemical effects of *Carbon Tetrachloride CCl4* with residual accumulation in Liver of mice

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Abstract:

Objective :- The aims of the present study are to determine the toxicopathological and pathophysiological effects of carbon tetrachloride (CCl4), with measurement residual quantity of CCl4 in liver tissue by (HSGC) method.

Methodology :- 40 Swiss strain white mice were used, average weigh about 30 –32 g , divided into three groups, the T1 and T2 group were administrated with (100 and 200 mg/kg/body weight CCl4 respectively , given via intraperitoneal injection two does weekly for 40 day). While the 3rd group served as control. Clinical signs were reported during the course of the study**,** then at day 40 post treatment , all animal were sacrificed, blood were collected at 20 and 40 days of treatment period for biochemical test and post mortem examination was done and any gross lesions were reported, liver section was taken for pathophysiological examination, also for measurement residual accumulated in tissue by Head-Space Gas Chromatographic analysis (HSGC) method.

Results :- All results showed that CCl4 caused significant increase of serum alanine aminotransferase and aspartate aminotransferase activity. Histopathological picture of liver, showed damage of liver parenchyma, disappear arrangements of hepatocyte, loss of hepatic cord, with congestion and dilatation of blood vessel also coagulative necrosis and apoptotic cells. Infiltration of inflammatory cells (macrophages &lymphocyte). Also high quantity from CCl4 was residual in hepatic tissue.

Conclusion :- The present study investigated that the CCl4 affected on the liver tissue, and causes histopathological lesion with elevation of liver enzymes specially ALT& AST , with accumulative effect in liver tissue after measurement by GC system. And the degree of influence depended on the concentration of the toxic dose .

Key words : *CCl4, Toxicopathological effects of* liver, ALT&AST, Gas Chromatographic analysis.

التأثيرات المرضيه السميه و الكيميائية الحياتيــة لرابع كلوريد الكاربون وقياس تراكمه في كبد الفئران البيضاء

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الخلاصة:
أجريت هذه الدراسة لبيان تأثير سمية محلول رابع كلوريد الكاربون المحقون في غشاء الخلب ، من خلال دراسة التغيرات المرضية السمية مع ملاحظة التأثيرات الكيميائية الحياتيــة وقياس نسب المتبقيات المحلول بوساطة في كبد الفئران البيضاء تجريبيا .

استخـدمت في الدراسة (40) من ذكـور الفئران البيضاء بعمـر البـلوغ و بأوزان تراوحت (30 – 35 غم) وأعمارها بين 6-8 اسابيع حيث قسمت الى ثلاث مجاميع حقنت المجموعة الأولى بـ (100 ملغم/كغم من محلول رابع كلوريد الكاربون) بينما ، حقنت المجموعة الثانية بـ(200 ملغم/كغم من محلول رابع كلوريد الكاربون) يومياً ولمدة 40 يوم بوساطة حقنها داخل غشاء الخلب ، بينما أعطيت المجموعة الثالثة المحلول الفيزيولوجي واعتبرت مجموعة السيطرة . أجريت عملية سحب الدم من قلب الفئران مباشرة التي تم التضحية بها وذلك بقتل (5 فأرمن كل مجموعه كل 20 يوم) طيلة فترة التجـربة لدراسة مستويات أنزيمات الكبـد (ALT,AST) كما تم رفع عضو الكبد وأجراء الصفة التشريحية له و تسجيل التغيرات المرضية الحاصلة في كل فترة، كما تم متابعـة المتبقيـات المتراكمة نتيجة التعرض لمحلول رابع كلوريد الكاربون في نهاية التجربة باستخدام تقانة

أوضحت الدراسة ان تأثير سميـة لرابع كلوريد الكاربون يـزداد بزيـادة الجـرعـة وفتـرة التعـرض حيث أظهرت النتـائج وارتفاع معنوي ($P\leq0.05$) في تركيز (ALT,AST) ، ووجـد أن لمحلول رابع كلوريد الكاربون فعـالية تراكميـة في الفئران البيضاء وخاصة في الكبـد.

بيـن الفحص ألمـرضـي ألمجهري لنسيج الكبد المدروس تدرج الآفــات والتي تزداد بزيـادة الجرعة المتناولة خلال فترة التجـربة والتي تبـدأ من التنكس ألخلـوي الحاد وحدوث التلـف والنخر في الخلايـا الكبديه المفحوصة مع النزف والاحتقـان الشديد داخل متن النسيج.

