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GC/MS-Based Metabolomics Profiling Approach and Determination of Ameliorative Effect of *Chiliadenus Montanus* Extract towards CCl_4 Induced Hepatotoxicity in Albinol Rats

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Abstract

The current study aimed at evaluating the GC/MS profiling of *Chiliadenus montanus* leaves extract which consists of 48 compounds, nine of them are major and represented 52.55 % of the total peak areas. The ameliorative effect towards the CCl_4-induced hepatotoxicity in male Wistar rats and endogenous antioxidants content in the liver were analyzed. CCl_4 diluted 1:9 (v/v) in olive oil was injected intraperitoneally followed by oral administration of methanolic plant extract (200 mg/kg body weight). The CCl_4-treated rats showed a significant decline in the studied hematological parameters, the serum levels of high-density lipoprotein (HDL), albumin (A) as well as the hepatic levels of glutathione (GSH) and activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR).This was accompanied by a significant elevation in the levels of total lipids (TL), triglycerides (TG), total cholesterol (TC), low-density lipoproteins (LDL), globulin (G), total bilirubin (TBil) and the activities of alanine and aspartate aminotransferase (ALAT and ASAT) and alkaline phosphatase (ALP) as well as the hepatic levels of malondialdehyde (MDA). In contrast, the administration of methanol extract, notably improved all the studied parameters. This study showed that CCl_4 administration to Wistar rats, at a high dose level, could induce a hepatic injury in addition to certain hematologic and metabolic alterations.The work was extended to examine tissue histopathology.

In conclusion, *C. montanus* leaves methanol extract, resulted in an attractive candidate for ameliorating of hepatotoxicity induced by CCl_4 through scavenging free radicals, improved liver functions, and normalizing the liver histopathological architecture. Further studies are required in order to identify the molecules responsible of the pharmacological activities. Yet, the treatment with methanol extract could ameliorate these alterations via their antioxidative effect.

Keywords: *Chiliadenus montanus*, GC/Mass, CCl_4-induced hepatotoxicity

1. Introduction

Chiliadenus montanus (Vahl.), Brullo, a herb endogenous to the Sinai region of Egypt (Saint Katherine area), where it grows at high elevation in the selected sites in fissures of mountains on alkaline soil with high bicarbonate and organic matter content, and low percentage of silt and clay and subsequently, low moisture content **[1]**. *C. montanus* is a member of the family (Asteraceae) **[2]**, popularly known as Haneida, is common in the Sinai Peninsula. This medicinal plant is traditionally used for chest diseases, diarrhea, renal troubles and stomachache. Moreover, evidences for their hypoglycemic, antioxidant and anticholestatic activities have been recently investigated **[3]**. Previous phytochemical researchers have identified the presence of active constituents in the aerial parts, including phenolic compounds that give *C. montanus* their medicinal values **[4]**. *C. montanus* is used as a herbal tea for the treatment of renal troubles and select chemical components have been shown to exhibit antimicrobial, antiatherogenic, antibacterial, antifungal and antiobesity activities **[5].**

Recently, studies investigated the phytochemistry of aerial parts of *C. montanus* which revealed the presence of new metabolities nature products **[6,7]**. The HPLC and HPLC-MS analysis showed individual polyphenolic compounds. Among all the isolates, metabolite (kaempferol-3-O-(6"-O-acetyl)-

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β-D-glucopyranoside) exhibits antimicrobial activity through inhibiting growth of Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Alcaligenes faecalis and Candida albicans. As with other flavonol structures (kaempferol-3-O-(6"-O-acetyl)-β-Dglucopyranoside) may contain an appropriate structural configuration to complex with bacterial cell walls and inhibit microbial growth **[8].**

Furthermore, the cytoprotective effect of *Chiliadenus montanus* hydroalcoholic extracts in an in vitro oxidative stress model and the human astrocytoma U373-MG cell line showed that *C. montanus* hydroalcoholic extracts exert a protective action by decreasing cell death and by inhibiting intracellular ROS production **[9]**. The antioxidant activity of the hydro alcoholic extract of *C. montanus* revealed that *C. montanus* extracts are active radical scavengers.

Recently a lot of studies have been conducted to identify natural compounds for prevention of the development and recurrence of cancers from cirrhosis or fibrosis of liver. The liver is responsible for metabolism and detoxification of the most of components that enter the body. Carbon tetrachloride (CCl_4) is a highly toxic chemical agent, the most famous chemical compound used to induce liver damage experimentally. Histopathological sectioning of the liver tissues indicated that, CCl_4 induced fibrosis, cirrhosis and hepatocarcinoma.

The toxic effect of CCl_4 is attributed to trichloromethyl radical produced during oxidative stress **[10]**. The number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer cells are significantly increased after liver injury induced by hepatotoxins such as CCl_4. It induced activation of liver resident macrophages and/or chemo attraction of extra hepatic cells **[11]**; e.g. neutrophils and lymphocytes. The activated macrophages are released and contributed to liver fibrosis, inflammation and injury **[12]**. Once the liver became injured, its efficacy became limited **[13]**. Due to the previously reported phenolic compounds **[14-16]** in plant extract, this study is interested in using this plant as an alternative treatment of hepatic disease.

2. Experimental

Material and Methods

Plant Material

C. montanus, samples were collected from Al-Tofaha valley ,Saint Catherine, South Sinai, Egypt in September 2019. It was identified by Prof. Dr. Alaaeldin Sayed Ewase, Ministry of Environment; Nature Conservation Sector, Biodiversity administration, Cairo, Egypt.

Extraction and Isolation

Part of plant samples were cleaned, air-dried at lab-temperature and grounded to fine powder and exhaustively extracted with (H_2O / MeOH v/v), to be used for phytochemical screening, hepatoprotective activities and other plant part (4 g) of *C. montanus* powder was extracted using diethyl ether (1 : 10, w/v) for three times (15 minutes each time) with the assistance of ultrasonic. The obtained turbid solution was filtrated and the solvent of filtrate was removed by rotary evaporation under reduced pressure. Then the extract was diluted with 1 mL of anhydrous ethyl alcohol: hexane (1 : 1, v/v) and was filtered through a 0.22 µm membrane filter. 1 µL of subsequent filtrate was injected to GC/MS for analysis **[17].**

For Phytochemical Screening

The methanol extract of C. montanus was made in two dimensional paper chromatographic in BAW and sprayed after drying with ($FeCl_3$), some spots convert to dark blue colour which indicated to contain phenolic and flavanoids compounds.

