

Original Article

Spicatic acid: A 4-carboxygentisic acid from *Gentiana spicata* extract with potential hepatoprotective activityHeba Handoussa¹, Natalia Osmanova², Nahla Ayoub^{3,*}, Laila Mahran^{4,5,*}¹ Department of Pharmaceutical Biology, Faculty of Pharmacy and Biotechnology, German University in Cairo, Egypt;² Department of Pharmaceutical Biology, Institute of Pharmacy, Hamburg University, Germany;³ Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt;⁴ Department of Pharmacology and Toxicology, Faculty of Pharmacy and Biotechnology, German University in Cairo, Egypt;⁵ Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

ABSTRACT: Due to our interest in bioactive plant derived materials, the hepatoprotective activity of the aqueous alcoholic extract of *Gentiana spicata* AEGS (Gentianaceae) on carbon tetrachloride treated rats was investigated. CCl₄ used at a concentration of 1 mL/kg.b.wt. significantly increased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). However, pre-treatment with AEGS and its individual components significantly prevented the increase in these enzymes, which are the major indicators of liver injury. Biochemical assays of liver homogenate showed that AEGS and its components restored reduced glutathione (GSH) depletion reduced the level of thiobarbituric acid reactive substances (TBARS). Furthermore, liver histological observation also showed an obvious amelioration in liver cell necrosis, liver lesions, and fatty changes in pre-treated groups. Phytochemical investigation of the extract showed high phenolic content and led to the isolation and identification of the new carboxygentisic acid, 1,4-dicarboxy 2,5-dihydroxybenzene, for which we suggest the name spicatic acid, together with the known flavonoids, quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)1'''' \rightarrow 6'''] 3-acetyl- β -galactopyranoside and quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)1'''' \rightarrow 6''']-4-acetyl- β -galactopyranoside, epicatechin, catechin and their gallolyated derivatives. All structures were elucidated on the basis of conventional analytical methods and confirmed by high resolution ESIMS, 1D- and

2D-NMR data. The new phenolic 4-carboxygentisic acid, spicatic acid is of special interest as it represents the first phenolic acid in nature which bears two carboxyl functions in one aromatic ring.

Keywords: *Gentiana spicata*, phenolics, hepatoprotective, 4-carboxygentisic acid, spicatic acid

1. Introduction

Gentiana spicata (Gentianaceae) is common in the tropics and subtropics of both hemispheres in sandy and loamy soils (1). It is commonly used as folk medicine for treating stomach disorders, hypertension, renal colic, rheumatic pains, and for elimination of stones from the kidney and the ureters (2-4).

Phytochemical studies reported the isolation of a number of metabolites from *Gentiana spicata*, quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)-(1 \rightarrow 6)]-3,4-diacetyl- β -galactopyranoside (5), quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)-(1 \rightarrow 6)]- β -galactopyranoside, quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)1'''' \rightarrow 6''']-3 acetyl- β -galacto-pyranoside, and quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)1'''' \rightarrow 6''']-4-acetyl- β -galactopyranoside (6), secoiridoid glucosides. sweroside (I), swertiamarin (II) and gentiopicroside (7) and alkaloids (4).

Liver damage in rats was induced using carbon tetrachloride (8) which causes severe xenobiotic-hepatotoxic effects. As it is metabolized in the body to a highly reactive trichloromethyl free radical (CCl₃) it leads to lipid peroxidation. Cell lysis leads to leakage of the two enzymes AST and ALT (9), and also depletion of cellular glutathione.

Administration of AEGS and its components (spicatic acid and flavonoids mixture) prior to CCl₄ intoxication has been used as a model to test the

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potential preventive role of phytochemicals against acute oxidative stress as flavonoids (10), and catechins (11) besides spicatic acid, the phenolic compound which resembles salicylic acid (12), were reported to protect against liver injury.

The present study was conducted to examine the hepatoprotective activities of the extract, flavonoids and spicatic acid, against CCl₄-induced hepatotoxicity in rats, to evaluate the therapeutic claims of *Gentiana* species in the traditional practice of treating liver disorders.

2. Materials and Methods

2.1. Instruments and materials

¹H NMR spectra were measured using a Jeol ECA 500 MHz NMR spectrometer 500 MHz NMR spectrometer, at 500 MHz. ¹H chemical shifts (δ) were measured in ppm, relative to TMS and ¹³C NMR chemical shifts to DMSO-d₆ and converted to the TMS scale by adding 39.5. Typical conditions: spectral width 8 kHz for ¹H and 30 kHz for ¹³C, 64 K data points and a flip angle of 45. FTMS spectra were measured on a Finnigan LTQFTMS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität zu Berlin). UV recordings were made on a Shimadzu UV-Visible-1601 spectrophotometer. [a]²⁵_D were measured on a Kruss polarimeter-8001 (A. Kruss Optronic, Germany). Paper chromatographic analysis was carried out on Whatman No. 1 paper, using solvent systems: (1) H₂O; (2) 6% HOAc; (3) BAW (*n*-BuOH-HOAc-H₂O, 4:1:5, upper layer). Solvent 3 was used for PPC.

