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Hepatoprotective Effect of *Carduus getulus* Pomel in relation to its metabolite fingerprint as analyzed *via* UPLC-MS technique

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UPLC/PDA/ESI-qTOF-MS technique was employed for the analysis of *C. getulus* extract that led to the detection of 32 metabolites, flavonoids of apigenin and luteolin-*O*-glycosides, caffeoylquinic acid isomers and fatty acids which were reported for the first time in *C. getulus*. Further, chromatographic techniques and spectral analyses provided unequivocal assignment of a total of 6 flavonoids *viz*., 3 luteolin conjugates, 2 apigenin derivatives and a tricin-glycoside. In addition, toxicity and hepatoprotective activity of the analyzed extract were determined. The extract demonstrated a significant improvement in the biochemical and antioxidant parameter levels with a slight improvement effect on liver tissue. Thus, this study suggests a moderate hepatoprotective effect of *C. getulus* likely mediated *via* its enrichment in luteolin and apigenin conjugates.

Keywords: *Carduus getulus*; UPLC-MS analysis; chromatography; flavonoids; hepatoprotective; toxicity.

INTRODUCTION

Liver injury i.e. Hepatitis C is a worldwide problem that is endemic in Egypt with no effective safe natural drug recognized for its treatment till now except for the drug ''silymarin''isolated from *Carduus marianus* (Morazzon and Bombardelli, 1995). Consequently, identification of other natural anti-oxidants that are of plant origin, from *Carduus* members, may help in healing or improving hepatic injuries. *Carduus* genus encompasses 90 species of thistles in Asteraceae (Azizi *et al.*, 2013), one of them is *Carduus getulus* Pomel growing in western Mediterranean coastal region of Egypt (Abdel Majeed *et al.*, 2009). *Carduus getulus*, known as hashroof by natives in Egyptian desert, is a wild annual herb with few phytochemical and biological reports. Among reported biological effects include antimicrobial screening and liver function for *C. getulus* (Abdel Rahman et al., 2011, El Sohafy et al., 2013) respectively. Sterols including taraxasterol acetate, taraxasterol, *β*-sitosterol and its glucoside, and three flavonoids (luteolin-4'-methoxy-7-*O*-glucoside, quercetin-3,3'-di-methoxy-7-*O*-glucoside and kaempferol-3-methoxy-7-*O*-rhamnoside) were isolated from the same plant (Abdel-salam et al., 1983).

Other *Carduus* members exhibit a myriad of biological activities i.e. antimicrobial activity of *C. pycnocephalus* attributed to its flavonoids content (Abd-Ellatif et al., 2011, Hassan et al., 2015), antioxidant activity (Hassan et al., 2015, Jeong et

al., 2008), anti-inflammatory, anti-nociceptive properties (Messay et al., 2012, Al-Shammari et al., 2015) and cytotoxic activity (Hassan et al., 2015). Classes of secondary metabolites identified in *Carduus* sp. include phenolic acids (Kozyra et al., 2017), lignans, flavonoids and triterpenes (Fernández et al., 1991, Tundis et al., 2000, Zhang et al., 2001, Messay et al., 2012, Elwekeel et al., 2013, Kozyra et al., 2013, Hassan et al., 2015,), alkaloids (Xie et al., 2004) and volatile constituents (Esmaeili et al., 2005).

This study included metabolites profiling and isolation attempt for *C. getulus* secondary metabolites in addition to the estimation of LD_{50}, sub-chronic toxicity and assessment of antihepatotoxic activity. To provide detailed coverage of *C. getulus* metabolome ultra performance liquid chromatography coupled to high resolution mass spectrometry was employed and leading to the annotation of 32 metabolites.

MATERIALS AND METHODS

1 Plant material and animals

Carduus getulus aerial parts were collected during April 2012 from Elqasr- 10 km, Agibaa-Elobayed road- Mersa Matroh, Egypt. The plant material was identified by Dr. Attia Mohammed, Desert Research Center-Egypt (voucher No.: 5/3/2015-A, Fac. of Pharmacy, Cairo Univ.) and the plant name has been checked with (http://www.theplantlist.org). In the current study, rats weighing 200-250 g and mice weighing 25-30 g of both sexes aged 10-16 weeks were purchased from the Laboratory animal colony, Ministry of Health and Population, Helwan, Cairo, Egypt. Rats were maintained under standard laboratory conditions at the animal center, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. They were fed with basal diet pellets (El-Nasr Company, Abou- Zaabal- Cairo- Egypt), supplied with water ad libitum and kept in a temperature-controlled environment (20-22°C) and 40%–60% relative humidity with an alternating 12 h light-dark cycle. The experiments were conducted in accordance with the ethical guidelines for research with laboratory animals and comply with the Guide for the Care and Use of Laboratory Animals.

2. Chemicals, reagents and materials

Acetonitrile and formic acid (LCMS grade) were obtained from J.T. Baker (Deventer, Netherlands), milliQ water was used for LC analysis. Umbelliferone (P98%), the used standard, was obtained from St. Louis, MO, USA. Ethanol, glacial acetic acid, n-butanol, neutral formalin, HCl, *o*-phosphoric acid, potassium chloride, sodium chloride, sodium dibasic phosphate were obtained from El-Nasr Pharm. Chem. Co. (Cairo, Egypt), anhydrous potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Fluka Lab. Chem. and Anal. Reagents Co. (Steinheim, Germany) and H_2O_2 were from Fisher scientific (Fair lawn, NJ, USA.). Polyamide and sephadex LH20 were obtained from (CarlRoth, Sweden) and (Pharmacia fine chemicals AB, Sweden) respectively. Shift reagents and chemicals for UV spectroscopic analysis of flavonoids (Mabry *et al.*, 1970) (NaOMe, $AlCl_3$, HCl, NaOAc and H_3BO_3) were obtained from Sigma Aldrich (USA). Silymarin, Ellman's reagent, thiobarbituric acid (TBA), reduced glutathione (GSH), catalase enzyme (CAT), 1,1,3,3-tetraethoxypropane, pyrogallol, trichloroacetic acid (TCA) and EDTA-Na_2 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA), while (CCl_4) was obtained from Loba-chemie Co. (Mumbai, India). Kits for the assessment of: ALT, AST, ALP, urea and creatinine (Maheshwari, 2008), total bilirubin and direct bilirubin, total proteins, albumin, HDL-cholesterol, total cholesterol, triglycerides (Ramakrishman and Sulochana, 2012) were provided from Spectrum Diagnostics TM and Diamond TM kits (Cairo, Egypt).