For GC/MS analysis and Identification for Volatile Components

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.251mm, 0.1 mm film thickness). For GC/MS detection , an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1ml/min. The injector and MS transfer line temperature was set at 280°C. (USA) The oven temperature was programmed at an initial temprature 50°C (hold 2 min to 150°C at an increasing rate of 7 °C /min. then to 270 at an increasing rate 5°C / min (hold 2 min) then to 310 as a final temperature at an increasing rate of 3.5°C/min (hold 10 min) **[18, 19].**

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed when possible to confirm GC retention times.

Assessment of Biological Activities

In vivo Hepatoprotective Activities

Animals

Male Wistar albino rats (100 to 120 g) were selected for this study. They were obtained from the Animal House, National Research Center, Egypt. All

animals were kept in controlled environment of air and temperature with access of water and diet ad libitum. Anesthetic procedures and handling with animals complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt.

Experimental design

18 male rats were used in this study. Animals were divided into 3 groups: Group 1, served as normal healthy control rats. Group 2, Rats were intraperitoneally injected with 500 microliters of CCl4 diluted 1: 9 (v/v) in olive oil that were injected intraperitoneally (0.1 ml) twice a week for 6 consecutive weeks. Group 3, Rats were intraperitoneally injected with CCl_4 (0.1 ml) followed by oral administration of *C. montanus* methanolic extract (200 mg/kg body weight).

Hematological and Biochemical Studies

Sample Preparations

Blood was collected from each animal by puncture of sublingual vein. Blood samples were divided into two parts. The first part was collected on EDTA for hematological analyses. The second part was collected into dry test tubes and then centrifuged at 3000 rpm in order to separate serum. The sera were kept at -20° C for further biochemical analysis. In order to collect the hepatic tissues, rats were immediately dissected. The liver was homogenized with 10% w/v ratio in ice-cold 50 mM Tris HCl buffer at pH 7.4 and then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and kept in deepfreeze at -20°C for further analyses.

Estimation of Hematological Parameters

The hematological parameters including red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT) count, hemoglobin (Hb) content and packe cell volume (PCV) were analyzed using Medonic M-Series analyzer (Clinical Diagnostics solutions Inc, Florida, USA).

Estimation of Serum Biochemical Parameters

In the serum of all the experimental groups, the levels of total lipids (TL), total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total proteins (TP), albumin (A), globulin (G), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), total bilirubin (TBil) and direct bilirubin (DBil) were measured colorimetrically using Biodiagnostics kits (Dokki, Giza, Egypt).

Non-enzymatic and Enzymatic Antioxidant Assay

The concentrations of non-enzymatic (glutathione, GSH) as well as enzymatic (catalase, CAT, superoxide dismutase, SOD, glutathione reductase, GR) antioxidants were estimated in the homogenate of the liver of control and treated rats. The method by which GSH content was measured was based on the reaction of 5, 5'-Dithiobis-2-nitrobenzoic acid with GSH **[21]**. The CAT activity was estimated in accordance to the method described **[22]**. The SOD activity assessment was based on the ability of SOD to inhibit the reduction reaction of nitrobluetetrazolium dye mediated by phenazine methosulphate **[23]**. The principle for measuring the GR activity was based on its ability to catalyze the reduction of glutathione (GSSG) **[24]**.

Determination of Serum Caspase-3 Activity

Lipid peroxide assay: The level of malondialdehyde (MDA) in the liver homogenate was assayed according to the described technique [20].The principle of this method depends on the reaction of the liberated MDA after lipid peroxidation (LPO) of the cell membranes with thiobarbituric acid in acidic medium. Serum caspase-3 activity was measured by a quantitative ELISA technique using the kit provided by R and D systems (MN, USA) according to the manufacturer's instructions **[25]**.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) for Analysis of Serum Bax and Bcl2 mRNA Levels:

Total RNA was extracted from serum using RNeasy mini kit (Qiagen, CA, USA). The isolated RNA was quantified using UV spectrophotometer (Beckman, USA) and the purity of RNA was verified with 260/280nm ratio ranging from 1.9-2.1. The integrity of RNA was assessed by gel electrophoresis. The total RNA (0.5–2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (Fermentas, USA). Real-time qPCR amplification and analysis were performed using an

Table (1): The Primer Sequences of the Studied Genes

Gene name	Primer sequence	Primer Size (bp)
Referactin	Forward primer:5′-CAG GAT GGC GTG AGG GAG AGC-3′ Reverse primer:3′-AAG GTG TGA TGG TGG GAA TGG-5′	263
Bax	Forward primer: 5'-TTC CGA GTG GCA GCT GAG ATG TTT-3' Reverse primer: 3'-TGC TGG CAA AGT AGA AGA GGG CAA-5'	194
Bcl-2	Forward primer:5'-CAT GCC AAG AGG GAA ACA CCA GAA-3' Reverse primer:3'-GTG CTTTGCATT CTTGGA TGA GGG-5'	224

Applied Biosystem with software version 3.1 (Step One™, USA). The primer sequences shown in **(Table 1)** were provided by Shine Gene, China.

Determination of Percent of DNA Damage by Comet Assay in Liver Tissues:

Single cell gel electrophoresis assay (also known as comet assay) was performed **[26]**.This test is a rapid, sensitive and simple method for detecting DNA damage. In this method, cellular DNA is detected by the migration of DNA fragments from the cell nucleus through an agarose gel using fluorescent dyes, under the influence of an electric field, resulting in a comet-like shape. With increasing number of breaks, DNA pieces migrate freely into the tail of the comet. The tail length and the percentage of total DNA in the tail reflect DNA damage, which is directly related to the frequency of breaks over a wide range of damage. All steps of the comet assay were conducted under dimmed light to prevent additional DNA damage. Image analysis was performed with a Leitz Orthoplane Pi fluorescence microscope (magnification 200) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software, Perspective Instruments). One hundred randomly selected cells per slide were scored.