2.2. Plant material, extraction and isolation

Plant material of *Gentiana spicata* is a wild plant collected from northern Sinai (Arab Republic of Egypt). The authenticity of species was confirmed by Professor Dr. Abdel Salam Mohamed Al-Nowiahi, Professor of Taxonomy, Faculty of Science, Ain-Shams University, Egypt. The aerial parts (1 kg) were exhaustively extracted with distilled water (5 L). The extract was evaporated *in vacuo* at low temperature until dryness followed by ethanolic extraction. The ethanol soluble extract was evaporated *in vacuo* until dryness. The dry residual powder of aqueous ethanolic extract (8 g) was fractionated using column chromatography (60 L × 4.5 ID cm) using Sephadex LH-20 (50 g) as a stationary phase. Elution started with water, and was then followed by water/ethanol mixtures of decreasing polarities. The elution process was monitored with UV light. Eluted fractions were screened using 2-DPC analysis on Whatmann paper No. 1, using BAW for the first direction, followed by 6% AcOH for the second direction. Elution with H₂O led to desorption of fraction I. Compound (1) was purely isolated (150 mg) through

crystallization of the material of fraction I from MeOH. Fraction II eluted with 50% MeOH was fractionated on precoated silica gel plates developed with EtOAc-HOAc-HCOOH-H₂O (30:0.8:1.2:8) (upperphase); separated bands were eluted with CHCl₃-MeOH (85:15) and (80:20) and subjected to repeated column chromatography on Sephadex LH-20 using MeOH as eluent to give (40 mg) pure sample of compound (2), and (35 mg) of compound (3).

Fraction III was applied onto a polyamide S₆ column using methanol: toluene: H₂O (60:38:2) as an eluent which led to desorption of four sub-fractions. Compounds (4-7) were isolated as pure sample through preparative PC of the eluted sub-fractions using BAW as eluent. The detected bands from the dried preparative paper chromatograms eluted by ethanol gave pure compounds (4) 15 mg, (5) 10 mg, (6) 45 mg and 30 mg of compound (7).

2.3. Spectral data of the new natural 1,4-dicarboxy 2,5-dihydroxybenzene (spicatic acid), compound (1)

Faint yellow amorphous powder, R_f values (×100): 84 (H₂O), 78 (6% AcOH), 45 (BAW). UV λ_{\max} (nm) in MeOH: 222 and 354. UV λ_{\max} (nm) in MeOH after acid hydrolysis: 222 and 354. IR (cm⁻¹): 3440, 1705, and 1605 cm⁻¹. ESI/MS *m/z* = 197.123 [M-H]⁻. EI/MS *m/z* = 198. ¹H-NMR (DMSO-d₆) δ (ppm): 7.07 (2H, s, H-3 and H-6). ¹³C-NMR (DMSO-d₆) δ (ppm): 119.46 (dd, ³*J* = 7.5 Hz and ²*J* = 2.5 Hz, C-1 and C-4), 151.99 (dd, ³*J* = 8 Hz and ²*J* = 2.2 Hz, C-2 and C-5), 117.36 (d, *J* = 160 Hz, C-3 and C-6), 170.37 (d, ³*J* = 6 Hz, C-7 and C-8).

2.4. Animals

Adult male Wistar albino rats, each weighing (200-250 g.) bred at National Scientific Research Center Laboratory, Giza, Egypt, were kept in groups of six per cage under controlled environmental conditions at a temperature of 25 ± 1°C and left for two weeks for acclimatization before use. The rats received standard laboratory chow and water.

2.5. Chemicals for biochemical assays

Carbontetrachloride (CCl₄), Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid)(DTNB), Ethylene diamine tetracetic acid disodium salt (EDTA), 1,1',3,3'-tetramethoxypropane thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.6. Biochemical assays

ALT and AST activities were analyzed according to the method described by Reitman and Frankel (13).

In brief, blood samples were first centrifuged at 4,000 rpm at 4°C for 20 min to obtain the serum. The serum collected was then analyzed for ALT and AST activities.

Liver homogenate was prepared in ice cold 0.1 M potassium chloride (KCl), was homogenized with 1.15% potassium chloride to make 10% w/v homogenate, and the supernatant developed after centrifugation was used to determine reduced glutathione (14). The other part of this homogenate was centrifuged at 1,000 rpm for 10 min at 4°C and in the formed supernatant; lipid peroxidation was assayed as malondialdehyde (MDA) (15).

