Biochemistry & Analytical Biochemistry
ISSN: 2161-1009

Sharmaa et al., Biochem Anal Biochem 2016, 5:2
DOI: 10.4172/2161-1009.1000267

Research Article Open Access

Protective Effect of a Standardized Fraction from *Vitex negundo* Linn. Against Acetaminophen and Galactosamine Induced Hepatotoxicity in Rodents

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Abstract

The use of *Vitex negundo* Linn. (Family: verbenaceae) is well documented in ayurveda and traditional Indian system of medicine for variety of diseases and liver ailments. The aim of the study was to investigate liver protective efficacy of a standardized bioactive fraction (SF) from *Vitex negundo* Linn. against acetaminophen (APAP) and galactosamine (GalN) hepatotoxicity. SF was tested at doses 12.5, 25, 50 and 100mg/kg, p.o. using both prophylactic and curative treatment schedule against APAP and GalN hepatotoxicity in mice and rats respectively. Isolated markers agnuside and negundoside were tested against APAP toxicity. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin and albumin were estimated in serum and triglycerides (TG), total protein, glutathione (GSH) and lipid peroxidation (LP) in liver homogenate. Histopathological studies were carried out against APAP induced hepatotoxicity. SF exhibited significant hepatoprotection against APAP and GalN evident by restoration of ALT, AST, LDH, ALP, bilirubin, TG, albumin and total protein. Levels of LP and GSH also exhibited significant dose dependent recovery when treated with SF. Agnuside and negundoside also exhibited dose dependent protection against APAP induced hepatotoxicity. Microscopic examination of histopathological sections of liver confirmed hepatoprotective potential of SF. Results of the study suggest significant value of SF as hepatoprotective with sufficient safety margin as no mortality and change in general gross behavior was observed up to 2000 mg/kg, p.o. Hepatoprotective mechanism of SF may be due to its antioxidant activity exhibited by protection against increased lipid peroxidation and maintained glutathione status. It is apparent from the present study that agnuside and negundoside are the active ingredients in SF and can be responsible for the activity of SF.

Keywords: *Vitex negundo*; Hepatoprotective; Acetaminophen; Galactosamine; Agnuside; Negundoside

Abbreviations

SF: Standardized bioactive Fraction; APAP: Acetaminophen; GalN: Galactosamine; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; LDH: Lactate Dehydrogenase; TG: Triglycerides; GSH: Glutathione; LP: Lipid peroxidation

Introduction

Vitex negundo Linn (Family: verbenaceae), commonly known as Five- Leaved Chaste Tree in English and Nirgundi in Sanskrit is 2-5 feet high aromatic annual shrub with bluish purple flowers, found throughout India, East Africa, and Japan. Seeds and leaves are bitter and leaves are used in fever, inflammation, vermifuge, headache and cough. Decoction of nirgundi has been used in steam bath for arthritis or joint pains [1]. It is also used as tonic in indigenous system of medicine. Flowers are astringent, used in diarrhea, fever and liver complaints [2]. Patients with rheumatoid arthritis exhibited encouraging results when treated with the plant [3]. Leaves and seeds are reported for anti-inflammatory, analgesic, anti-arthritic activities [4,5]. Significant anti-fungal [6] and antibacterial activity against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* has been reported [7]. Alcoholic extract of the seeds is reported for hepatoprotective activity against CCl_4 [8], leaves against anti-tubercular drugs and thioacetamide induced hepatotoxicity [9,10].

A large number of flavonoids and irridoids have been isolated from this plant. The reported flavonoids include casticin, orientin, isoorientin, luteolin, luteolin-7-0-glucoside, corymbosin, gardenins A and B, 3-0-desmethylartemetin, 5-0-desmethylnobiletin, 3',4',5,5',6,7,8-heptamethoxyflavone, 3',5-dihydroxy-4',7,8- trimethoxy flavanone and 3',5-dihydroxy-4',6,7-trimethoxyflavanone [11-13]. Five flavone glycosides viz., 6β-glucopyranosyl–7-hydroxy-3',4',5',8-tetramethoxyflavone–5-O-α-L-rhamnopyranoside, 3', 7-dihydroxy-4',6,8-trimethoxyflavone-5-O-(6"-O- acetyl- β- D- glucopyranoside), 3,3',4',6,7-pentamethoxy- flavone 5'-O-(4"-O-β-D-glucopyranosyl-α-rhamnopyranoside, 4', 5, 7- tri-hydroxyflavone-8- (2"- caffeoyl - α -glucopyranoside) and 3', 5, 5',7-tetrahydroxy-4-methoxyflavone-3'-O-(4"-O-α-D-galactopyranosyl) galactopyranoside have also been reported [14,15]. Major constituents of the leaves are irridoid glycosides, five of which have been isolated and identified as aucubin, agnuside, negundoside, 6′-p-hydroxybenzoyl mussaenosidic acid and nishindaside [16,17].

Our interest for evaluation of hepatoprotective activity led us to the identification of a standardized bioactive fraction (SF) from the ethanol extract of aerial parts of *Vitex negundo*. We have investigated the hepatoprotective activity of SF in detail and found that SF isolated from the ethanol extract of *Vitex negudo* has significant hepatoprotective potential.

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Received: February 03, 2016; **Accepted:** April 18, 2016; **Published** April 21, 2016

Citation: Sharmaa N, Suric J, Chandana BK, Singha B, Sattib N, et al. (2016) Protective Effect of a Standardized Fraction from *Vitex negundo* Linn. Against Acetaminophen and Galactosamine Induced Hepatotoxicity in Rodents. Biochem Anal Biochem 5: 267. doi:10.4172/2161-1009.1000267

Materials and Methods

Chemicals

Galactosamine, Acetamenophen and silymarin were purchased from Sigma chemicals Co., USA. All other chemicals used were of analytical grade and purchased locally.

Botanic material

Aerial parts of the plant *Vitex negundo* Linn, identified and authenticated by taxonomist of the Institute were collected locally during August to October. A voucher specimen (collection no. 17814) has been deposited in Herbarium of Indian Institute of Integrative medicine, Jammu.

