Hepatorenal protective effect of Antistax® against chemically-induced toxicity

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ABSTRACT

Background: Antioxidant natural products and chemoprevention are considered nowadays as an effective approach against health various disorders and diseases induced by oxidative stress or free radicals. **Objective:** The aim of this study was to assess the hepato- and nephroprotective activity of a standardized red vine leaf aqueous extract AS195 (Antistax®). **Methods:** The protective activity of AS195 (100 mg/kg) was investigated on carbon tetrachloride (CCl_4)-intoxicated rats in comparison with silymarin. The flavonoid/proanthocyanidin nature of AS195 was identified by phytochemical and nuclear magnetic resonance (NMR) analyses, while its total phenol/proanthocyanidin/flavonoid content and antioxidant activity were determined by Folin-Ciocalteau, vanillin-sulfuric acid, $AlCl_3$, and 2,2-diphenyl-2-picrylhydrazyl radical scavenging assays, respectively. **Results:** Relative to the control CCl_4–intoxicated group, pretreatment with AS195 could significantly suppressed the elevated serum levels of alanine aminotransferase, alkaline phosphatase, γ-glutamyl transferase, total cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, triglycerides, bilirubin, creatinine, uric acid, and calcium, whereas it significantly increased the diminished serum levels of high-density lipoprotein cholesterol, albumin and total protein. Moreover, AS195 significantly decreased malondialdehyde formation in the tissues of liver and kidney, whereas it significantly elevated and nonprotein sulfhydryl groups, compared with the intoxicated control. The improvement in biochemical parameters by AS195 was obviously observed and further confirmed by restoration of normal histological features in the two organs. **Conclusions:** The results of the present study revealed the capacity of AS195 to enhance the recovery from xenobiotic-induced hepatorenal toxicity initiated by free radicals.

Key words: Antioxidant, Antistax®, flavonoids, hepatorenal protective, proanthocyanidin, *Vitis vinifera*

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INTRODUCTION

Antistax® is a dried aqueous extract of red vine leaf (folium *Vitis vinifera* L., var. tinctoria, Vitaceae), AS195 (4–6:1, water), which has been used for treatment or prevention of symptoms of chronic venous insufficiency grades I and II, heavy tired swollen legs and feet, and varicose vein since decades. The preclinical studies suggested that the protective effect of the extract on venous system could be attributable to the flavonoid and proanthocyanidin contents.[1] Moreover, the results of *in vitro* and *in vivo* studies revealed the anti-herpetic,[2] antidiabetic,[3] and diuretic[4] activities of the aqueous extract of red vine leaf. Nevertheless, other bioactivities were reported for extracts and fractions obtained by organic solvents, which may contain poorly water-soluble or hydrophobic components, e.g. sterols, resveratrol, phenylacrylic acid derivatives.[1] Vine leaves contain proanthocyanidins (up to 4%), flavonoids, e.g. quercetin- and kampferol-3-O-glycosides (up to 3.5%), anthocyanins (up to 1.5%), phenolic acids, and sugars, e.g. raffinose, which are likely to be carried over to the aqueous extract.[5] However, AS195 was adjusted to contain 4-7% of flavonol glycosides.[1]

Liver and kidney are the key organs of metabolism and excretion and they are continuously exposed to free radical-liberating xenbiotics. Liver diseases and

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nephrotoxicity represent serious health problems.[6,7] Since synthetic drugs used in treatment of liver and kidney diseases are inadequate or have serious adverse effects, search of safe and readily available natural products or nutraceuticals could be a preferred alternative solution. The high level of polyphenols claimed in AS195 was thus prompted us to extend the medicinal value of this aqueous extract, on the basis of its expected antioxidant power, by evaluating its efficacy in combating chemically-induced hepatorenal injury.

MATERIALS AND METHODS

Chemicals and analytical instruments

All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Antistax® capsules (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) contained 180 mg of the dry aqueous extract of red vine (*V. vinifera*) per capsule (batch No. B03820003, manufacturing date 2/2011, expiry date 2/2014) and coded in this study as AS195. The ^{13}C NMR spectrum [Figure 1 and Table 1] was recorded in DMSO-d_5 on a Bruker Avance DRX-500 spectrometer (Central Lab., College of Pharmacy, KSU). Precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for analytical thin layer chromatography (TLC). The absorbance was measured on a Specord 40 UV-VIS instrument (Jena Analytik AG, Germany) in the 2,2-diphenyl-2-picrylhydrazyl (DPPH) antioxidant, vanillin-sulfuric acid, and Folin-Ciocalteu assays; on a Reflotron® Plus Analyzer (Roche Diagnostics GmbH, Mannheim, Germany) for estimation of serum biochemical markers; and on a Shimadzu UV mini-1240 spectrophotometer (Shimadzu Europe, Milano, Italy) for the other measurements.

