Cytotoxic, hepatoprotective and antioxidant activities of *Silybum marianum* variety *albiflorum* growing in Egypt

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Abstract

Silymarin prepared from the fruits of *Silybum marianum* (L.) Gaertn. (Asteraceae) has long been used for the treatment of liver disorders. This study was carried out to evaluate the protective effect of the fruit extract of white-flowered *S. marianum* variety *albiflorum* Eig. (WSE) against paracetamol–induced liver toxicity in rats. Silyhermin, isosilandrin A/B were identified as the major flavonolignans in WSE. Cytotoxic activities of WSE and isolated flavonolignans compared to silymarin were carried out using sulforhodamine B assay. WSE, silyhermin and isosilandrin had no obvious harmful effect on normal human cell line compared to silymarin with IC$_{50}$ values 78.95, 84.34, 72.14 and 16.83 µg/ml, respectively. The hepatoprotective activity of WSE at dose 50 mg/kg was comparable to silymarin (100 mg/kg). These data were supplemented with histopathological studies on liver sections. The hepatoprotective effects of WSE on oxidative stress induced by administration of paracetamol are probably associated with its antioxidant properties.

**Key words:** *Silybum marianum* variety *albiflorum*, silyhermin, isosilandrins, cytotoxicity, hepatoprotective, oxidative stress.

1. Experimental

1.1. General

Silica gel 60 (Fluka, St. Louis, MO, USA, particle size 0.063-0.2 mm, 70-230 mesh) and sephadex LH-20 (Sigma, USA, bead size 25-100 µm) were used for column chromatography. Pre-coated silica gel TLC plates with fluorescent indicator 254 nm; layer thickness 0.2 mm (Fluka) were used for analysis of fractions and isolated compounds. UV-visible spectrophotometer Shimadzu UV 240 was used for scanning UV spectra of isolated compounds. The preparative HPLC instrument consists of Agilent 1260 Infinity pump (G1361A), Agilent 1260 diode array detector VL (G1315D), and Agilent 1260 Infinity preparative auto sampler (G2260A). Separation was conducted on
Agilent column (9.4 x 250 mm, 5 µm), USA. The NMR spectrometer used was Bruker model AVANCE III HD (Fälladen, Switzerland) operating at the basic frequency of 400.13 MHz (O1). The solvents used in this study were of analytical grade. Methanol used for HPLC preparative separation of isolated compounds was of HPLC grade (Sigma-Aldrich, Steinheim, Germany). Standard silymarin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2. Plant material

*S. marianum* variety *albiflorum* Eig. fruits were collected from Assiut city, Upper Egypt on 15 April 2015. Botanical authentication was carried out by Dr. Abdel Halim Mohamed, Flora and Phytotaxonomy Department, Agricultural Research Center, Cairo, Egypt. The fruits were manually separated from the heads and freed from their pappus.

1.3. Isolation of major flavonolignans from *S. marianum* variety *albiflorum*

The fruits *S. marinaum* variety *albiflorum* (150 g) were extracted with methanol according to a reported method (AbouZid et al. 2016). The pericarp of the fruits (67.1 g) was extracted with methanol (x3, 100 ml) to yield 5.8 g crude extract. The extract was monitored by TLC using methylene chloride : acetone : formic acid 7.5:1.6:0.9, under short ultraviolet light and sprayed with 10% sulfuric acid spray reagent. The crude extract (5.8 g) was loaded on silica gel (10 g), dried and applied onto a silica gel column (200 g). The column was developed using methylene chloride (0.1% formic acid) as a mobile phase and increasing the polarity by acetone (0-50%). Twenty three fractions (50 ml) were collected, concentrated under reduced pressure and monitored by TLC. Fractions 16 and 17 eluted from the previous column with 40% acetone in methylene chloride (0.1% formic acid) were pooled, evaporated to dryness (0.5 g) and further purified on sephadex LH-20 column (50 g) using methanol. Fifteen sub-fractions were collected, concentrated and monitored by TLC, examined under short ultraviolet light short wavelength and sprayed with 10% sulfuric acid spray reagent. Sephadex LH-20 column fractions 8 and 9 showed one spot at R_f value 0.6. The two fractions were pooled, evaporated to dryness (184.4 mg), analyzed by ^1^H NMR and identified as silyhermin (Biedermann et al. 2016).
Fractions 13-16 from the first silica gel column were pooled, evaporated to dryness (2.9 g) and further purified on sephadex LH-20 (50 g) column using methanol as a mobile phase. Fourteen sub-fractions (40 ml) were collected, concentrated under reduced pressure, and monitored by TLC, examined under short ultraviolet light short wavelength and sprayed with 10% sulfuric acid spray reagent. Sub-fractions 7-10 showed one spot. These fractions were pooled and evaporated to dryness (199.7 mg). The pooled fractions were further purified by preparative HPLC using methanol: water containing 0.1% formic acid 55:45, at 3 ml/min. Two compounds were isolated, analyzed by $^1$H NMR spectroscopy and identified as isosilandrin A ($R_t = 54.18$ min) and isosilandrin B ($R_t = 57.46$ min).