Introduction:

The liver occupies a vital role in the main functions of the organism. It is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogeneity (1). Carbon tetrachloride (CCl4) has long been known as a model toxicant and has been the focus of many in vitro and in vivo toxicological studies (2). Pharmacokinetic data suggested that corn oil vehicle resulted in slower absorption , also it is distributed to all major organs as a function of blood flow and fat content of the tissues, with higher concentrations in fat, liver, kidney, brain, lung, bone marrow, and adrenals (3)and (4). The metabolism occurs primarily in liver, although it may also occur in other tissues , studies in human liver, mice and rats, cytochrome P450 (CYP2 E1) is primarily responsible for the bio-activation of CCl4 (5) and (6). From other hands CC14 is metabolized to toxic components by the mixed function oxidase system, and free radicals are produced during the metabolism of CC14 and free radicals may be important mediators of the toxicity of these two halomethanes (7).

Material and method:

Forty, Swiss strain white mice , with ages about 8 – 12 weeks and body weight ranged between (30 – 35g) were obtained from animal house of the pathology department at college of veterinary medicine, Baghdad university. Animals were randomly divided into three equal treated groups. 1st group (T1) and 2nd group (T2) treated with 100 and 200 mg/kg/body weight CCl4, via

intraperitoneal injection at two does weekly for 40 days. While the 3rd group, served as control and were given mammalian physiological saline. During the course of study**,** any clinical signs were reported**,** then at day 45 post treated , all animal were sacrificed and blood samples were collection for biochemical tests according to (8) for further determination of aspartate amino transferase (AST) and alanine amino transferase (ALT) according to (8). Via cardiac puncture technique, collected in test tubes without anticoagulant which allowed to stand and coagulate for 15 minutes in refrigerator, The serum was separated from coagulated blood samples by centrifugation at 2000rmp for 15 minutes, a spirited and frozen at -20C° until using. Post mortem examination was done and any gross lesion were reported**,** than the liver specimens were taken for fixed in 10% formalin for 72hour and processed according to (9), the histopathological changes were observed under light microscope. And another piece, made for residual amount of CCl4 in liver was done by using head-space gas chromatographic analysis (HSGC) according to (10).

Statistics analysis :-

Data were expressed as(Mean ± Stander Division) . The statistical analysis was carried out by one way analysis of variance (ANOVA) and made the comparison by Dunnett's T-test. ($P \leq 0.05$) was considered statistically significant.

Results:

Biochemical analysis :- The results of AST (U/L) are listed in (table: 1) showed significant increase ($p \leq 0.05$) of AST activity in the two treated groups (284.2 ± 2.51; 303.20 ± 3.07) in comparison with control (224.02 ±1.12) respectively, also there are significant increased ($P \leq 0.05$) between the values of T1 and T2 group along the period of experiment **(**284.2 ± 2.51 ; 303.20 ± 3.07 **).** There are a significant increased ($P \leq 0.05$) in AST in G1 between the 20 and 40 days (252.82±1.93; 284.2±2.51) and significant increased ($P \leq 0.05$) in G2 between the 20 and 40 days (294.93±2.59; 303.20±3.07). Also there are a significant increased ($P \leq 0.05$) in AST between G1 and G2 during 20 days (252.82±1.93; 294.93±2.59) and (284.2±2.51; 303.20±3.07) respectively.

Table:1 Effect of CCl4 toxicity on AST(U/L)values in serum of mice.

Group / *Parameters and period*	Control	1st Group	2nd Group	***L.S.D***
AST at 20 day	**227.16±1.14** a C	**252.82±1.93** **a B**	**294.93±2.59** **a A**	**4.94**
AST at 40 day	**224.02±1.12** **a C**	**284.2±2.51** **b B**	**303.20±3.07** **b A**	**5.15**
L.S.D	**3.64**	**4.07**	**4.33**	

Values are presented as means ±SE. (n= 5 mice /group)
Different capital letters means significant (P≤0.05) results between groups.
Different small letters means significant (P≤0.05) results between days.