2.7. Histopathological examination of rat liver

CCl₄-induced liver necrosis was evaluated using hematoxylin and eosin stain (16). After collecting the blood under ether anaesthesia, the rat liver was removed and fixed in 10% formalin. The liver was dehydrated with serial dilutions of alcohol (methyl, ethyl and absolute ethyl), cleared in xylene and embedded in paraffin. Paraffin bees wax tissue blocks were prepared for sectioning at 4 micron thickness. The tissue sections obtained were collected on glass slides, deparaffinized and stained using hematoxylin and eosin stain (16) for histopathological examination through the light microscope.

2.8. Statistical analysis

Data were expressed as means ± standard deviation. Differences between the studied groups were evaluated using a one-way ANOVA test. A value of $p < 0.05$ was considered as statistically significant (17).

2.9. Determination of the median lethal dose (LD₅₀)

LD₅₀ was determined according to the procedure described by Paumgarten *et al.* (18).

3. Results

3.1. Isolation and structure elucidation

Following column chromatographic fractionation of the *G. spicata* extract, compounds (1-7) were isolated. Conventional and spectral analysis using NMR spectroscopy and mass spectrometry indicated that one of these compounds (1) was found to be new natural product (*see Appendix*).

Compound (1), isolated as a light yellow amorphous powder, it exhibited in EI-MS, a molecular ion [M]⁺ at m/z : 198. The characteristic chromatographic properties (yellow spot on PC under UV light) UV absorption data in methanol [λ_{\max} : 222 (inflection), 354 nm] and the result of acid hydrolysis the compound was recovered unchanged after being refluxed with 2 N aqueous HCl,

100°C for 3 h which suggested that (1) is most probably a phenolic carboxylic acid derivative. The ambiguity of the structure of this new compound was solved using the following aids; IR analysis which showed strong absorptions at 3440, 1705, and 1605 cm⁻¹, consistent with the presence of OH, carboxylic carbonyl and benzenoid C=C groups, respectively. The ¹H NMR spectrum (DMSO-d₆) of (1) disclosed a singlet aromatic resonance at δ ppm 7.07.

This result possesses a symmetrical tetra substituted benzene ring such that each of the two symmetrical protonated carbons is connected to a hydroxylate carbon from one side and to a carboxylated carbon on the other side 117.36 (d, $J = 160$ Hz). The site of attachment of the four substitutions attached to the benzene nucleus of (1) has been unraveled by performing de-coupled and ¹H coupled ¹³C NMR spectral analysis. The spectra revealed the presence of carbon resonances at δ ppm 170.37 (d, $^3J = 6$ Hz) attributable to the two carbonyl carbons of the two symmetrical carboxyl groups at C-7 and C-8.

It revealed also a resonance at 151.99 δ ppm (dd, $^3J = 8$ Hz and $^2J = 2.2$ Hz) assigned to the two symmetrical oxygenated carbons (C-2 and C-5). The two quaternary carbons appeared in this spectrum at δ ppm 119.46 (dd, $^2J = 2.5$ Hz and $^3J = 7.5$ Hz), while the two symmetrical protonated carbons had a resonance at δ ppm 117.36 (d, $J = 160$ Hz).

Results showed that AEGS contained 0.27% of 1,4-dicarboxy 2,5-dihydroxybenzene (1), for which we suggest the name spicatic acid (Figure 1), in addition to 0.13% of acetylated flavonoids; quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)-1''' \rightarrow 6'']-3-acetyl- β -galactopyranoside (2) and quercetin 3-*O*-[(2,3,4-triacetyl- α -rhamnopyranosyl)-1''' \rightarrow 6'']-4-acetyl- β -galactopyranoside (3) were identified. The isolation and characterization of four known catechins: epicatechin (4), catechin (5), epigallo-catechin 3-*O*-gallate (6), gallic catechin 3-*O*-gallate (7) are reported for the first time from this species.

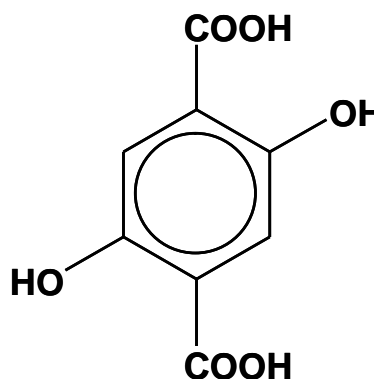


Figure 1. 4-Carboxygentisic acid (Spicatic acid).

3.2. Evaluation of the biological activity

3.2.1. Determination of the median lethal dose (LD₅₀)

The median lethal dose (LD₅₀) of the tested extract was 6.7 g/kg.b.wt.