Table 1: Diagnostic ^{13}C NMR data (ppm) of protonated carbons of AS195 proanthocyandins and flavonoid glycosides in comparison to common proanthocyanidins and flavonol glycosides

Carbon atom	Common proanthocyanidins[a]	Flavonol glycosides[b]	AS195 phenolics
C-2	76.4-82.0	-	76.7-82.2
C-3	66.2-73.1	-	67.7-73.0
C-4	28.0-37.2	-	29.0
C-6	96.1-108.6	99.1-103.0	96.8-104.1
C-8	95.6-108.1	94.0-98.0	96.8-104.1
Sugar C-1	-	96.9-102.2	96.8-102.0
Sugar C-2-C6	-	60.6-77.8	60.2-76.7

[a]Porter *et al.*[30]; [b]Pereira *et al.*[31]

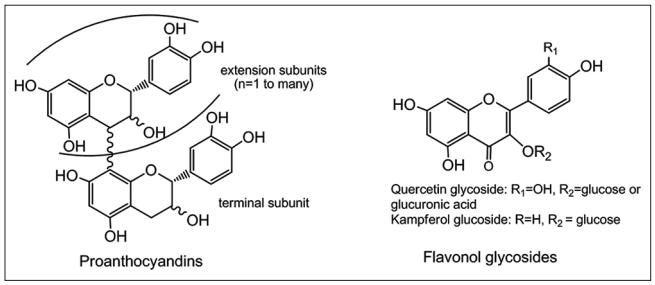

Figure 1: Structure of common proanthocyandins and flavonol glycosides in AS195

Phytochemical analysis of AS195

To 10% methanolic solution of the extract, 3 mg magnesium powder was then added followed by few drops of concentrated HCl (Shinoda's test). An orange coloration indicated the presence of flavonoids.

To 10% methanolic solution of the extract, a few drops of 10% ferric chloride solution were added. A brownish-green coloration indicated the presence of proanthocyanidins.[8] The proanthocyanidin content was further detected by the red color developed with vanillin-HCl reagent.[9]

Two analytical Si gel TLC plates were developed with EtOAc-MeOH-H_2O (100:16.5:13.5) for further phytochemical investigation using spray regents. Spraying a TLC plate with 1% methanolic $AlCl_3$[10] disclosed yellow fluorescent chromatographic bands at 366 nm and indicated the presence of several flavonoids; R_f values at 0.77, 0.60, and 0.50 (correspondent to three minor flavonoid glycosides) and at 0.20 and 0.15 (related two major more polar flavonoid glycosides). The brownish red bands appeared in the second TLC plate, after spraying with 5% vanillin-HCl reagent/visible light, revealed the presence of proanthocyandins (R_f values at < 0.15).

Determination of total phenolic content

Total phenols in AS195 was estimated using Folin-Ciocalteu assay according to a procedure described by Singleton and Rossi[11] with some modifications. Samples were sonicated in methanol followed by centrifugation and each supernatant was separated, evaporated to dryness, and then reconstituted in methanol to make up a stock solution of 1 mg/mL. Briefly, aliquots (1.0 mL) of the diluted extract or standard solutions (gallic acid and quercetin) in methanol were mixed with 2.5 mL 0.2 N Folin–Ciocalteu reagent. After 5 min, the reaction mixture was neutralized with sodium carbonate (2 mL, 7.5% w/v) solution. After incubation (2 h/RT), absorbance "A" of the resulting blue color was measured at 765 nm. Calibration curves were prepared using "A" of different concentrations (6.25, 12.5, 25, 50, 100, and 200 μg/mL, $n = 3$) of standards. Total phenolic content was expressed as mg gallic acid equivalent (GAE) or quercetin equivalent (QUE) per g extract [Table 2] by the following equation: $T = C \times V/M$. C is the concentration of gallic acid or quercetin established from the calibration curve in

Table 2: Total phenol/proanthocyanidin/flavonoid content and antioxidant activity of AS195

Index	Value
Phenol content[a]	
mg GAE/g	149.93±0.35[e]
mg QUE/g	134.48±0.39[f]
Proanthoyanidin content[b]	
mg CTE/g	62.8±0.8[g]
Flavonoid content[c]	
mg QUE/g	103.06±4.9[h]
Antioxidant activity (IC_{50} mg/mL)[d]	13.4 (5.2)[i]

Indices were measured by [a]Folin-Ciocalteau; [b]Vanillin-sulfuric acid; [c]$AlCl_3$, and [d]DPPH radical scavenging assays. The linear regression equations was [e]y=0.0212x+0.0548, r2=0.998; [f]y=0.0193x+0.1714, r2=0.993; [g]y=0.0024x−0.0077, r2=0.998; and [h]y=23.696x+0.0763, r2=0.999. [i]A value obtained by a reference antioxidant flavonoid (rutin). GAE: Gallic acid equivalent; QUE: Quercetin equivalent; CTE: (+) catechin equivalent; IC_{50}: Inhibitory concentration 50%

mg/mL, V is the volume of the extract solution in mL, and M is the weight of the extract in g.

Determination of proanthocyanidin content

Proanthocyanidins in AS195 were estimated using vanillin-sulfuric acid assay according to a method described by Sun, *et al*.[12] with some modifications. To one mL of (+)-catechin (CT) solutions (18.75, 37.5, 75, 150, or 300 μg/mL methanol) or test solution (200 μg/mL methanol) in a test tube, 2.5 mL of 1% vanillin solution in methanol, and 2.5 ml of 3.6 N H_2SO_4 in methanol were added. After incubation (30°C/20 min), "A" of each reaction mixture was measured at 500 nm and calculated using the equation: $A = (A_s–A_b)–(A_c – A_0)$. A_s, A_b, A_c, and A_o are absorbances of solutions with the test sample, without the test sample, without vanillin, and with only H_2SO_4 (total volume, 6 mL), respectively. Proanthocyanidin content was thus calculated from a calibration curve prepared by using A of CT dilutions and the above equation as mg (+)-catechin equivalent (CTE) per g extract [Table 2].