The results of ALT (U/L) are listed in (table: 2) showed significant increase ($p \leq 0.05$) along the period of treated groups (85.32 ± 3.71 ; 94.51 ± 4.93) in comparison with control (53.70 ±0.77) respectively, also there are significant increased ($p \leq 0.05$) between the values of T1 and T2 group along the period of experiment (85.32 ± 3.71 ; 94.51 ± 4.93). There are a significant increased ($P \leq 0.05$) in ALT in G1 between the 20 and 40 days (73.78±2.10; 85.32±3.71) and significant increased ($P \leq 0.05$) in G2 between the 20 and 40 days (86.23±3.40; 94.51±4.93). Also there are a significant increased ($P \leq 0.05$) in ALT between G1 and G2 during 20 days (73.78±2.10; 86.23±3.40) and (85.32±3.71; 94.51±4.93) respectively.

Table:2 Effect of CCl4 toxicity on ALT(U/L)values in serum of mice.

Group / *Parameters and period*	Control	1st Group	2nd Group	***L.S.D***
ALT (at 20 day)	**51.84±0.71 a C**	**73.78±2.10 b B**	**86.23±3.40 b A**	**4.06**
ALT (at 40 day)	**53.70±0.77 a C**	**85.32±3.71 a B**	**94.51±4.93 a A**	**5.18**
L.S.D	**3.93**	**4.03**	**4.85**	

Values are presented as means ±SE. (n= 5 mice /group)
Different capital letters means significant ($P \leq 0.05$) results between groups.
Different small letters means significant ($P \leq 0.05$) results between days.

Histopathological examination :-

The specific structural lesions observing in the liver parenchyma exposed to 100 and 200 mg/L concentrations showed various degrees of hydropic swelling with fatty changes of hepatocyte were observed clear space with vacuolar degenerative of hepatocyte, disappear arrangements of hepatocyte with loss of hepatic cord infiltration of kupfer cell with extensive karyolysis & karyorrhexis (Fig:1) , severe congestion and dilatation of blood vessel, with mild aggregation of MNCs around central vein which appear in (Fig:2 , 3 & 4).

The severity of injury parenchyma, appear in 200 mg/L more than 100 mg/L with presence apoptotic cells, focal area of coagulative necrosis, accompanied with sever infiltration of mono nuclear cell (macrophages &lymphocyte). In addition to neutrophile are present in liver parenchyma and capsule (Fig: 5 & 6).

Residual assay :-

The results of residual assay CCl4 by (GCs) in liver, appear in (Fig:8). While the (Fig: 7) show the stander assay of CCl4, and from this figure the Ret. Time was (7.431), and the area & area% under the peak was (17849734 ; 2.1012%), while the Ret. Time and area under peak of CCl4 that acumination in liver was (7.271 ; 494522) respectively. Which it's important for describe the substantial the item, also important for concentration of these item and

percentage in sample. By used the equation :-

$$\text{Concentration sample} = \frac{\text{Concentration stander} \times \text{Area of stander}}{\text{Area of sample}}$$

$$\text{Concentration of CCl4 in liver} = \frac{\text{Concentration sample} \times \text{Volume of sample}}{\text{Weight of organ}}$$

which equal (8.021 ppm) that conceder a highly concentration in living tissue.