Histopathological Study

Liver tissues were excised from sacrificed animals, individually weighed, and, from them, 5 µm thickness slices were cut, fixed in 10% paraformaldehyde, and embedded in paraffin wax blocks. Tissue sections of 5 µm thick were stained with hematoxylin and eosin (H&E).

Statistical analysis

Data were statistically analyzed by the aid of Statistical Package of the Social Sciences, SPSS version 23 (copyrighted by IBM SPSS software, USA). Data were expressed as a mean ± standard error of mean (SEM).

3. Result and Discussion:

The GC/MS analysis:

Diethyl ether fraction consists of 48 compounds. The total peak areas of the detected compounds is 98.60 % **(Figure 1)**.

The probabilities of the structures of the detected compounds are listed in **(Table 2)**. The major compounds **(Figure 2)** are Borneol $C_{10}H_{18}O_2$ (11.28%), (Isoborneol) $C_{10}H_{18}O$

(5.21%), phenol,2,6-bis(1,1-dimethylethyl)-4-methyl $C_{15}H_{24}O$ (5.17%), bicyclo [3.2.0] heptan-2¬-one,5-formyl methyl-¬6-¬hydroxy¬ 3,3'-dimethyl-6-vinyl $C_{13}H_{18}O_3$ (4.72%), longifolen aldehyde C_{15} $H_{24}O$ (5.49%), isoaromadendrene epoxide $C_{15}H_{24}O$ (4.74%), spathulenol $C_{15}H_{24}O$(4.60%), cis5,8,11,14, 17 - eicosapentaenoic acid $C_{20}H_{30}O_2$ (5.55%) and 3-cyclo hexyl propionic acid methyl ester $C_{10}H_{18}O_2$ (5.79%) for which represented 52.55 % of the total peak areas.

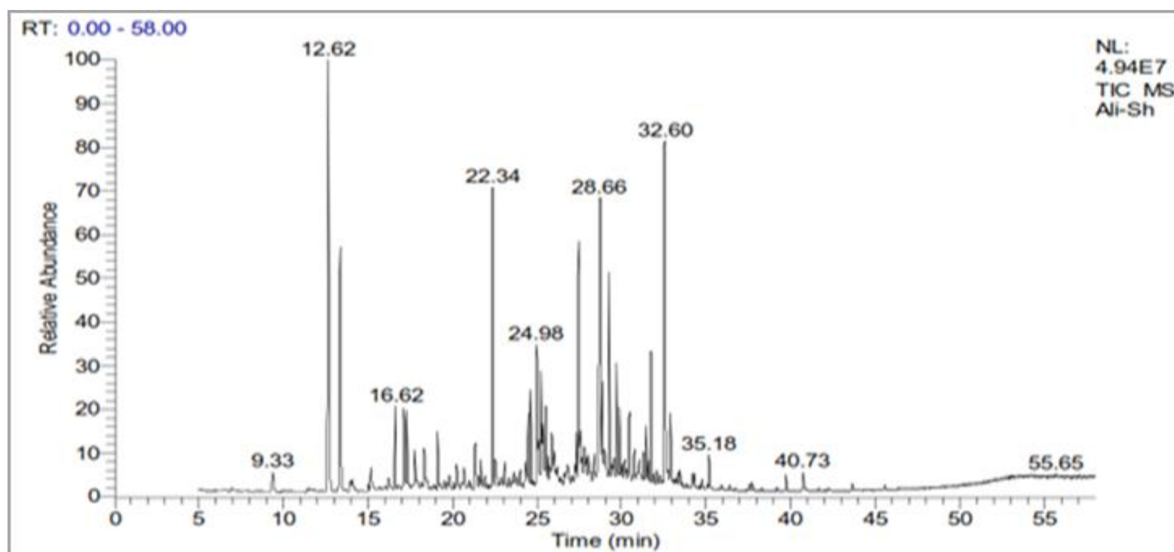

Fig. (1): GC/MS Analysis of *C. montanus*.

Table (2): GC/MS Analysis of Diethyl Ether Fraction of *C. montanus*:

NO	Identified Compounds	R_t min.	MW	MF	Area %
1	1,8-Cineole	9.33	154	$C_{10}H_{18}O$	0.61
2	1,7,7-Trimethylbicyclo[2.2.1] heptan-2-ol, (Borneol)	12.62	170	$C_{11}H_{18}O$	11.28
3	1,7,7-Trimethyl, exo, bicyclo [2.2.1] heptan-2-ol (Isoborneol)	13.33	154	$C_{11}H_{18}O$	5.91
4	α-Citronellol	15.16	156	$C_{10}H_{20}O$	0.61
5	Bornyl acetate	16.62	196	$C_{12}H_{20}O_2$	1.72
6	3-Methyl-3-(4-methyl-3-pentenyl), oxiranemethanol	17.13	170	$C_{10}H_{18}O_2$	1.57
7	Verbenol	17.73	152	$C_{10}H_{16}O$	1.27
8	2-Pentadecyn-1-ol	18.32	224	$C_{15}H_{28}O$	0.89
9	1-Methyl-4-(2methyl-oxiranyl) - *p*- Menthane, 1,2:8,9 -diepoxy, 7-Oxabicyclo[4.1.0] heptane	19.12	168	$C_{10}H_{16}O_2$	1.11
10	9,12,15-Octadecatrienoicacid, 2-(acetyloxy)-1-[(acetyloxy) methyl]ethyl ester	20.21	436	$C_{25}H_{40}O_6$	0.74
11	Cis *p*-Mentha17,8-dien-2-ol	20.68	152	$C_{10}H_{16}O$	0.73
12	α-Bisabolol	21.37	222	$C_{15}H_{26}O$	1.96
13	cis à-Bisabolene epoxide	21.67	220	$C_{15}H_{24}O$	0.52
14	2,6bis (1,1Dimethylethyl)4-methyl phenol	22.34	220	$C_{15}H_{24}O$	5.17
15	Corymbolone	23.09	236	$C_{15}H_{24}O_2$	2.31
16	E- Farnesene epoxide,	24.31	220	$C_{15}H_{24}O$	0.62
17	Cis-Farnesol	24.47	222	$C_{15}H_{26}O$	1.58
18	Isoshyobunone	24.61	220	$C_{15}H_{24}O$	1.73
19	Caryophyllene oxide	24.98	220	$C_{15}H_{24}O$	2.41
20	Ledene oxide(II)	25.05	220	$C_{15}H_{24}O$	1.10
21	γ-Eudesmol	25.13	222	$C_{15}H_{26}O$	0.58
22	10-[(Tetrahydro-2-Hpyran-2-yl)oxy], 1-decanol	25.29	258	$C_{15}H_{30}O_3$	1.64
23	1-(Hydroxymethyl)2,5,5,8-Atetrametyldecahydro 2-naphthalenol	25.53	240	$C_{15}H_{28}O_2$	1.32
24	Lilac alcohol D	25.88	170	$C_{10}H_{18}O_2$	0.52
25	9,12,15-Octadecatrienoic acid, 2,3dihydroxypropyl ester,	25.94	352	$C_{21}H_{36}O_4$	0.63
26	Ledene oxide(II)	26.74	220	$C_{15}H_{24}O$	0.48
27	2,6-Dimethyl-8-tetrahydropyranyloxy-2,6-octadien-1-ol	27.32	254	$C_{15}H_{26}O_3$	0.84
28	5-Formylmethyl-6-hydroxy 3,3-dimethyl-6-vinyl, Bicyclo [3.2.0] heptan-2-one	27.46	222	$C_{13}H_{18}O_3$	4.72
29	Bisabolol oxide A	27.61	238	$C_{15}H_{26}O_2$	0.50
30	Hexadecadienoic acid, methyl ester	27.81	266	$C_{17}H_{30}O_2$	0.62
31	4-(6,6-Dimethyl-2-methylenecyclohex-3-enylidene) pentan-2-ol	28.40	206	$C_{14}H_{22}O$	0.90
32	Longifolen aldehyde	28.66	220	$C_{15}H_{24}O$	5.49
33	Isoaromadendrene epoxide	28.74	220	$C_{15}H_{24}O$	4.74
34	Trans Z-α-Bisabolene epoxide	28.84	220	$C_{15}H_{24}O$	1.28
35	Spathulenol	29.24	220	$C_{15}H_{24}O$	4.60
36	1-(3-Hydroxypropyl)5,5,8-α-trimethyl decahydro naphthalen-2-ol	29.55	254	$C_{16}H_{30}O_2$	0.61
37	9,10-Dimethyltricyclo[4.2.1.1(2,5)] decane-9,10-diol	29.69	196	$C_{12}H_{20}O_2$	2.24
38	2,5-Octadecadiynoic acid, methyl ester	29.88	290	$C_{19}H_{30}O_2$	1.26
39	*n*-Decanol tetrahydro pyran ether	30.20	242	$C_{15}H_{30}O_2$	0.49
40	Methyl arachidonate	30.47	318	$C_{21}H_{34}O_2$	2.20
41	Geranyl isovalerate	30.77	238	$C_{15}H_{26}O_2$	1.04
42	Tetrahydro isovelleral	31.29	236	$C_{15}H_{24}O_2$	0.50
43	Soaromadendrene epoxide	31.41	220	$C_{15}H_{24}O$	1.38
44	Nerolidolepoxyacetate	31.73	296	$C_{17}H_{28}O_4$	2.32
45	Cis-5,8,11,14,17-Eicosapentaenoic acid	32.53	302	$C_{20}H_{30}O_2$	5.55
46	(S)-3-Cyclohexyl-2-hydroxy-propionic acid methyl ester	32.60	170	$C_{10}H_{18}O_2$	5.79
47	2-Methyl-E,E-3,13-octadecadien-1-ol	32.89	280	$C_{19}H_{36}O$	1.71
48	1- (1-Hydroxy-1-heptyl)-2-methylene-3-pentyl cyclopsropane	35.18	238	$C_{16}H_{30}O$	0.82
	Total identified compounds				98.61%
	Total unidentified compounds				1.39%

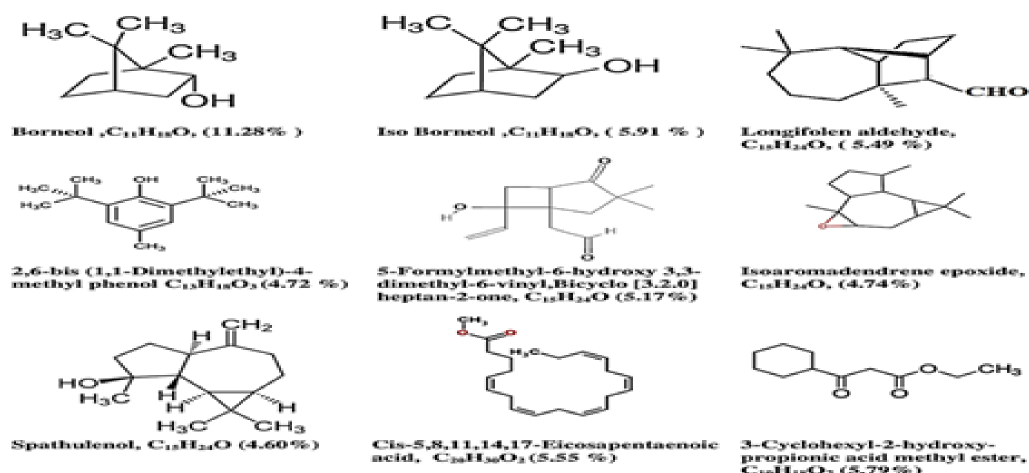

Fig. (2): Major compounds identified by GC-MS analysis of *C.montanus*

Hepatoprotective Activities

Impact on Hematological Parameters

The consequences of hematological parameters in **(Table 3)** revealed that the kind of treatment fundamentally influenced all the read blood parameters aside from the PLT check that didn't show any critical contrasts among all the examined groups. Rats of CCl_4-managed bunch indicated a prominent decrease in the RBC and WBC checks, Hb substance and PCV, as compared with the controls. When contrasted with the rats of CCl_4-treated group, the rats administered *C. montanus* methanolic extract after CCl_4 administration showed critical heights in the RBC, WBC tallies, Hb substance and PCV. In accordance with our results, the information **[27]** revealed that rats treated with CCl_4 for 45 days fundamentally diminished the red platelet tally (RBC), white platelet check (WBC), stuffed cell volume (PCV), and Hb levels while Nigella sativa treatment altogether expanded the decreased RBC, WBC, PCV, and Hb levels.