3. Extraction procedure for *C. getulus* aerial parts

To profile *C. getulus via* high resolution UPLC–PDA–MS analysis, freeze dried plant was ground with a pestle in a mortar using liquid nitrogen. 20 mg was then homogenized with 4 ml 70% MeOH containing 10 µg/ml umbelliferone (as internal standard) using an ultrasonic bath for 30 min. Extract was then vortexed and centrifuged at 10,000g for 10 min to remove plant debris and filtered through 22 µm Millipore filter. For large scale isolation and biological assays, other quantity (2 kg) of shade dried grinded sample was extracted on cold using 70 % aqueous ethanol till exhaustion. Alcoholic extract was evaporated under reduced pressure at 50 ºC till dryness to give 200 g of resinous residue.

4. Plant analysis via UPLC/PDA/MS

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100- 1.0 mm, particle size 1.8 µm; Waters) applying the following elution

binary gradient at a flow rate of 150 µL min^{-1}: 0–1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1–16 min, linear from 5–95% B; 16–18 min, isocratic 95% B; 18–20 min, isocratic 5% B. The injection volume was 3.1µL (full loop injection). C18 bonded phase used for the HSS T3 sorbents is compatible with 99.9% aqueous mobile phase and provides ultra-low MS bleed, while promoting superior polar compound retention which has been successfully used for profiling of similar plant matrices (Farag and Wessjohann, 2012, Farag et al., 2013). Eluted compounds from UPLC were detected from *m/z* 100 to 1000 using a MicroTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo-II electrospray ion source in negative ion modes using the following instrument settings: nebulizer gas, nitrogen,1.6 bar; dry gas, nitrogen, 6 l min^{-1}, 190 ºC; capillary,5500 V (+4000 V); end plate offset, 500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp. MicroTOF-Q: Precursor ions were selected in Q1 with an isolation width of ±2 D and fragmented in the collision cell applying collision energies in the range of 10–30 eV. Argon was used as collision gas. Product ions were detected using the following parameter settings: collision RF 150/400 Vpp (timing 50/50); transfer time, 70 ls; pre pulse storage, 5 ls; pulser frequency, 10 kHz; spectra rate, 1.5 Hz. For CID of in-source fragment ions (pseudo-MS3), in-source CID energy was increased from 0 to 100 V. Ion Trap MS: MSn mass spectra were also obtained from a LCQ Deca XP MAX system (ThermoElectron, San Jose, USA) equipped with a ESI source (electrospray voltage 4.0 kV, sheath gas: nitrogen; capillary temperature: 275-C) in negative ionization modes. The Ion Trap MS system is coupled with the exact Waters UPLC setup and using same elution gradient. The MSn spectra were recorded by using the following conditions: MS/MS analysis with starting collision-induced dissociation energy of 30 eV and an isolation width of ±2 D in a data dependent, negative ionization mode. UPLC–MS files were converted to netcdf file format using the File Converter tool in Bruker Daltoniks software and further processed using AMDIS software to assist in adjacent peak deconvolution and background subtraction (Halket et al., 1999). Metabolites were characterized by their UV–Vis spectra (220–600 nm), retention times, mass spectra and phytochemical dictionary of natural products database and reference literature. UV spectroscopy used for detecting λmax in MeOH and shift reagents (analytikjena, specord 200 spectrophotometer).

5. Chromatographic isolation of the constituents

130 g of the resinous semidried 70% ethanol extract was suspended in a small amount of water and applied on polyamide column (600 g) following gradual elution starting with 100 % water and increasing ethanol gradually (5, 10, 20 and 30% till 100%). Similar fractions were pooled together after investigation by PC using BAW (S1) and 15 % acetic acid (S2) as solvent systems, resulting in collection of 4 main fractions (Cg F1-F4) eluted with 20 %, 30-50 %, 60-80 % and 85-95 % ethanol/H_2O respectively. Fraction Cg F1(2.6 g) was further purified on sephadex LH-20 column by distilled water then on a preparative PC by BAW, followed by elution of the major band with ethanol, filtration and concentration of the filtrate under vacuum. Then, it was chromatographed again on sephadex LH-20 column by saturated n-butanol with water to obtain C_1 (6 mg). Whereas, Cg F2 (1.3 g) was applied to sephadex LH-20 column using 30% methanol/water to obtain 6 sub-fraction (Sf 1-6) of two main collectives (Sf 2-3, 0.4 g & Sf 4-5, 0.3 g). Both of them were reapplied separately on sephadex column using 30% methanol/water to obtain C_2 (17 mg) and C_3 (10 mg) respectively. Additionally, Fraction Cg F3 (0.9 g) was applied later on sephadex LH-20 column using saturated butanol with water to yield 2 main sub-fractions (Sf 4; 0.12 g & Sf 5-7; 0.3 g) containing two compounds C_4 and C_5 respectively. Each one of them was applied separately again on sephadex LH-20 column using 50% methanol/water for purification (C_4 9 mg & C_5 6 mg). Finally, Cg F4 (1.2 g) was chromatographed on sephadex LH-20 column using 60% methanol/water to obtain C_6 (12 mg).

6. NMR spectra measurement

^1H and ^{13}C spectra were recorded at 25 °C in CD_3OD and DMSO-d_6 on Bruker Avance III 400 & 100 MHz respectively. Chemical shifts were referenced to the solvent residual peaks at δH 3.33 and 2.50 for ^1H and δC 49.30 and 39.5 for ^{13}C for CD_3OD and DMSO-d_6, respectively.

7. Acute and subchronic toxicity

Lethality test (LD_{50}) was estimated in mice orally according to OECD-OECD guidelines No. 420. In a preliminary test, animals in four groups each of 5 animals received one of 5, 50, 300 and

2000 mg/ kg b. wt. of the tested extract suspended in the vehicle (3% v/v Tween 80). Animals were observed for 24 h for signs of toxicity and number of deaths. From these results, extra doses of 5, 7.5, 15 g/ kg b. wt. of the tested extract were administered. Control animals were received the vehicle and were kept under the same conditions without any treatments. Signs of toxicity and number of deaths per dose in 24 h were recorded and the LD_{50} was calculated as the geometric mean of the dose that results in 100% mortality and that which cause no lethality. 10 mature rats of both sexes (200- 250 g) were divided into two groups, for sub-chronic toxicity study, each of 5 animals. The first group, control rats received the vehicle. The second was orally administered *C. getulus* ethanol extract (500 mg/ kg b. wt.). Extracts were administered to animals orally by gastric intubation for 4 weeks.

8. Hepatoprotective activity

A total of 40 rats were divided into four groups; ten animals each and the schedule of treatment were adopted as follows, Group 1: rats were given corn oil (2 ml/ kg b. wt., s. c.) twice a week for two consecutive weeks and served as negative control group. Group 2: rats were injected CCl_4 (2 ml/ kg b. wt., s. c.) diluted in corn oil (50 % v/v) twice a week for two consecutive weeks. Group 3, 4: rats were treated with silymarin orally (50 mg/ kg b. wt.) and alcoholic extract of *C. getulus* orally (500 mg/ kg b. wt.), respectively daily for three consecutive weeks and injected CCl_4 (2 ml/ kg b. wt., s. c.) diluted in corn oil (50 % v/v) twice a week for two consecutive weeks (beginning from the 2nd week of the study). Extract and silymarin were administered to the animals orally by gastric intubation for 3 weeks (Dutta et al., 2013).