3.2.2. Effects of aq. EtOH extracts of *G. spicata*, its components and silymarin on serum ALT and AST levels in rats subjected to CCl₄ as hepatotoxin

Intraperitoneal dose of CCl₄ significantly elevated the levels of serum ALT and AST in comparison to the untreated group; 153% and 88.9%, respectively. Pretreatment with either the tested extract; its components (flavonoids mixture and spicatic acid), suppresses the leakage of serum enzyme activities of ALT; with values of 41.6%, 44%, and 46.5% levels, respectively, (Figure 2A) and AST with values of 60.8%, 35.3%, and 38.2%, respectively (Figure 2B). Silymarin as a standard reference resulted in a significant inhibition of cellular leakage of ALT and AST; 55.6% and 41.7%, respectively.

3.2.3. Effects of aq. EtOH extracts of aerial parts of *G. spicata* on liver contents of thiobarbituric acid reactive substances (TBARS) levels in rats subjected to CCl₄

Administration of CCl₄ led to a significant increase in the levels of tissue lipid peroxidation marker TBARS. The MDA was significantly increased to 155% compared to the untreated group. However, this increase in TBARS was inhibited by pretreatment of rats with the tested extract and its components (flavonoids mixture and spicatic acid) with 57.9%, 68.5%, and 63.5%, respectively comparable to the CCl₄ treated group (Figure 2C). The extract has shown to impart significant hepatoprotective activities by modulation of free radical-induced lipid peroxidation where lipid peroxidation and reactive oxygen species are associated with hepatic injury. That hepatoprotective effect was assured when compared to the reference standard drug silymarin.

3.2.4. Effects of aq. EtOH extracts of aerial parts of *G. spicata* on liver contents of reduced glutathione levels in rats subjected to CCl₄

Hepatic tissue GSH levels were significantly decreased in animals treated with CCl₄ as compared to the untreated group (vehicle-treated) with values equal to 45%. The decreased level of liver GSH was significantly ameliorated by pretreatment of rats with the investigated extract and its components (flavonoids mixture and spicatic acid) with values of 75%, 52.9%, and 64.7%, respectively, compared to the CCl₄ group (Figure 2D).

