A publication of Phcog.Net
Pharmacognosy Magazine

ORIGINAL ARTICLE
Year : 2019 | **Volume** : 15 | **Issue** : 65 | **Page** : 659--670

Metabolic profiling of *Solanum villosum* Mill subsp. *miniatum* (bernh. ex willd.): Hepatoprotective and antifibrotic activity in a rat model of liver fibrosis

Alaa El-Din E. Abdel-Hamid[1], Riham Salah El Dine[1], Jandirk Sendker[2], Soheir M El Zalabani[1], Meselhy R Meselhy[1], Elena Jimenez-Negro[2], Ashraf B Abdel-Naim[3],

[1] Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt

[2] Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, Münster, Germany

[3] Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

Correspondence Address:
Ashraf B Abdel-Naim
Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah
Saudi Arabia

Abstract

Aim/Background: To assess the liver antifibrotic action of ethanolic extract of aerial parts of *Solanum villosum* Mill subsp. *miniatum* (Bernh. ex Willd.) (SVE) and correlate this activity with its high performance liquid chromatography-quadrupole time of flight (HPLC-qTOF) Electrospray Ionisation Mass Spectrometry (ESIMS) phytochemical profile. **Materials and Methods:** The median lethal dose of SVE was determined, and a rat model of carbon tetrachloride (CCl_4)-induced liver fibrosis was used to evaluate its antifibrotic activity. Markers for hepatotoxicity, fibrosis, and oxidative stress were assessed, and histopathological features of liver tissues were examined. Metabolite profiling of SVE was achieved via HPLC-qTOF-ESIMS coupled with Photodiode array (PDA). Liver fibrosis was induced in rats by oral administration of $CCl_4$for 6 weeks. Silymarin (positive control) and SVE (100 and 250 mg/kg) were orally administered daily for 6 weeks. **Results:** Compared to CCl_4-intoxicated group, administration of SVE obviously ameliorated fibrosis of the hepatic capsule associated with aggregation of multiple focal fat cells formation. Both silymarin and SVE ameliorated the rise in serum markers of hepatotoxicity (alanine transaminase, aspartate transaminase, and alkaline phosphatase), markedly attenuated CCl_4-induced oxidative stress. The antifibrotic activity of SVE was evidenced by inhibiting the rise in hepatic hydroxyproline content and accumulation of collagen. This was confirmed by the ability of SVE to inhibit alterations in expression of the fibrosis-related genes Collagen Iα, matrixmetalloproteinase-2 (MMP-2), tissue inhibitor matrix metalloproteinase-2, and transforming growth factor beta 1. HPLC-qTOF-ESIMS analysis of SVE revealed the presence of 47 metabolites, among which 33 were tentatively identified. **Conclusion:** SVE exhibited hepatoprotective and antifibrotic activities in rats by enhancing the antioxidant capacity and regulating expression of fibrogenic mediators.

How to cite this article:
E. Abdel-Hamid AE, El Dine RS, Sendker J, El Zalabani SM, Meselhy MR, Jimenez-Negro E, Abdel-Naim AB. Metabolic profiling of *Solanum villosum* Mill subsp. *miniatum* (bernh. ex willd.): Hepatoprotective and antifibrotic activity in a rat model of liver fibrosis.Phcog Mag 2019;15:659-670

How to cite this URL:
E. Abdel-Hamid AE, El Dine RS, Sendker J, El Zalabani SM, Meselhy MR, Jimenez-Negro E, Abdel-Naim AB. Metabolic profiling of *Solanum villosum* Mill subsp. *miniatum* (bernh. ex willd.): Hepatoprotective and antifibrotic activity in a rat model of liver fibrosis. Phcog Mag [serial online] 2019 [cited 2019 Sep 29];15:659-670
Available from: http://www.phcog.com/text.asp?2019/15/65/659/267175

Full Text

SUMMARY

Compared to carbon tetrachloride (CCl4)-intoxicated group, administration of ethanolic extract of aerial parts of Solanum villosum Mill subsp. miniatum (Bernh. ex Willd.) (SVE) significantly decreased fibrosis of the hepatic capsule associated with aggregation of multiple focal fat cells formation. Both silymarin and SVE reversed the biochemical markers of hepatotoxicity (alanine transaminase, aspartate transaminase, and alkaline phosphatase), markedly attenuated CCl4-induced oxidative stress by ameliorating the decline in the activities of superoxide dismutase, catalase, and glutathione reductase and impeding accumulation of lipid peroxidation products. SVE inhibited alterations in expression of the fibrosis-related genes Collagen Iα, MMP-2, tissue inhibitor matrix metalloproteinase-2, and transforming growth factor beta 1. This was further confirmed by inhibiting the rise in hepatic hydroxyproline content. High performance liquid chromatography-quadrupole time of flight ESIMS analysis of SVE revealed the presence of 47 metabolites, among which 33 were tentatively identified. In conclusion, the metabolic profile of the investigated extract indicated the presence of a mixture of bioactive components, especially steroidal saponins, steroidal glycoalkaloids, and polyphenols that possibly contribute to observed hepatoprotective and antifibrotic activities in rats.

[INLINE:1]

Abbreviations used: µl: Microliter; µM: Micrometer; µmol: Micromole; ALP: Alkaline phosphatase; ALT: Alanine transaminase; ANOVA: Analysis of variance; AST: Aspartate transaminase; CAT: Catalase; CCl4: Carbon tetrachloride; cDNA: Complementary deoxyribonucleic acid; Col-1α: Collagen alpha 1; DAD: Diode array detector; DM: Diabetes mellitus; DMSO: Dimethylsulfoxide; DNA: Deoxyribonucleic acid; ECM: Extracellular matrix; ESI: Electrospray ionization; eV: Electron volt; Fig: Figure; GHS: Globally Harmonized System of Classification and Labeling of Chemicals; GR: Glutathione reductase; GSH: Glutathione reduced; H and E: Hematoxylin and eosin; HPLC: High performance liquid chromatography; Hz: Hertz; Kg: Kilogram; LD50: Median lethal dose; LDH: Lactate dehydrogenase; m/z: Mass per charge; M*: Molecular ion peak; MDA: Malondialdehyde; MeOH: Methanol; Mg: Milligram; mg/dl: Milligram per deciliter; mg/ml: Milligram per milliliter; min: Minute; Ml: Milliliter; mM: Millimolar; mmol: Millimole; MMP-2: Matrix metalloproteinase-2; MS: Mass spectroscopy; MS/MS: Mass spectrometry/Mass spectrometry; NADPH: Reduced nicotinamide adenine dinucleotide phosphate; Nm: Nanometer; OECD: Organisation for Economic Co-operation and Development; P: Probability; p.o: Orally; PCR: Polymerase chain reaction; PDA: Photodiode array; Rf: Retardation factor; RNA: Ribonucleic acid; Rpm: Revolutions per minute; rRNA: Ribosomal ribonucleic acid; SE: Standard error; Ssp: Subspecies; SVE: Solanum villosum Ethanolic Extract; TBA: Thiobarbituric acid; TBARS: Thiobarbituric acid reactive substances; TGF-β: Transforming growth factor beta; TIMP-2: Tissue inhibitor matrix metalloproteinase-2; TOF: Time of flight; UV: Ultraviolet; V: Volt; v/v: Volume per volume; VBA: Visual basic for application; VLC: Vacuum liquid chromatography; β-actin: Beta-actin.