Determination of flavonoid content

Total flavonoid content was determined using $AlCl_3$ method as previously described[13] using quercetin as a standard. Briefly, an aliquot of 2 mL methanolic solution of AS195 (0.1 mg/mL) or standard solution was mixed with the same volume of 2% $AlCl_3$ in methanol and after 30 min the absorbances were read at 415 nm using a methanol blank. The total flavonoid content is determined by using a standard curve with quercetin (0–0.25 mg/ml) and the results were expressed as mg QUE per g AS195.

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay[14]

The antioxidant activity of AS195 or the reference flavonoid rutin was determined at 250, 50, 10, 2, and 0.4 μg/mL concentrations in methanol. Each solution (1 mL) was mixed with freshly prepared DPPH methanolic solution (125 μL of 1 μM) and 375 μL methanol. After incubation (25°C, 30 min), the decrease in absorbance (A) was measured at λ 517 nm. Inhibition percent of DPPH radical was calculated from the equation: $I\% = [(A_{blank}–A_{sample})/A_{blank}] \times 100$ [Table 2].

Animals and monitoring

Healthy male adult Wistar albino rats (150–170 g) were obtained from the Experimental Animal Care Center, College of Pharmacy, KSU. After a 1-week adaptation period, rats were randomly divided into groups (6 rats/cage) and kept at 22°C ± 2°C, 55% humidity, and 12/12 h light–dark cycle. The animals were provided with Purina chow rat diet (UAR-Panlab, Barcelona, Spain) and drinking water *ad libitum*. All treatment protocols for this study were approved by the Ethics Committee of the Experimental Animal Care Society, KSU.

Pretreatment and carbon tetrachloride (CCl_4)-induced hepatorenal toxicity

Five groups (I–IV) of animals were used. Group I was kept as normal control. Group II received carbon tetrachloride (CCl_4) and served as a CCl_4-intoxicated control. Groups III and IV were assigned as treatment groups. Group III was pretreated with AS195 at doses of 100 mg/kg/rat orally whereas group IV was pretreated with silymarin (SYL) at 10 mg/kg/rat orally, for 17 days. Group I and II animals received a similar volume of vehicle once daily orally. At the 16th day, groups II–IV received CCl_4 in liquid paraffin (1:1) at a dose of 1.25 mL/kg/rat intraperitoneally. After 48 h, following CCl_4 challenge, the blood was collected by cardiocentesis and serum was obtained by centrifugation at 1000 × *g* for 20 min at 4°C. The liver and kidney were removed for biochemical and histological assessment.

Analyses of serum hepatic biochemical and lipid profile

Serum alanine aminotransferase (ALT) and AST, γ-glutamyl transferase (GGT), alkaline phosphatase (ALP) were determined calorimetrically by methods of Reitman and Frankel,[15] Fiala, *et al*.,[16] and King and Armstrong,[17] respectively, whereas bilirubin was determined by the method of Stiehl[18] Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were measured by the methods of Demacher and Hijamaus,[19] Burstein and Scholnick,[20] and Foster and Dunn,[21] respectively, using Roche kits (Roche Diagnostics GmbH). low-density lipoprotein cholesterol (LDL-C) and the very low-density lipoprotein-cholesterol (VLDL-C) levels were calculated from the formula: LDL-C = TC-HDL-C-VLDL-C; VLDL-C = TG/5.27.

Analyses of serum creatinine, calcium, urea, and uric acid

Serum creatinine was measured by Jaffe reaction method[22] using CS604 kit (Crescent Diagnostics, Jeddah, Saudi Arabia). Calcium was determined by o-cresolphthalein method described by Gitelman[23] using CE500 kit (Crescent Diagnostics). Urea and uric acid were determined by urease and uricase methods described by Munan, *et al*.[24] and Fossati, *et al*.,[25] respectively, using Roche kits (Roche Diagnostics GmbH).

Determination of lipid peroxidation

A modified method of Utley, *et al*.[26] was used. The liver (or kidney) was homogenized in 0.15 M KCl at 4°C; and the homogenate (10% w/v, 1 mL) was transferred into a centrifuge tube and incubated at 37°C for 3 h. Aqueous trichloroacetic acid (TCA, 10%, 1 mL) was then added and the mixture was centrifuged at 800 × *g* for 10 min. The supernatant (1 mL) was removed and mixed with aqueous thiobarbituric acid (TBA, 1 mL, 0.67%) and placed in a boiling water bath for 10 min. The mixture was cooled, diluted with 1 mL distilled water, and "A" was read at 535 nm. The lipid peroxidation was expressed as malondialdehyde (MDA) in nmol/g wet tissue using a standard curve of MDA dilutions according to the following equation: "A" of sample × 72.06 × dilution factor/weight of sample in *g* × slope of standard curve.