Table (3): Impact of oral administration of CCL_4 alone or with various *C. montanus* methanolic extract, on certain hematological parameters of male albino rats

Parameters	Experimental groups		
	Control	**CCL_4**	***C .montanus* MeOHextrat+ CCL_4**
Red blood cell count (×1012 L^{-1})	5.99 ± 0.31	6.81 ± 0.6	6.02 ± 0.3
White blood cell count (×109 L^{-1})	5.9 ± 0.82	16.5 ± 1.9	13.4 ± 0.7
Platelet count (×109 L^{-1})	471 ± 43.3	793.5± 43.6	452.6 ± 30.8
Hemoglobin content (g d L^{-1})	12.6 ± 0.38	10.3 ± 0.3	11.3 ± 0.9
Packed cell volume (%)	34.2 ± 1.23	40.1 ± 1.9	37.1 ± 3.4

Data are represented as mean ± standard error.

Effect on Serum Biochemical Parameters

The lipid profile of the exploratory creatures as influenced by the administration of CCl_4 alone, *C. montanus* methanolic extract in addition to CCL_4 are were showed in **(Table 4)**. The serum levels of TL, TC, TG, LDL-C and HDL-C of the rats were especially affected by the sort of treatment. In contrast with control group, all the contemplated lipid profile parameters of CCL_4-treated group were altogether raised aside from the degrees of HDL-C that were quite diminished. Then again, rats treated *C. montanus* methanolic extract in addition to CCL_4 showed a checked decrease in the levels of TL, TC, TG and LDL-C, as contrasted and the CCL_4-treated group. After the effects of the current investigation have additionally settled that CCl_4 treatment might have influenced the lipid digestion of liver (fatty oil and cholesterol levels). This is confirmed from the current perceptions in which CCl_4 caused a critical ($p < 0.05$) increment in the degrees of lipid parameters. In this association **[28]**, CCl_4 inebriation is like hepatitis in the occurrence of the fatty

substances catabolism. This circumstance could be additionally credited to the decrease of lipase action, which could prompt decline in fatty substance hydrolysis **[29]**. Then again, hypercholesterolemia in CCl_4 inebriated rats may be due to harm of hepatic parenchymal cells that lead to aggravation of lipid digestion in liver **[30]**. Be that as it may, rats treated with *C. montanus* methanolic extract demonstrated a significant ($p < 0.05$) decrease in triacylglycerol and cholesterol esteems contrasted with CCl_4-inebriated rats. The system of lipid bringing down impacts of *C. montanus* methanolic extract may be credited to an inhibitory action on microsomal acyl coenzyme A: cholesterol acyltransferase in vitro. This chemical is answerable for acylation of cholesterol to cholesterol esters in liver **[31].**

Table (4): Effect of oral administration of CCL_4 alone or with various *C. montanus* methanolic extract, on the groupings of serum all out lipid (TL), all out cholesterol (TC), fatty substances (TG), low thickness lipoprotein cholesterol (LDL-C) and high-thickness lipoprotein cholesterol (HDL-C) of male albino rats

Parameters	Experimental groups		
	Control	CCL_4	*C. montanus* MeOH extract + CCL_4
TP ($g\ d\ L^{-1}$)	6.68 ± 0.22	6.27 ± 0.3	6.38 ± 0.7
A ($g\ d\ L^{-1}$)	4.42 ± 0.13	3.2 ± 0.2	3.9 ± 0.1
G ($g\ d\ L^{-1}$)	2.46 ± 0.24	3.9 ± 0.1	2.9 ± 0.1
A/G ratio	1.72 ± 0.16	0.95 ± 0.07	1.35 ± 0.09

Data are represented as mean ± standard error.

Serum protein profile of various groups of rats in **(Table 5)** was observably influenced by the kind of treatment as rats controlled CCL_4 alone showed stamped decreases in the degrees of albumin concurrent with a critical expansion in the degrees of globulin, when contrasted with the controls. Along these lines, the A/G proportion of this group was astoundingly diminished. Then again, the rats of *C. montanus* extract in addition to CCL_4 - treated groups showed a checked expansion in the degrees of albumin and A/G proportion however a stamped decline in the degrees of globulin, when contrasted with the CCL_4-treated group.

Table (5): Impact of oral administration of CCL_4 alone or with various *C. montanus* extract, on the centralizations of serum complete protein (TP), albumin (A), globulin (G) and A/G proportion of male albino rats

Parameters	Experimental groups		
	Control	CCL_4	*C. montanus* MeOH extract + CCL_4
TL ($mgdL^{-1}$)	512.04± 43.06	663.6 ± 42.3	540.80 ± 31.76
TC ($mgdL^{-1}$)	118.20± 2.97	230.8 ± 20.3	180.8 ± 4.5
TG ($mgdL^{-1}$)	104.40± 7.34	171.8 ± 14.5	121.40 ± 8.3
LDL-C ($mgdL^{-1}$)	61.20 ± 9.87	161.4 ± 16.7	78.8 ± 4.9
HDL-C ($mgdL^{-1}$)	36.60 ± 6.40	28.6 ± 3.5	32.8 ± 4.3

Data are represented as mean ± standard error.

In this examination the critical ($p < 0.05$) decline in serum albumin of rats treated with CCl_4 when contrasted with control may shows helpless liver capacities or debilitated union, either essential as in liver cells harm or optional to decreased protein consumption and diminished assimilation of amino acids brought about by a malabsorption conditions or ailing health, or misfortune protein in pee, because of nephritic disorder and constant glomerulonephritis **[32]**.Then again, a huge ($p < 0.05$) increment in grouping of serum egg whites was seen in rats got *C. montanus* methanolic extract in addition to CCl_4 in contrast with rats got CCl_4 alone. The expansion of egg whites fixation after treatment with *C. montanus* methanolic extract might be credited to the diminishing in lipid peroxidation cycles and expansion in the activities of plasma protein thiols because of the treatment **[33].**

Liver capacity markers, as impacted by the administration of CCL_4 - *C. montanus* extract alone and blended, were introduced in **(Table 6)**. The activities of ASAT, ALAT and ALP and TBil, in serum of rats were altogether influenced by the kind of treatment, though the serum levels of DBil were not influenced by any of the contemplated factors. In contrast with the controls, the CCL_4-treated rats demonstrated critical rises in the activities of ASAT and ALAT and ALP just as the degrees of TBil. Despite what might be expected, the activities of ALP, ASAT and ALAT just as the degrees of TBil and DBil of *C. montanus* extract in addition to CCL_4 - treated rats were not essentially not quite the same as those of the benchmark group.