Extraction and fractionation

The shade dried and powdered leaves (1kg) of *V. negundo* were soaked in 95% ethanol (5L) and kept overnight. The percolate was filtered and concentrated under reduced pressure below 50°C. The extraction procedure was repeated three times using three liters ethanol each time. The combined ethanol extract (yield, 141g) was stirred with water (500 ml) for 1 hour and filtered through Celite. The aqueous solution was concentrated at 50°C and finally dried in vacuum desiccator to give standardized bioactive fraction SF (yield, 106g).

Isolation of agnuside and negundoside

The standardized bioactive fraction (SF) of *Vitex negundo* (50 g) was adsorbed over silica gel (100 g) to make slurry which was packed over a column of silica gel (1 kg) packed in chloroform. Elution was done with chloroform followed by mixture of chloroform and methanol. Elution with 10% methanol in chloroform gave agnuside followed by mixture of agnuside and negundoside and then negundoside. The compounds were identified on the basis of NMR and Mass spectral data and compared with authentic samples (Figure 1).

NMR Data

Agnuside, obtained as crystalline compound. M^+ : 466, 1H NMR (200 MHz, CD3OD) δ 6.35 (dd, J= 2 and 6 Hz, H-3), 5.12 (dd, J= 4 and 6 Hz, H-4), 2.70 (m, H-5), 4.48 (m, H-6), 5.82 (s, H-7), 2.94 (m, H-9), 5.05 (s, H-10), 4.69 (d, J=8 Hz, H-1'), 3.65 (m, H-2'), 7.92 (dd, J = 2 and 7 Hz, H-2" ,H-6"), 6.84 (dd, J = 2 and 7 Hz, H-3"); 13CNMR δ 97.92 (C-1), 141.50 (C-3), 105.35 (C-4), 46.04 (C-5), 82.58 (C-6), 132.09 (C-7), 142.55 (C-8), 46.04 (C-9), 63.46 (C-10), 100.07 (C-1'), 74.56 (C-2'), 77.61 (C-3'), 71.01 (C-4'), 77.82 (C-5'), 62.49 (C-6'), 121.81 (C-1"), 132.73 (C-2", C-6"), 116.08 (C-3", C-5"), 163.50 (C-4"), 167.70 (CO).

Negundoside, obtained as crystalline compound. M+ : 496, 1H NMR (200 MHz, CD3OD) δ 5.48 (J= 3 Hz, H-1), 7.09 (s, H-3), 2.95 (m, H-5), 2.19 (m, H-9), 1.25 (s. H-10), 6.80 (dd, J= 2 and 7 Hz, H-3", H-5"), 7.83 (d, J= 2 and 7 Hz, H-2", H-6"), 4.99 (d, J=7 Hz, H-1'), 4.69 (d, J=7 Hz, H-2"); 13CNMR δ 122.99 (C-1), 133.67 (C-2", C-6"), 116.92 (C-3", C-5"), 164.11 (C-4"), 168.08 (CO), 95.83 (C-1), 151.98 (C-3), 116.92 (C-4), 30.96 (C-5), 31.98 (C-6), 42.04 (C-7), 80.64 (C-8), 53.19 (C-9), 25.15 (C-10), 170.71 (C-11), 98.64 (C-1'), 76.85 (C-2'), 75.70 (C-3'), 72.55 (C-4'), 79.30 (C-5'), 63.54 (C-6'). (NMR graphs submitted as supplementary data)

Chemical analysis and standardization

The Standardized bioactive fraction (SF) on TLC and HPLC analysis was found to contain two irridoid glycosides, agnuside and negundoside as major components which have been reported earlier from this plant [16,17]. Quantitative HPLC analysis of the fraction was carried out for determining the contents of agnuside and negundoside using a Waters HPLC system consisting of two pumps, (Waters 515 pumps with Waters pump control module-II), an automatic sampling unit (Waters 717 plus auto sampler), a column oven, a photodiode array detector (Waters 2996) and temperature control module-II. Waters empower software was used for data analysis and data processing. The samples were run at 30°C on a Merck LiChrospher® RP-18 Column (5 µm, 250 x 4.00 mm, 5 µM) in a linear elution mode with 1mL/min flow rate using methanol : 2.0% acetic acid in water (3:7) as solvent system and detected by UV detection at 340 nm. An equilibration period of 10 min was used between the runs.

All the solvents used were of HPLC grade, filtered over 0.45µm Millipore filter paper and degassed through in line degasser AF from waters.

The accurately weighed quantities of the active fraction were dissolved in a known volume of HPLC grade methanol. The samples were filtered through a millipore micro filter (0.45 µm) and then injected into the HPLC system. Stock solutions of the standards were prepared in HPLC grade solvents. From the stock solutions, working solution was prepared by mixing requisite quantity of each marker and dilution with HPLC grade solvent for further analysis.

The compounds exhibited linear responses in the calibration curves, which were prepared by using the multipoint calibration curve method. Working solution of the standards was injected in different amounts (negundoside 0.22-1.1 µg and for agnuside 0.26-1.3 µg) and calibration curve was obtained for negundoside (r2= 0.999569) and agnuside (r2=0.998843). The content of negundoside and agnuside in SF on the basis of HPLC results was found to be 2.318% and 21.947% respectively (Figure 2).

Experimental animals

Male wistar rats (160-180 g) and swiss albino mice (20-25g) were housed under standard conditions (23.0 ± 2°C, 60-70% humidity, and 12 h photo period) and allowed free access to food (Ashirwad India Ltd., Chandigarh, India) and water. The experiments were conducted according to the ethical norms approved by Institutional Animal Ethics Committee guide lines for animal care and were adhered to as recommended by Institutional Animal Ethics Committee IIIM, Jammu.

Grouping and treatment

Seven groups with six mice per group were used in study against APAP induced hepatotoxicity. First four groups were fed with SF

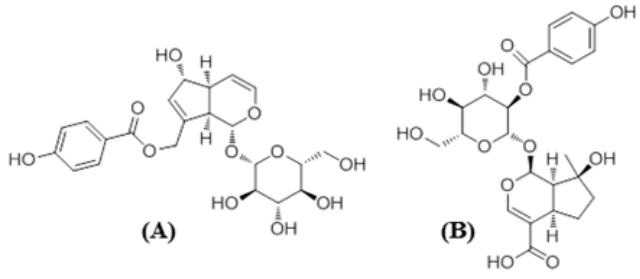

Figure 1: (A) Agnuside. (B) Negundoside.