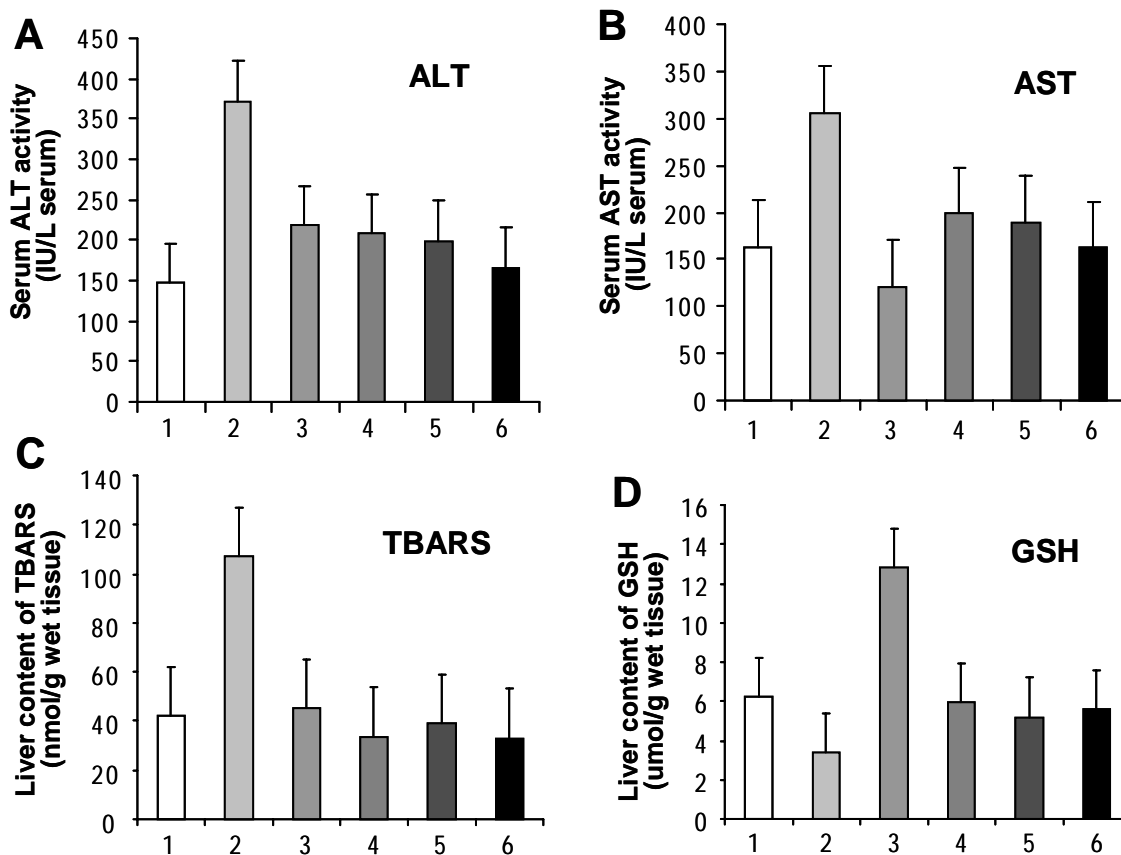


Figure 2. Effect of AEGS and its components on various enzymes in liver homogenate. 1, Untreated animals; 2, Control animals given only CCl₄; 3-6, CCl₄ (1 mL/kg)-intoxicated rats intraperitoneally administered for 14 successive days with aqueous ethanolic extract of aerial parts of *Gentiana spicata* (3), Flavonoids mixture (4), 4-carboxygentisic acid (spicatic acid) (5), silymarin (6). A, ALT; B, AST; C, TBARS; D, GSH.

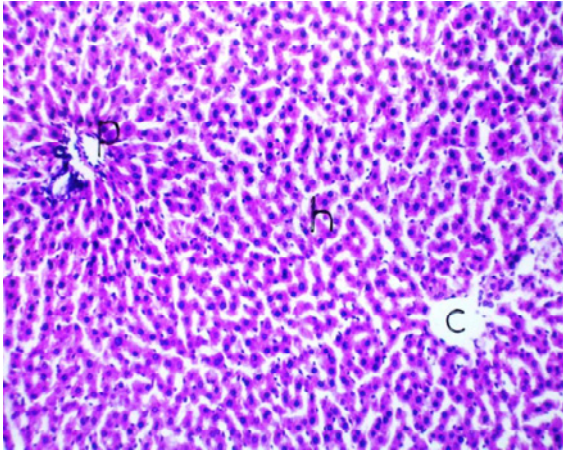


Figure 3. Liver of rat in control group showing the normal histological structure of the central vein (C), Portal area (P) and surrounding hepatocytes (H).

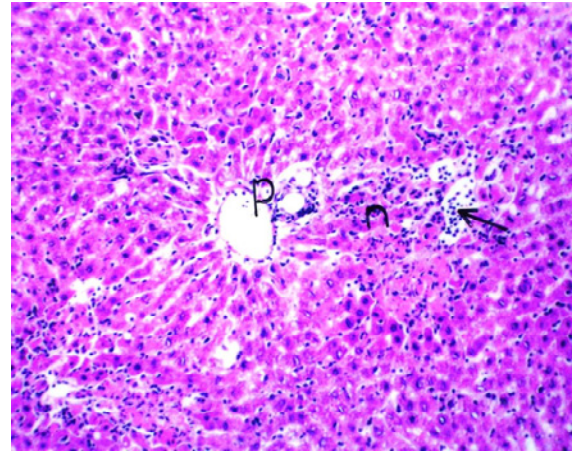


Figure 4. Liver of rat administrated spicatic acid and carbon tetrachloride. The photo shows few focal areas of coagulative necrosis (n) associated with focal inflammatory cells infiltration (arrow).

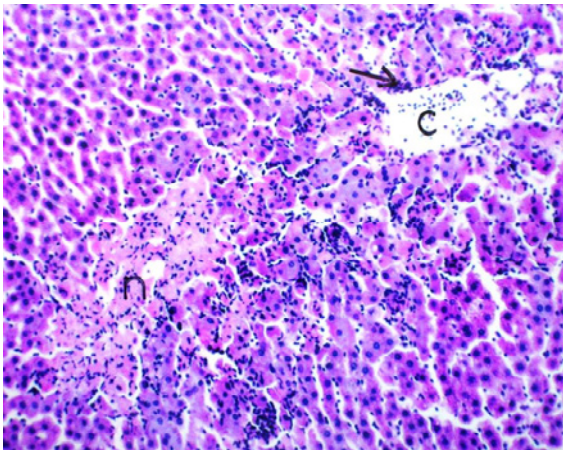


Figure 5. Liver of rat administrated flavonoids mixture and carbon tetrachloride. The photo shows focal cogulative necrosis (n) with inflammatory cells infiltration between the hepatocytes.