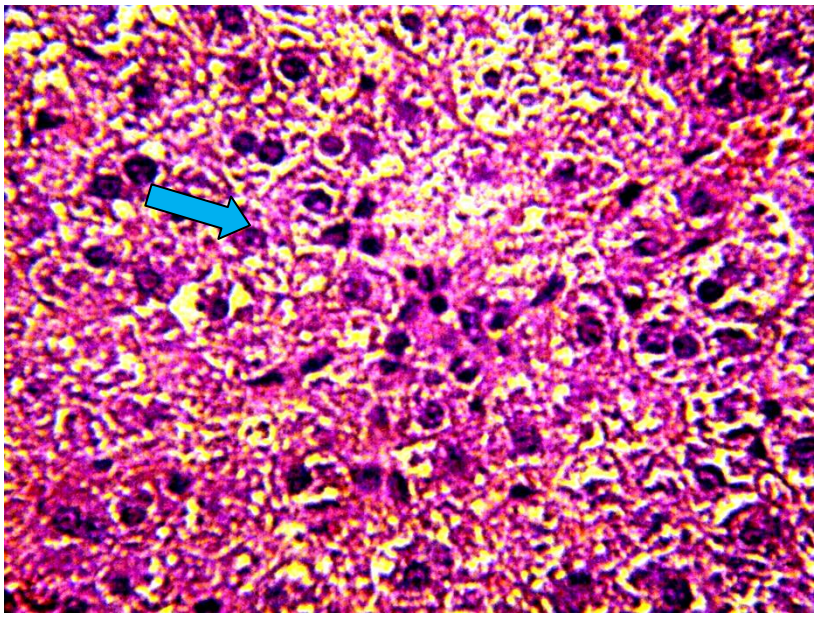


Fig 1:- Histopathological picture of mice liver at 20 days treated with 100mg/k. show disappear arrangements of hepatocyte with loss of hepatic cord. infiltration of kupfer cell with ➡ extensive karyolysis & karyorrhexis (H&E stain 40X)

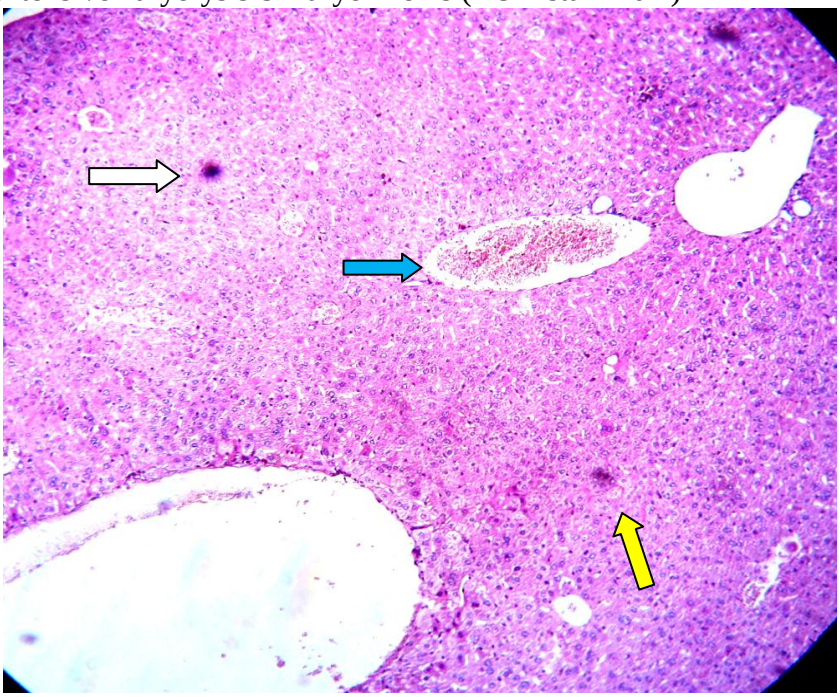


Fig 2:-Histopathological picture of mice liver at 40days treated with 200mg/k. show sever degeneration with loss of sinusoid ⇨with sever necrosis⇨also congestion and dilatation of blood vessel⇨ (H&E stain 4X)

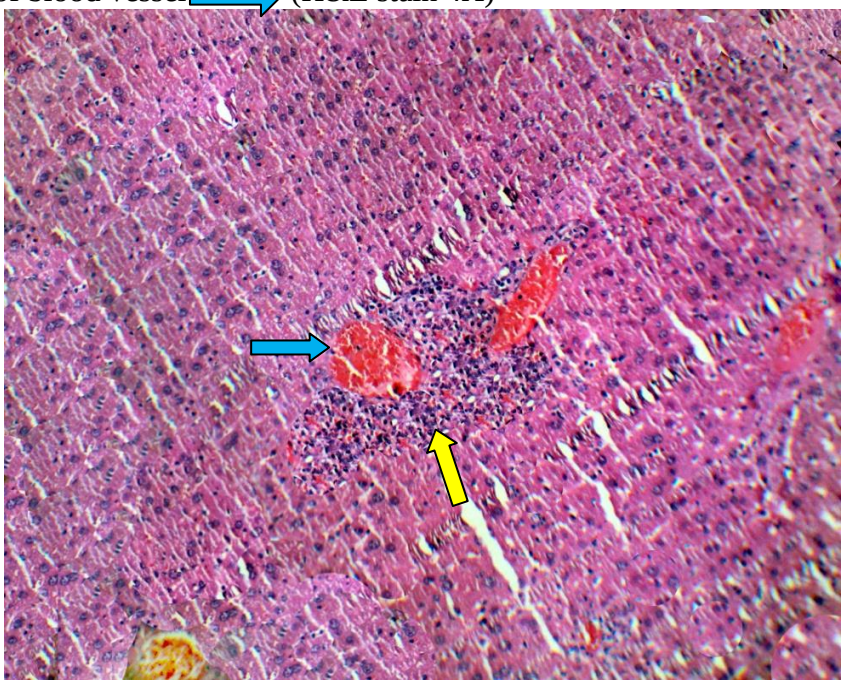


Fig 3:-Histopathological picture of mice liver at 40 days treated with 100mg/k. show sever aggregation of MNCs ⇨with congestion of blood vesse ⇨ (H&E stain 4X)