Introduction

Solanum is a large and diverse genus of annual and perennial plants; the majority of which are used in folk medicine[1] and some are important foodstuffs. Plants of this genus are rich in steroidal saponins, glycoalkaloids,[2] and phenolic compounds.[3]Solanum villosum Mill subsp. miniatum (Bernh. ex Willd.) is distributed and the most predominant in Africa[4] and used traditionally in the treatment of inflammatory swelling as well as liver and spleen enlargement, sore eye, and is easily available to the local people.[5] Leaves of this plant are also eaten as boiled salad, and its orange berries are consumed as fruit.[6] The antioxidant capacity and free radical scavenging activity of the ethanol extract of S. villosum as well as the effects related to its bioactive phytochemical constituents were evaluated.[7] The chloroform: methanol extract (1:1 v/v) of mature green leaves showed potent larvicidal activity against the fourth instar larval form of Culex quinquefasciatus,[6] while green, mature fruits extract showed potential biocontrol agent against Stegomyia aegypti, a common vector of dengue fever.[8]

Liver fibrosis is not an independent disease but rather a histological change caused by liver inflammation and characterized by imbalance in the production and degradation of extracellular matrix (ECM) components in the liver.[9] Under chronic stress, the localized fibrosis expands, eventually leading to cirrhosis, increased risk of liver cancer, and the need for a liver transplant in advanced stages. Extensive progresses toward better understanding of the cellular and molecular mechanisms of liver fibrosis have greatly motivated the development of potential antifibrotic agents.[10] Unfortunately, drugs used to reduce fibrosis (such as corticosteroids or penicillamine) are usually either too toxic to be taken for long time or ineffective.[11],[12] On the contrary, the broad spectrum of biological activities offered by natural products and herbal medicines has increased interest in their potential for treating liver fibrosis.[13]

Materials and Methods

Chemicals

Carbon tetrachloride (CCl4), dimethylsulfoxide (DMSO), corn oil, p-nitrophenol, p-nitrocatechol, Ellman's reagent, chloramine-T, p-dimethylaminobenzaldehyde, indocyanine green, and thiobarbituric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Reduced nicotinamide adenine dinucleotide phosphate was supplied by Sorachim Chemicals (Lausanne, Switzerland). All other chemicals were of commercial grade.

Plant material

The aerial parts of S.villosum Mill subsp. miniatum (Bernh. ex Willd.) were collected during fruiting stage in April 2014 from plants cultivated at the Experimental Plants Station, Faculty of Pharmacy, Cairo University, Giza, Egypt. Identity of the plant material was verified by Dr. Reem Samir Hamdy, Associate Professor of Taxonomy and Flora, Department of Botany, Faculty of Science, Cairo University. A Voucher specimen (No 2014.6.2a) was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extraction

The powdered air-dried aerial parts of S.villosum (1.5 kg) were extracted thrice with 95% ethanol (4.5 L), at room temperature. The solvent was evaporated under reduced pressure to yield 200 g of dark green semisolid residue. This residue (referred to herein as SVE) was kept at 4°C until further use.

High performance liquid chromatography-quadrupole time of flight ESIMS analysis

Chromatographic separation was performed by means of a Dionex Ultimate 3000 RS Liquid Chromatography System, on a Dionex Acclaim RSLC 120, C18 column (2.1 mm × 100 mm, 2.2 μm). Sample injection volume was 2 μL. The binary elution system consisted of A: H2O with 0.1% formic acid and B: MeCN with 0.1% formic acid, at 0.4 mL/min. A stepwise elution gradient was adopted: 0–5 min, 5% B; 5–37 min, linear 5%–100% B; 37–47 min, isocratic 100% B; 47–48 min, linear 100%–5% B; 48–55 min, isocratic 5% B. Detection of eluted compounds was performed with a Dionex Ultimate diode array detector-3000 RS, over a wavelength range of 200–400 nm and a Bruker Daltonics micro TOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source in positive mode at 3 Hz over a mass range of m/z 50–1500. The instrument settings were adjusted as follows: nebulizer gas N2, 4 bar; dry gas N2, 9 L/min, 200°C; capillary voltage, −4500 V; end plate offset, −500 V; transfer time, 100 μs; prepulse storage, 6 μs; collision gas, N2; collision energy, 8 eV (FullMS) or 40 eV (mass spectroscopy [MS]/MS); collision RF 130 Vpp. MS/MS scans were triggered using AutoMS2 settings that select up to two ions, within an m/z range of 200–1500 and a minimum intensity of 2000 counts as precursor ions for two successive MS/MS scans, unless the same ion has been either selected as precursor ion within 10s earlier in time or identified as constant background signal. Internal dataset calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10 mM solution of sodium formate in 50% isopropanol that was infused during liquid chromatography (LC) re-equilibration using a divert valve equipped with a 20 μL sample loop.

Sample preparation for high performance liquid chromatography-quadrupole time of flight-ESIMS

A known weight of the SVE was dissolved in methanol (MeOH) by ultrasonication for 10 min. The solution was centrifuged, and the supernatant was transferred and diluted with MeOH to a final concentration of 5 mg/mL. This solution was used for LC-MS analysis.

