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Synergistic effect of silymarin and standardized extract of *Phyllanthus amarus* against CCl_4-induced hepatotoxicity in *Rattus norvegicus*

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Abstract

In search of the effective and standardized hepatoprotective combination therapy, silymarin and standardized extract of *Phyllanthus amarus* has been evaluated against CCl_4-induced hepatotoxicity in rats. Eight groups of rats were used. The animals of group A served as normal and were given only vehicle. The animals of group B served as toxin control and were administered with CCl_4 (50% solution of CCl_4 in liquid paraffin, 2 ml/kg b.w., intraperitoneally). The animals of groups C–H received silymarin (100 mg/kg b.w.), *Phyllanthus amarus* aqueous extract (100 mg/kg b.w.), *Phyllanthus amarus* ethanolic extract (100 mg/kg b.w.), silymarin (50 mg/kg b.w.) + *P. amarus* aq. ext. (50 mg/kg b.w.), silymarin (50 mg/kg b.w.) + *P. amarus* eth. ext. (50 mg/kg b.w.) and marketed formulation (M.F.) 5 ml/kg b.w. for 6 days orally as well as CCl_4 (2 ml/kg b.w.) on 4th day intraperitoneally. The test materials were found effective as hepatoprotective as evidenced by plasma and liver biochemical parameters. The combination of silymarin and *Phyllanthus amarus* showed synergistic effect for hepatoprotection and silymarin with ethanolic extract of *P. amarus* showed better activity due to the higher concentration of phyllanthin in ethanolic extract in comparison to aqueous extract of *P. amarus* as estimated by HPLC.

Keywords: *Phyllanthus amarus*; Silymarin; CCl_4; Synergistic effect; Hepatotoxicity

Introduction

Living in a world of inadequately controlled environmental pollution and expanding therapy with potent drugs, the liver, which is the key organ of metabolism and excretion, is continually exposed to a variety xenobiotics and therapeutic agents. Thus the disorders associated with this organ are numerous and varied. An actual curative therapeutic agent has not yet been found. In fact, most of the available remedies support or promote the process of healing or regeneration of the liver.

Silymarin is a flavonoid extracted from the milk thistle *Silybum marianum*. It has been reported to prevent liver injuries induced by various chemicals or toxins including ethanol (Farghali et al., 2000; Halim et al., 1997; Lieber et al., 2003). Although the hepatoprotective properties of silymarin have been reported both from *in vitro* and *in vivo* studies, its

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mechanism of action still has not been established. Two major mechanisms that have been proposed include functioning as an antioxidative scavenger of free radicals (mainly active toward HO and HOCl and less so for H_2O_2 or O_2^-) and as a regulator of immune functions by modulating cytokine production (Ferenci et al., 1989; Luper, 1998).

A standardized extract from the milk thistle *Silybum marianum* contains as its main constituents the flavonoids silybin, silydianin and silychristin (Flora et al., 1996). The flavonoid silibinin, which constitutes 60–70% of silymarin, has been identified as the major active ingredient (Wagner et al., 1974; Sonnenbichler and Zetl, 1986). Silymarin enhances the activity of hepatocyte RNA-polymerase I (Sonnenbichler and Zetl, 1986), complexes toxic-free iron (Pietrangelo et al., 1995), protects the cell membrane from radical-induced damage (Mira et al., 1994), and blocks the uptake of toxins such as *Amanita phalloides* toxin (Hruby et al., 1983; Desplace et al., 1975). A potent scavenger, it prevents lipid peroxidation and normalizes the lipid profile of hepatocyte membranes (Muriel and Mourelle, 1990). Silymarin has also been reported to provide liver protection against CCl_4 and paracetamol-induced liver damage in rat models (Mourelle et al., 1989; Muriel et al., 1992).