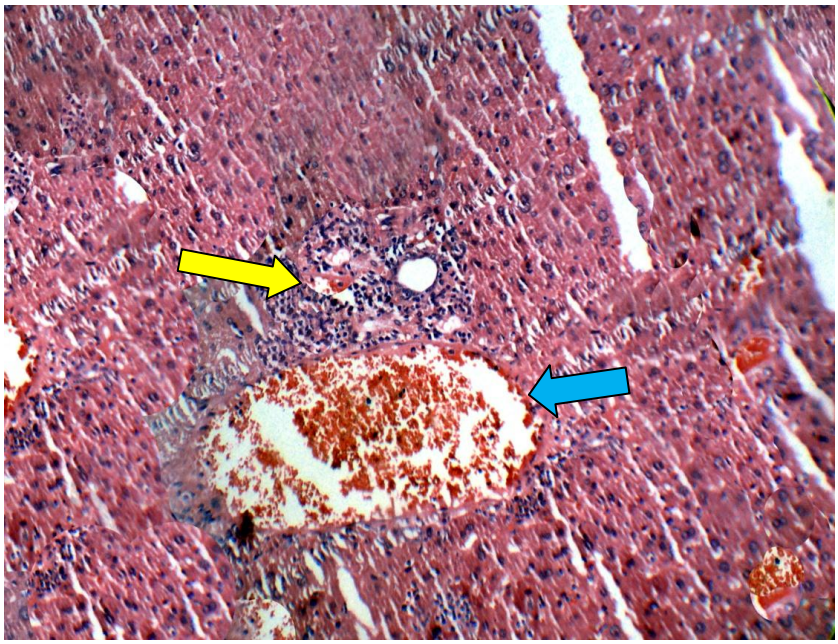


Fig 4:-Histopathological picture of mice liver at 40 treated with 200mg/k. show sever aggregation of MNCs around central vein⇨with congestion and dilatation of blood vessel ⇨ (H&E stain 10X)

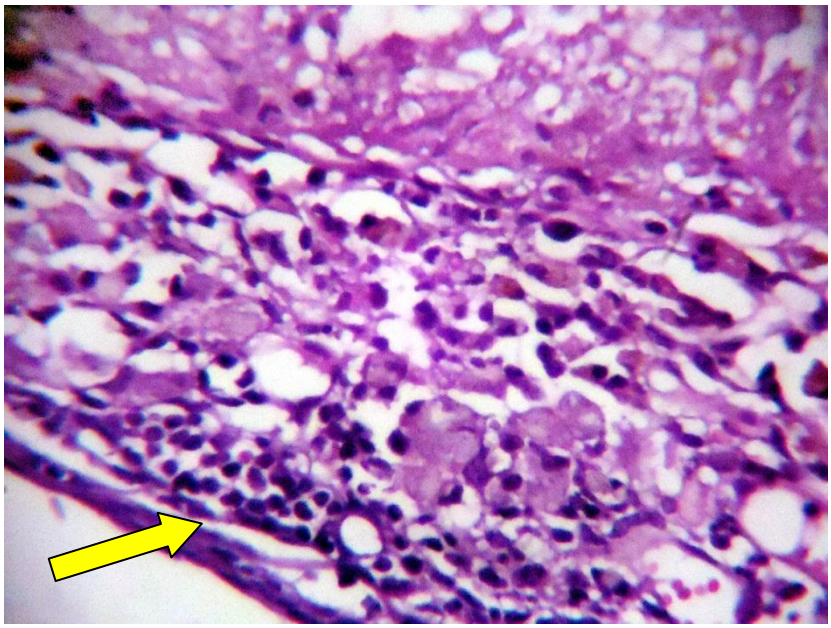


Fig 5:- Histopathological picture of mice liver at 40 treated with 200mg/k show accumulation of PMNCs in the liver capsule (H&E stain 10X)