Estimation of nonprotein sulfhydryl groups

Hepatic (or renal) nonprotein sulfhydryl (NP-SH) groups were measured according to the method of Sedlak and Lindsay[27] after homogenization in ice-cold ethylenediaminetetraacetic acid (EDTA, 0.02 M). The homogenate (5 mL) was mixed with distilled water (4 mL) and TCA (50%, 1 mL), shaken for 10 min and then centrifuged. A supernatant (2 mL) was mixed with Tris buffer (4 mL, 0.4 mol/L, pH 8.9) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.1 mL) was then added and shaken. "A" was measured within 5 min at 412 nm against a reagent blank without homogenate. NP-SH in nmol/g wet tissue is calculated according to the following equation: Corrected "A" of sample × dilution factor/weight of sample in g.

Determination of total protein and albumin

Serum albumin; and serum and tissue total protein (TP) were estimated according to the method of Doumas[28] by CS600 and CS610 kits (Crescent Diagnostics), respectively. The principle is based on the formation of a blue/violet complex when protein peptide bonds react with Cu (II) ions in alkaline solution (biuret reaction). KNa tartrate and KI Solutions were added as stabilizers. "A" was measured at 546 nm and protein was calculated as $(A_{sample}/A_{standard}) \times$ concentration of standard.

Histopathological study

The liver and kidney samples were fixed in 10% neutral buffered formalin for 24 h and processed using a VIP tissue processor. The processed tissues were then embedded in paraffin blocks and sections (5 μm thickness) were cut by a rotary microtome (American Optical, Buffalo, NY, USA). Sections were stained with hematoxylin and eosin,[29] and then examined microscopically for pathomorphological changes.

Data analysis

Values are presented as arithmetic means ± standard error of the mean. Data were statistically analyzed by using One-way Analysis of Variance (ANOVA), followed by Dunnett's multiple comparison tests. The $P < 0.05$ was taken as a criterion for a statistically significant difference.

RESULTS

Qualitative and quantitative determination of phenolic contents and antioxidant capacity of AS195

The pink color developed by Shinoda's test revealed the presence of flavonoids, which were visualized as five yellow fluorescent bands on Si gel TLC using $AlCl_3$ spray reagent at 366 nm.[10] Moreover, the cherry-red and brownish-green colors produced with vanillin-HCl[9] and $FeCl_3$[8] test reagents, respectively, indicated the presence of proanthocyanidins in AS195. The ^{13}C NMR spectrum of AS195 measured in DMSO-d_6 [Figure 2] showed a group of protonated carbon signals consistent with those of common proanthocyanidin oligomers[30] and flavonol glycosides[31] [Table 1]. The total phenolic content [Table 2] in the extract was quantified by Folin-Ciocalteu assay as 149.93 ± 0.35 mg GAE or 134.48 ± 0.39 mg QUE per g extract. Moreover, the proanthocyanidin and flavonoid portions of phenolic content were further quantified with modified vanillin and $AlCl_3$ methods to be 62.8 ± 0.8 mg CTE and 103.06 ± 4.9 mg QUE per g extract, respectively. Furthermore, the antioxidant activity of AS195 was estimated using DPPH radical scavenging assay. The extract was able to reduce the blue DPPH radical methanolic solution (125 μL of 1 μM) into the yellow stable DPPH at IC_{50} 13.4 μg/mL [Table 2], close to 40% of potency of the standard rutin (IC_{50} 5.2 μg/mL). On the basis of above findings, it was expected that AS195 would exhibit a considerable protective activity against the oxidative stress initiated by the free radicals-liberating xenobiotic such as CCl_4 *in vivo*.

Effect of AS195 on liver-related serum and tissue markers

Pretreatment with AS195 (groups III) significantly reduced the CCl_4-induced elevated levels of serum ALT ($P < 0.001$),

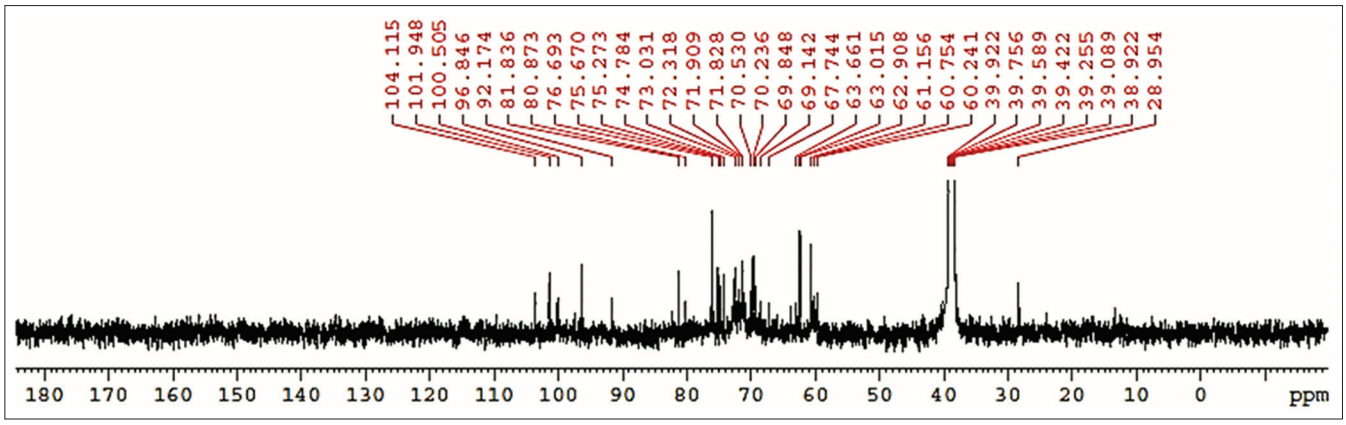

Figure 2: ^{13}C NMR spectrum (125 MHz, DMSO-d_6) of AS195 (25 mg). NS = 3000, AQ = 1.091241