Phyllanthus amarus and related species are Indian plants that contain phyllanthin, hypophyllanthin and polyphenols with antiviral properties. *Phyllanthus amarus* whole plant powder administered at a dosage of 0.66 g/kg in rat showed hepatoprotective activity against CCl_4-induced liver damage (Sane et al., 1995). An aqueous extract inhibited woodchuck hepatitis virus DNA polymerase and surface antigen expression (Venkateswaran et al., 1987; Ott et al., 1997) and several protein kinases such as cAMP-dependent protein kinase, protein kinase C and myosin light-chain kinase in rat liver (Polya et al., 1995). A nonrandomized clinical study showed a remarkable 59% (22 of 37 patients) clearance of HBsAg in chronic carriers who were treated for 30 days compared with only 4% (1 of 23 patients) given placebo (Thyagajaran et al., 1988). Sane et al. in 1997 reported the efficacy of the plant in inhibiting the hepatitis B virus. The leaves were found to possess antipyretic activity (Mehrotra et al., 1991). Nirtetralin and niranthin are reported to possess hepatoprotective and anti-inflammatory activities. Cytotoxic effect of nirtetralin, niranthin and phyllanthin on two human leukemia cell lines, K-562 and Lucena-1 suggests a potential action of Phyllanthus lignans as multidrug resistance (MDR) reversing agents (Srivastava et al., 2008).

In search of an effective, standardized and safe hepatoprotective combination therapy, we used the silymarin and standardized extract of *Phyllanthus amarus* against CCl_4-induced hepatotoxicity in rats.

Materials and methods

Phyllanthus amarus whole plant was collected from the medicinal conservatory of the Central Institute of Medicinal and Aromatic Plants (CIMAP), and dried in the shade. This plant material was minced and soaked in water and ethanol separately with occasional shaking at room temperature for 18 h. The mixtures were centrifuged (4000 rpm × 30 min) and the supernatant were evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator (Buchi B-480). The yields of dried residues were 19.95% and 5.22%, respectively. These extracts were solubilized using 0.7% carboxymethylcellulose (CMC) suspension. Silymarin containing silybin (32%) was procured from Sigma, USA and solubilized using 0.7% CMC suspension. The phyllanthus extracts were evaluated for phyllanthin content by HPLC. For HPLC studies, column-Waters Symmetry Shield^{TM} RP-18 (4.6 × 150 mm; 5 μm) with mobile phase methanol:water (0.1% TFA) – 70:30 at flow rate – 0.7 ml/min was used. Photodiode array detector used for data acquisition and the calculation was performed at 220 nm to quantify the phyllanthin content using extracts and phyllanthin standard. The representative HPLC fingerprint of ethanolic and aqueous extracts *P. amarus* thus obtained have been shown in Fig. 1. The reference silymarin (1.0 mg/ml) was chromatographed (Fig. 2) using the conditions (column-Waters symmetry C_{18}, 250 mm × 4.6 mm, 5 μm; mobile phase – MeOH: water (30:70); flow rate 1.5 ml/min; detection $\lambda = 288$ nm) reported earlier (Ding et al., 2001).

Animals

Male *Rattus norvegicus* (150 ± 10 g) of Charles Foster strain were the experimental subjects maintained under controlled conditions (temperature 25 ± 2 °C; relative humidity 50 ± 5%; 12 h light/dark cycle). The animals were maintained on certified pelleted rodent diet (Dayal Industries, Lucknow, India). Water was provided *ad libitum*. Institutional Animal Ethics Committee approved the animal experiments and the guidelines for Animal care were followed as recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

In vivo hepatoprotective activity studies

The model described by Yadav and Dixit (2003) was employed with some modifications. The rats were divided into eight groups consisting of five animals each. The animals of group A served as normal and were given only vehicle (0.7% CMC suspension 1 ml/kg b.w.) for 6 days. The animals of group B served as toxin control and administered with CCl_4