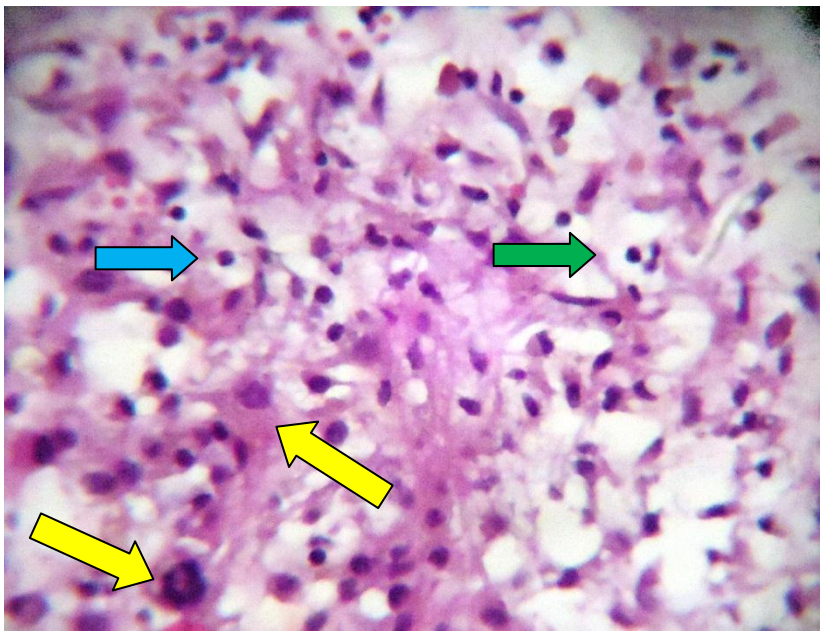
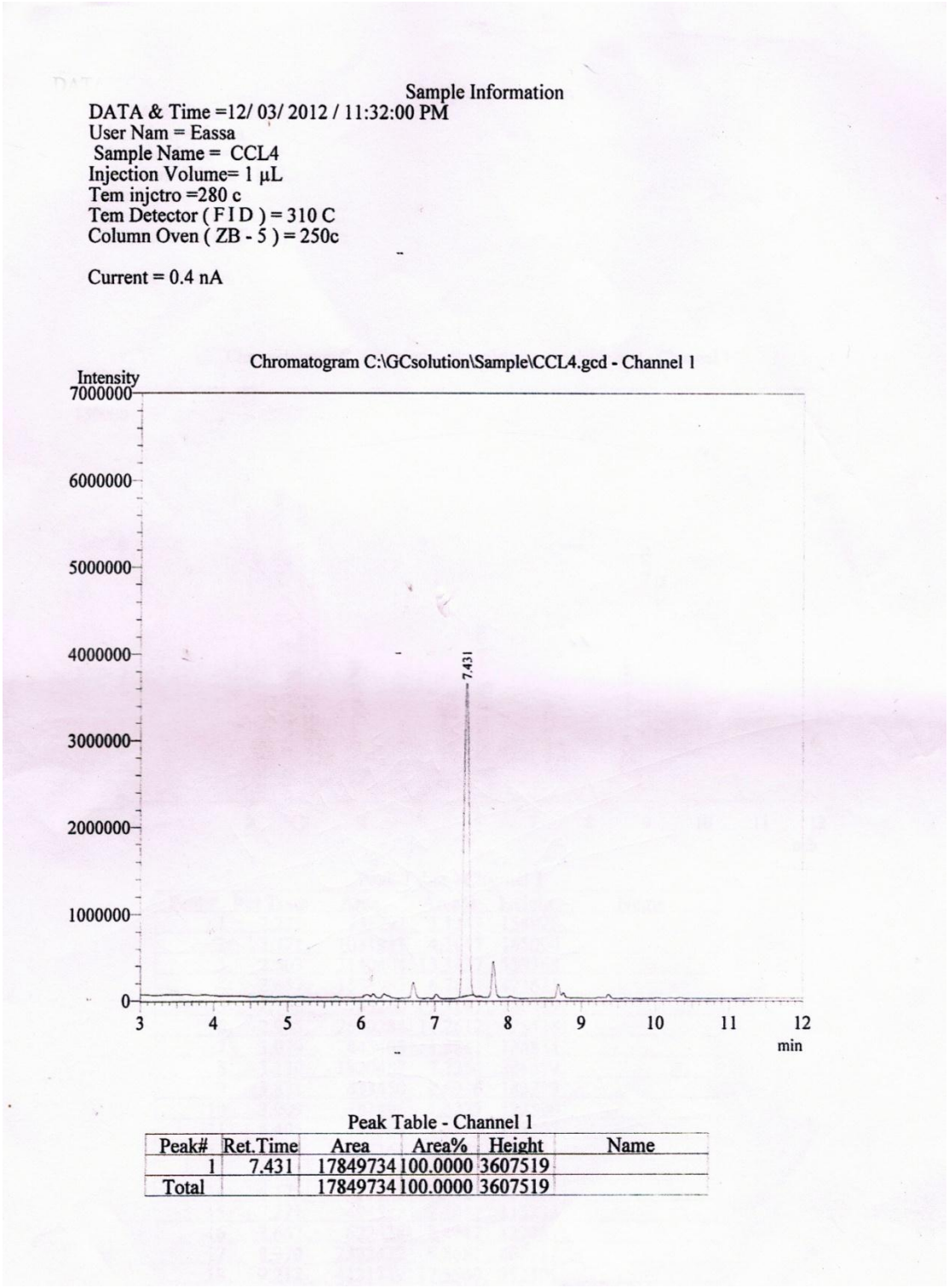