In the current investigation serum hepatic biomarkers, AST and ALT activities were extraordinarily expanded ($p < 0.05$) in rats treated with the CCl_4 contrast with control. As in the current examination, past investigations have demonstrated that $CC1_4$ expanded essentially serum ALP levels, and absolute protein and egg whites levels **[34,**

35].The expanded serum levels of hepatic markers have been credited to the liver injury, in light of the fact that these proteins are found in cytoplasmic region of the cell and they are delivered into flow if there should be an occurrence of cell harm **[36].** Then again, treatment with C. montanus extract in addition to CCL_4 was found to stifle ($p < 0.05$) the expansion of serum AST and ALT activities. As per the current outcomes, numerous other plant removes were accounted for to have impressive restorative impacts on liver injury actuated by substance specialists, for instance, administration of poly phenolic extricates from chicory (Cichorium intybus) came about in completely standardization of the serum AST and ALT levels in mice presented to thioacetamide, a hepatotoxic organosulfur compound **[37]**. Comparative impacts from barberry extractupon administration to CCl_4 actuated hepatotoxic creatures have additionally revealed **[38]**. These finding infers that challenge to shield liver tissue from CCl_4 injury.

Table (6): Impact of oral administration of CCL_4 alone or with various *C. montanus* extract, on the activities of serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and antacid phosphatase (ALP) and the degrees of complete bilirubin (TBil) and direct bilirubin (DBil) of male albino rats

Parameters	Experimental groups		
	Control	**CCL_4**	***C. montanus* MeOH extract + CCL_4**
ASAT ($U\ L^{-1}$)	33.02 ± 1.30	109.3 ± 25.9	72.6 ± 10.6
ALAT ($U\ L^{-1}$)	25.60 ± 1.50	69.6 ± 9.7	46.9 ± 8.3
ALP ($U\ L^{-1}$)	55.30 ± 3.84	79.2 ± 11.8	63.4 ± 10.1
TBil ($mg\ d\ L^{-1}$)	0.66 ± 0.02	0.94 ± 0.05	0.81 ± 0.04
DBil ($mg\ d\ L^{-1}$)	0.11 ± 0.005	0.15 ± 0.009	0.11 ± 0.005

Data are represented as mean ± standard error.

Impact on the hepatic lipid peroxidation and endogenous cancer prevention agents: The impacts of CCL_4 alone or with *C. montanus* methanolic extract administration on the degrees of hepatic MDA and GSH and the activities of endogenous cell reinforcement proteins were appeared in **(Table 7**). The hepatic degrees of MDA and GSH just as the activities of CAT, SOD and GR were essentially impacted by the kind of treatment. In the liver of rats regulated CCL_4 alone, there was an important height in the degrees of MDA joined by a stamped decrease in the GSH substance, SOD and GR activities when contrasted with those of controls. In the rats of *C. montanus* methanolic extract plus CCL_4 - treated groups, the mean estimations of hepatic MDA fixation were fundamentally lower than those of CCL_4-treated rats and were not essentially not the same as those of the controls. Then again, the mean estimations of hepatic GSH substance of *C. montanus* extract in addition to CCL_4 - treated rats were essentially higher than those of CCL_4-treated group. When contrasted with the CCL_4-treated group, the rats directed *C. montanus* extract in addition to CCL_4 indicated a stamped height in the activities of CAT and SOD and GR, that didn't essentially contrast from those of the controls.

Information of the current investigation is as per the discoveries of different specialists, for example, the scientist **[39]** who revealed that hepatotoxic impacts by CCl_4 are lipid peroxidation root, and are to a great extent because of its dynamic metabolite CCl_3 (This metabolite can extract hydrogen from unsaturated fats, starting the lipid peroxidation), lead to cell injury, lastly liver harm. Additionally, the another scientist **[40]** expressed that the adequacy of any hepatoprotective medication is subject to its ability of either diminishing the hurtful impact or reestablishing the ordinary hepatic physiology that has been dispersed by a hepatotoxin. In this association, the current investigation uncovered that *C. montanus* methanolic extract decreased ($p < 0.05$) CCl_4 prompted raised catalyst levels in tried groups, showing the assurance of primary uprightness of hepatocytic cell film or recovery of harmed liver cells. As recently noted and like the outcomes accomplished for different plants in the writing **[41, 42]** our perceptions and discoveries can be ascribed to the cell reinforcement elements of *C. montanus* extract that most likely repress lipid peroxidation and thusly restraint of oxidative pressure. Subsequently, the cell films stay unblemished and thus cells are forestalled to enter the corruption step.

Table (7): Effect of oral administration of CCL_4 alone or with various *C. montanus* methanolic extract, on the degrees of hepatic malondialdehyde (MDA) and glutathione (GSH) and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) of male albino rats

Parameters	Experimental groups		
	Control	**CCL_4**	***C. montanus* MeOH extract + CCL_4**
MDA ($nmol\ g^{-1}$ liver)	4.48 ± 0.11	9.8 ± 0.6	5.8 ± 0.4
GSH ($mg\ g^{-1}$ liver)	40.04 ± 5.10	21.7 ± 7.8	34.1 ± 9.1
CAT ($U\ g^{-1}$ liver)	104.3 ± 17.1	45.4 ± 9.7	91.6 ± 16.4
SOD ($U\ g^{-1}$ liver)	9.56 ± 0.17	5.6 ± 0.9	7.9 ± 0.5
GR ($U\ g^{-1}$ liver)	73.20 ± 2.71	34.8 ± 2.8	62.6 ± 3.3

Data are represented as mean ± standard error.

Assurance of Serum Caspase-3 Action:

The information in **(Table 8)** uncovered that CCl_4 inebriation created a critical height in serum caspase-3 action arriving at 324% of the benchmark group. Then again, administration of *C. montanus* methanolic extract altogether decreased serum caspase-3 movement when contrasted with CCl_4-inebriated group arriving at 215 and 182% of the benchmark group, separately.