Data processing

LC-MS data were processed using the Dissect Compounds function of Data Analysis 4.1 SR1 (Bruker Daltonics) using an internal s/n threshold of 5 and a maximum number of overlapping compounds of 5. The monoisotopic molecular weight of each compound was determined through an in-house visual basic for application script from the most intense adduct ion found in its full MS spectrum. Compounds were tentatively identified or characterized based on accurate m/z values of adduct ions, fragment ions, or neutral losses observed in full MS or preferably MS/MS spectra and whenever possible, ultraviolet (UV) spectra. Identification was aided through METLIN and KNApSAcK databases.[14],[15]

Assessment of antifibrotic activity

Animals

Male Wistar rats, weighing 200–250 g, were purchased from the animal house of King Fahd Medical Research Center, King Abdulaziz University. Animal handling and experimental protocol were approved by the Unit of Biomedical Ethics at King Abdulaziz University, Jeddah, Saudi Arabia (Reference # 157-14) and were housed in a well-ventilated, temperature-controlled room at 22°C ± 3°C with 12 h light/dark cycle. Food consisted of normal rat chow and water provided ad libitum.

Acute oral toxicity study (median lethal dose)

The acute toxicity of SVE was tested in male SWR mice (6 mice) according to Organisation for Economic Co-operation and Development (OECD) guidelines, Annex 2d.[16] Animals were fasted overnight and the extract administered orally using a gastric needle at a dose up to 5000 mg/kg (in 1% DMSO in saline, 10 ml/kg dosing volume).

Experimental protocol

Rats were randomly divided into five groups of six animals each. Group A (control group) received 1% DMSO in saline (1 ml/kg, P.O., daily) as well as corn oil (1 ml/kg, P.O., 3 times/week). Group B (fibrosis model group) received 1% DMSO in saline (1 ml/kg, P.O., daily) as well as CCl4 mixed with corn oil 1:1 (0.5 ml/kg, P.O., 3 times/week). Dose and regimen of CCl4 were based on data published in literature with little modifications.[17] Group C was given silymarin in 1% DMSO (50 mg/kg, P.O., daily) along with CCl4 mixed with corn oil 1:1 (1 ml/kg, P.O., 3 times/week). Groups D and E were given P.O. daily SVE dissolved in 1% DMSO in saline (100 or 250 mg/kg, respectively) together with CCl4 mixed with corn oil 1:1 (1 ml/kg, P.O., 3 times/week). The choice of these two doses was based on a preliminary experiment as well as high safety of the extract as indicated by median lethal dose (LD50) testing.

All animals were treated over a period of 6 weeks; then, blood samples were collected from the retro-orbital plexus. Animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Sera (separated by centrifugation at 1000 rpm, for 10 min) were used for assessment of liver functions. Rats were sacrificed by decapitation 24 h after the last treatment and livers were dissected out and weighed. Each liver was divided into three parts: the first was kept in formalin for histopathological examination, the second was saved for homogenization in phosphate buffer saline (0.1 M, pH 7.4), and the last part was kept as such in ribonucleic acid (RNA) stabilization solution for 24 h. Both second and third portions were then kept at −80°C for subsequent analyses.

Hepatotoxicity markers

Aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activities were assessed colorimetrically using commercial assay kits and following the instructions indicated by the manufacturer (Biodiagnostics, Dokki, Giza, Egypt).

Evaluation of oxidative status

Generation of lipid peroxides was assessed by measuring the concentration of thiobarbituric acid reactive substances calculated as malondialdehyde (MDA) in homogenates according to previous methods.[17] Superoxide dismutase (SOD) and catalase (CAT) activities and H2O2 content were assessed in liver homogenates using commercial assay kits according to the manufacturer's instructions (Biodiagnostic, Dokki, Giza, Egypt). Assessment of glutathione reductase (GR) was according to previous procedures.[18]

Determination of hydroxyproline

Total hydroxyproline content (pg/mg liver tissue) was determined according to previous reports[19] and used as a quantitative measure of collagen deposition and fibrosis.

Assessment of gene fibrosis markers by real-time polymerase chain reaction

The gene markers, collagen 1α, MMP-2, tissue inhibitor matrix metalloproteinase-2 (TIMP-2), and transforming growth factor beta 1 (TGF-β1) were assessed by real-time polymerase chain reaction (PCR) (RNA preparation, reverse transcription, and quantitative real-time PCR) according to previous methods.[20] Total RNA was extracted from liver tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's protocol. Genomic DNA was eliminated by a DNase-on-column treatment supplied with the kit.

The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the ratio of the absorbance at 260 and 280 nm was used to assess the purity of RNA. Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 μg RNA using a high capacity cDNA reverse transcription kit (Promega Benelux Biotechnology, Leiden, The Netherlands). Real-time quantitative PCR was performed with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems Inc., Carlsbad, California, USA) to detect the expression levels of collagen alpha 1, matrix metalloproteinase2 (MMP-2), TIMP-2, and TGF-β. Beta-actin was used as an internal control. Each 10 μl reaction contained 5 μl SYBR Green Master Mix (Applied Biosystems Inc., Carlsbad, California, USA), 0.3 μl gene-specific forward and reverse primers (10 μM), 2.8 μl cDNA, and 1.9 μl nuclease-free water. The sequences of PCR primer pairs used for each gene were recorded, and data were analyzed with the ABI Prism sequence[20] from PE Applied Biosystems (Foster City, CA, USA) and expressed as the mean fold change ± standard error (SE) for three independent amplifications. Relative expression of studied genes was calculated using the comparative threshold cycle method.

Histopathological analysis

For histopathological examination, samples of rat livers from control and experimental groups were fixed in 10% neutral-buffered formaldehyde for 24 h. The standard method of dehydration, immersion in xylene and embedding in paraffin at 56°C in hot air oven for 24 h was used. Sections, 4-μm thick, were prepared by means of a sledge microtome and stained with hematoxylin and eosin (H and E) then with Masson's Trichrome stain to allow visualization of collagen fibers in the fibrotic tissues.[21] Stained slides were examined and photographed using a Nikon TE 2000 microscope (Nikon, Japan).

Statistical analysis

Data are presented as mean ± SE. Multiple comparisons were performed using one-way analysis of variance followed by Tukey–Kramer as a post hoc test (GraphPad Prism 6.01, GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were considered significant at $P < 0.05$.