Isosilandrin A, UV: $\lambda_{\text{max}}$(MeOH) nm (log ε): 322 (3.52), 287 (4.12), 229 (4.25). $^1$H NMR spectral data (DMSO-$d_6$): $\delta = 2.70$ (br d, $J = 16.8$ Hz, 1H, 3-H$_{eq}$), 3.16 (s, 1H, 3-H$_{ax}$), 3.76 (s, 3H, OCH$_3$), 4.17 (br s, 1H, β-H), 4.89 (d, $J = 7.9$ Hz, 1H, α-H), 5.45 (br d, $J = 12.0$ Hz, 1H, 2-H), 5.85 (s, 1H, 8-H), 5.86 (s, 1H, 6-H), 6.78-7.09 (m, 6H, 2′,2″,5′,5″,6′,6″). Signals of γ1-H and γ2-H were obscured by water signal.

Isosilandrin B, UV: $\lambda_{\text{max}}$(MeOH) nm (log ε): 322 (3.52), 287 (4.12), 229 (4.25). $^1$H NMR spectral data (DMSO-$d_6$): $\delta = 2.72$ (dd, $J = 17.2$, 2.4 Hz, 1H, 3-H$_{eq}$), 3.16 (s, 1H, 3-H$_{ax}$), 3.78 (s, 3H, OCH$_3$), 4.16 (m, 1H, β-H), 4.90 (d, $J = 8.0$ Hz, 1H, α-H), 5.46 (br d, $J = 12.4$ Hz, 1H, 2-H), 5.87 (s, 1H, 8-H), 5.88 (s, 1H, 6-H), 6.78-7.07 (m, 6H, 2′,2″,5′,5″,6′,6″). Signals of γ1-H and γ2-H were obscured by water signal.

1.4. Cytotoxic activity

In vitro cytotoxic activity was carried out using the sulphorhodamine B assay. Doxorubicin was used as a positive control. Normal human cell line (HFB-4) and human cancer cell line (Hep-G2) were seeded in 96 well microtiter plates at a concentration of 1,000–2,000 cells/well, 100 µl/well. After 24 h, cells were incubated for 72 h with various concentrations of tested samples. For each concentration, three wells were used. The plates were incubated for 72 h. The medium was discarded. The cells were fixed with 150 µl cold trichloroacetic acid for 1 h at 4°C. The plates were washed with distilled water using a Tecan automatic washer (Crailsheim, Germany) and stained with 50 µl 0.4% SRB dissolved in 1% acetic acid for 30 min at room temperature in the dark. The
plates were washed with 1% acetic acid to remove unbound dye and air-dried for 24 h. The dye was solubilized with 150 µl/well of 10 mM tris base (pH 7.4) for 5 min on a shaker at 1,600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 490 nm with an ELISA microplate reader. The percentage of cell survival was calculated as follows: Surviving fraction = O.D. (treated cells)/O.D. (control cells) x100.

1.5. Ethics Statement

All procedures in the present study were approved by the ethics committee of the Faculty of Pharmacy, Beni-Suef University. The guidelines declared by U.S.A. national institute of health for care and use of laboratory animals were followed in all experimental procedures.