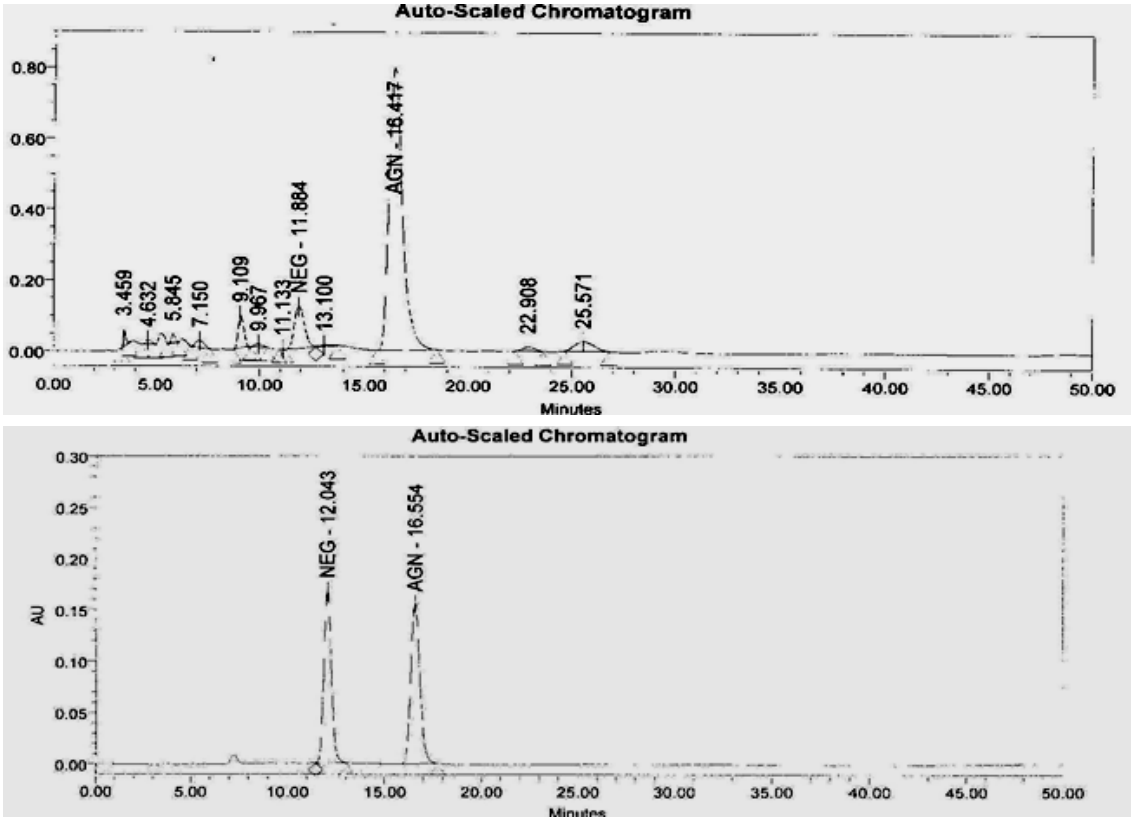

Figure 2: (A) HPLC graph of Fraction. (B) HPLC graph of marker compounds negundoside and agnuside.

12.5, 25.0, 50.0 and 100 mg/kg, p.o. respectively and the fifth group was administered reference drug silymarin (50 mg/kg, p.o.). Group six and seven were given proportionate volume of vehicle (distilled water). APAP was administered to first six groups as per treatment schedules (prophylactic and curative) and group seven was administered vehicle only to serve as vehicle control group. Separate set of experiments were used for agnuside and negundoside using same procedures and doses as were used for SF. In case of study against GalN induced hepatic injury, grouping and treatment is same except for, wistar rats were used in place of mice.

Induction of liver injury

Acetaminophen (APAP): Liver injury was induced by administering APAP (200 mg/kg, i.p.) in mice six hours after exposure to diethyl ether [18].

Galactosamine (GalN): Liver injury was induced by administering GalN (300 mg/kg,s.c.) in rats.

Effect against acetaminophen (APAP) induced hepatic injury in mice

Prophylactic study: Test, reference drug and vehicle were given 74h, 50h, 26h, 2h before and 6h after ether exposure. APAP was administered 6 hours after ether exposure. Liver and blood samples were collected 18 hours after APAP administration [19].

Curative study: Test, reference drug and vehicle were given 6h, 24h, and 48h after exposure with ether. APAP was administered 6h after exposure to ether. Liver and blood samples were collected 2 hours after last treatment with the test material [19].

Effect against GalN induced hepatic injury in rats

Prophylactic study: Test, reference drug and vehicle were given 74 h, 50 h, 26 h, 2 h before and 6 h after GalN (300 mg/kg, s.c). Liver and blood samples were collected 24 hours after GalN administration [19].

Curative study: Test, reference drug and vehicle were given 6 h, 24 h, and 48 hours after GalN (300 mg/kg, s.c) administration. Liver and blood samples were collected 50 hours after GalN administration [19].

Blood biochemistry

Blood samples were collected in glass tubes from orbital sinus to obtain haemolysis free clear serum for the analysis of ALT and AST, ALP, LDH, bilirubin and albumin by standard methods.

Preparation of liver homogenate

All the animals were sacrificed and livers were quickly excised washed and perfused with chilled normal saline, dried on filter paper, weighed, minced and homogenized in ice bath in chilled 10mM Tris-HCl buffer (pH 7.4) to obtain 10% liver homogenate for the estimation of total protein [20],TG [21], GSH [22], and LP [23].

Hepatoprotective activity percent

The hepatoprotective activity percentage protection (H) for each parameter was calculated as follows:

$$H = (1 - \frac{T - V}{C - V})100$$

Where 'T' is mean value of drug and toxin. 'C' is mean value of toxin alone and 'V' is the mean value of vehicle treated group.

Overall hepatoprotective activity = Mean ± SE of (H) of all the parameters

Histopathology

The animals used in the curative study against APAP induced hepatotoxicity were sacrificed and liver tissue was examined grossly. A small portion of liver tissue of all the animals was fixed in 10% formaline saline, processed and embedded in paraffin wax to obtain 4 to 5 micron thick sections using a microtome. Finally the sections were subjected to histopathological examination after processing in alcohol - xylene series and haematoxylin and eosin staining [24].