ALP, GGT, and bilirubin ($P < 0.01$) [Figure 3 and Table 3], demonstrating a capacity to restore the normal functional status of the injured hepatocytes. Moreover, a significant increase in the levels of serum albumin ($P < 0.001$) and TP ($P < 0.01$) lowered by CCl_4 liver-injury was observed in rats pretreated with the extract at 100 mg/kg. Furthermore, administration of the extract at 100 mg/kg to the intoxicated rats markedly ameliorated the values of TC, TG, VLDL-C ($P < 0.001$), HDL-C and LDL-C ($P < 0.01$), with an effect on TG and VLDL-C similar to that attained by the standard hepatoprotective flavonolignan SYL at 10 mg/kg [Figure 4]. The level of hepatic MDA, an end product of lipid peroxidation, was significantly increased in CCl_4-intoxicated rat liver compared to that in normal control [Table 4]. However, pretreatment with AS195 significantly ameliorated ($P < 0.001$) the abnormal level of MDA. In the same experiment, the extract significantly increased ($P < 0.001$) the level of TP diminished by CCl_4-intoxication. Moreover, AS195 markedly increased the hepatic NP-SH level ($P < 0.01$).

Table 3: Effect of AS195 on CCl_4-induced hepatic injury-linked serum parameters in rats

Groups (*n*=6)	Bilirubin (mg/dL)	Albumin (U/L)	TP (g/dL)
I: Normal	0.58±0.01	4.94±0.22	9.58±0.57
II: CCl_4	2.66±0.15***[a]	1.73±0.16***[a]	4.47±0.34***[a]
III: CCl_4 + AS195 (100 mg/kg)	1.87±0.10**[b]	3.14±0.27***[b]	6.26±0.39***[b]
IV: CCl_4 + SYL (10 mg/kg)	1.05±0.06***[b]	3.49±0.28***[b]	8.02±0.38***[b]

All values represent mean±SEM. Data were statistically analyzed by using ANOVA, followed by Dunnett's multiple comparison test. **P<0.01; ***P<0.001; [a]As compared to normal group; [b]As compared to CCl_4 only group. TP: Total protein; CCl_4: Carbon tetrachloride; SEM: Standard error of mean

Table 4: Effect of AS195 on parameters of liver tissue in CCl_4-intoxicated rats

Groups (*n*=6)	TP (g/L)	MDA (nmol/g)	NP-SH (nmol/g)
I: Normal	110.97±7.21	0.85±0.15	7.24±0.69
II: CCl_4	51.09±2.52***[a]	8.39±0.39***[a]	3.36±0.46***[a]
III: CCl_4 + AS195 (100 mg/kg)	76.24±3.11***[b]	4.10±0.23***[b]	5.52±0.45**[b]
IV: CCl_4 + SYL (10 mg/kg)	82.63±4.04***[b]	2.89±0.39***[b]	7.03±0.52***[b]

All values represent mean±SEM. Data were statistically analyzed by using ANOVA, followed by Dunnett's multiple comparison test. **P<0.01; ***P<0.001. [a]As compared to normal group; [b]As compared to CCl_4 only group. TP: Total protein; CCl_4: Carbon tetrachloride; SEM: Standard error of mean; MDA: Malondialdehyde; NP-SH: Nonprotein sulfhydryl

Effect of AS195 on kidney-related serum and tissue markers

Administration of AS195 at 100 mg/kg was found to significantly ($P < 0.001$) inhibited the CCl_4-induced high level of creatinine comparable to SYL at 10 mg/kg. The inhibitory effect of this extract on the CCl_4-induced elevated uric acid, and calcium level were also significant ($P < 0.001$) [Figure 5]. In comparison with the CCl_4-intoxicated kidney, pretreatment with AS195 significantly lowered the level of renal MDA and exerted a moderate increase in TP and NP-SH levels [Table 5].

Effect of AS195 on the histopathological features of liver and kidney

Results of histopathological assessment of hepatic tissues [Figure 6] were found to be correlated with the above-mentioned biochemical findings. In the control CCl_4-intoxicated group, the lobular architecture of liver tissue was deformed and showed an evidence of extensive pericentral vein necrosis and fatty changes with ballooning of hepatocytes and infiltration of inflammatory cells. However, these alterations were minimized and liver while parenchyma were considerably returned to its normal status upon pretreatment with AS195 at 100 mg/kg. The kidney in the control CCl_4-intoxicated group also showed necrosis, loss of tubular details, degeneration/shrinkage of glomeruli and Bowman's capsules, and infiltration of inflammatory cells [Figure 7]. However, rats pretreated with AS195 exhibited noticeable correction of renal injury

as demonstrated by the apparent improvement of tubular and glomerular architecture and reduction of inflammatory cells. Therefore, a substantial histological recovery of liver and kidney was attained by AS195 at 100 mg/kg on comparison with SYL at the dose of 10 mg/kg.