Results

High performance liquid chromatography-PDA-ESIMS analysis of ethanolic extract of Solanum villosum

Analysis of the chemical constituents of SVE by high performance liquid chromatography-quadrupole TOF-ESIMS revealed the presence of 47 metabolites [Table 1] and [Figure 1]. Tentative identification of 33 was made possible with the aid of METLIN and KNApSAcK databases.[14],[15] The identities, retention times, UV characteristics, and observed molecular and fragment ions for individual components are depicted in [Table 2]. The identified metabolites included 1 coumarin (Scopoletin), 3 flavonoids, 1 phenolic acid (Phaseolic acid), 1 fatty acid (3-(1-hydroxymethyl-1-propenyl) glutaric acid), 6 N-phenylpropenoyl amides, 10 steroidal saponins, and 11 steroidal alkaloids.{Table 1}{Figure 1}{Table 2}

Steroidal alkaloids

N-containing [M*H]* ions, corresponding [M*H*Na]2* ions, and a fragmentation pattern indicating a glycosidic moiety [Figure 2]. Among these, three different glycosylation patterns were observed, which match chacotriose (neutral loss of 454 μ), solatriose (neutral loss of 470 μ), and their hydrolysis product (leading to β1- or β2-compounds, neutral loss of 308 μ), all of them being common features of Solanum glycoalkaloids [Table 2].[22] Each glycosylation pattern occurred with three different C27-aglycon masses [Table 2]. In addition, compounds 42 and 45 were detected as free aglycones of these compounds. The aglycones of all eleven compounds were in turn characterized by analyzing the product ion spectra of either their [M*H]* or of the aglycone fragment if available (pseudo-MS3). Steroidal alkaloids of spirosolan-type (solasodine and solasodine glycosides) and of solanidan-type (solanidine glycosides) have been reported to occur in S. villosum.[23],[24] Solasodine-type steroidal alkaloids can be recognized by diagnostic fragments resulting from the cleavage of their F ring, which is an ion at m/z 114 and a neutral loss of 143 u (C8H17 NO) for compounds without F ring modifications such as solasodine. The absence of a fragment ion at m/z 161 suggests that all eleven compounds have a 5, 6 double bond.[25]{Figure 2}

An ion at m/z 114 was found in all product ion spectra except those of 12, 15, and 19, which were indistinguishable with regard to their aglycone fragments. These three compounds have two more oxygens than solasodine, and their product ion spectra showed a neutral loss of 159 u (C8H17 NO2 by accurate mass) between the aglycone fragment at m/z 446 and its fragment ion at m/z 287. We therefore suggest that 12, 15, and 19 are dihydroxysolasodine glycosides with a hydroxylated F ring, which apparently prevented the formation of the diagnostic fragment at m/z 114. The remaining hydroxyl group must be attached to rings A – D as indicated by the fragment ions at m/z 287 and two fragment ions at m/z 269 and 251, which represent the elimination of one or two water molecules from m/z 287, respectively.

Mass spectra of 30, 32, 38, and 45 each showed an aglycone signal at m/z 414; their product ion spectra were indistinguishable with regard to their diagnostic aglycone fragments at m/z 114, 271, and 253. The product ion spectra of m/z 414 were identical to reference spectra of solasodine[26] (MID 41805). Thus, 30 was identified as α-solasonine, 32 as α-solamargine, and 45 as solasodine; all three are known constituents of S. villosum.[23],[24] Compound 38 can be taken as the result of either cleaving one glucosyl moiety from 30 (β1-solasonine) or one rhamnosyl moiety from 32 (β1-or β2-solamargine).

Mass spectra of 21, 22, 26, and 42 each showed an aglycone signal at m/z 430 with an accurate mass indicating one more oxygen atom than solasodine. The product ion spectra of 21, 26, and 42 were indistinguishable with regard to their diagnostic aglycone fragments: m/z 114 indicates an unmodified F ring as found in solasodine; as aforementioned. The fragment ions at m/z 287, 267, and 251 indicate an additional hydroxyl group at rings A – D. Compound 21 was tentatively assigned as 12-hydroxysolasonine, which is a known constituent of S. lycocarpum.[14] In contrast, 22 shows the fragment ions of solasodine, at m/z 114, 271, and 253 [Figure 3]. Thus, the only remaining options for the additional oxygen atom are hydroxylation of C-20 or the adjacent methyl group C-21 [Figure 3]. The only known compound with this structural feature is 21-hydroxysycophantine, which has been isolated from Solanum sycophanta Dunal.[27] As this compound has a different sugar chain, 22 should be a new natural compound, most likely 3-O-chacotriosyl-21-hydroxysolasodine. The specific assignments of 21, 22, 30, 32 and 45 to particular compounds are based on the assumption that stereochemistry and linkage positions within the sugar chains are identical to previously described ster oidal alkaloid glycosides of S. villosum or other Solanum species and insofar inductive.[23]{Figure 3}

N-phenyl propenoyl amides

Compound 41 is another nitrogenous compound with a molecular formula of C34H37N3O6 whose product ion pattern resembles that of a steroidal alkaloid glycoside. The accurate masses of its product ions however indicate the presence of three coumaroyl moieties attached to a C7H19N3 skeleton [Figure 4]. A prominent m/z signal of the molecule indicates that none of the three nitrogen atoms has basic properties. This supports that the three nitrogens are part of N-coumaroyl amide partial structures. Thus, tricoumaroylspermidine is a very reasonable candidate for 41. This compound has been described as a constituent of safflower and peanut flowers in the form of different geometric isomers.[28],[29]{Figure 4}

Compounds 7, 16, 20, 25, and 29 were identified as N-phenyl propenoyl amides based on their accurate masses, isotope patterns, and product ion spectra, the latter ones being identical with reference spectra found in the METLIN database.[15]N-phenyl propenoyl amides are wide-spread herbal constituents.[30]

Steroidal saponins

Ten compounds (27, 33, 34, 36, 39, 40, 50-53) were detected with odd-numbered [M*H]* and [M*Na]* ions and fragmentation patterns pointing toward a glycosidic moiety. Their accurate aglycone masses and the presence of at least low intensity fragment ions at m/z 115 and– except for 39– at m/z 273 or 271 indicate that these compounds are spirostan analogs of the aforementioned steroidal alkaloids.