Safety evaluation

Acute toxicity study was conducted using OECD guide lines No. 423. Three female mice of same age and weight group were taken in a single dose up to the highest dose of 2000 mg/kg orally. The animals were observed continuously for 1 hour and then hourly for 4 hours and finally after every 24 hours up to 15 days for any mortality or gross behavioral changes.

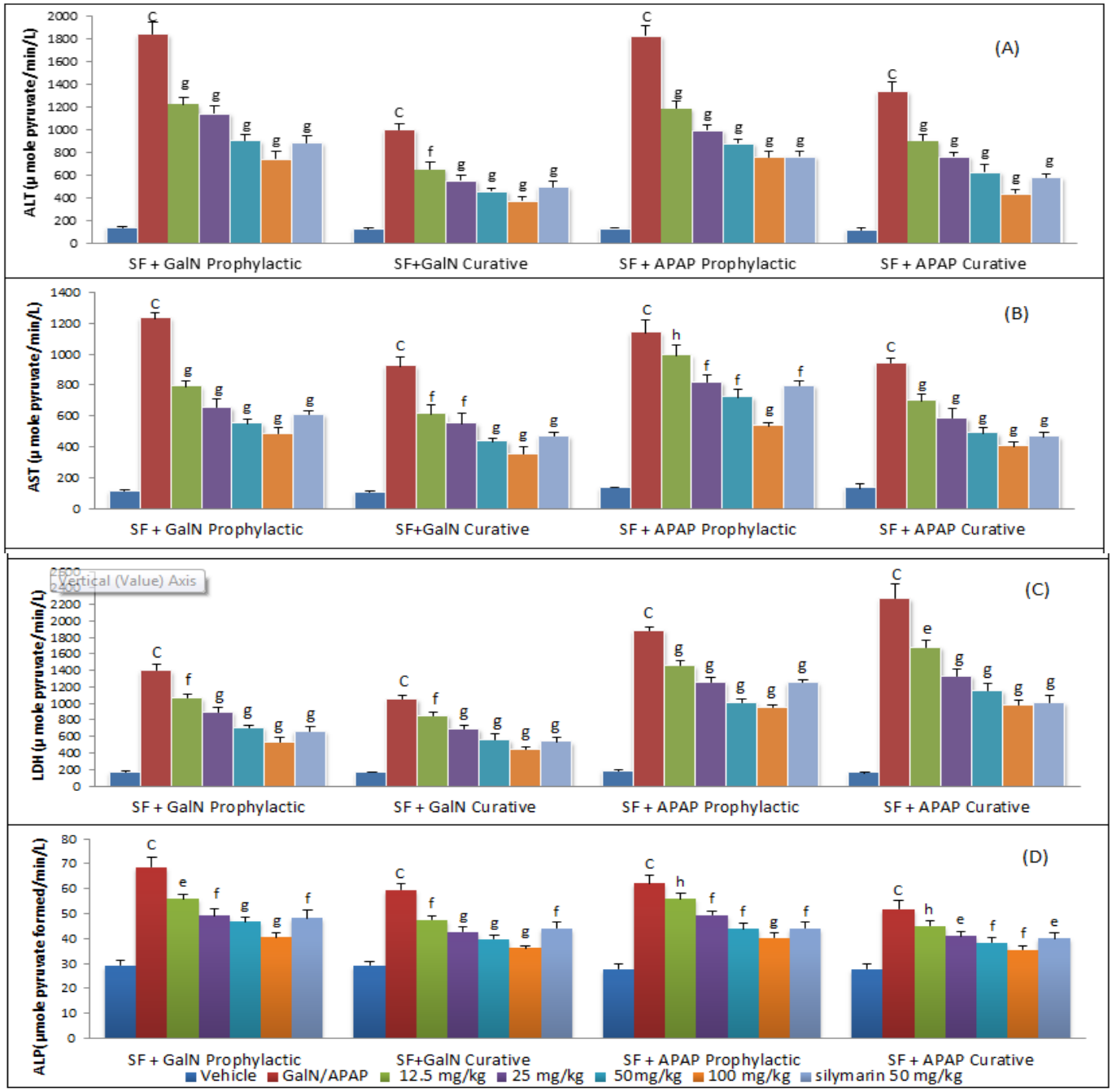

Figure 3: Effect of SF on serum ALT, AST, LDH and ALP against GalN 300mg/kg, s.c., APAP 200mg/kg, i. p. (Prophylactic & Curative study). (Values represent the mean ± SE of six animals in each group. P Value Vs Vehicle- C< 0.001; P Value Vs CCl4- e< 0.05, f< 0.01, g< 0.001, h not significant).

Statistical analysis

The data obtained is presented as Mean ± SE of six animals in each group and were analyzed by One Way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using computerized program. P-value< 0.05 was taken as the criterion of significance.

Results

Effect against acetaminophen (APAP) induced hepatic injury in mice

Significant (P<0.001) increase in the levels of ALT, AST, LDH, ALP, TG and lipid peroxidation and decrease in the level of albumin, protein and GSH were observed after administration of APAP. Prophylactic and curative treatments of SF at 12.5, 25.0 50.0 and 100 mg/kg exhibited significant reversal of above biochemical parameters in a dose dependant manner. A similar effect was observed with silymarin (Figures 3-5).