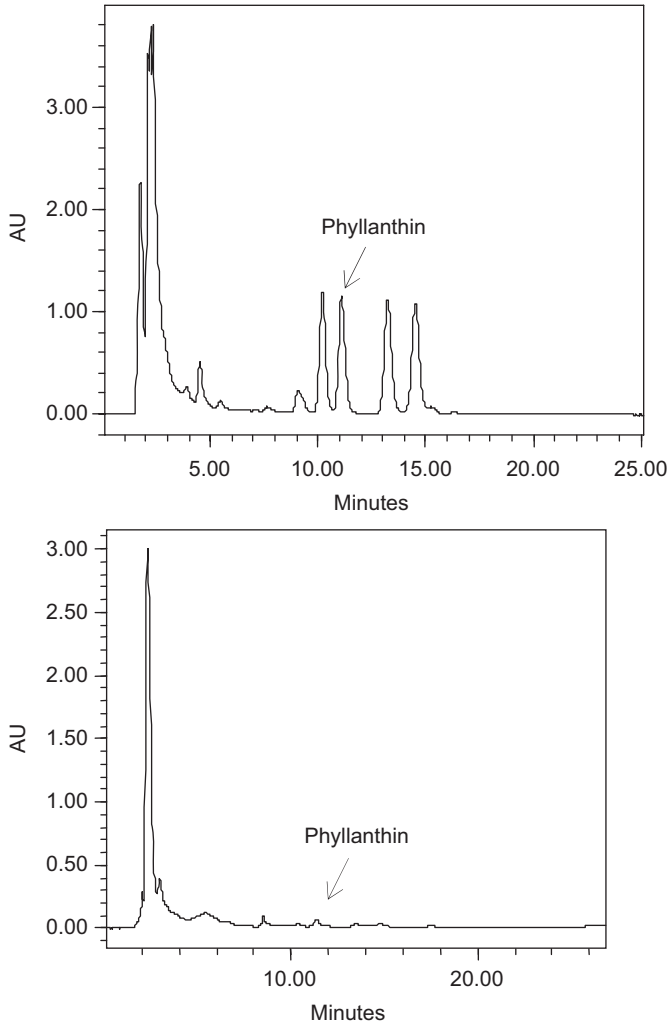

Fig. 1. HPLC Chromatograms of ethanolic and aqueous extracts of *P. amarus*.

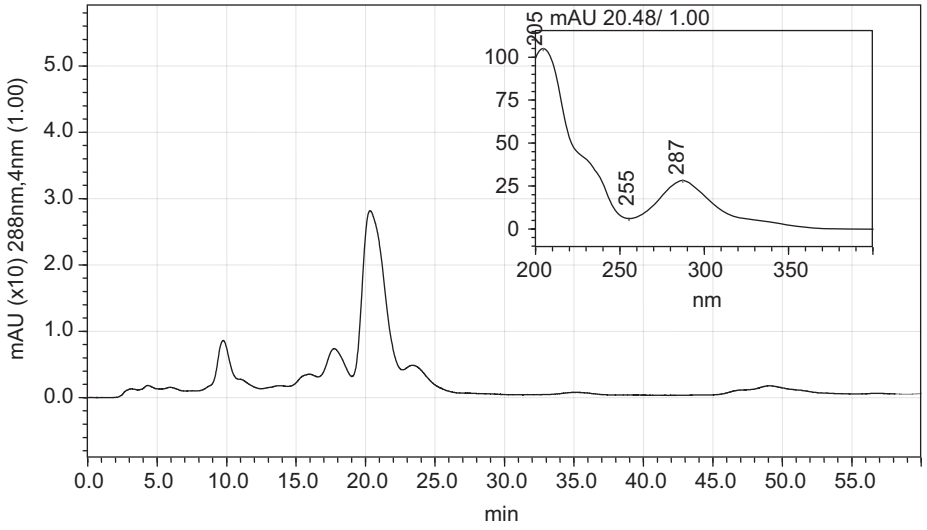

Fig. 2. HPLC chromatogram of silymarin reference substance (UV spectra of major component is shown in insight).

(50% solution of CCl_4 in liquid paraffin, 2 ml/kg b.w., intraperitoneally (i.p.)) on 4th day and with vehicle on rest of the days. The animals of groups C–H received silymarin (100 mg/kg b.w.), *Phyllanthus amarus* aqueous extract (100 mg/kg b.w.), *Phyllanthus amarus* ethanolic extract (100 mg/kg b.w.), silymarin (50 mg/kg b.w.) + *P. amarus* aq. ext. (50 mg/kg b.w.), silymarin (50 mg/kg b.w.) + *P. amarus* eth. ext. (50 mg/kg b.w.) and marketed formulation (M.F.) 5 ml/kg b.w. for 6 days orally as well as CCl_4 (2 ml/kg b.w.) on 4th day i.p. The detailed dosage regimen is given in Table 1.