9. Serum and Tissue Preparation

Blood samples were collected from retro-orbital venous plexus under light ether anesthesia in non-heparinized tubes and the serum was separated for measuring bio-chemical parameters. Animals were later sacrificed with liver sample divided into two parts; first was formalin fixed for histopathological examination and the second part was homogenized for the determination of liver MDA and GSH contents as well as CAT and SOD enzymatic activities. Kidney samples for the chronic toxicity module were also kept for histopathological examination

10. Histopathological examination of the liver and kidney

Autopsy samples were taken from the liver and kidney of rats in different groups and fixed in 10% neutral buffered formalin for 24 h and decalcification was done in formic acid. Washing was performed with tap water, and then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µ thicknesses by sledge microtone. The obtained tissue sections were collected on glass slides; deparaffinized, stained by hematoxylin and eosin stain, and then examination was done through the light electric microscope (Banchroft et al., 1996).

11. Statistical analysis of data

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad prisim version 5 (GraphPad, San Diego, CA). Group differences were analyzed using one-way analysis of variance ANOVA followed by Tukey-Kramer for multiple comparison tests. The difference was considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

1. Metabolites profiling *via* UPLC-qTOF-MS

The comprehensive metabolite profile of *C. getulus* led to the identification of 32 metabolites. The identities, retention times, UV characteristics, and observed molecular and fragment ions for individual components are presented in Table 1.

Flavonoids (flavone, flavonols, and flavanones) and/or their conjugates, *O*-glycosides, were previously reported in *Carduus* sp. and MS spectral examination of *C. getulus* was in agreement with previous reports (Fernández et al., 1991, Zhang et al., 2001, Messay et al., 2012, Elwekeel et al., 2013, Kozyra et al., 2013, Hassan et al., 2015). UV-spectra revealed a flavone backbone for most detected flavonoid peaks except for that of peak 16. All flavonoids were of the *O*-glycoside type (Mabry et al., 1970). In MS analysis, the nature of the sugars could be revealed by the elimination of sugar residue, that is, 176 amu (hexouronic acid), 162 amu (hexose; glucose or galactose), 132 amu (pentose) and 146 amu (monodeoxyhexose).

Table (1): Peak assignments of metabolites in *C. getulus* 70% methanol extract analysed *via* UPLC–PDA-MS in negative ionization mode.

Peak	Rt (s)	UV	(M-H)- (m/z)	Formula	Error "ppm"	MSn product ions	Identification
1	116	274.9	315.1081	$C_{14}H_{19}O_8$	1.7	153 , 135, 123	Hydroxytyrosol hexoside
2	151.1	287.9, 326.3	353.0875	$C_{16}H_{17}O_9$	2	315	1-*O*-caffeoylquinic acid
3	196.5	287.9, 326.3	353.0877	$C_{16}H_{17}O_9$	2.4	191, 179,173	5-*O*-caffeoylquinic acid
4	201.2	287.9, 326.3	353.0876	$C_{16}H_{17}O_9$	0.5	191, 316.9	*O*-Caffeoylquinic acid isomer
5	208	Nd	623.1261	$C_{27}H_{27}O_{17}$	-1.2	447, 285	Luteolin-*O*-hexosyl-*O*-glucuronide
6	237	264.4, 314.8	607.1295	$C_{27}H_{27}O_{16}$	1.6	431, 285	Luteolin-*O*-glucuronide rhamnoside
7	244	268.4, 320.3	609.1443	$C_{27}H_{29}O_{16}$	3	447, 285	Luteolin-*O*-dihexoside
8	250.1	268.4, 327.8	563.1401	$C_{26}H_{27}O_{14}$	0.4	269	Apigenin-*O*-hexosyl -*O*-pentoside
9	261	253.4, 266.4, 343.3	579.1349	$C_{26}H_{27}O_{15}$	1	447, 285	Luteolin-*O*-hexosyl -*O*-pentoside
10	277	202.5, 268.4, 343.8	593.1503	$C_{27}H_{29}O_{15}$	1.6	447, 285	Luteolin-7-*O*-rutinoside (scolymoside)
11	285.1	206.5, 220, 253.9, 349.3	447.0925	$C_{21}H_{19}O_{11}$	1.8	296	Luteolin-7-*O*-glucoside (cynaroside)
12	290	269.9, 331.8	557.0591	$C_{25}H_{17}O_{15}$	-2.9	515, 447, 237	Unknown flavone glycoside
13	293.9	270.9, 321.3	541.0639	$C_{25}H_{18}O_{14}$	-1.9	201, 269, 307, 447	Unknown flavone glycoside
14	297	265, 345	591.1352	$C_{27}H_{27}O_{15}$	0.7	415 ,269	Apigenin-*O*-hexouronide -rhamnoside.
15	298.9	265.4, 337	577.1187	$C_{26}H_{25}O_{15}$	1.9	491	Tricin-*O*- malonyl hexoside
16	305	264.9, 346.3	447.0939	$C_{21}H_{19}O_{11}$	-0.6	285	Astragalin
17	312	264.9, 335.3	461.1077	$C_{22}H_{21}O_{11}$	2.6	285	Luteolin-*O*-glucuronide
18	314.4	266.4, 335	445.0766	$C_{21}H_{17}O_{11}$	1.8	269	Apigenin-*O*-Hexouronide
19	334.3	262, 343.8	431.0993	$C_{21}H_{19}O_{10}$	0.5	285	Luteolin-*O*-rhamnoside

20	336	263.4, 335.3	461.1081	$C_{22}H_{21}O_{11}$	1.8	299, 447	Chrysoeriol-*O*-hexoside
21	345.7	266.9, 311.3	431.0975	$C_{21}H_{19}O_{10}$	2.7	269	Apigenin-*O*-glucoside
22	374.3	266, 346	285.0401	$C_{15}H_9O_6$	1.7	285	Luteolin
23	415.4	266.9, 336.8	269.0462	$C_{15}H_9O_5$	1.7	269	Apigenin
24	426.2	267.4, 345.8	329.0669	$C_{17}H_{13}O_7$	0.2	174.9, 269, 299	Tricin.
25	449.1	Nd	329.2332	$C_{18}H_{33}O_5$	0.4		Unknown
26	649.1	Nd	293.2111	$C_{18}H_{29}O_3$	1.9	112.9, 248	Hydroxy-Octadecatrienoic acid
27	689.9	Nd	295.2279	$C_{18}H_{31}O_3$	0.7		Hydroxyoctadecadienoic acid
28	757.6	Nd	295.2276	$C_{18}H_{31}O_3$	1.1	112.9, 248.9	Hydroxyoctadecadienoic acid
29	832.7	Nd	277.2172	$C_{18}H_{29}O_2$	0.1	112.9, 211	Linolenic acid
30	884.2	Nd	279.2333	$C_{18}H_{31}O_2$	1.5	261	Linoleic acid
31	925	Nd	255.2327	$C_{16}H_{31}O_2$	0.4	112.9	Palmitic acid
32	941.8	Nd	281.2487	$C_{18}H_{33}O_2$	0		Oleic acid