1.6. Animals

Wistar male rats weighing 150-180 g were obtained from the animal house of Nahda University in Beni-Suef (NUB), Beni-Suef, Egypt. Animals were kept under the following conditions: temperature (25 ± 2°C), humidity (60 ± 10%), a 12/12 h light-dark cycle and allowed to access water and food freely.

1.7. Experimental design

Fifty six rats were divided into seven groups (eight rats each). First group in which the rats received vehicle only for 7 days (Normal). Second group in which the rats received WSE (200 mg/kg, p.o.) for 7 days (W200). Third group in which the rats received vehicle for 7 days and paracetamol (600 mg/kg, p.o.) at the 7th day of the experiment (APAP). Fourth group in which the rats received standard silymarin (100 mg/kg, p.o.) for 7 days and paracetamol (600 mg/kg, p.o.) at the 7th day of the experiment (P100+APAP). Fifth group in which the rats received WSE (50 mg/kg, p.o.) for 7 days and paracetamol (600 mg/kg, p.o.) at the 7th day of the experiment (W50+APAP). Sixth group in which the rats received WSE (100 mg/kg, p.o.) for 7 days and paracetamol (600 mg/kg, p.o.) at the 7th day of the experiment (W100+APAP). Seventh group in which the rats received WSE (200 mg/kg, p.o.) for 7 days and paracetamol (600 mg/kg, p.o.) at the 7th day of the experiment (W200+APAP). All rats were fasted for 18 h before and 24 h after paracetamol administration with free access to water ad libitum.
1.8. Blood and tissue preparation

At the end of the experiment blood samples were withdrawn and serum was prepared by centrifugation at 3000 r.p.m. for 15 min at 4°C. The serum was used for assessment of ALT, AST, albumin, direct and total bilirubin levels. The animals were sacrificed and livers were dissected out immediately and washed with cold saline. One part of the liver was immersed in 10% formal saline for histopathological examination. Another part was homogenized in 4 volumes of phosphate buffered saline, centrifuged at 1000 g for 15 min at 4°C and the supernatants were then stored at -80°C till analysis. Liver homogenates were used for determination of reduced GSH and MDA contents as well as SOD activity.

1.9. Biochemical tests

ALT and AST estimation was carried out according to the procedures of the assay kits (Randox, UK). The assay depends on colorimetric measurement of the reaction at 546 nm. Serum albumin and bilirubin were determined colorimetrically using test reagent kits. Liver tissue homogenates were used for the determination of reduced GSH content using BlueGene Biotech assay kit (BlueGene Biotech, China). Hepatic content of MDA was estimated according to the kit instruction (Lifespan Bioscience, USA). Liver SOD activity was determined by superoxide dismutase kit (MyBio-source, USA).

1.10. Histopathological Examination

The isolated kidneys were washed using normal saline, fixed in 10% formal saline, dehydrated in ascending grades of alcohol and embedded in paraffin wax. Sections were taken at thickness of 5 μm. Staining was done by hematoxylin and eosin (H&E). The section fields were examined under light microscope by a histopathologist (Bancroft et al. 1996).

1.11. Statistical analysis

The present results are illustrated as mean ± SEM. All the statistical analyses were performed using one way analysis of variance (ANOVA) test with Tukey’s post hoc comparison test. Significance was based on $P$ value < 0.05. Data analysis was done using Prism 5 (GraphPad Software, USA).

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**Figure Captions**

Fig. S1. Chemical structures of flavanolignans isolated from the white-flowered *Silybum marianum* growing in Assiut, Upper Egypt.

Fig. S2. Expanded region (0-8 ppm) of $^1$H NMR (400 MHz, CD$_3$OD) spectra of extract from the white-flowered *Silybum marianum* fruits (black, lower), silyhermin (green, middle) and isosilandrins (red, upper).