Assessment of hepatoprotective activity

On 7th day, the blood was collected from the retro orbital plexus of each animal and serum was separated which was analyzed for various biochemical parameters, i.e. serum glutamyl oxalacetic acid transaminase (SGOT), serum glutamyl pyruvate transaminase (SGPT) (Reitman and Frankel, 1957), alkaline phosphatase (ALKP) (Kind and King, 1954), serum bilirubin (SBLN) (Malloy and Evelyn, 1937), total protein and creatinine by using enzymatic reagents (Bayer Diagnostics, India).

Liver glycogen

After collecting the blood from each animal, they were sacrificed by cervical dislocation and liver was separated, washed in Ringer's solution and soaked in filter paper. Immediately the liver was stored at −20 °C temperature and used for estimation of liver glycogen and liver triglycerides.

Liver triglyceride content determination

The liver triglyceride content was determined as per the method described by Zhenyuan Song et al. (2006). For this purpose, 50–100 mg of liver was homogenized in 4 ml of chloroform: methanol (2:1). A total of 0.8 ml of 50 mM NaCl was added to each sample. Samples were then centrifuged and the organic layer was removed and dried. The resulting pellet was dissolved in phosphate-buffered saline containing 1% Triton X-100 and the triglyceride contents were determined by using an enzymatic reagent (Bayer Diagnostics, India).

Statistical analysis

Results are presented as mean ± S.E.M. Statistical analysis was performed using student's *t*-test and one-way analysis of variance (ANOVA), wherever appropriate with the help of Graph Pad Prism 4.0 software. Difference between groups were considered to be

Table 1. Dosage regimen of test samples (extracts) and toxin to experimental groups

Groups	Day						
	1	2	3	4	5	6	7
A	Veh	Veh	Veh	Veh	Veh	Veh	Blood was collected, animals were sacrificed and liver was isolated
B	Veh	Veh	Veh	Veh + CCl_4	Veh	Veh	
C	Sily	Sily	Sily	Sily + CCl_4	Sily	Sily	
D	P.A.(Aq.)	P.A.(Aq.)	P.A.(Aq.)	P.A.(Aq.) + CCl_4	P.A.(Aq.)	P.A.(Aq.)	
E	P.A.(Eth.)	P.A.(Eth.)	P.A.(Eth.)	P.A.(Eth.) + CCl_4	P.A.(Eth.)	P.A.(Eth.)	
F	Sily + P.A.(Aq.)	Sily + P.A.(Aq.)	Sily + P.A.(Aq.)	Sily + P.A.(Aq.) + CCl_4	Sily + P.A.(Aq.)	Sily + P.A.(Aq.)	
G	Sily + P.A.(Eth.)	Sily + P.A.(Eth.)	Sily + P.A.(Eth.)	Sily + P.A.(Eth.) + CCl_4	Sily + P.A.(Eth.)	Sily + P.A.(Eth.)	
H	M.F.	M.F.	M.F.	M.F. + CCl_4	M.F.	M.F.	

Veh = vehicle, Sily = silymarin, P.A. = *Phyllanthus amarus*, Aq. = aqueous, Eth. = ethanolic, M.F. = marketed formulation.

statistically significant at $p<0.05$ (Kulkarni, 1993; Ostle, 1966).

Results

The results obtained in the biological experiment are presented in Table 2. Liver glycogen and liver triglycerides are presented in Figs. 3 and 4.

Administration of CCl_4 to rats caused significant increase in serum enzymes like SGOT, SGPT, ALKP, SBLN, compared to treated rats. It also caused the significant change in biochemical parameters estimated from liver homogenate, like liver triglyceride and liver glycogen.

Results presented in Table 2 indicate that the levels of serum enzymes namely SGOT, SGPT, ALKP and SBLN and total protein were affected after treatments of rats with silymarin, *P. amarus* aq. ext., *P. amarus* eth. ext., *silymarin* + *P. amarus* aq. ext., *silymarin* + *P. amarus* eth. ext. and marketed formulation (M.F.) when compared with toxin control group. SGOT levels were significantly ($p \leqslant 0.01$) reduced in all treated groups. It was most significantly reduced in silymarin + *P. amarus* eth. ext.-treated group of animals.