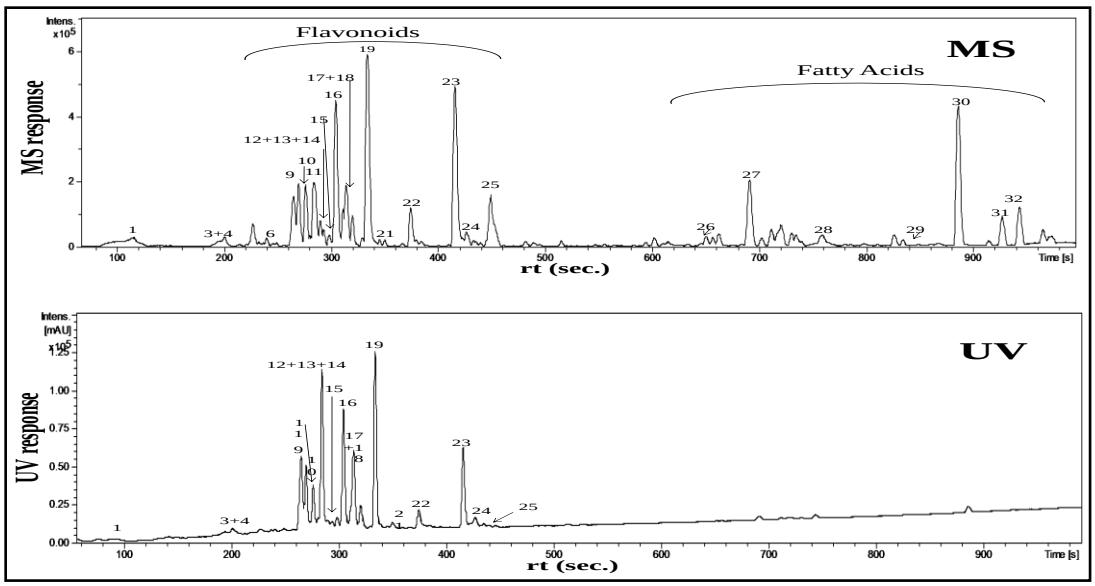
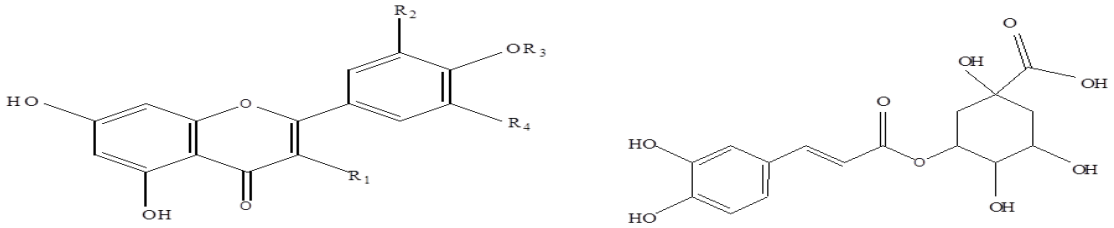


Figure. (1) Representative UPLC–UV–MS traces of *C. getulus* methanol extract characterized by two main regions: (100–450 s) with peaks principally due to caffeoylquinic acid isomers and flavonoid conjugates and a region (640–940) for fatty acids. Peak numbers follow those listed in (Table 1) for metabolite identification using UPLC–UV–MS.



Cpd.	R_1	R_2	R_3	R_4
Apigenin	H	H	H	H
Luteolin	H	OH	H	H
Tricin	H	OCH_3	H	OCH_3
Astragalin	O-glu	H	H	H
Chrysoeriol	H	OCH_3	H	H

Fig. (2) Flavonoids subclasses and caffeolquinic acid identified in *C. getulus* extract, with selected compound(s) discussed in the text.

Tandem MS spectra of *C. getulus* peaks identified luteolin as its aglycone (*m/z* 285.04, $C_{15}H_9O_6)^-$ in peaks (5-7, 9-11, 17, 19 and 22) and apigenin (*m/z* 269.044, $C_{15}H_9O_5)^-$ in peaks (8, 14, 18, 21 and 23), (Table 1 & Fig. 1). In contrast, tricin, a methylated derivative of tricetin, with *m/z* 329.0669 amu and molecular formula $(C_{17}H_{13}O_7)^-$ was detected in peaks 15 and 24. Fragmentation pattern of peak 15 showed neutral losses of 86 amu (-malonyl group), 162 amu (-hexose) and 60 amu (-2OCH_3), identified as tricin-*O*-malonyl-hexoside. Similar mass losses of 162 and 30 amu were observed in peak 20 with *m/z* 461.1081, $C_{22}H_{21}O_{11}^-$ identified as chrysoeriol-*O*-hexoside. The only detected flavonol was a kaempferol conjugate assigned in peak 16 $[M-H]^-$, (*m/z* 447.0939, $C_{21}H_{19}O_{11})^-$ showing fragment at *m/z* 285 of its aglycone. UV absorbance confirmed the flavonol moiety from its λ_{max} at 346 nm and suggestive for a 3-*O*-glycosylated kaempferol (Mabry et al., 1970) assigned as astragalin. The

main nucleus structures of these compounds were depicted in Fig. 2.

Another class of compounds that was detected includes phenolic derivatives, hydroxytyrosol hexoside was recognized from its $[M-H]^-$, *m/z* 315.1081 detected at R_t 116 s (peak 1) and showing losses of 18 amu and 162 amu for water and hexose moieties, respectively. Additional fragment appearing at 123 amu for the loss of $[M+-CH_2O]^-$ in hydroxytyrosol was also detected.

Positional isomers of 1-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid, structure depicted in Fig. 2) and another isomer were identified for peaks 2, 3 and 4 respectively (Fig. 1). UV spectra with λmax 326 nm is diagnostic to caffeoylquinic acid isomers (Clifford et al., 2003) in addition to predominant fragment of 191 amu for quinic acid found in the tandem MS spectra of peaks 2, 3 and 4 (Fig. 1 & Table 1).

Fatty acids were also characterized in the negative ion MS spectra and unsaturated fatty acids, linolenic (peak 29), linoleic (peak 30) and oleic acid (peak 32) were identified from their respective $[M-H]^-$ mass appearing at *m/z* 277.2172, 279.2333 and 281.2487 amu. A major saturated fatty acid detected in extract was palmitic acid (31) and showing $[M-H]^-$ *m/z* 255.2327 amu with a predicted molecular formula of $C_{16}H_{31}O_2^-$. MS spectral analysis also revealed for several other hydroxylated fatty acids in peaks 26, 27 and 28 with $[M-H]^-$ appearing at *m/z* 293.2111, 295.2279 and 295.2276 amu respectively, with predicted molecular formulas of $C_{18}H_{29}O_3^-$ and $C_{18}H_{31}O_3^-$, respectively. Peak 28 exhibited similar MS spectra to that of 27, albeit with a mass difference of 2 amu indicative of an extra double bond. The two peaks were tentatively identified as hydroxy-octadecatrienoic (26) and hydroxy-octadecadienoic acid (27, 28).