DISCUSSION

Antioxidant natural products and chemoprevention are considered nowadays as an effective approach against health hazards induced by oxidative stress. Various studies have been shown that several xenobiotics cause generation of free radicals, which play a major role in initiation of oxidative stress-related diseases. Extensive *in vivo* studies on rodents have demonstrated that CCl_4 causes injuries to liver,[6] kidney,[7] and many organs *via* generation of electrophilic trichloromethyl (CCl_3) and peroxy trichloromethyl ($OOCCl_3$) radicals.[32] Such reactive free radicals produced from halogenated alkanes by the metabolizing activity of cytochrome P450 2E1 (CYP2E1) was reported to induce hepato-and nephrotoxicity[33] as they start to initiate lipid peroxidation and protein deterioration.[32] A subsequent alteration of cell membrane permeability and function, leakage of intracellular enzymes into serum[34] and other abnormal biochemical and histopathological changes in tissues occur.[35] Moreover, the increase in concentration of free peroxide radical and unsaturated fatty acid peroxide can induce alterations in the cholesterol profile and lipid metabolism along with induction of oxidative DNA damage, including the formation of DNA adducts and chromosomal alterations.[36] The induced lipid peroxidation and damage of hepatocyte membrane was also reported to be followed by the release of a myriad of growth factors, inflammatory mediators and prostaglandins from activated hepatic macrophages, which potentiate CCl_4-induced hepatic injury by further generation of a variety of reactive oxygen species (ROS).[37]

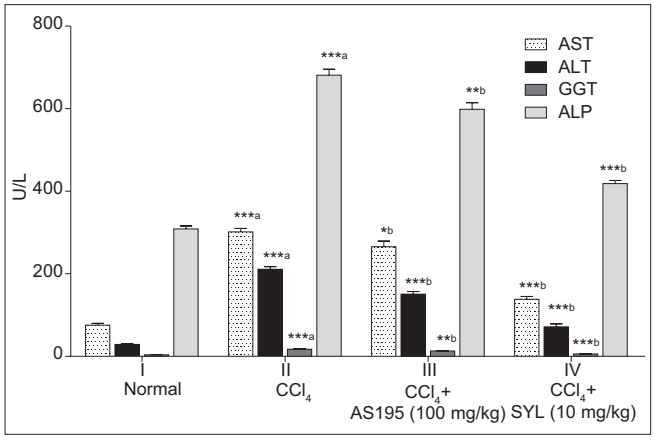

Figure 3: Effect of AS195 on carbon tetrachloride (CCl_4)-induced hepatic injury-linked serum parameters in rats. All values represent mean ± standard error of the mean data were statistically analyzed by using Analysis of Variance, followed by Dunnett's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [a]As compared to normal group. [b]As compared to CCl_4 only group

Table 5: Effect of AS195 on kidney tissue parameters in CCl_4-intoxicated rats

Groups (*n*=6)	TP (g/L)	MDA (nmol/g)	NP-SH (nmol/g)
I: Normal	96.20±3.68	0.65±0.13	9.61±0.41
II: CCl_4	36.32±2.42***a	9.08±0.58**a	4.95±0.40***a
III: CCl_4 + AS195 (100 mg/kg)	48.70±3.25*b	4.36±0.39***b	6.68±0.52*b
IV: CCl_4 + SYL (10 mg/kg)	71.05±3.99***b	2.27±0.22***b	8.63±0.37***b

All values represent mean±SEM. Data were statistically analyzed by using ANOVA, followed by Dunnett's multiple comparison test. **P*<0.05; ***P*<0.01; ****P*<0.001. [a]As compared to normal group; [b]As compared to CCl_4 only group. TP: Total protein; CCl_4: Carbon tetrachloride; SEM: Standard error of mean; MDA: Malondialdehyde; NP-SH: Nonprotein sulfhydryl

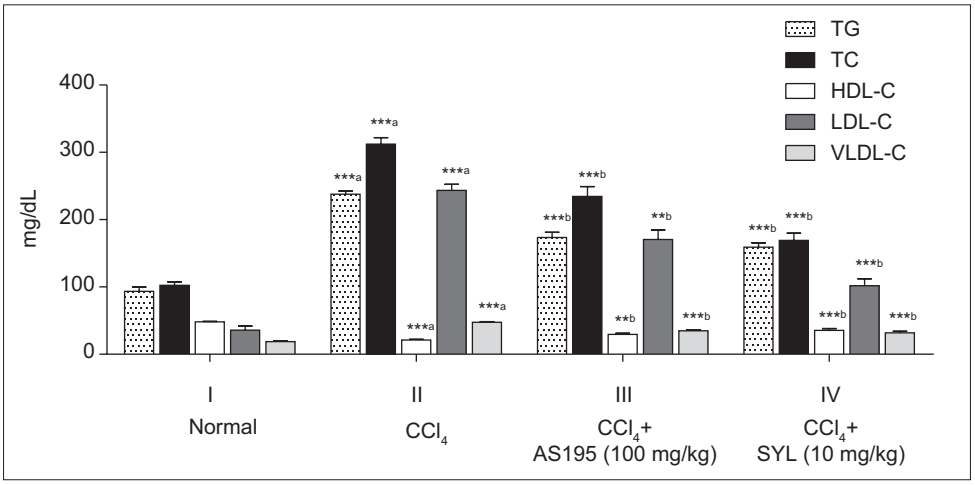

Figure 4: Effect of AS195 on carbon tetrachloride (CCl_4)-induced lipid profile change in rats. All values represent mean ± standard error of the mean Data were statistically analyzed by using Analysis of Variance, followed by Dunnett's multiple comparison test. **$P < 0.01$; ***$P < 0.001$. [a]As compared to normal group. [b]As compared to CCl_4 only group