Levels of SGPT were also reduced in all treated groups, but it was significantly reduced only by marketed formulation followed by silymarin + *P. amarus* eth. ext. treatment. Similar results were obtained, when ALKP was estimated in all groups of animals, where alkaline phosphatase was significantly reduced by marketed formulation (M.F.) followed by *P. amarus* eth. ext. treatment. Serum bilirubin was significantly reduced in all treated groups except in *P. amarus* eth. ext.-treated group. It is almost coming to normalization by *silymarin* + *P. amarus* aq. ext. and *silymarin* + *P. amarus* eth. ext. treatment. Total protein was also estimated from serum, which exhibit significant reduction in *silymarin* + *P. amarus* aq. ext., *silymarin* + *P. amarus* eth. ext. and marketed formulation (M.F.)-treated animals.

Plasma creatinine levels did not show any significant change in any of the groups, when compared with normal control group. Liver triglycerides estimated from liver homogenate showed significant reduction in *silymarin* + *P. amarus* aq. ext., *silymarin* + *P. amarus* eth. ext. and marketed formulation (M.F.)-treated animals, where as liver glycogen was significantly reduced only in *silymarin* + *P. amarus* eth. ext.-treated animals, when compared with toxin group of animals.

The quality of the standardized *P. amarus* extracts were ensured using phyllanthin content as well as the reproducibility of other chemical profile of HPLC fingerprint. The phyllanthin content was found to be 1.98% and 0.08% in ethanolic and aqueous extract,

Table 2. Serum parameters for the hepatoprotective combination studies

Parameters	Control	Toxin	Silymarin	*P. amarus* aq. ext.	*P. amarus* eth. ext.	Sily + *P. amarus* aq. ext.	Sily + *P. amarus* eth. ext.	Marketed formulation
SGOT (U/l)	41.08 ± 7.50	137.58^{a} ± 13.35	60.77^{b} ± 9.89	76.71^{b} ± 8.42	69.76^{b} ± 10.3	59.69^{b} ± 8.8	47.04^{b} ± 4.37	57.44^{b} ± 7.67
SGPT (U/l)	30.25 ± 4.26	70.05^{a} ± 14.46	39.25 ± 5.13	56.89 ± 5.93	44.23 ± 5.64	37.74 ± 7.14	36.92 ± 6.63	33.28^{b} ± 3.83
ALKP (U/l)	66.17 ± 6.02	117.73^{a} ± 13.79	80.24 ± 10.26	99.52 ± 8.43	76.44 ± 7.06	98.45 ± 9.71	84.68 ± 13.34	68.22^{b} ± 9.86
Total bilirubin (mg/dl)	0.27 ± 0.02	0.77^{a} ± 0.13	0.38^{b} ± 0.04	0.33^{b} ± 0.08	0.55 ± 0.03	0.28^{b} ± 0.02	0.27^{b} ± 0.02	0.28^{b} ± 0.02
Total protein (g/dl)	7.52 ± 0.16	9.34 ± 0.45	7.84 ± 0.54	9.12 ± 0.43	8.17 ± 0.36	6.19^{b} ± 0.97	6.74^{b} ± 0.24	6.78^{b} ± 0.3
Creatinine (g/dl)	2.56 ± 0.18	3.4 ± 0.34	3.2 ± 0.33	3.34 ± 0.35	3.11 ± 0.39	2.55^{b} ± 0.5	2.27^{b} ± 0.4	3.67^{b} ± 0.51

For each group $n = 5$, the values are mean ± SEM.
Values with 'a' exhibit significant ($p<0.05$) changes from control.
Values with 'b' exhibit significant ($p<0.05$) changes when compared to toxin.