Six compounds (34, 40, and 50-53) showed the diagnostic fragments of a fully saturated spirostan system at m/z 417, 273, 255, and 161 [Figure 5].[25] The same signals occur in the fragment ion spectra of its acetylated epimer neotigogenin,[15] (MID 43696). The indistinguishable aglycones of 34, 40 and 50-53 can be considered as tigogenin, which has been found in a hydrolyzed extract of S. villosum.[31]{Figure 5}

Product ion spectra of 27, 33, and 36 showed a signal of the intact aglycone at m/z 415 and thus should include a double bond. The absence of m/z 161 in 33 and 36 suggest a Δ5,6 double bond, which is in accordance to the presence of prominent product ions at m/z 271 and 253.[25] The aglycone of 33 and 36 can thus be considered as diosgenin, which only differs from tigogenin by its double bond.[32] In contrast, m/z 161, 273, and 255 are product ions of 27 and allow for locating its double bond at Δ20, 21 but not in the F ring as the product ion at m/z 115 indicates a fully saturated F ring.

The aglycone of 39 shows an accurate mass at m/z 431 indicating a molecular formula with one more oxygen atom than 27, 33, and 36. Structural information for 39 could only be deduced from the product ions at m/z 115 and the absence of m/z 161. Thus, 39 should have a saturated F ring and a Δ5, 6 double bond, whereas no indication for the position of the additional oxygen atom was found. It seems likely that a modification in the A–D ring region prevented formation of the otherwise prominent product ions at m/z 273, 271, 255, 253, or any other comparable fragment.

The sugar chains of the ten steroidal saponins showed no overlap with the sugar chains of the aforementioned steroidal alkaloid glycosides [Table 2]. Low signal intensities or homogenous sugar composition prevented the analysis of the sugar chains of 36, 50, 52, and 53 beyond their composition.

The sugar chains of 33, 34, and 39, each constituted of four hexose and one pentose unit, were analyzed on the basis of product ion and neutral loss spectra of their respective protonated molecules and found indistinguishable. We propose a linear chain of four hexose units with the pentose unit attached to the second hexose [Figure 5]. Thus, 34 was tentatively identified as tigonin, which has been vaguely reported to occur in family Solanaceae; Cestrum diurnum[33] and a very similar compound in Capsicum annuum.[34] Compound 27 was– at least by its limited MS data – indistinguishable from 33; both compounds were tentatively identified as Δ5, 6-dehydrotigonin.

Compound 40, consists of three hexose, one pentose and one deoxypentose unit. Its product ions suggest a twofold branched arrangement comparable to the neotigogenin glycoside tribulosin found in the Zygophyllaceae Tribulus cistoides.[35]

The neutral loss spectrum of protonated 51 shows the first significant signal at −456 μ, indicative for the loss of two hexose and one deoxyhexose unit. The corresponding signal at m/z 579 in the product ion spectrum represents the aglycone conjugated with a hexose unit. A reasonable candidate for 51 is degalactotigonin, which has been reported to occur in different Solanum species.[36],[37] The specific assignments of 33, 34, 36, 40, and 50–53 to particular compounds or their aglycones are based on the assumption that stereochemistry and linkage positions within the sugar chains are identical to previously described steroidal glycosides of related species and insofar inductive.

Other compounds

Any assignment of compounds belonging to other compounds are based on their accurate masses, isotope patterns, UV data, and either product ion spectra that were identical with reference spectra found in METLIN database[15] or mz Cloud (re3data.org 2017) or alternatively occurrence in genus Solanum as reported in KNApSAcK database.[14]

Acute oral toxicity study (median lethal dose)

No mortality was observed in the tested animals 24 h after oral administration of 5000 mg/kg SVE. The same result was obtained on repeating the test using three additional animals at the same dose. According to the Acute Toxic Class Method reported in OECD guidelines No. 423,[16] SVE is considered by Globally Harmonized System of Classification and Labeling of Chemicals to be unclassified with LD50 cutoff >5000 mg/kg.

Hepatotoxicity markers

Activities of liver enzymes, ALT, AST, and ALP, in sera were used as biochemical markers for hepatotoxicity. Challenging rats with CCl4 led to significant increase in serum AST, ALT, and ALP activities compared to the control group (A), as shown in [Table 3]. However, daily administration of SVE to rats (Groups D and E) attenuated the hepatotoxic insult of CCl4 and significantly restored the levels of AST, ALT, and ALP to values comparable to those in the control group and better than those in Group B [Table 3] and [Figure 6]. Similarly, administration of silymarin over 6 weeks (Group C) significantly decreased serum AST, ALT and ALP activities by 3.4, 3.9 and 1.9 folds, respectively.{Table 3}{Figure 6}

Oxidative stress markers

Administration of CCl4(Group B) resulted in a significant decrease in liver SOD, CAT, and GR activities alongside with a significant increase in MDA content as compared to the control group [Table 4] and [Figure 7]. Liver content of H2O2 was not significantly altered. Administration of SVE (Groups D and E) led to a significant protection against the depletion in SOD, CAT and GR activities as they exhibited almost normal values. Further, accumulation of lipid peroxidation products indicated by MDA by CCl4 challenge was significantly prevented and showed values comparable to those of the control animals. Recovery of all oxidative stress markers was also observed in Group C receiving silymarin.{Table 4}{Figure 7}

Histopathological examination (hematoxylin and eosin stain)

The photomicrographs of H and E stained liver tissues of rats in control and experimental groups are depicted in [Figure 8]. Sections of liver tissues from animals in the control group

showed normal histological structure of the central vein and surrounding hepatocytes in the parenchyma [Figure 8]a. Samples from the CCl4-treated group [Figure 8]b showed significant thickening in the hepatic capsule and fibrosis associated with aggregation of multiple focal fat cells formation underneath the capsule, which was embedded in the parenchyma as a lipoma. Tissue samples from rats treated with silymarin (Group C) or SVE (Groups D and E) [Figure 8]c, [Figure 8]d, [Figure 8]e exhibited mild fibrosis in the capsule with fat cells aggregation underneath hepatic parenchyma as compared to CCl4-treated group [Figure 8]b.{Figure 8}

Antifibrotic activity

Histopathological examination (Masson's Trichome stain) and hydroxyproline content