Prophylactic treatments of SF at 12.5, 25.0 50.0 and 100mg/kg exhibited 20.88 ± 3.3.37%, 37.53 ± 2.45%, 48.00 ± 1.77% and 58.46 ± 1.51% overall hepatoprotective activity and with curative treatment it was 28.14 ± 3.98%, 43.82 ± 2.67%, 56.45 ± 2.57%, 67.58 ± 3.54% respectively. Prophylactic and curative treatments with

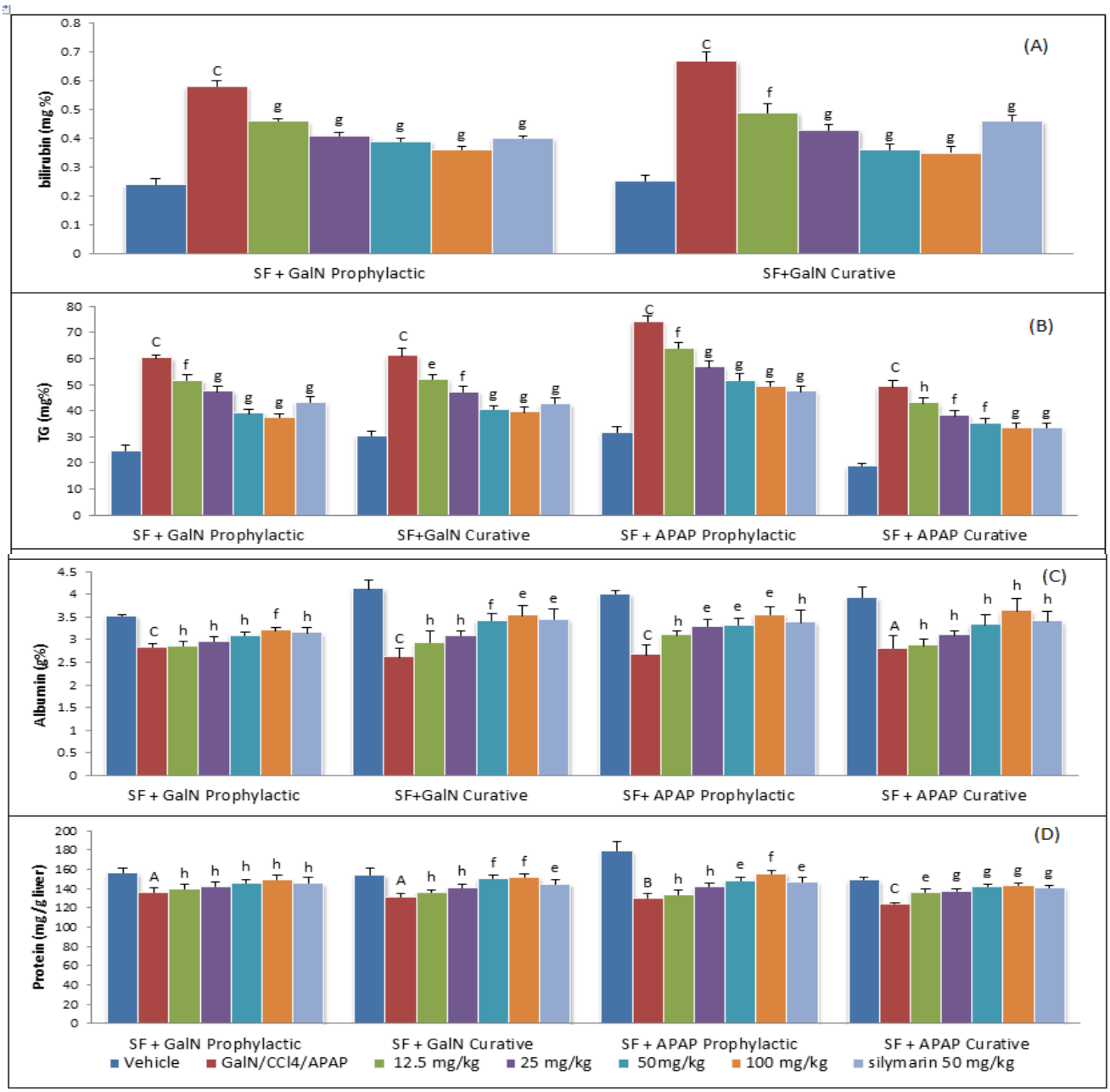

Figure 4: Effect of SF on bilirubin, triglycerides, albumin and protein against GalN 300mg/kg, s.c., APAP 200mg/kg, i. p. (Prophylactic & Curative study). (Values represent the mean ± SE of six animals in each group. P Value Vs Vehicle-A< 0.05; C< 0.001; P Value Vs CCl4- e< 0.05, f< 0.01, g< 0.001, h not significant).

silymarin at 50mg/kg, p.o. exhibited 50.46 ± 4.05% and 57.76 ± 1.87% hepatoprotection respectively, which was comparatively less than SF (100 mg/kg) and the difference was significant (P<0.05) in case of curative study and insignificant in case of prophylactic study.

Prophylactic treatment with agnuside and negundoside at 12.5, 25.0 50.0 and 100mg/kg ameliorates APAP toxicity and the overall hepatoprotective activity exhibited by agnuside was 24.37 ± 4.78%, 40.49 ± 4.12%, 55.82 ± 3.64% and 67.82 ± 4.38% and with negundoside it was 27.36 ± 5.47%, 43.84 ± 3.21%, 59.61 ± 2.10% and 69.87 ± 3.17% respectively (Tables 1-2).

Histopathology

Microscopic examination of the vehicle treated animals showed normal architecture of hepatic parenchyma. Treatment with APAP alone showed extensive centrilobular necrosis of hepatic parenchyma, along with evidence of hepatic steatosis and scattered lymphomononuclear (LMN) cell infiltrate. Treatment with SF showed dose dependent hepatoprotective effect when given after APAP administration in the form of centrilobular necrosis involving smaller foci of hepatic parenchyma and evidence of regeneration in the form of binuclear hepatocytes adjacent to area of necrosis. The hepatoprotective effect of SF 100mg/kg was comparable to that of silymarin (Figures 6A-6F)).

Effect against GalN induced hepatic injury in rats

Significant (P<0.001) increase in the levels of ALT, AST, LDH, ALP, bilirubin, TG and lipid peroxidation and decrease in the level of Albumin, protein and GSH were observed after administration of GalN (Figures 3-5).

Treatments with SF at 12.5, 25.0 50.0 and 100 mg/kg, p.o exhibited significant normalization in the altered levels of all biochemical parameters in a dose dependant manner. A similar effect was observed with silymarin (Figures 3-5). Prophylactic treatment with SF at 12.5, 25.0, 50.0 and 100 mg/kg, p.o against GalN induced hepatotoxicity exhibited 25.83 ± 3.40%, 39.87 ± 3.39%, 54.78 ± 2.21% and 67.11 ± 2.00% overall hepatoprotection respectively. SF 100mg/kg exhibited significantly (p< 0.001) better activity than silymarin (53.27 ± 1.91%). Curative treatment at above doses exhibited 30.81 ± 3.14%, 46.00 ± 2.71%, 63.44 ± 2.2.75%, 73.38 ± 2.2.24% overall protection respectively. SF (50 and 100 mg/kg) exhibited significantly (p<0.05 and p<0.001) better activity than silymarin (55.96 ± 1.18%).