Table (8): Effect of *C. montanus* methanolic extract on serum caspase-3 action following CCl_4 inebriated rats

Groups \ Parameters	Serum Caspase-3 (µg/ml)
Control	1.6 ± 0.18
CCl_4 alone	5.7 ± 0.17
CCl_4 + *C. montanus* MeOH extract	4.1± 0.9

Values are expressed as mean ± S.E.M. (n=10)

Quantitative converse record polymerase chain response (qRT-PCR) for investigation of serum Bax and BCl2 mRNA levels:

The CCl_4 inebriation created an increased guideline of Bax mRNA serum level alongside an undeniable down-guideline of Bcl2 mRNA level by practically 11.22 and 0.19 folds of the benchmark group, individually **(Table 9)**. Administration of one or the other *C. montanus* methanolic extract reduced the serum level of Bax mRNA, however raised that of Bcl2 mRNA when contrasted with CCl_4-inebriated group arriving at 4.31 and 0.55 folds of the benchmark group, individually for *C. montanus* methanolic extract- treated group and 6.08 and 0.65 folds of the benchmark group, individually for *C. montanus* methanolic extract treated group.

Our outcomes are in fortuitous event with those of different specialists who announced that particular activator of peroxisome proliferator-enacted receptor-α (PPARα) **[43, 44]**. Such Activation causes an upregulation of mRNA and protein levels of various peroxisomal and non-peroxisome-related compounds and primary proteins **[45]**.

Table (9): Effect of *C. montanus* methanolic extract on the serum mRNA level of Bax and BCl_2 in CCl_4 inebriated rats

Groups \ Parameters	Serum Bax mRNA	Serum BCl_2 mRNA
Control	1.02 ± 0.01 fold	1.15 ± 0.003 fold
CCl_4 alone	11.098 ± 1.290 fold	0.17 ± 0.02 fold
CCl_4+*C.montanus* MeOH extract	5.6 ± 0.18 fold	0.55 ± 0.04 fold

Values are expressed as mean ± S.E.M. (n=10).

Likewise, the current outcomes at the sub-atomic level, indicated a generously high articulation of the favorable to apoptotic protein (Bax) in the liver tissues of CCl_4-inebriated rats, while the counter apoptotic protein; Bcl-2 was significantly down-controlled. Together, this suggests that the Bax/Bcl-2 proportion, a primary list for apoptosis, may be significantly raised; demonstrating CCl_4–upgraded apoptosis in the livers of inebriated rats. Likewise, the current investigation indicated a critical hindrance of serum caspase-3 action in CCl_4-inebriated group. This might be ascribed to the over creation of ROS which actuated a huge rise of caspase¬3 movement prompting apoptotic condition **[46]**.

Our discoveries are upheld by past reports that specific compound specialists incited apoptosis in the rat kidney and liver straightforwardly through the change of apoptotic quality articulation **[47]**. Group confirmations have demonstrated that these specialists could actuate cell apoptosis by focusing on the mitochondrial apoptotic pathway **[48]**. This might be through the enactment of cytochrome c delivery starting from the mitochondria, guideline of Bcl-2 articulation, up-guideline of Bax articulation, movement of Bax into the mitochondrial film, and initiation of caspase-3 **[49]**.

Our examination uncovered that treatment of CCl_4-inebriated rats with the *C. montanus* methanolic extract down-regulated the declaration of Bax and up-managed Bcl-2 articulation. The current outcomes additionally announced huge hindrance of caspase-3activity in groups treated with *C. montanus* methanolic extract.

Assurance of percent of DNA damage by comet assay in liver tissues:

The information in **(Table 10)** and figures 3-4 uncovered that CCl_4 liver inebriation delivered a huge height in tail second contrasted with control group of rats. Then again, administration of one or the other *C. montanus* methanolic extract plus CCl_4 altogether decreased tail second and thusly critical decrease in the percent of DNA harm when contrasted with CCl_4-inebriated group in contrast with the benchmark group.

Table (10): Effect of *C. montanus* methanolic extract on the level of DNA harm in the liver tissue of CCl_4 – inebriated rats

Groups	Tailed cell (%)	Untailed (%)	Tail Length (µm)	DNA Tail (%)	Tail Moment units
Control	5.2 ± 1.2	94.8 ±1.1	1.7 ± 0.2	1.8 ± 0.15	4.6 ± 0.8
CCl_4 alone	31.7 ±3.7	68.3 ±4.5	3.9 ±0.07	4.3 ± 0.2	12.1 ±0.3
CCl_4+*C.montanus* MeOH extract	18.6 ±1.3	81.4 ±0.73	2.6 ±0.07	3.5 ± 0.1	9.7 ± 0.6
CCl_4+ *C.montanus* MeOH extract	18.6 ±1.3	81.4±0.73	2.6 ±0.07	3.5 ± 0.1	9.7 ± 0.6

Values are expressed as mean ± S.E.M. (n=10).

Histopathological Results

Brain:

Tiny examination of control cerebrum areas of rats show profoundly dynamic neurons which having colossal pale-recolored cores, atomic chromatin and unmistakable nucleoli vanished. The glial cells encompassed the neurons and backing it. These cells have little thickly recolored cores with dense chromatin and no obvious nucleoli. Neuropil or foundation substances are appeared in the cortex **(Fig. 5)**. Assessment of areas of mind cortex of rats regulated with CCl_4 alone demonstrated dull neurons with unpredictable shape and glial cells that showed up inside white vacuoles. Neurofibrillary tangles recolored with red tone and seeming as though flares were established. The knot shows up as long pink fibers in the cytoplasm. The neuropil is seemed vacuolated **(Fig. 6)**. Photomicrograph of area in mind cortex of rat regulated with CCl_4 and *C. montanus* methanolic extract indicating the structure of neurons showed up pretty much like typical and customary

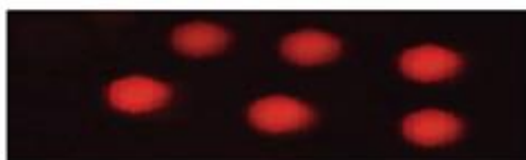

Figure 3: Visual score of DNA damage (class 0) using comet assay in liver tissues of untreated rats.