Fig. S3. Cytoxicity of white-flowered *Silybum marianum* fruits extract, silyhermin, isosilandrins and silymarin against normal human cell line (HFB-4) and human cancer cell line (Hep-G2) using the sulphorhodamine B assay.
Fig. S4. Photomicrographs for sections from normal group (A) showing normal histological structure of hepatic tissue with intact hepatocytes and central vein; W200 group (B) showing apparent normal hepatic parenchyma and few activated Kupffer cells; APAP group (C and D) showing centrilobular necrosis of hepatocytes associated with periportal infiltration of inflammatory cells and degenerative changes of scattered hepatocytes; P100+APAP group (E) showing apparent normal hepatocytes with scattered binucleated cells (arrow head), mild sinusoidal dilatation; W50+APAP group (F) showing portal triad with mild proliferation of bile ducts, mild infiltration of inflammatory cells as well as mild central vein congestion and dilation; W100+APAP group (G) demonstrating many apparent intact hepatocytes with vesicular nuclei (arrow), activated Kupffer cells (arrow head) and moderate sinusoidal dilatation; W200+APAP group (H) showing apparent intact many hepatocytes with mild congestion of hepatic blood vessels and few activated Kupffer cells (H&E X 400).
Table S1

Effects of oral administration of white-flowered *Silybum marianum* fruit extract on liver functions and oxidative stress biomarkers in paracetamol-induced hepatotoxicity in rats (n = 8 rats per group). Each bar represents the mean ± SEM of the group.

| Group                                      | Serum ALT (U/l) | Serum AST (U/l) | Serum total bilirubin (mg/dl) | Serum direct bilirubin (mg/dl) | MDA (ng/g wet tissue) | GSH (pg/g wet tissue) | SOD (U/g wet tissue) |
|--------------------------------------------|-----------------|-----------------|------------------------------|-------------------------------|----------------------|----------------------|----------------------|
| Normal                                     | 19.65 ± 0.51    | 117.20 ± 3.25   | 0.87 ± 0.06                  | 0.06 ± 0.00                   | 6.10 ± 0.30          | 38.70 ± 2.50         | 15.35 ± 0.95         |
| WSE (200 mg/kg)                            | 21.53 ± 2.04    | 129.10 ± 9.23   | 0.93 ± 0.04                  | 0.05 ± 0.01                   | 6.67 ± 0.59          | 43.20 ± 2.00         | 16.80 ± 0.50         |
| Paracetamol (600 mg/kg)                    | 111.90 ± 7.59*  | 300.60 ± 15.52* | 1.84 ± 0.08*                 | 0.35 ± 0.03*                  | 27.10 ± 1.00*        | 3.75 ± 0.35*         | 2.10 ± 0.20*         |
| Silymarin (100 mg/kg) + APAP               | 59.01 ± 5.79*   | 193.60 ± 12.51* | 1.24 ± 0.06*                 | 0.10 ± 0.01*                  | 14.53 ± 0.59*        | 14.50 ± 0.47*        | 5.97 ± 0.52*         |
| WSE (50 mg/kg) + APAP                      | 53.53 ± 5.58*   | 177.60 ± 14.73* | 1.27 ± 0.07*                 | 0.08 ± 0.00*                  | 18.70 ± 0.61*        | 14.70 ± 0.89*        | 5.47 ± 0.35*         |
| WSE (100 mg/kg) + APAP                     | 36.09 ± 3.43ab  | 146.40 ± 14.37ab | 1.17 ± 0.08*                 | 0.06 ± 0.01*                  | 14.37 ± 0.59*        | 23.60 ± 1.04ab       | 8.77 ± 0.56ab        |
| WSE (200 mg/kg) + APAP                     | 29.67 ± 4.10ab  | 139.00 ± 8.19ab | 1.13 ± 0.07*                 | 0.05 ± 0.00*                  | 10.10 ± 0.17ab       | 30.30 ± 0.76ab       | 11.53 ± 0.41ab       |

*Significantly different from normal group at $P < 0.05$.

*a* Significantly different from paracetamol group at $P < 0.05$.

*b* Significantly different from silymarin group at $P < 0.05$. 
Fig. S1

Silyhermin

Isosilandrin A

Isosilandrin B
Fig. S2

[Chemical Shift (ppm) graph]
Fig. S3

Viability % of HFB4 cells

Viability % of Hep-G2 cells

Concentration (µg/ml)