Safety evaluation

No mortality or any sign of gross behavioral changes were observed in mice initially for 72 hours and finally up to 15 days when fed with SF up to 2000 mg/kg, p.o.

Discussion

The present study has demonstrated that standardized bioactive fraction (SF) from *Vitex negundo* Linn. has significant hepatoprotective effect against two mechanistically different hepatotoxins i.e., acetaminophen and galactosamine. Acetaminophen toxicity is manifested by a highly toxic electrophile n-acetyl-p-benzoquinone-imine (NAPQI) which is readily detoxified by enzymatic conjugation with hepatic glutathione (GSH). In the events of NAPQI accumulation, it binds covalently to tissue macromolecules and probably also oxidizes lipids, alters homeostasis of calcium causing severe hepatic damage [25]. The increased levels of serum enzymes (ALT, AST, LDH and ALP)

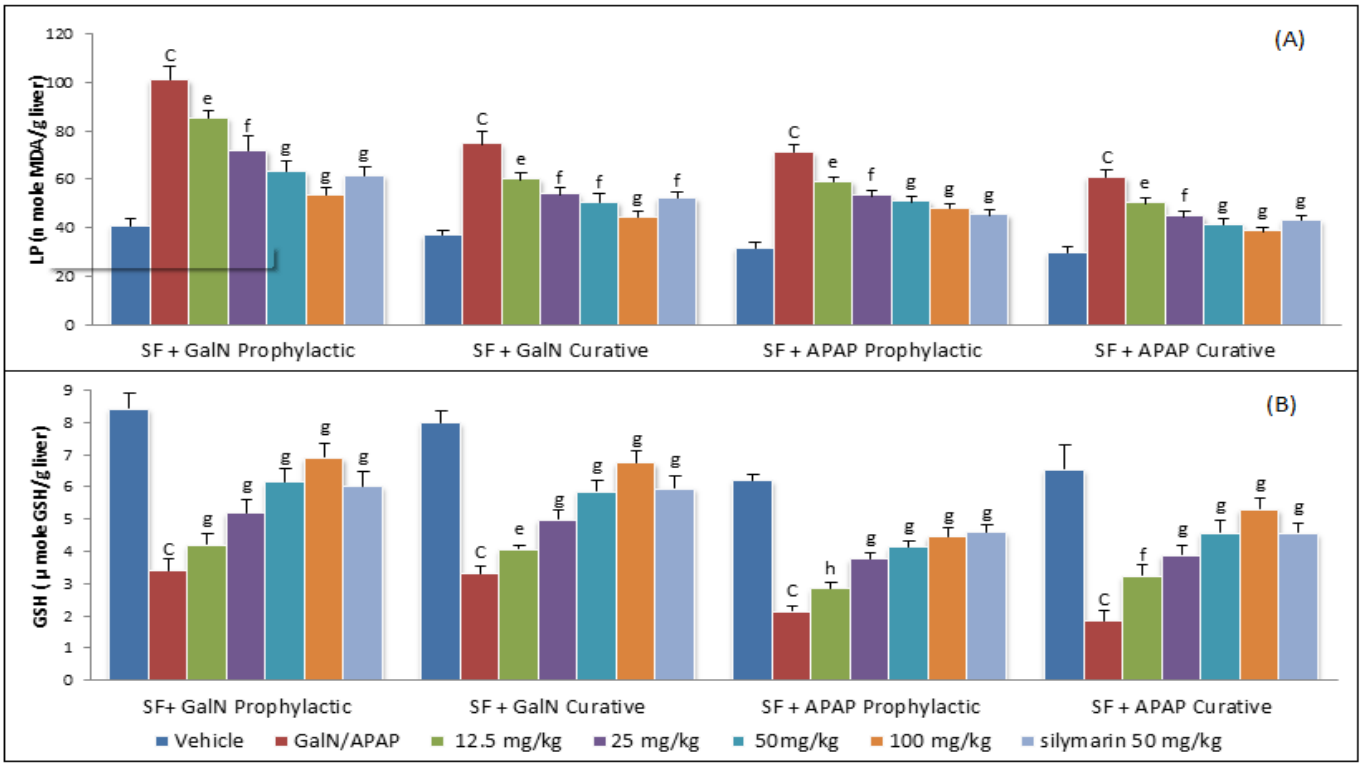

Figure 5: Effect of SF on lipidperoxidation (LP) and GSH against GalN 300mg/kg, s.c., APAP. (Values represent the mean ± SE of six animals in each group. P Value Vs Vehicle- C< 0.001; P Value Vs CCl4- e< 0.05, f< 0.01, g< 0.001, h not significant).