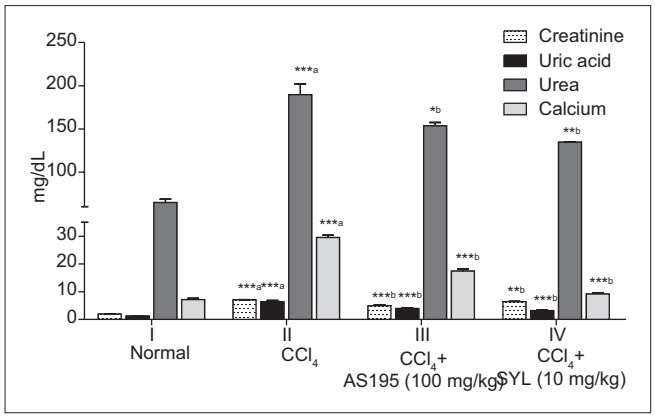

Figure 5: Effect of AS195 on kidney tissue parameters in carbon tetrachloride (CCl_4)-intoxicated rats. All values represent mean ± standard error of the mean Data were statistically analyzed by using Analysis of Variance, followed by Dunnett's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [a]As compared to normal group. [b]As compared to CCl_4 only group

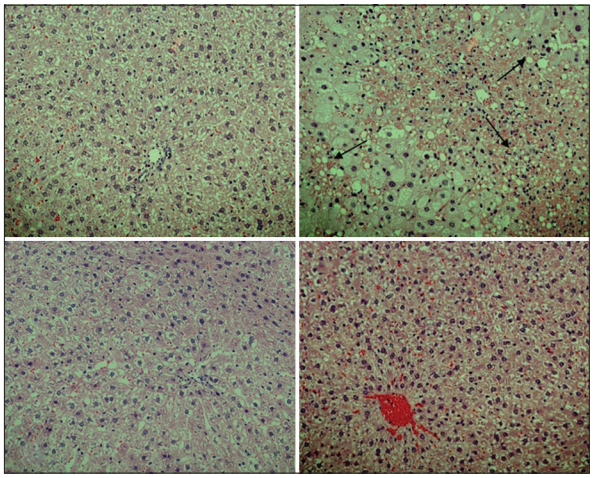

Figure 6: Photomicrography of hematoxylin and eosin stained sections of liver

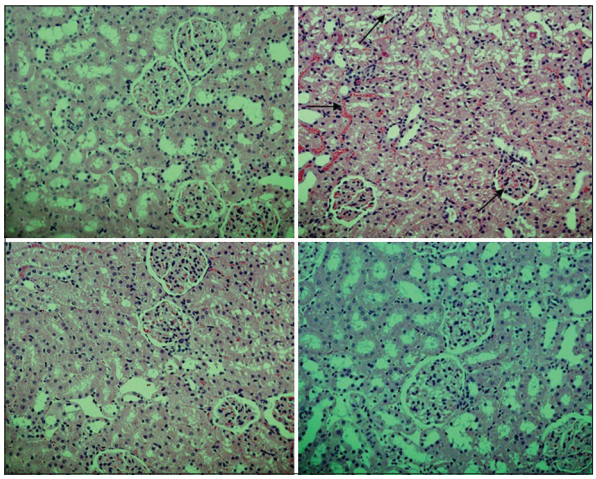

Figure 7: Photomicrography of hematoxylin and eosin stained sections of kidney

In addition, the level of NP-SH, e.g. glutathione (GSH), the nonenzymatic part of the antioxidant defense, and antioxidant enzymes (SOD, GPX, and CAT) decrease in the tissues due to their rapid consumption after combatting free radical-induced oxidative stress.[38] Therefore, inhibition and/or scavenging of intracellular ROS would play a critical role in preventing liver and kidney diseases.

Flavan-3-ols (catechins or their oligomers: Proanthocyanidins) and flavonols (especially quercetin) are the most abundant 2-phenylchroman-based natural polyphenols in our food which usually exist in the form of aglycones and diverse glycosides, respectively. These natural products possess powerful antioxidant properties due to their phenolic hydroxyl groups which serve as electron or hydrogen donors to terminate the free radical chain reaction yielding a stable phenolic radical[39] and consequently therapeutic benefit against oxidative stress-related diseases. Antistax® (AS195), a standardized aqueous extract of red vine leaves which has been used for many decades in treatment or prevention of symptoms of chronic venous insufficiency, was selected in this study for its high phenolic content. It contains mainly flavonoids and proanthocyanidins [Figure 1 and Table 2], as identified by phytochemical and nuclear magnetic resonance spectroscopic analyses [Tables 1 and 2 and Figure 2], in considerable amounts. Thus, the extract exhibited a substantial *in vitro* (DPPH) free radical scavenging capacity [Table 2]. This prompted us to evaluate the protective potentiality of AS195 against the hepatorenal toxicity induced by the free radicals-liberating xenobiotic CCl_4 on the basis of biochemical and histopathological evidences.