On treating liver fibrotic tissues with Masson's Trichrome stain, bundles of collagen fibers accumulated around portal tracts and central veins are stained dark blue. In the current study, Masson's Trichrome-stained liver tissues of control rats (Group A), silymarin, and SVE-treated groups (Groups C, D, and E) did not show intense collagen fibers areas although these were visible in stained liver tissues of CCl4-treated rats (Group B) [Figure 9]a, [Figure 9]b, [Figure 9]c,[Figure 9]d, [Figure 9]e. Further, the antifibrotic activities of SV (at both doses) were confirmed by the observed inhibition of liver content of hydroxyproline as compared to CCl4 group [Figure 9]f.{Figure 9}

Assessment of expression of fibrosis-related genes

Administration of CCl4 increased the expression of collagen 1α and TIMP-2 by approximately 822% and 700%, respectively, as compared to A ($P < 0.05$). However, the combined administration of CCl4 with SVE (at doses of 100 and 250 mg/kg) significantly ameliorated the rise in the expression of both genes highlighting the ability of SVE to interfere with the early cellular events leading to fibrosis [Table 5]. Further, mRNA expression of MMP2 was significantly inhibited by 75% CCl4 challenge (Group B) [Figure 10]. This reduction was prevented by both silymarin and SVE treatment. The effect of CCl4 on the expression of TGF-β1 was significantly elevated by 1028% as shown in [Figure 10]d. Interestingly, administration of silymarin (Group C) or SVE (at both dose levels 100 and 250 mg/kg, Groups D and E) led to significant reduction in TGF-β1expression. The observed reduction ($P < 0.05$) amounted to 48.5% in the silymarin-treated group (C) and 71.7 and 76.9% in D and E, respectively.{Table 5}{Figure 10}

Discussion

Although several specific therapies for patients with different liver diseases have been successfully developed, specific and effective antifibrotic therapy remains elusive.[38] Preclinical studies revealed that combination therapies that address liver fibrosis in a multipronged approach hold much promise for future treatment. Several therapies can target specifically fibrosis, soluble mediators, the ECM, and its receptors, and others target a more general component of the liver disease process such as oxidative stress.[39]

Lipid peroxidation and oxidative stress induce inflammation, cause hepatocytes necrosis, and promote the progression of liver fibrosis through the activation of various cytokines.[40],[41],[42] Therefore, a rationale existed to explore the potential antioxidant activity of SVE and relate it to its the overall antifibrotic activity. CCl4 is one of the most hepatotoxins used to induce liver fibrosis in experimental rats and to investigate liver injury associated with oxidative stress and free radicals. In this study, rats treated with CCl4 developed significant hepatic damage and oxidative stress as evidenced by substantial increase in the activities of the liver enzymes (AST, ALT, and ALP) and decrease in the activities of antioxidant enzymes; CAT, SOD, and GR. These findings suggested loss of functional integrity of hepatocyte cell membrane.[43] Nevertheless, on treatment with SVE, the activities of these enzymes were significantly protected. Furthermore, the effect of SVE on lipid peroxidation was evidenced by its ability to prevent MDA accumulation in liver tissues.[44] Such observations highlight the role of the antioxidant potential of SVE in protecting against CCl4-induced oxidative stress.

It was observed that CCl4 administration enhances gene expression of collagen 1α and the hydroxyproline content in hepatic tissues. This is consistent with previous data on CCl4 experimental liver fibrosis.[2] This eventually increases collagenous matrix deposition in liver and subsequently leads to liver fibrosis.[45],[46] In the present study, administration of SVE at both doses as well as silymarin decreased the expression of collagen 1α and reduced the hepatic content of hydroxyproline. These observations gained support by our histopathological examination of the liver slices obtained from the different treatment groups.

Moreover, the impact of SVE on TGF-β1 gene expression was assessed. It is well documented that TGF-β1 is a potent fibrotic factor in hepatic tissues[47] and is known to regulate not only the deposition of the ECM but also pathological fibrosis.[48] It is worth mentioning that two important proteins: metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs) regulate ECM degradation and are associated with liver fibrosis.[49] Previous data reported increase in the expression of TGF-β1 mRNA in animals with liver fibrosis.[50],[51] In the current study, an increase in TGF-β1, TIMP-2 expression, and hydroxyproline content as well as a decrease in MMP-2 expression was observed in the liver tissues obtained from CCl4 treated group. However, coadministration with SVE (or silymarin) induced significant reduction in the expression of TGF-β1and TIMP-2. Accordingly, it is suggested that the ability of SVE to modulate the expression of MMP-2 and TIMP-2 is a crucial for understanding the observed antifibrotic activity.

Several members of genus Solanum were reported to exert hepatoprotective activity;[52] among these are Solanum alatum,[53]Solanum nigrum,[54] and Solanum melongena.[55] Fractions rich in steroidal saponins from both Solanum xanthocarpum and S. nigrum were found to exhibit potent hepatoprotective and antioxidant effects in paracetamol-induced hepatotoxic rats.[56] Moreover, saponin-rich fractions from other plants like Dioscorea nipponica[57] and Asparagus officinalis[58] demonstrated a significant hepatoprotective effect in mice; and diosgenin was reported to be efficient as liver antifibrotic.[59] In addition, several steroidal alkaloids previously isolated from Solanum species showed significant hepatoprotective activity against CCl4-induced liver damage.[60] In fact, capsimine and isocapsicastrine, two steroidal alkaloids isolated from Solanum capsicastrum exhibited strong hepatoprotective effects.[61] Besides, the presence of a number of antioxidant phenolic and flavonoid compounds in SVE greatly correlate with its hepatoprotective and antifibrotic efficacy.[62],[63],[64],[65]

Conclusion

S. villosum aerial parts extract (SVE) was found to exhibit a significant protective activity against CCl4-induced liver fibrosis in rats. This beneficial effect appeared partly mediated by its ability to reserve liver integrity, attenuate CCl4-induced oxidative stress, modulate mRNA expression of gene-related fibrosis, and ameliorate collagen deposition in liver tissues. Besides, the metabolic profile of the investigated extract indicated the presence of a mixture of bioactive components, especially steroidal saponins, steroidal glycoalkaloids, and polyphenols that possibly contribute synergistically to its antifibrotic effect.