Treatment	Dose mg/ kg	Serum parameter[a]					Hepatic parameters			Overall % Protection
		ALT (Units)	AST (Units)	ALP[b]	LDH (units)	Albumin (g %)	LP[c]	GSH[d]	Protein (mg %)	
Vehicle	-	115.36 ± 6.48	120.25 ± 8.55	29.55 ± 2.66	170.99 ± 6.36	3.99 ± 0.15	43.10 ± 2.42	7.36 ± 0.29	165.42 ± 6.98	-
Veh+ APAP	-	1714.79 ± 88.49^{C}	1617.14 ± 72.04^{C}	54.78 ± 4.16^{C}	1671.82 ± 109.03^{C}	2.85 ± 0.12^{C}	95.68 ± 6.45^{C}	3.22 ± 0.29^{C}	134.27 ± 4.06^{B}	-
A side+ APAP	12.5	1289.64 ± 78.73^{f} (26.58)	1218.61 ± 84.39^{f} (26.62)	46.42 ± 2.47^{h} (33.13)	1194.95 ± 72.12^{f} (31.77)	3.17 ± 0.12^{h} (28.07)	74.18 ± 3.81^{e} (40.89)	3.52 ± 0.20^{h} (7.24)	134.48 ± 4.52^{h} (0.67)	24.37 ± 4.78^{k}
A side + APAP	25	1122.45 ± 83.05^{g} (37.03)	1119.01 ± 64.90^{g} (33.27)	44.01 ± 1.50^{e} (42.68)	1091.59 ± 64.16^{g} (38.66)	3.51 ± 0.26^{e} (57.89)	65.50 ± 3.81^{f} (57.39)	4.34 ± 0.32^{e} (27.05)	143.60 ± 4.82^{h} (29.95)	40.49 ± 4.12^{i}
A side + APAP	50	906.62 ± 34.28^{g} (50.52)	865.55 ± 48.46^{g} (50.21)	40.14 ± 2.04^{e} (58.02)	856.69 ± 37.80^{g} (54.31)	3.71 ± 0.27^{e} (75.00)	60.99 ± 2.24^{g} (65.97)	4.99 ± 0.33^{f} (42.75)	149.80 ± 6.13^{h} (49.85)	55.82 ± 3.64^{h}
A side + APAP	100	765.31 ± 4`1.22^{g} (59.36)	686.27 ± 48.69^{g} (62.18)	38.32 ± 2.73^{f} (65.23)	682.20 ± 53.58^{g} (65.94)	3.92 ± 0.04^{g} (93.85)	54.39 ± 2.14^{g} (78.52)	5.58 ± 0.29^{g} (57.00)	153.13 ± 6.18^{e} (60.54)	67.82 ± 4.38^{i}
Sily+ APAP	50	742.72 ± 33.40^{g} (60.77)	737.10 ± 66.51^{g} (58.79)	42.05 ± 1.63^{e} (50.45)	847.76 ± 47.43^{g} (54.90)	3.49 ± 0.15^{f} (56.14)	8.86 ± 3.48^{f} (51.00)	5.01 ± 0.35^{f} (43.23)	151.34 ± 5.23^{e} (54.79)	53.75 ± 1.94

Values represent mean ± SE of six animals in each group; within parenthesis is % protection; Units: Each unit is µ mole pyruvate/min /L; b: µ mole of p-nitrophenol formed /min/L; c: lipid peroxidation (n mole MDA/g liver); d: glutathione (mole GSH/g liver). P Value Vs. Vehicle- A< 0.05P, B< 0.01, C< 0.001; P Value Vs. CCl_4- e< 0.05, f< 0.01, g< 0.001, h not significan; P Value Vs silymarin i< 0.05, j< 0.01, k< 0.001, h not significant.

Table 1: Hepatoprotective potential of Agnuside against APAP (200mg/kg i.p.) induced hepatic injury in mice (Prophylactic study).

Treatment	Dose mg/ kg	Serum parameter[a]					Hepatic parameters			Overall % Protection
		ALT (Units)	AST (Units)	ALP[b]	LDH (units)	Albumin (g %)	LP[c]	GSH[d]	Protein (mg %)	
Vehicle	-	102.48 ± 5.92	107.50 ± 8.36	26.00 ± 2.21	155.82 ± 7.55	3.63 ± 0.26	34.62 ± 2.97	6.60±0.35	143.68 ± 7.76	-
Vehicle+APAP	-	1449.44 ± 70.45^{C}	1442.95 ± 66.26^{C}	56.24 ± 2.41^{C}	1883.07 ± 92.46^{C}	2.07 ± 0.30^{B}	66.09 ± 3.75^{C}	2.71±0.27^{C}	115.44 ± 4.76^{A}	-
N side+ APAP	12.5	1138.39 ± 59.44^{f} (23.08)	1041.84 ± 51.39^{g} (30.03)	47.45 ± 1.81^{f} (29.97)	1531.26 ± 117.12^{e} (20.36)	2.99 ± 0.21^{e} (32.09)	55.73 ± 2.51^{e} (32.90)	3.64±0.28^{e} (23.48)	124.00 ± 4.91^{h} (30.31)	27.77 ± 1.93^{k}
N side+ APAP	25	960.56 ± 69.10^{g} (36.28)	884.59 ± 37.56^{g} (41.80)	42.81 ± 2.16^{f} (45.12)	1456.26 ± 127.43^{e} (24.70)	3.11 ± 0.19^{e} (44.26)	52.70 ± 2.19^{e} (42.54)	4.12±0.33^{f} (35.64)	127.55 ± 5.97^{h} (42.90)	39.15 ± 2.40^{k}
N side+ APAP	50	806.54 ± 31.89^{g} (47.72)	687.58 ± 29.13^{g} (56.55)	40.72 ± 2.31^{g} (51.95)	1181.29 ± 39.20^{g} (40.62)	3.34 ± 0.23^{f} (69.71)	49.48 ± 2.19^{f} (52.75)	4.53±0.46^{f} (46.12)	130.59 ± 5.42^{h} (53.65)	52.38 ± 3.04^{h}
N side+ APAP	100	674.01 ± 36.77^{g} (57.55)	610.59 ± 30.99^{g} (62.32)	38.77 ± 1.99^{g} (58.31)	963.61 ± 75.74^{g} (53.22)	3.43 ± 0.19^{f} (78.48)	48.46 ± 1.03^{f} (56.55)	4.79±0.36^{g} (52.64)	133.01 ± 4.64^{e} (62.54)	60.20 ± 2.90^{h}
Sily+ APAP	50	662.46 ± 44.93^{g} (58.42)	660.36 ± 49.40^{g} (58.59)	40.67 ± 2.30^{g} (52.12)	911.64 ± 69.67^{g} (56.23)	3.16 ± 0.20^{e} (49.63)	0.93 ± 2.07^{f} (48.14)	4.55±0.36^{g} (46.62)	135.18 ± 5.23^{e} (69.90)	54.95 ± 2.68

Values represent mean ± SE of six animals in each group; within parenthesis is % protection; Units: Each unit is µ mole pyruvate/min /L; b: µ mole of p-nitrophenol formed /min/L; c: lipid peroxidation (n mole MDA/g liver); d: glutathione (µ mole GSH/g liver). P Value Vs. Vehicle- A< 0.05P, B< 0.01, C< 0.001; P Value Vs. CCl_4- e< 0.05, f< 0.01, g< 0.001, h not significan; P Value Vs silymarin i< 0.05, j< 0.01, k< 0.001, h not significant.