UPLC–MS profiling of *C. getulus* crude extract revealed for the enrichment of flavone type glycosides in the plant. Additional purification was thus attempted to isolate them to provide unequivocal assignment of its structure *viz*., sugar attachment position or type using NMR. Fractionation and isolation attempts for 70% ethanol aerial parts extract led to the isolation of 6 flavonoids.

3.2. Spectral data and chemical investigation of isolated compounds from 70% ethanol extract

Compound 1; luteolin-7-*O*-neohesperidoside (6 mg): R_f values in S1: 0.26 and S2: 0.30. UV λ_{max}, nm: MeOH: 257, 352; +NaOMe: 267, 402; +$AlCl_3$: 275, 431; +$AlCl_3$/HCl: 276, 367 sh., 392; +NaOAc: 262, 361; +NaOAc/H_3BO_3: 262, 377.

Compound 2; tricin-5-*O*-β-D-glucoside (17 mg): R_f values in S1: 0.44 and S2: 0.80. UV λ_{max}, nm: MeOH: 267, 346; +NaOMe: 267, 412; +$AlCl_3$: 280, 362; +$AlCl_3$/HCl: 280, 362; +NaOAc: 274, 316 sh., 364; +NaOAc/H_3BO_3: -, 347. MS data: $[M-H]^-$ 491.12, $(C_{23}H_{23}O_{12})^-$; fragments at *m/z* 329 $(C_{17}H_{13}O_7)^-$, 314 $(C_{16}H_{10}O_7)^-$ and 299 $(C_{16}H_{10}O_6)^-$.

Compound 3; luteolin-7-*O*-β-D-glucoside (10 mg): R_f values in S1: 0.44 and S2: 0.15. UV λ_{max}, nm: MeOH: 253, 265, 346; +NaOMe: 263, 300sh, 394; +$AlCl_3$: 274, 298sh, 329, 432; +$AlCl_3$/HCl: 273, 294sh, 358, 387; +NaOAc: 259, 266sh, 365sh, 405; +NaOAc/H_3BO_3: 259, 372.

Compound 4; luteolin (9 mg): R_f values in S1: 0.78 and S2: 0.90. UV λ_{max}, nm: MeOH: 252, 265, 290sh, 347; +NaOMe: 266sh, 329sh, 401; +$AlCl_3$: 274, 300sh, 328, 426; +$AlCl_3$/HCl: 266sh, 275, 294sh, 355, 385; +NaOAc: 269, 326sh, 384; +NaOAc/H_3BO_3: 259, 301sh, 370, 430sh.

Compound 5; apigenin-7-*O*-β-D-glucoside (7 mg): R_f values in S1: 0.52 and S2: 0.21. UV λ_{max}, nm: MeOH: 265, 333; +NaOMe: 270, 389; +$AlCl_3$: 275, 302, 345, 385; +$AlCl_3$/HCl: 277, 299, 341, 382; +NaOAc: 267, 355, 385; +NaOAc/H_3BO_3: 268, 337.

Compound 6; apigenin (12 mg): R_f values in S1: 0.88 and S2: 0.11. UV λ_{max}, nm: MeOH: 265, 290sh, 336; +NaOMe: 275, 324, 392; +$AlCl_3$: 276, 301, 348, 384; +$AlCl_3$/HCl: 276, 299, 340, 381; +NaOAc: 274, 301, 376; +NaOAc/H_3BO_3: 268, 302sh, 338.

NMR spectral data of isolated compounds (1-6) were presented in table 2.

UV spectral data of the 6 isolated flavonoids revealed their flavone structures from their λmax in MeOH (<350 nm) (Mabry et al., 1970). ^{1}H NMR analysis further confirmed the compounds structure, sugar type stereochemistry and attachment position. Concerning compound 1, the ^{1}H NMR spectrum, showed the structure of (C_1) to be luteolin-7-*O*-β-D-glucorhamnoside (Li et al., 2008). ^{1}H NMR revealed signals of at δ ppm 7.29 (1H, broad s, H- 2'), 7.27 (1H, d, J= 8 Hz, H- 6'), 6.8 (1H, d, J= 8 Hz, H-5'), 6.61 (1H, broad s, H-8), 6.47 (1H, s, H-3), 6.39 (1H, broad s, H-6) and signal appeared as doublet at δ 4.93 ppm (1H, d, J= 6.8 Hz, H-1'' of glucose) assigned for the anomeric proton of the sugar moiety. Moreover, another one appeared at 4.62 (1H, broad s, H-1''' of rhamnose) which is confirmed by the presence of doublet peak at 1.1 (3H, J= 7.2 Hz, methyl group of rhamnose).

Table (2): NMR spectral data of flavonoids $C_{(1–6)}$ isolated from 70% ethanol *C. getulus* aerial parts extract.