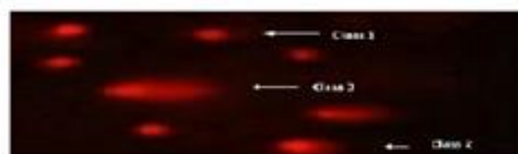

Figure 4: Visual score of DNA damage (classes 1, 2 and 3) using comet assay in liver tissues of treated rats.

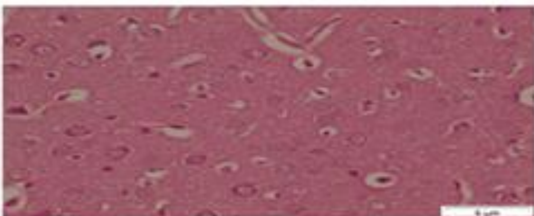

Figure 5: Photomicrograph of section in brain cortex of control rat shows the nerve cells (neurons) that having pale-stained huge nuclei, disappeared nuclear chromatin and prominent nuclei, surrounding support cells (glial cells) having small nuclei with densely stained, condensed chromatin with no visible nucleoli, background substance (neuropil) and perivascular space are shown in the cortex (H and E. Scale bar 5 µm).

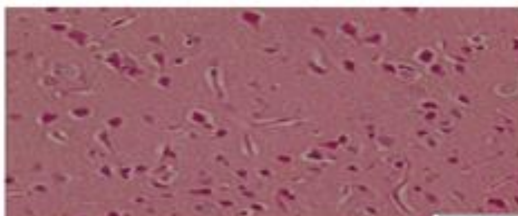

Figure 6: Photomicrograph of section in brain cortex of rat administered with CCl_4 alone showing dark neuron with irregular shape and glial cells that appeared inside white vacuoles. Neurofibrillary tangles stained with magenta color and looking like flames were founded. The tangle appears as long pink filaments in the cytoplasm. The neuropil is appeared vacuolated (H and E. Scale bar 5 µm).

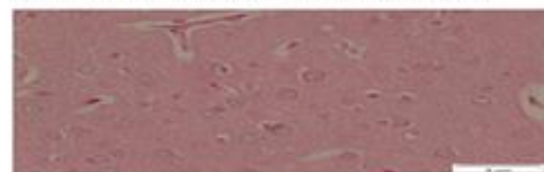

Figure 7: Photomicrograph of section in brain cortex of rat administered with CCl_4 and *C. montanus* methanolic extract showing the normal structure of neuron with regular shape and glial cells). Notice few dark neurons are found (H and E. Scale bar 5 µm).

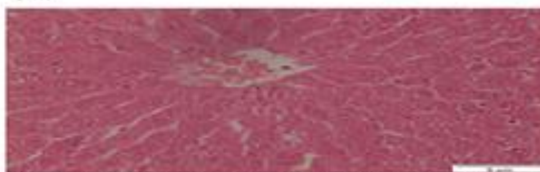

Figure 8: Photomicrograph of section in liver of control rat shows normal histological structure of hepatic lobules central vein, hepatocytes, blood sinusoids, and nuclei (H&E, ×400).

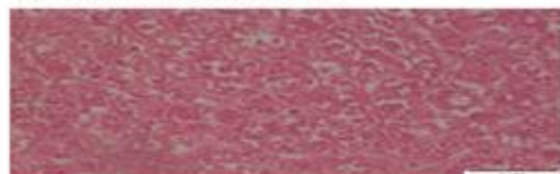

Figure 9: Photomicrograph of section in liver of rat administered with CCl_4 alone showing disruption of the liver tissue with loss of lobular arrangement, bridging fibrosis with collagenous septa formation expanded portal tract to central vein with mononuclear cells, vacuolar degeneration and necrosis of hepatocytes. Dilated and congested central vein was observed and pyknotic nuclei (H&E, ×400).

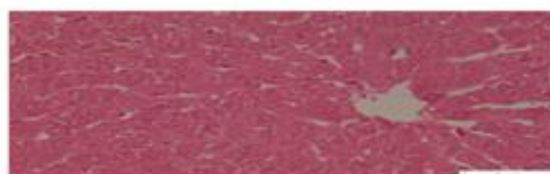

Figure 10: Photomicrograph of section in liver of rat administered with CCl_4 and *C. montanus* methanolic extract showing showing mild inflammatory cells infiltrations around central vein (arrow), vacuolar degeneration, and necrosis of hepatocytes (star). Binucleated and activated Kupffer cells were noticed (H&E, ×400).

shape **(Fig. 7).**

Liver:

Minuscule assessments of areas of liver from ordinary control rats show the typical engineering of hepatic lobules. The focal veins lies at the focal point of the lobules encompassed by ropes of hepatocytes. Between the strands of hepatocytes, the hepatic sinusoids are seen **(Fig. 8)**. Histopathological examination of liver from rats regulated with CCl_4 alone demonstrating interruption of the liver tissue with loss of lobular plan, spanning fibrosis with collagenous septa development extended gateway lot to focal vein with mononuclear cells, vacuolar degeneration and corruption of hepatocytes (Fig. 9). Liver segments of rats directed with CCl_4 and *C. montanus* methanolic extract demonstrating gentle incendiary cells invasions around focal vein, vacuolar degeneration, and putrefaction of hepatocytes. Binucleated and actuated Kupffer cells were seen **(Fig. 10).**

In the current examination, the biochemical discoveries were likewise affirmed by histopathological perceptions. The progressions generally incorporate hepatocellular putrefaction or apoptosis, greasy group, provocative cells invasion and other histological appearances which were likewise reliable with the discoveries of different creators .

4. Conclusions

GC/MS of *C. montanus* showed 48 compounds; nine of them are major and represent more than 50% of the total components and hepatoprotective effects of *C. montanus* methanolic extract on CCl_4-induced hepatic damage in male Wistar rats were observed in the present study. Probably, antioxidative properties of the extract helped hepatic cells to obviate CCl_4-induced necrosis and inflammation which can be also observed in histopathological findings.

The results obtained here and the reports from previous studies suggest that *C. montanus* methanolic extract may function as a good candidate for the treatment or prevention of liver failure. However, further investigations are required to unveil the molecular identification of the active ingredients and elucidation of the mechanisms involved in the effect.

5. Conflicts of interest

6. Formatting of funding sources

National research center

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