Fig 6:-Histopathological picture of mice liver at 40 days treated with 100mg/k. show fatty degeneration also sever distraction of liver parenchyma with infiltration of MNC with presence apoptotic cells (H&E stain 10X)

Fig 7:- Normal carves of stander CCl4 by use gas chromatographic analysis.



Peak Table - Channel 1

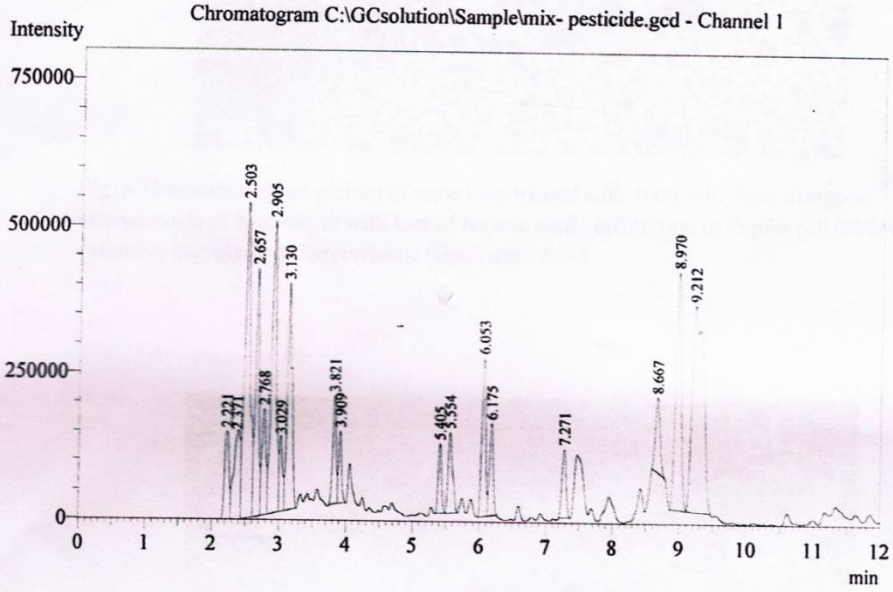
Peak#	Ret.Time	Area	Area%	Height	Name
1	7.431	17849734	100.0000	3607519	
Total		17849734	100.0000	3607519	

Fig 8 :- Residual carves of CCl4 in live by use gas chromatographic analysis .