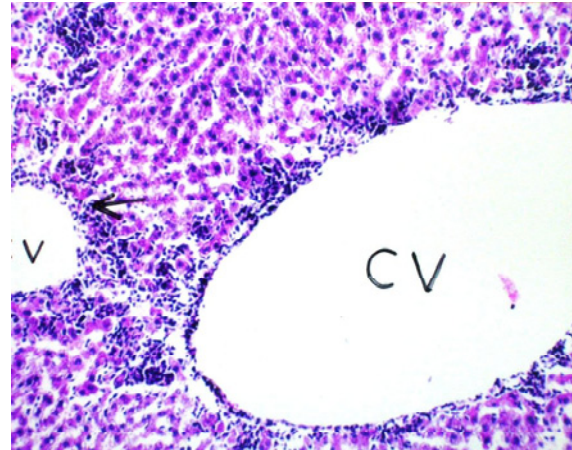


Figure 6. Liver of rat administrated silymarin and carbon tetrachloride. The photo shows massive numbers of inflammatory cells infiltration surrounding the dilated central vein (CV).

3.3. Histopathological conditions of liver

Figure 3 shows a representative photomicrograph of the protective effect of AEGS against CCl_4 -induced liver injury in rats. Rats treated with normal saline showed no necrosis, inflammation, or vascular degeneration (Figure 3). Rats treated with AEGS and its components (spicatic acid and flavonoids) showed few focal areas of coagulative necrosis with inflammatory cell infiltration in the hepatic tissue with mild tubular dilatation (Figures 4 and 5, respectively), when compared to those treated with only silymarin used as a standard reference (Figure 6). In rats administered CCl_4 alone, focal necrosis was seen with degeneration associated with inflammatory cell infiltration and severe tubular dilatation in the central vein (Figure 7) and middle zones prominent with many kupffer cells around the lesions.

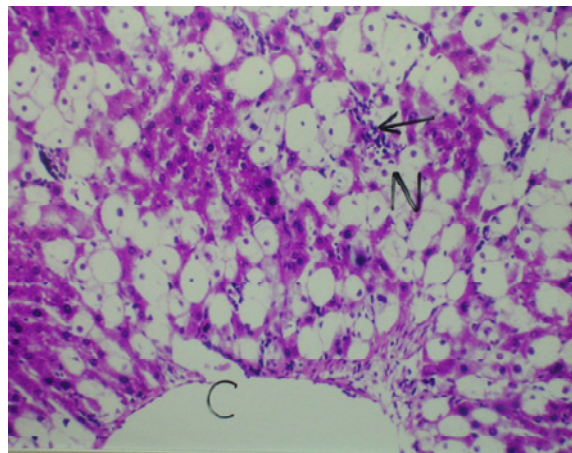


Figure 7. Liver of rat administrated carbon tetrachloride. The photo shows severe dilatation of central vein (C) with necrosis (N) and inflammatory cells infiltration (arrow) in the surrounding hepatocytes.

4. Discussion

A huge effort has been exerted to discover new antioxidants from natural compounds (19,20). Complementary and alternative medicine is becoming popular among patients with liver diseases (21). Different phenolic compounds used as folk medicine for several centuries have proven to ameliorate some inflammatory ailments as hepatitis in China (22).

Serum transferases (ALT and AST) are reliable markers of liver function (23). These enzymes are released into the blood as a result of cell membrane damage. The ease of liberation from liver hepatocytes of ALT and AST is considered a very sensitive indicator of necrotic lesions within the liver. Indeed they were significantly increased in control animals given only CCl₄; in group 2, administration of CCl₄ to rats induced severe xenobiotic-hepatotoxic effects. CCl₄ is metabolized in the body to a highly reactive trichloromethyl radical (CCl₃) which is responsible for initiation of a series of complex chain reactions. These reactions result in production of lipid peroxides which attack membrane phospholipids and stimulate lipid peroxidation and cell lysis, a decrease in membrane integrity and a loss in selective permeability (9).

In the present study, CCl₄-induced hepatotoxic effects could be attributed to free radical generation noted by an increased MDA level, one of several byproducts of the lipid peroxidation process. The increase in TBARS (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals.

Treatment with the ethanolic extract of *G. spicata* and its components group (3); aqueous ethanolic extract of aerial parts, group (4) flavonoids mixture, and group (5) 4-carboxgentisic (spicatic acid), significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of *G. spicata* is due to its antioxidant activity owing to the presence of polyphenolics contents. It was proven that the decreased MDA level was due to the flavonoids mixture in which it was comparable to silymarin. Flavonoids are known to exhibit a hepatoprotective effect (24). Flavonoid compounds were proven to protect against carbon tetrachloride-induced injury (25) and that was proven in this study as *Gentiana* is rich in flavonoids.