Table 2: Hepatoprotective potential of Negundoside against APAP (200mg/kg i.p.) induced hepatic injury in mice (Prophylactic study).

may be interpreted as a result of liver cell destruction or changes in membrane permeability. These enzymes are characteristic of liver cell damage; therefore their release into serum after APAP administration confirmed liver damage [26]. Administration of APAP causes significant increase in lipid peroxidation, depletion of hepatic glutathione (GSH), hypoalbuminemia and hypoproteinemia [27,28]. In our study a significant increase in levels of serum enzymes, lipid peroxidation and decrease in the levels of GSH, albumin and protein were observed. Treatment with SF and isolated markers agnuside and negundoside significantly reversed and restored altered levels of these parameters towards normal and exhibited comparative hepatoprotective potentials with silymarin against the toxic implication of acetaminophen.

Microscopic examination of histopathological section confirms dose dependent hepatoprotective efficacy of SF against APAP. This was evident as smaller area of necrosis, and regeneration in the form of binucleation of hepatocytes at margin of area of necrosis. The hepatoprotective efficacy of SF at higher doses i.e. 100mg/kg was comparable to that of silymarin supported by the biochemical and histopathological findings.

In our study administration of GalN caused a significant increase in the levels of serum enzymes (ALT, AST, LDH and ALP), bilirubin, and lipid peroxidation, depletion of hepatic glutathione, hypoalbuminemia and hypoproteinemia. This was substantiated by the findings of other authors [27]. SF treatment ameliorated levels of serum enzyme,

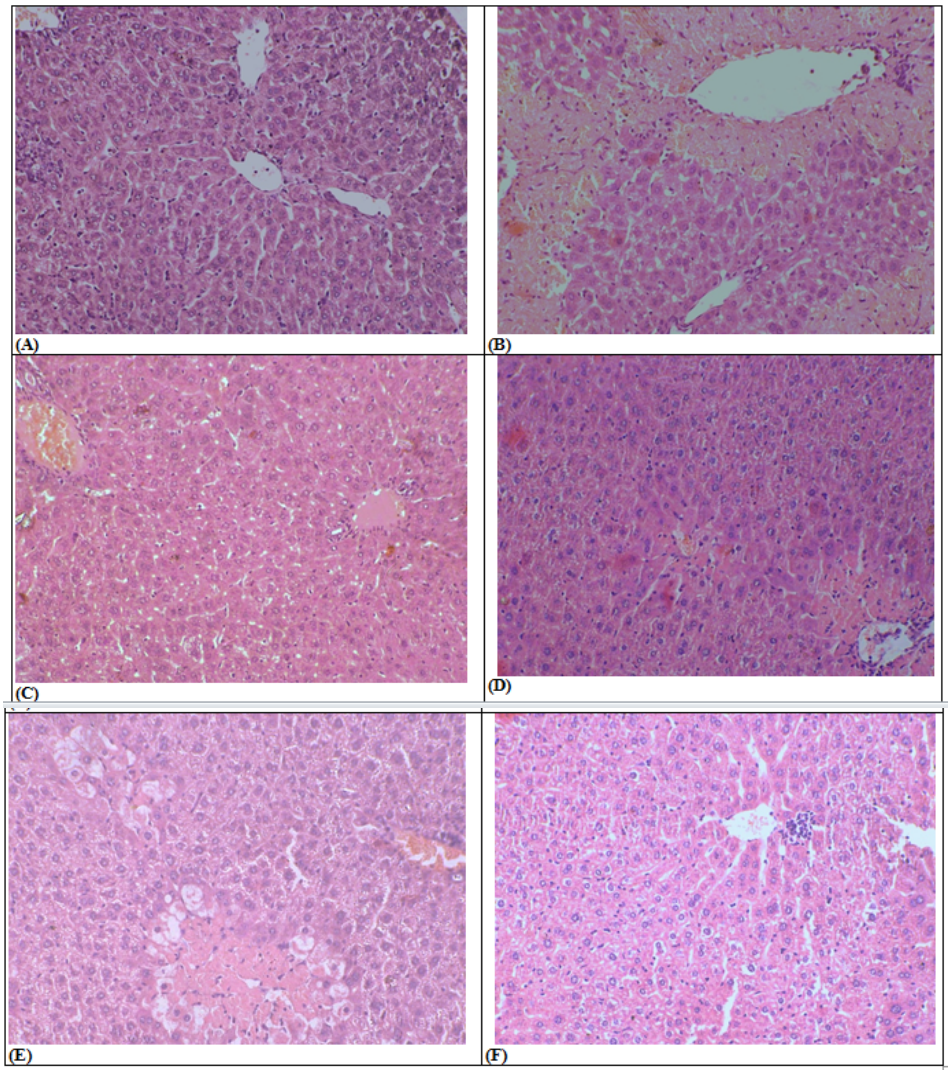

Figure 6: The photomicrographs of liver sections stained with haematoxylin and eosin (20x).

(A) Normal mouse liver section. (B) APAP treated mouse liver section. (C) SF 100mg/kg. + APAP treated mouse liver section. (D) SF 50mg/kg. + APAP treated mouse liver section. (E) SF 25mg/kg. + APAP treated mouse liver section. (F) Silymarin 50mg/kg. + APAP treated mouse liver section.

bilirubin, lipid peroxidation, GSH, albumin and protein by protecting from liver cell damage induced by D-GalN dose dependently.

Conclusion

It is apparent from the present study that SF and individual marker compounds agnuside and negundoside isolated from SF exerts significant hepatoprotective activity suggesting presence of agnuside and negundoside in SF responsible for its efficacy. APAP models rely on the cytochrome P-450 system to produce reactive metabolites NAPQI. Therefore the possible hepatoprotective mechanism of SF may be due to its antioxidant activity exhibited by protection against increased lipid peroxidation and maintained glutathione status. Protection against galactosamine induced liver injuries may be by inhibiting the UDP sugar derivatives, enhancing biosynthesis of glycoprotein, and or by stabilizing the cell membrane. However, further mechanistic studies are warranted before we could conclude on the exact mechanism(s) involved in the hepatoprotective activity. However, it appears that SF being orally absorbed, exhibiting significant efficacy, well tolerated and safe up to 2000 mg/kg, p.o. may be of significant therapeutic value in the management of hepatic diseases.

Acknowledgement

The authors are thankful to the staff of animal house for the care and supply of the experimental animals. The project was financially supported by the council of Scientific and Industrial Research (CSIR), New Delhi.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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