Acknowledgements

The authors express their deep thanks and gratitude to Prof. Essam Abdel-Sattar, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, for his great support with his kind advice, helpful suggestions and valuable comments throughout this study. Also, the authors express their deep gratitude to Prof. Dr. Adel Baker Kholoussy, Pathology Department, Faculty of Veterinary Medicine, Cairo University, for performing the histopathological studies.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Silva TM, Nascimento RJ, Batista MM, Agra MF, Camara CA. Brine shrimp bioassay of some species of *Solanum* from Northestern Brazil. Rev Bras Farmacognosia 2007;17:35-8.
2 Cronquist A. An Integrated System of Classification of flowering Plants. New York: Columbia University Press; 1982.
3 Oszmiański J, Kolniak-Ostek J, Wojdyło A. Characterization of phenolic compounds and antioxidant activity of *Solanum scabrum* and *Solanum burbankii* berries. J Agric Food Chem 2014;62:1512-9.
4 Edmonds JM, Chweya JA. Black nightshades. *Solanum nigrum* L. and related species. Promoting the Conservation and use of Underutilized and Neglected Crops. Rome, Italy: Gatersleben/International Plant Genetic Resources Institute, Institute of Plant Genetics and Crop Plant Research; 1997.
5 Shah VV, Shah ND, Patrekar PV. Medicinal plants from solanaceae family. Res J Pharm Technol 2013;6:143-51.
6 Chowdhury N, Bhattacharjee I, Laskar S, Chandra G. Efficacy of *Solanum villosum* Mill. (Solanaceae: Solanales) as a biocontrol agent against fourth instar larvae of *Culex quinquefasciatus* say. Turk J Zool 2007;31:365-70.
7 Venkatesh R, Kalaivani K, Vidya R. Analysis of phytochemicals and free radical scavenging activity of *Solanum villosum* (Mill). – A traditional medicinal plant in Southern India. World J Pharm Pharm Sci 2014;3:741-55.
8 Chowdhury N, Ghosh A, Chandra G. Mosquito larvicidal activities of *Solanum villosum* berry extract against the dengue vector *Stegomyia aegypti*. BMC Complement Altern Med 2008;8:10.
9 Starkel P, Leclercq IA. Animal models for the study of hepatic fibrosis. Best Pract Res Clin Gastroenterol 2011;25:319-33.
10 Dhiman A, Nanda A, Ahmad S. A recent update in research on the antihepatotoxic potential of medicinal plants. Zhong Xi Yi Jie He Xue Bao 2012;10:117-27.
11 Daba MH, El-Tahir KE, Al-Arifi MN, Gubara OA. Drug-induced pulmonary fibrosis. Saudi Med J 2004;25:700-6.
12 Richeldi L, Davies HR, Spagnolo P, Luppi F. Corticosteroids for Idiopathic Pulmonary Fibrosis. The Cochrane Library; 2003.
13 Zhang A, Sun H, Wang X. Recent advances in natural products from plants for treatment of liver diseases. Eur J Med Chem 2013;63:570-7.
14 Afendi FM, Okada T, Yamazaki M, Hirai-Morita A, Nakamura Y, Nakamura K, *et al.* KNApSAcK family databases: Integrated metabolite-plant species databases for multifaceted plant research. Plant Cell Physiol 2012;53:e1.
15 Zhu ZJ, Schultz AW, Wang J, Johnson CH, Yannone SM, Patti GJ, *et al.* Liquid chromatography quadrupole time-of-flight mass spectrometry characterization of metabolites guided by the METLIN database. Nat Protoc 2013;8:451-60.
16 OECD. 423: Acute Oral Toxicity – Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals (section 4: health effects); 2001. p. 1-14.
17 Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271-8.
18 Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. Cancer Res 1984;44:5086-91.
19 Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996;29:225-9.
20 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C (T)) method. Methods 2001;25:402-8.
21 Bancroft J, Stevens A, Turner D. Theory and Practice of Histological Techniques. New York: Churchill Livingstone; 1996. p. 766.
22 Friedman M, McDonald GM, Filadelfi-Keszi M. Potato glycoalkaloids: Chemistry, analysis, safety, and plant physiology. Crit Rev Plant Sci 1997;16:55-132.
23 Mohy-Ud-Din A, Khan ZU, Ahmad M, Kashmiri MA. Chemotaxonomic value of alkaloids in *Solanum nigrum* complex. Pak J Bot 2010;42:653-60.
24 Ripperger H. *Solanum* steroid alkaloids – An update. Alkaloids Chem Biol Perspect 1998;12:103-85.
25 Claeys M, Van den Heuvel H, Chen S, Derrick PJ, Mellon FA, Price KR. Comparison of high- and low-energy collision-induced dissociation tandem mass spectrometry in the analysis of glycoalkaloids and their aglycons. J Am Soc Mass Spectrom 1996;7:173-81.
26 Zhou X, He X, Wang G, Gao H, Zhou G, Ye W, *et al.* Steroidal saponins from *Solanum nigrum*. J Nat Prod 2006;69:1158-63.
27 Usubillaga A, Aziz I, Tettamanzi MC, Waibel R, Achenbach H. Steroidal alkaloids from *Solanum sycophanta*. Phytochemistry 1997;44:537-43.
28 Sobolev VS, Sy AA, Gloer JB. Spermidine and flavonoid conjugates from peanut (*Arachis hypogaea*) flowers. J Agric Food Chem 2008;56:2960-9.
29 Zhao G, Qin GW, Gai Y, Guo LH. Structural identification of a new tri-p-coumaroylspermidine with serotonin transporter inhibition from safflower. Chem Pharm Bull (Tokyo) 2010;58:950-2.
30 Hensel A, Deters AM, Müller G, Stark T, Wittschier N, Hofmann T. Occurrence of N-phenylpropenoyl-L-amino acid amides in different herbal drugs and their influence on human keratinocytes, on human liver cells and on adhesion of *Helicobacter pylori* to the human stomach. Planta Med 2007;73:142-50.
31 Carle R. Investigations on the content of steroidal alkaloids and sapogenins within *Solanum* sect. *Solanum* (= sect. Morella) (Solanaceae). Plant Syst Evol 1981;138:61-71.
32 Schreiber K, Aurich O, Osske G. *Solanum*-alkaloids: XVIII. Thin layer chromatography of Solanum steroidal alkaloids and steroidal sapogenins. J Chromatog A 1963;12:63-9.
33 Chakravarti RN, Datta S, Mitra MN. Tigogenin and ursolic acid from *Cestrum diurnum* linn. Experientia 1964;20:200.
34 Yahara S, Ura T, Sakamoto C, Nohara T. Steroidal glycosides from *Capsicum annuum*. Phytochemistry 1994;37:831-5.
35 Mahato SB, Sahu NP, Ganguly AN, Miyahara K, Kawasaki T. Steroidal glycosides of Tribulus terrestris linn. J Chem Soc Perkin Transactions 1 1981: 2405-10. DOI: 10.1039/P19810002405.
36 Amir M, Kumar S. Possible industrial applications of genus Solanum in twenty first century – A review. J Sci Ind Res 2004;63:116-24.
37 Jia Y, Sun C, Sun Y, Yang W, Sun L. Simultaneous determination of trillin and desgalactotigonin contents in *Solanum lyratum* by HPLC-ELSD. Zhongguo Zhong Yao Za Zhi 2012;37:323-6.
38 Lam P, Cheung F, Tan HY, Wang N, Yuen MF, Feng Y. Hepatoprotective effects of Chinese medicinal herbs: A focus on anti-inflammatory and anti-oxidative activities. Int J Mol Sci 2016;17:465.
39 Rockey DC. Current and future anti-fibrotic therapies for chronic liver disease. Clin Liver Dis 2008;12:939-62, xi.
40 Basu S. Carbon tetrachloride-induced lipid peroxidation: Eicosanoid formation and their regulation by antioxidant nutrients. Toxicology 2003;189:113-27.
41 Krawczyk M, Bonfrate L, Portincasa P. Nonalcoholic fatty liver disease. Best Pract Res Clin Gastroenterol 2010;24:695-708.
42 Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007;45:27-37.
43 Vilei MT, Granato A, Ferraresso C, Neri D, Carraro P, Gerunda G, *et al.* Comparison of pig, human and rat hepatocytes as a source of liver specific metabolic functions in culture systems – Implications for use in bioartificial liver devices. Int J Artif Organs 2001;24:392-6.
44 Akomolafe SF, Oboh G, Akindahunsi AA, Afolayan AJ. Antiperoxidative activity of *Tetracarpidium conophorum* leaf extract in reproductive organs of male rats. Evid Based Complement Alternat Med 2015;2015:798491.
45 Fan X, Zhang Q, Li S, Lv Y, Su H, Jiang H, *et al.* Attenuation of CCl_4-induced hepatic fibrosis in mice by vaccinating against TGF-β1. PLoS One 2013;8:e82190.
46 Hanauske-Abel H. Fibrosis of the liver: Representative molecular elements and their emerging role as anti-fibrotic targets. In: Zakim D, Boyer TD, editors. Hepatology: A Textbook of Liver Disease. 4th ed. Philadelphia: W.B. Saunders; 2003. p. 347-94.
47 Al-Rasheed NM, Attia HA, Mohamad RA, Al-Rasheed NM, Al-Amin MA, Al-Onazi A. Aqueous date flesh or pits extract attenuates liver fibrosis via suppression of hepatic stellate cell activation and reduction of inflammatory cytokines, transforming growth factor- β 1 and angiogenic markers in carbon tetrachloride-intoxicated rats. Evid Based Complement Alternat Med 2015;2015:247357.
48 Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci 2002;7:d793-807.
49 Duarte S, Baber J, Fujii T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biol 2015;44-46:147-56.
50 Chen WX, Li YM, Yu CH, Cai WM, Zheng M, Chen F. Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. World J Gastroenterol 2002;8:379-81.
51 Lin WC, Lin WL. Ameliorative effect of ganoderma lucidum on carbon tetrachloride-induced liver fibrosis in rats. World J Gastroenterol 2006;12:265-70.
52 Rao AV, Gurfinkel DM. The bioactivity of saponins: Triterpenoid and steroidal glycosides. Drug Metabol Drug Interact 2000;17:211-35.
53 Lin CC, Lin WC, Yang SR, Shieh DE. Anti-inflammatory and hepatoprotective effects of *Solanum alatum*. Am J Chin Med 1995;23:65-9.
54 Sultana S, Perwaiz S, Iqbal M, Athar M. Crude extracts of hepatoprotective plants, *Solanum nigrum* and *Cichorium intybus* inhibit free radical-mediated DNA damage. J Ethnopharmacol 1995;45:189-92.
55 Akanitapichat P, Phraibung K, Nuchklang K, Prompitakkul S. Antioxidant and hepatoprotective activities of five eggplant varieties. Food Chem Toxicol 2010;48:3017-21.
56 Gupta AK, Ganguly P, Majumder UK, Ghosal S. Hepatoprotective and antioxidant effects of total extracts and stereoidal saponins of *Solanum xanthocarpum* and *Solanum nigrum* in paracetamol induced hepatotoxicity in rats. Pharmacologyonline 2009;1:757-68.
57 Yu H, Zheng L, Yin L, Xu L, Qi Y, Han X, *et al.* Protective effects of the total saponins from *Dioscorea nipponica* makino against carbon tetrachloride-induced liver injury in mice