Cpd no.	1 (CD_3OD)	2 (CD_3OD)	3 (DMSO-d_6)	4 (DMSO-d_6)	5 (DMSO-d_6)	6 (DMSO-d_6)	2(CD_3OD)	3 (DMSO-d_6)	4 (DMSO-d_6)	5 (DMSO-d_6)	6 (DMSO-d_6)
C/H-agly.	δ H (m, J value-Hz)	δ H (m, J value-Hz)	δ H (m, J value-Hz)	δ H (m, J value-Hz)	δ H (m, J value-Hz)	δ H (m, J value-Hz)	δ C	δ C	δ C	δ C	δ C
2	-	-	-	-	-	-	164.9	164.5	163.8	164.8	163.8
3	6.47 (1H, s)	6.63 (1H, s)	6.7 (s)	6.60 (s)	6.85 (s)	6.96 (s)	105.7	103.1	102.8	103.4	102.8
4	-	-	-	-	-	-	178.9	181.9	181.6	182.4	181.5
5	-	-	-	-	-	-	158.7	161.1	157.3	162.3	161.3
6	6.39 (broad s)	6.71 (d, J=1.6 Hz)	6.4 (d, J = 2.2 Hz)	6.15 (d, J = 2.5 Hz)	6.45 (d, J = 2.5 Hz)	6.13 (d, J = 2 Hz)	103.9	99.5	98.9	100.0	98.7
7	-	-	-	-	-	-	162.6	162.9	164.4	163.4	163.6
8	6.61 (broad s)	6.8 (d, J= 1.6 Hz)	6.8 (d, J = 2.2 Hz	6.4 (d, J = 2.5 Hz)	6.83 (d, J = 2.5 Hz)	6.43 (d, J = 2 Hz)	98.2	94.7	93.8	95.3	93.9
9	-	-	-	-	-	-	159.4	156.9	161.5	157.4	157.2
10	-	-	-	-	-	-	107.5	105.3	103.6	105.8	103.6
1'	-	-	-	-	-	-	121.2	121.3	121.4	121.2	121.1
2'	7.29 (broad s)	7.23 (sharp s)	7.42 (d, J = 2.2 Hz)	7.40 (d, J = 2.1 Hz)	7.95 (d, J = 8 Hz)	7.86 (d, J = 8.8 Hz)	103.7	112.5	113.3	129.1	128.3
3'	-	-	-	-	6.95 (d, J = 8 Hz)	6.87 (d, J = 8.8 Hz)	148.3	145.8	145.8	116.6	116.8
4'	-	-	-	-	-	-	139.6	150.0	149.8	161.6	161.4
5'	6.8 (d, J= 8 Hz)	-	6.9 (d, J = 8.3Hz)	6.85 (d, J = 8.5 Hz)	6.95 (d, J = 8 Hz)	6.87 (d, J = 8.8 Hz)	148.3	115.9	116.0	116.6	116.8
6'	7.27 (d, J= 8 Hz)	7.23 (sharp s)	7.45 (dd, J = 8.3 , 2.2 Hz)	7.39 (dd, J = 8.5, 2.1 Hz)	7.95 (d, J = 8 Hz)	7.86 (d, J = 8.8 Hz)	103.6	119.2	118.9	129.1	128.3
-OCH3	-	3.9 (6H, sharp s)	-	-	-	-	55.7	-	-	-	-
Glu. 1"	4.93 (d, J= 6.8 Hz)	4.86 (d, J= 7.6 Hz)	5.08 (d, J = 6.6 Hz)	-	5.05 (d, J = 7.5 Hz)	-	103.6	99.8	-	100.4	-
Glu. 2"	3-4 (m)	3-4 (m)	-	3-4 (m)	-		73.3	72.1	-	73.6	-
Glu. 3"			-		-		75.9	76.4	-	76.9	-
Glu. 4"			-		-		69.8	69.5	-	70.1	-
Glu. 5"			-		-		77.2	77.1	-	77.7	-
Glu. 6"			-		-		61.1	60.6	-	61.1	-

Rham. 1'''	4.62 (broad s)	-	-	-	-	-	-	-	-	-	-
Remain-ing sugar protons	3-4 (m)	3-4 (m,	-	-	-	-	-	-	-	-	-
Rham. 6'''	1.1 (3H, d, J= 7.2 Hz)	-	-	-	-	-	-	-	-	-	-

The downfield shift of H-6 & H-8 in comparison to the aglycone signals was indicative of 7-*O*-glycosylation and the glycosidic linkage suggested to be (gluco 2" →1"' rhamnosyl) named neohesperidosyl moiety (Mabry et al., 1970).

While, compound C_2 exhibited $(M-H)^-$ at m/z 491.1201 and upon fragmentation it exhibited fragments *m/z* 329 of tricin (neutral loss of 162 amu characteristic to hexose) and *m/z* 299 (loss of 30 amu due to methoxy group). Moreover, ^{1}H NMR spectrum revealed aromatic signals at δ ppm 7.23 (2H, sharp s, H- 2', 6'), 6.8 (1H, d, J= 1.6 Hz, H-8), 6.71 (1H, d, J=1.6 Hz, H-6), 6.63 (1H, s, H-3). The signal of the sugar anomeric proton was found at δ 4.86 (1H, d, J= 7.6 Hz, H-1" of β- form). Additionally, another signal appeared at 3.9 (6H, sharp s, of two chemically identical methoxy groups substituted on aromatic ring) identified as tricin-5-*O*-β-D-glucoside (Li et al., 2016). 5-*O*-glycosylation was suggested based on the upfield shift of C-4 at δ 178 ppm due to disruption of the intramolecular H-bond interactions by the presence of sugar moiety at 5-OH gp (Agrawal, 1989). Resonances of the flavonoid moiety carbon signals were assigned by comparison with those published for tricin-5-*O*-β-D-glucoside (Li et al., 2016).

The ^{1}H NMR spectra of compounds 3 and 4 showed the same NMR signals for luteolin aglycone with sugar anomeric proton at δ 5.08 (d, J = 6.6 Hz, H-1" of β-D-glucose) found in spectrum of C_3. So, C_3 and C_4 were identified as luteolin-7-*O*-β-D-glucoside (cynaroside) (Wang et al., 2003) and luteolin (Zheng et al., 2008) respectively.

Incontrast, C_5 and C_6 signals showed the same NMR signals for apigenin aglycone with the presence of sugar anomeric proton at δ 5.05 (d, J = 7.5 Hz, H-1" of glucose) in the spectrum of C_5. Consequently, C_5 and C_6 were identified as apigenin-7-*O*-β-D-glucoside (Guvenalp et al., 2006) and apigenin (Chaturvedula and Prakash, 2013) respectively.

3.3. Acute and subchronic toxicity:

To assess for *C. getulus* extract safety margin, acute toxicity effect was monitored in animals by estimation of the lethality test (LD_{50}). Treated mice with different doses (up to 15000 mg/ kg b. wt.) were alive during 24 h of observation and showing no visible signs of acute toxicity. According to Hodge and Sterner toxicity scale (Sandu et al., 2012), the LD_{50} value of the extract is in the practically non-toxic category.

Similarly, sub-chronic toxicity study was performed by following complete blood picture, liver and kidney serum enzymes followed by histopathological sections after 4 weeks of administration. Urea and creatinine, biochemical markers used for diagnosis of renal damage (Maheshwari, 2008) showed no significant difference in the mean values of their serum level (39.8±3.1 and 0.68±0.03 mg dl^{-1}, respectively) from control animals (41.5±2.16 and 0.70±0.03 mg dl^{-1}, respectively) at ($p \leq 0.05$) and in agreement with histopathological sections showing normal nephron structure (Fig. 3). Additionally, no significant change was found in kidney weight ratio at dose of 500 mg/kg b. wt. of *Carduus* extract (0.64 ± 0.01%) when compared with control (0.6%). Moreover, oral dosing of *C. getulus* extract for 4 weeks did not show any significant effect on AST (23.4 ± 1.7 U/L), ALT (26.2 ± 3.4 U/L) and ALP (68.4 ± 5.7 U/L) or on liver weight ratio (2.9 ± 0.12%) as compared to control group (21.3 ± 1.5, 22.1 ± 1.9, 72.3 ± 4.8 U/L and 3.1 ± 0.09% respectively) that was confirmed by histopathological examination showing normal hepatocytes structure (Fig. 4B)

Finally, blood picture parameters for rats after extract administration were found at normal levels compared with the control. In conclusion, the plant extract was found to be safe on the liver, kidney and blood post 4-weeks of administration.