Glutathione is one of the most abundant biological antioxidants present in liver. It is a non protein cysteine reservoir in the liver and is involved in many cellular processes including the detoxification of endogenous and exogenous compounds. Glutathione is able to protect cellular constituents from the toxic effects of free radicals. This reflects the inability of liver cells to retain intracellular enzymes which indicates severe damage to the plasma membrane. Its functions are concerned with removal of free

radical species and cytotoxic active oxygen species including hydrogen peroxide, superoxide radicals, hydroxyl radical, hydrogen peroxide, nitric oxide and alkoxy radicals. It also removes non radical species such as hydrogen peroxide and singlet oxygen and is involved with maintenance of membrane protein thiols and as a substrate for glutathione peroxidase (GPx) (26). As long as there is homeostasis between the rate of radical generation and radical dissipation, the cellular generation of free radicals is not considered harmful (27). Reactive oxygen radicals are involved in cell growth, differentiation, progression, and death. Therefore, low concentrations of reactive oxygen radicals are considered beneficial and even indispensable in processes such as intracellular signaling and defense against microorganisms. Nevertheless, higher amounts of reactive oxygen radicals contribute to the aging process and many diseases (28). Several biological molecules that are involved in cell signaling and gene regulation systems are very sensitive to the redox status of the cell. ROS within cells act as secondary messengers in intracellular signaling cascades. ROS can induce as well cellular senescence and apoptosis and therefore function as anti-tumorigenic species (29). Oxidative stress induces a cellular redox imbalance that has been found in various cancer cells when compared to normal ones. The redox imbalance thus may be related to oncogenic stimulation. Oxidative DNA lesions have been noted in many tumors (29). Antioxidants induce gene expression of detoxifying enzymes and small molecules that mimic antioxidant enzymes are known to be tools for treatment of many diseases (30). Although antioxidants from plant origins have proven to protect against hepatotoxicities, it's not recommended to rely on herbal supplements for routine treatment of any chronic liver disease because of the relative paucity of clinical studies (21).

The close resemblance in structure between spicatic acid and salicylic acid might explain its antioxidant effect against the hepatotoxicity of CCl₄. Salicylic acid was proven to inhibit lipooxygenase-catalyzed lipid peroxidation at therapeutic concentrations. These findings suggest possible inhibitory activity against enzymatic lipid peroxidation in clinical settings (12). Iron is essential for lipooxygenase activity and salicylic acid is known for its interference with iron. The present investigation is the first to separate the promising antioxidant spicatic acid that proved hepatoprotective and which resembles salicylic acid in its ability to neutralize reactive oxygen species through nonenzymatic mechanisms.

Gentiana spicata contains a mixture of biologically active constituents belonging chemically to flavonoids (4) which have antioxidant properties through a radical scavenging mechanism (31-33).

The suggested mechanism could also be due to the presence of the isolated catechins (catechin,

epicatechin and their gallate ester derivatives) that act as antioxidants even more than vitamin C (34). These catechins isolated from *G. spicata* were similar to the ones extracted from green tea (35) and proved to mediate antioxidant and free radical scavenging activities. *Gentiana spicata* is considered to be an excellent antioxidant source, which could contribute to the prevention of many diseases related to oxidative stress.

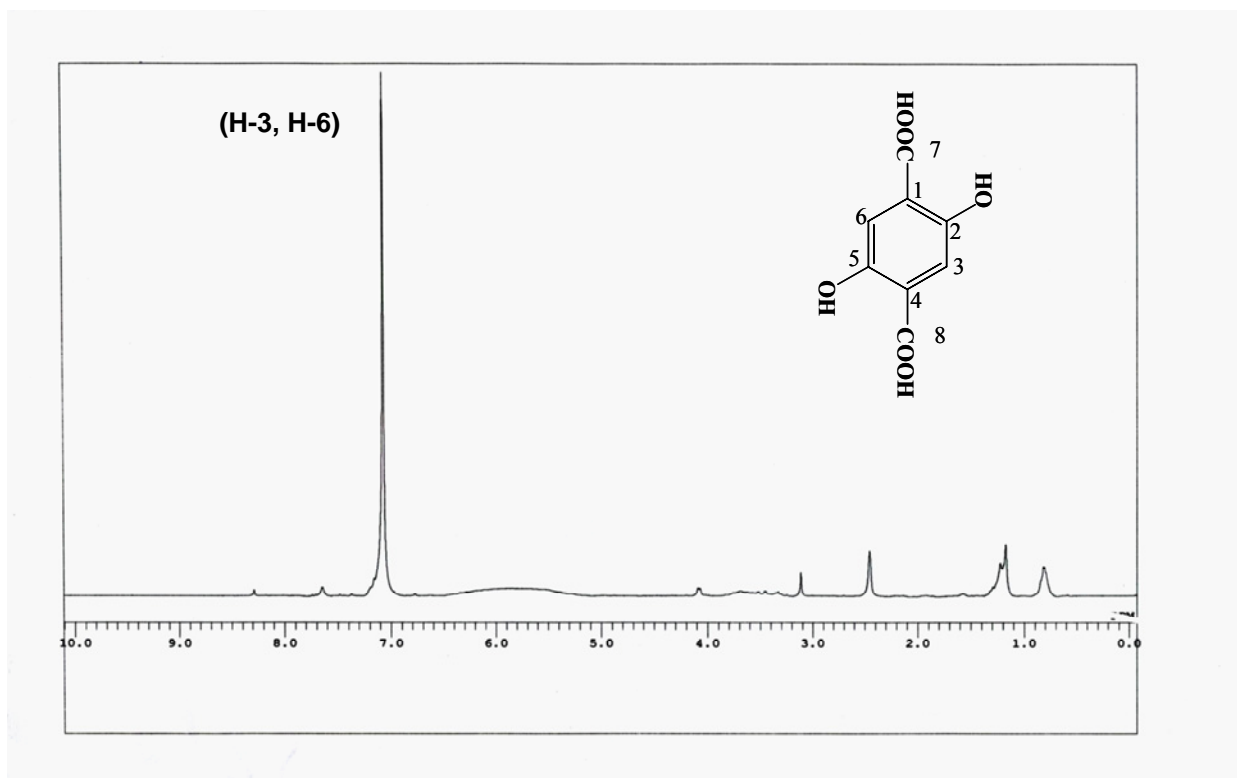
In conclusion, the results of the present study suggest that the aqueous ethanolic extract (250 mg/kg.b.wt.) of *Gentiana spicata* family Gentianaceae flavonoid mixture and spicatic acid possesses a potential activity to alleviate the hepatotoxic effects associated with CCl₄ administration. This was seen from normalization of serum ALT and AST. Liver MDA activity was highly reduced while liver reduced glutathione was elevated. This indicated a potent hepatoprotective activity against CCl₄-induced free radical species.