through suppression of apoptosis and inflammation. Int Immunopharmacol 2014;19:233-44.
58 Zhu X, Zhang W, Zhao J, Wang J, Qu W. Hypolipidaemic and hepatoprotective effects of ethanolic and aqueous extracts from *Asparagus officinalis* L. by-products in mice fed a high-fat diet. J Sci Food Agric 2010;90:1129-35.
59 Xie WL, Jiang R, Shen XL, Chen ZY, Deng XM. Diosgenin attenuates hepatic stellate cell activation through transforming growth factor-β/Smad signaling pathway. Int J Clin Exp Med 2015;8:20323-9.
60 Lin CN, Chung MI, Gan KH. Novel antihepatotoxic principles of *Solanum incanum*. Planta Med 1988;54:222.
61 Lin CN, Gan KH. Antihepatotoxic principles of *Solanum capsicastrum*. Planta Med 1989;55:48-50.
62 Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. Lancet 1993;342:1007-11.
63 Kim SM, Kang K, Jho EH, Jung YJ, Nho CW, Um BH, *et al.* Hepatoprotective effect of flavonoid glycosides from *Lespedeza cuneata* against oxidative stress induced by tert-butyl hyperoxide. Phytother Res 2011;25:1011-7.
64 Singab AN, Youssef DT, Noaman E, Kotb S. Hepatoprotective effect of flavonol glycosides rich fraction from Egyptian *Vicia calcarata* desf. Against CCl_4-induced liver damage in rats. Arch Pharm Res 2005;28:791-8.
65 Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: Antioxidants or signalling molecules? Free Radic Biol Med 2004;36:838-49.