3.4. Hepatoprotective activity:

The ability of alcoholic plant extract to protect against CCl_4 hepatotoxic effects was assessed in a rat animal model along with silymarin serving as a positive drug control. Treatment of animals with the hepatotoxic agent, CCl_4 , resulted in significant ($p \leq 0.05$) increase in the liver aminotransferases (AST and ALT) and ALP levels, indicative of hepatocytes damage, and decreases serum albumin and total protein. Severe jaundice was revealed from elevated bilirubin level in addition to elevated serum total cholesterol and triglycerides and accompanied by a decrease in serum HDL-cholesterol (Al-Assaf et al., 2013, El Sohafy et al., 2013). Additionally, CCl_4 administration led to significant increase in MDA (lipid peroxidation) and reduction of both GSH content and activity of antioxidant enzymes SOD and CAT later are endogenous protective antioxidants against free oxygen radicals in hepatic tissue (Balahoroğlu et al., 2008).

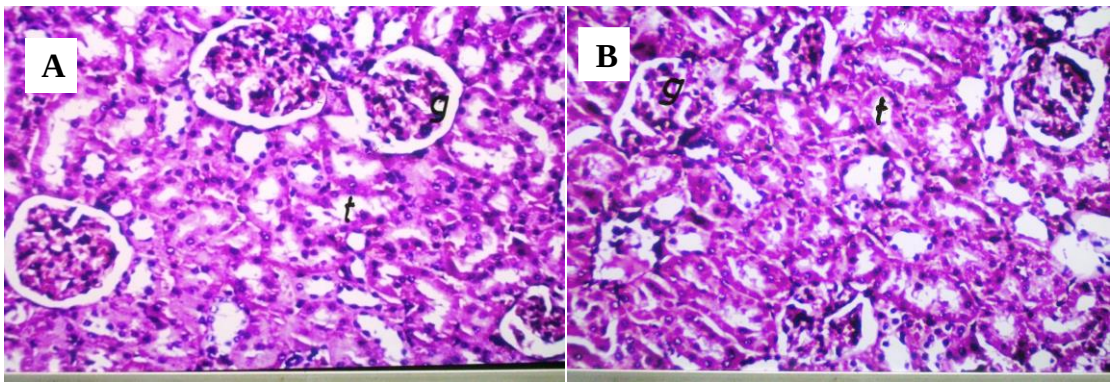


Figure. (3) Histology of kidney samples from (A) control, (B) treated group with *C. getulus* extract. (g) the glomeruli and (t) the tubules. Hematoxylin–Eosin staining, magnifications: ×40.

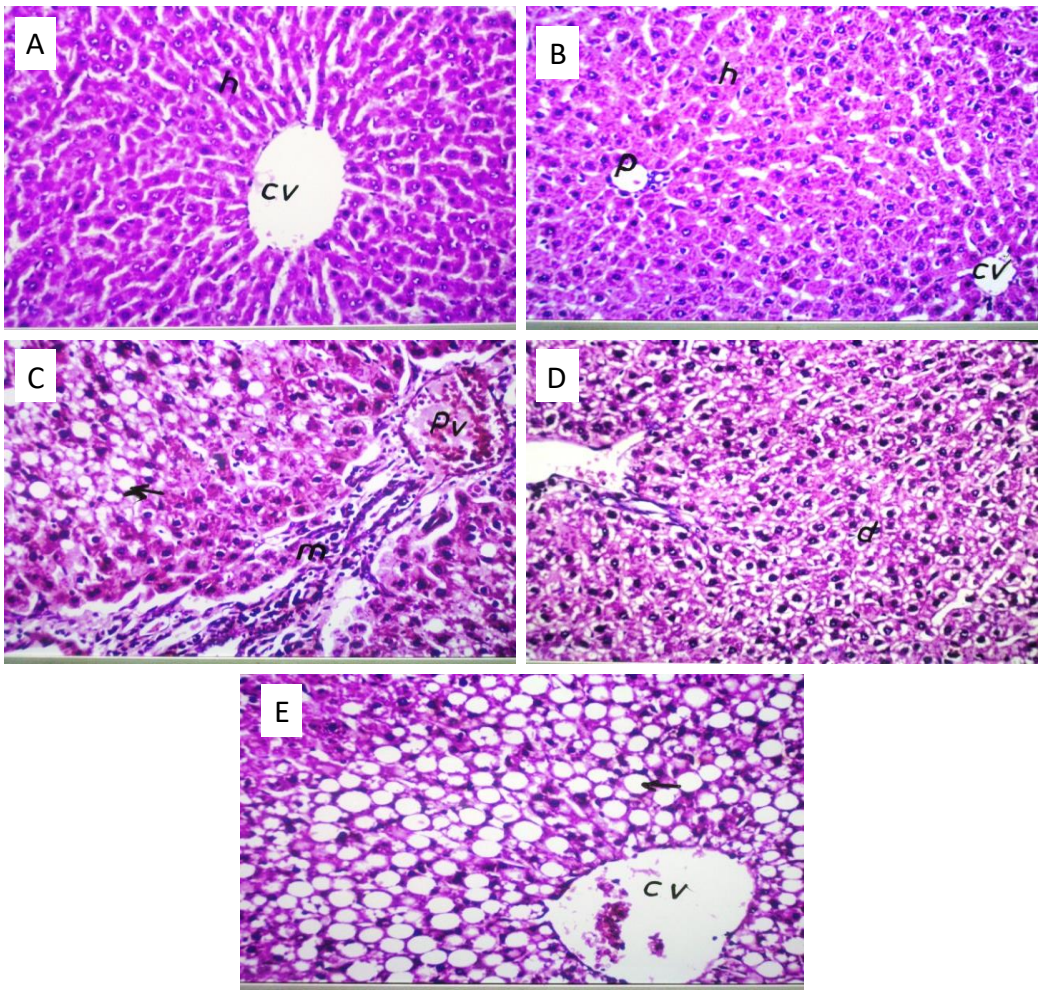


Figure. (4) Histology of liver samples from (A) control, (B) treated group with *C. getulus* extract for 4 weeks, (C) CCl_4-treated, (D) CCl_4 intoxicated group and treated with silymarin, (E) CCl_4 intoxicated group and treated with *C. getulus* extract. The central vein (CV), hepatocytes (h), fatty changes (arrow), inflammatory cells infiltration (m), mild degenerative change (d) and the portal vein (PV). Hematoxylin–Eosin staining, magnifications: ×40.