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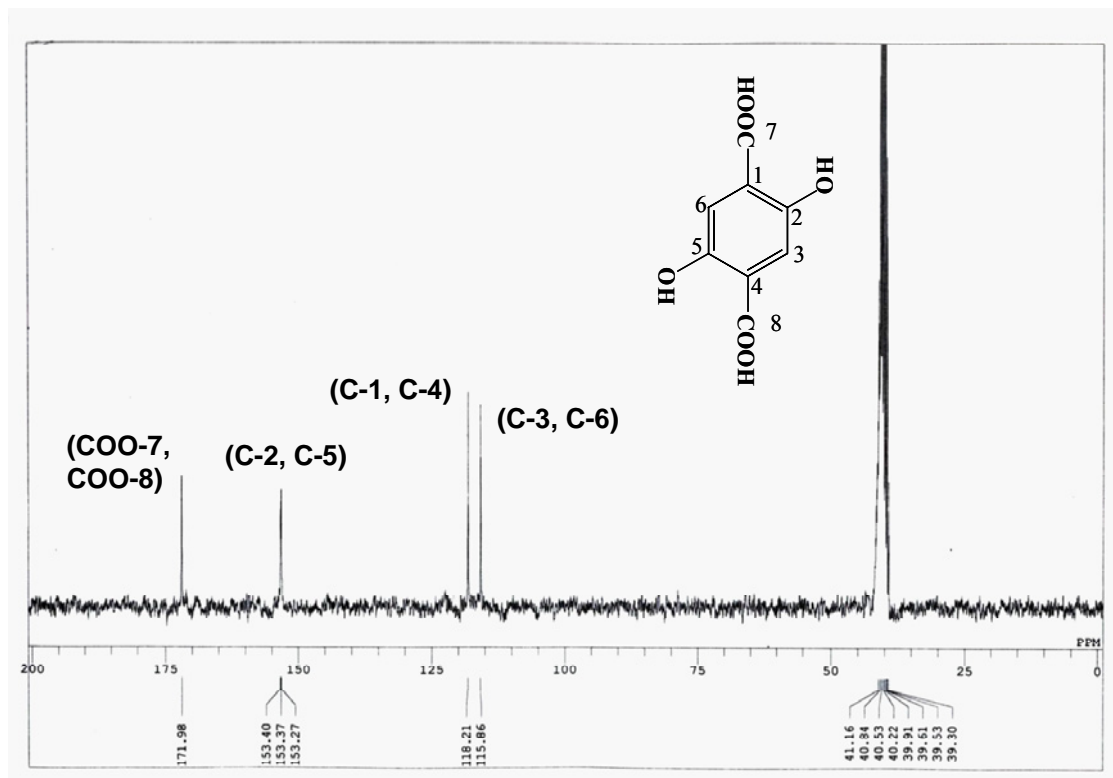
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Appendix 1



¹H-NMR of compound (1): 1,4-dicarboxy 2,5-dihydroxy benzene (spicatic acid).

Appendix 2



^{13}C -NMR of compound (1): 1,4-dicarboxy 2,5-dihydroxy benzene (spicatic acid).