Sample Information

DATA & Time =27/ 06/ 2012 / 8:58:30 PM
User Nam = Eassa
Sample Name = Liver
Injection Volume= 1 µL
Tem injctro =280 c
Tem Detector (F I D) = 310 C
Column Oven (ZB - 5) = 250c

Current = 0.4 nA



Peak Table - Channel 1

Peak#	Ret.Time	Area	Area%	Height	Name
1	2.221	735292	3.1243	154926	
2	2.371	1031933	4.3847	145090	
3	2.503	3140404	13.3437	539368	
4	2.657	1595983	6.7814	421621	
5	2.768	783260	3.3281	180369	
6	2.905	2650284	11.2612	498418	
7	3.029	443408	1.8841	124854	
8	3.130	1820489	7.7354	386589	
9	3.821	633450	2.6916	183719	
10	3.909	381896	1.6227	122500	
11	5.405	399964	1.6995	117207	
12	5.554	627160	2.6648	136245	
13	6.053	1084939	4.6100	269670	
14	6.175	635078	2.6985	156892	
15	7.271	494522	2.1012	115233	
16	8.667	622428	2.6447	127481	
17	8.970	2322432	9.8681	406917	
18	9.212	4131739	17.5560	353301	
Total		23534661	100.0000	4440400	

Discussion:

In liver, our study founded the CCl4 induced a severe damage in histopathological picture and alteration in biochemical level, also the CCl4 accumulation in liver tissue when given intrapretonial injection.

The alteration in AST and ALT may occur due to oxidative stress of CCl4 , and plays a role in detoxifying and produced free radicals are produced during the metabolism of CC14 and free radicals may be important mediators of the toxicity of these two halomethanes (11). Also bioactivities of carbon tetrachloride was occur by activation of cytochrome P450 (CYP2 E1), and this isoenzyme catalyses the reductive de-chlorination of carbon tetrachloride, forming the reactive tri-chloromethyl radical ($CCl3^{o}$). and trichloromethyl peroxy ($CCl3OO^{o}$) which is the one type of free radical emanated from CCl4 initiate peroxidation of membrane unsaturated fatty acids.(12). lead to generation of free radicals and produced cell injury demonstrated by increased leakage of alanine aminotransferase (ALT), aspartate aminotransferase (ALT). This evidence was in consistence with (13) who predicted that CCl4 caused significant increasing serum activity of alanine aminotransferase and aspartate aminotransferase of rats. Also the results were in agreement with (14**)** who investigated that the exposure to CCl4 caused increase in the level of ALT and ALT .

These free radical as demonstrated the histopathological effects, this radical can bind with lipids and proteins, under anaerobic conditions to produce chloroform (CHCl3), by react with hydrogen dimerism to form hexachloroethane, or undergo further reduction to producing carbon monoxide. This idea is in consistence with idea mentioned by (15) and (16). Also the tri-chloromethyl radical can react with oxygen, forming the trichloromethylperoxyl radical ($CCl3OO^{o}$) in aerobically condition (with oxygen) which its highly reactive radical, that may initiate lipid peroxidation or may react further, producing phosgene (COCl2) (17).

The severe damage and destruction to the liver tissue parenchyma and present edema may be due to severe inflammation that present in liver**,** with damage in the B.Vs, leading to escape of fluids from B.V to the hepatic tissues, that lead to increase the pressure on the liver tissue causes irreversible tissue and damage . This result agreed with (6) and (18) when reported severe cell injury in hepatocyte , that interfering with the functions of the liver cells during exposure to the CCl4.

The degenerative changes and necrosis, occur because the mitochondrial activity of liver cells is affected by $CCl3OO^{o}$ radicals with disruptive effects on hepatocyte and produced swelling and distortion of mitochondrial cristae, uncoupled energy metabolism, inhibited cellular respiration, and altered calcium kinetics follow, the organelles mediating cellular energy metabolism. The results were in agreement with (19) who investigated that Carbon tetrachloride c
ause hepatotoxicity and acute hepatocellular injury, due to the effect of CCl4 on glucose metabolism, during intraperitoneal injection of rat by carbon tetrachloride (1 ml/kg) for three month.

The residual of CCl4 in liver occur because of carbon tetrachloride's lipophilic solubility properties, most of the compound accumulates in tissues with high fat content. This result agreed with (20) who reported that highest in concentration in the adipose tissue followed by liver, brain, and spleen, and was lowest in gill regardless of the administration route. Also these result were in agreement with (10) who investigated that the CCl4 was highly sensitive to analysis when determination by Gas chromatography.

Conclusion:

The present study investigated that the CCl4 affected on the liver tissue, and causes histopathological lesion with elevation of liver enzymes specially ALT& AST , with accumulative effect in liver tissue after measurement by GC system. And the degree of influence depended on the concentration of the toxic dose .

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