Table (3): Effect of pretreatment with silymarin and *C. getulus* extract on changes in body weight, relative liver weight ratio, serum enzymes, total and direct bilirubin, total proteins, albumin and lipid profile levels, oxidative stress and activities of antioxidant liver enzymes of carbon tetrachloride-treated animals. (n = 10)

Group Parameter	Control	CCl_4		Silymarin + CCl_4		*Carduus* Extract + CCl_4	
		Mean ± S.E	% Deterioration [a]	Mean ± S.E	% Amelioration [b]	Mean ± S.E	% Amelioration[b]
Increase in body weight (g)	75.0 ± 3.0	35.3 ± 3.8[a]	53%**	36.1 ± 3.5[a]	-	46.9 ± 4.7[a]	-
Relative body-weight ratio (100×)	3.1 ± 0.09	4.7 ± 0.22[a]	52%*	4.0 ± 0.21[a,b]	29%**	4.2 ± 0.23[a]	-
ALT (U/L)	22.1 ± 1.9	66.1± 2.5[a]	199%*	30.6 ± 2.9[b]	54%**	30.7 ± 1.7[b]	54%**
AST (U/L)	21.3 ± 1.5	77.8± 3.2[a]	265%*	36.2 ± 2.6[a,b]	53%**	36.7 ± 2.5[a,b]	53%**
ALP (U/L)	72.3 ± 4.8	173 ± 13.6[a]	139%*	106 ± 7.8[b]	39%**	129 ± 7.6[b]	25%**
Total bilirubin (mg/dl)	1.1 ± 0.07	3.0 ± 0.35[a]	173%*	1.8 ± 0.07[b]	40%**	1.1 ± 0.25[b]	63%**
Direct bilirubin (mg/dl)	0.98 ± 0.1	2.4 ± 0.09[a]	145%*	1.6 ± 0.07[a,b]	33%**	1.5 ± 0.12[a,b]	38%**
Total protein (g/dl)	8.0 ± 0.09	5.7± 0.21[a]	29%**	6.9 ± 0.29[b]	21%*	7.2 ± 0.25[b]	26 %*
Albumin (g/dl)	6.8±0.18	4.6±0.32[a]	32%**	6.2±0.27[b]	35%*	6.5±0.30[b]	41%*
cholesterol (mg/dl)	210.0 ± 7.8	384.3 ± 11.7[a]	83%*	284.2 ± 14.9[a,b]	29%**	274.7 ±11.3 [a,b]	19%**
Triglycerides (mg/dl)	451.7 ± 21.9	596.7±27.1[a]	32%*	482.8 ± 19.7[b]	19%**	446.7 ± 22.3[b]	25%**
HDL (mg/dl)	74.9 ± 6.5	41.4 ± 3.5[a]	45%**	59.2 ± 4.7	43%*	63.9 ± 5.4	54%*
MDA(nmol/ mg protein)	1.0 ± 0.14	4.7 ± 0.48[a]	370%*	1.7 ± 0.2[b]	64%**	1.9 ± 0.17[b]	60%**
GSH (µmol/ml)	12.6 ± 0.76	6.9 ± 0.35[a]	45%**	10.8 ± 0.66[b]	57%*	9.4 ± 0.64 [b]	36%*
CAT (U/mg protein)	2.0 ± 0.20	0.74 ±0.07[a]	63%**	1.5 ± 0.09[b]	103%*	2.3 ± 0.26[b]	211%*
SOD (U/mg protein)	8.7 ± 0.92	3.5 ± 0.22[a]	60%**	6.8 ± 0.31[b]	94%*	6.3 ± 0.28[b]	80%*

[a] Significantly different from the control group.
[b] Significantly different from the CCL_4-treated group.
using one-way ANOVA followed by Tukey–Kramer method for multiple comparison at $p \leq 0.05$.
* elevation ** reduction

Both silymarin, well characterized anti-hepatotoxic agent and *C. getulus* extract ameliorated CCl_4-induced alterations (Table 3). *Carduus* extract induced significant reduction of ALT, AST, ALP, total bilirubin, direct bilirubin, total cholesterol and TG serum elevated levels concurrent with a significant increase in total proteins, serum albumin level and HDL-cholesterol in comparison to CCl_4-treated group. In addition, *Carduus* extract significantly mitigated the lipid peroxidation activity in the rat liver induced by CCl_4 as manifested by a decrease in MDA levels and restored GSH content, CAT and SOD activities. It should be noted that treatment with *Carduus* extract had no effect on improvement of CCl_4 effect on body weight or decreasing liver-body weight ratio as compared to CCl_4-treated group.

Furthermore, improvement in biochemical parameters following *C. getulus* treatment was also confirmed from liver histopathological study (Fig. 4). Liver section of control group showed no histopathological alterations, with a normal histological structure of the central vein and surrounding hepatocytes (Fig. 4A). In contrast, administration of CCl_4 revealed centrolobular fatty change as observed in most of the hepatocytes associated with inflammatory cells infiltration in the portal area and dilatation in the portal vein (Fig. 4C). Pretreatment with silymarin during CCl_4 administration showed mild degenerative change in some of the hepatocytes (Fig. 4D), while prior treatment with *C. getulus* extract and during CCl_4 administration showing dilatation in central vein with centrolobular fatty changes (Fig. 4E).

The hepatoprotective effect of *C. getulus* was examined previously (El Sohafy et al., 2013) and, with the administration of dose of 500 mg kg^{-1} exhibiting a mild protection effect on the level of bilirubin (10.69%), while other biochemical parameters were not improved (ALT, AST, ALP). Although, our results differs from that of (ALT, AST, ALP) it was in agreement with that of bilirubin level reduction (El Sohafy et al., 2013). It may be due to difference in climate, place and year of plant collection.

In summary, *C. getulus* extract showed improvement of hepatic function and confrontation

of oxidative stress, an action that is likely to be attributed to enrichment in flavonoids mainly of apigenin and luteolin-*O*-conjugates known by their antioxidant effect. The potent therapeutic action of apigenin and luteolin against hepatotoxicity was proved previously (Sandhar et al., 2011).

CONCLUSION

To the best of our knowledge, this study provides the first metabolite profile of *C. getulus via* UPLC-MS. Additionally, it is noteworthy that the majority of the identified isolated compounds were consistent with the results derived *via* the UPLC/PDA/qTOF/MS profiling analysis, posing UPLC-MS as an advanced technique for flavonoids profiling *in planta*. The approach utilized herein allowed for the identification of several flavonoids isolated for the first time in *C. getulus* aerial parts. All of the isolated compounds were reported previously in *Carduus* sp. and also detected in the UPLC-MS spectrum except for tricin-5-*O*-β-D-glucoside that is reported herein for the first time in the whole genus. Enrichment of flavonoids in plant extract is likely to mediate for its moderately safe hepatoprotective effect. Further testing of these metabolites will help provide more evidence for potential medicinal uses of *C*. *getulus*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

HET performed chromatographic isolation and purification of compounds, biological experiments, analyzed all the data and wrote the manuscript.
MAF performed LCMS analysis, revised peak assignments and reviewed the manuscript.
AME, SAE and KFA supervised the chromatographic experiments, revised interpretation of spectral data and reviewed the manuscript.
AMM designed and performed the biological experiments, revised the analysis of biological data.
All authors read and approved the final version.

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