The present study revealed that CCl_4 significantly increased ($P < 0.001$) the levels of serum ALT, GGT, and ALP which pointed out acute hepatocyte injuries, altered membrane integrity, and consequently enzyme leakage. However, on pretreatment with AS195, the pathological levels of these enzymes were markedly restored [Figure 3], indicating the ability of the extract to protect hepatocytes against the deleterious effect of CCl_4-derived free radicals. Therefore, we found that the CCl_4-induced enhanced lipid peroxidation, which is indicated by high levels of MDA, was significantly normalized in liver tissue when pretreated with the extract [Table 4]. Moreover, the observed decrease ($P < 0.01$) of serum bilirubin, on pretreatment of the intoxicated animals with AS195 [Table 3], indicated that bilirubin is rapidly and selectively taken up into the liver by a healthy hepatocyte membranes.

Administration of CCl_4 significantly increased serum TG, TC, LDL-C, VLDL-C while decreased the

HDL–C [Figure 4], which was found to be in agreement with previous studies.[29,36] Particularly, the increased serum concentrations of TG, TC, LDL-C, and VLDL-C, and the decreased level of HDL-C, were obviously ameliorated upon pretreatment with the AS195. Other studies with animal models reported that flavonoids and proanthocyanidins exhibited similar correction effects in comparison with the CCl_4 intoxicated animals.[36,40]

Furthermore, the present results revealed that the concentrations of serum albumin and TP in the serum and hepatorenal tissues were markedly decreased after CCl_4 challenge [Tables 3 and 4], in agreement with previous studies.[29] The interpretation for the depletion of proteins in CCl_4-intoxicated animals may be due to the relationship between the damage occurring to DNA *via* free radicals and consequently the protein expression. Therefore, due to the free radical scavenging properties of polyphenol content, pretreatment with AS195 significantly increased the expression of these protein. Better levels of liver function-related enzymes and MDA along with those of albumin, TP and lipid profile in hepatic tissue, have been thus achieved by administration of AS195 in intoxicated animals. These findings indicated a preservation of biomembrane integrity, induction of protein synthesis, and stability of cholesterol metabolism in hepatocytes against free radicals-induced oxidative stress, respectively. This subsequently led to a marked parallel restoration of the histological features of liver [Figure 6].

In the same study, the protective effects of AS195 against CCl_4-induced nephrotoxicity on relevant oxidative stress parameters was also investigated, including renal MDA level, renal injury serum biomarkers (creatinine, uric acid, urea, calcium), and renal histopathological findings. Nephrotoxicity was evidenced by a significant alteration of these parameters [Table 5 and Figures 5 and 7] in the CCl_4-intoxicated group when compared with those of normal control in the same way as previously reported.[29] Pretreatment with AS195 significantly corrected the levels of renal MDA; markedly decreased the elevated levels of creatinine, urea, uric acid, and calcium; and improved the histopathological features in renal tissue [Table 5 and Figures 5 and 7].

With regard to the antioxidant defense system in this study, CCl_4 treatment significantly reduced the total content of NP-SH (e.g. GSH), which is considered as an important nonenzymatic antioxidant defense against lipid oxidative damage in the liver and kidney eliminating the formed peroxyl radicals. Therefore, NP-SH dependent enzymes such as glutathione peroxidase, reductase, and S-transferase (GPx, GR, and GST, respectively) will also be expected to be negatively affected flowingly. In our study, the level of NP-SH content was markedly ($P < 0.01$) or partially ($P < 0.05$) restored in the liver and kidney tissues on pretreatment with AS195, respectively. However, this effect seems to be dose-dependent. Therefore, other studies proved that treatment with proanthocyanidins in a higher dose (400 mg/kg) or with pure related flavonol glycoside (70 mg/kg/rat) significantly increased GSH along with a marked restoration in GSH dependent antioxidant enzymes levels relative to the CCl_4-intoxicated rats.[36,38]

The overall hepatorenal protection may be explained by many characteristics of AS195. The extract contained high amount of polyphenols (149.93 ± 0.35 mgGAE/g) represented mainly by flavonoids and proanthocyanidins which conducted high antioxidant potential through their direct free radical scavenging activity[39] as revealed by the *in vitro* DPPH assay, preventing the cascade that causes oxidative stress. This lead to in *in vivo* inhibition of lipid peroxidation along with the restoration of protein expression and nonenzymatic antioxidant NP-SH system. Moreover, the proanthocyanidin content of the extract can effectively suppress CCl_4-induced cytosolic CYP2E1 expression, which prevent the initial step of generating free radicals from CCl_4.

CONCLUSION

The administration of AS195 remarkably protected against CCl_4-induced hepatorenal injury as revealed by a combination of biochemical and histopathological evidences. This protective effect could be attributed to the antioxidative activity of the proanthocyanidins and flavonoid glycosides, which can suppress the oxidative stress generated by the free radicals of xenobiotics. This effect can play a role in protecting or maintaining the normal physiological and histological features of the susceptible organs. The results of the present study suggest that AS195 can serve as an adjuvant therapy in the treatment of oxidative stress-related liver and kidney ailments in addition to its regular use in the management of chronic venous insufficiency.

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