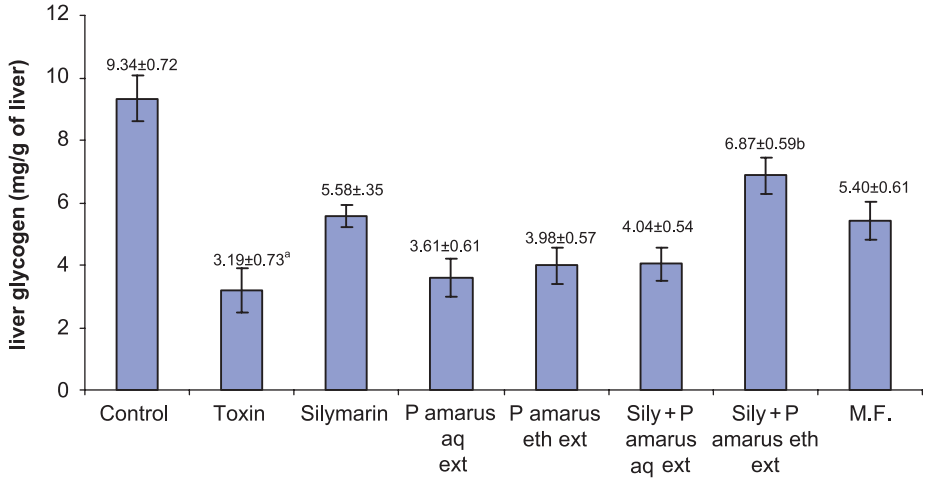

Fig. 3. Liver glycogen content in different groups. For each group ($n = 5$) the values are mean ± SEM. Values with 'a' exhibit significant ($p < 0.05$) changes from control. Values with 'b' exhibit significant changes when compared to toxin.

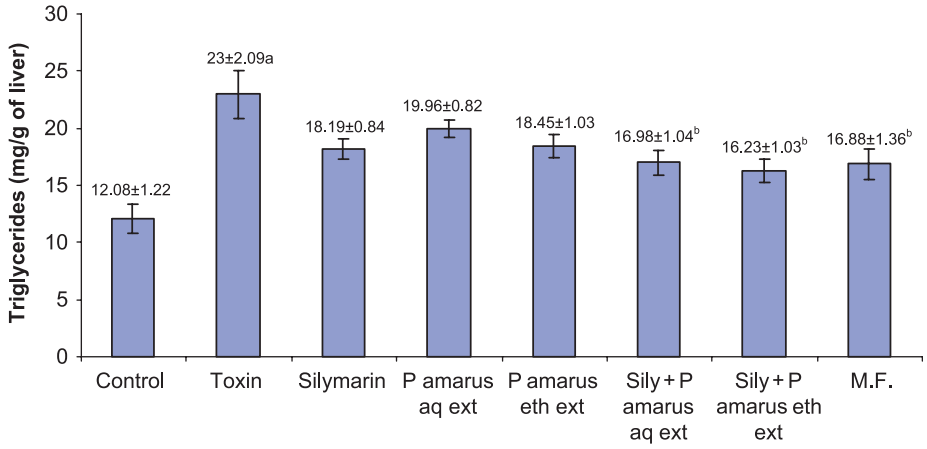

Fig. 4. Liver triglyceride content in different groups. For each group ($n = 5$) the values are mean ± SEM. Values with 'a' exhibit significant ($p < 0.05$) changes from control. Values with 'b' exhibit significant changes when compared to toxin.

respectively. The phyllanthin was detected using a PDA detector at 220 nm.

Discussion

The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by carbontetrachloride administration has been widely used as an indicator of liver protective activity of drugs in general (Clauson, 1989). Since the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962), CCl_4-mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450-dependent monooxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee et al., 1995). Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4-induced liver lesions (Castro et al., 1974).

Serum glutamyl oxalacetic acid transferase, serum glutamyl pyruvate transferase, alkaline phosphatase and total bilirubin in plasma have been reported to be sensitive indicator of liver injury (Molander et al., 1955). The disturbance in the transport function of the hepatocytes as a result of hepatic injury, causes the leakage of enzymes from cells due to altered permeability of membrane (Zimmerman and Seeff, 1970). This results in decreased levels of GOT, GPT and alkaline phosphatase in the hepatic cells and a raised level in

Table 3. Percent Recovery of Serum parameters by different test samples

Parameters	Silymarin	*P. amarus* aq. ext.	*P. amarus* eth. ext.	Sily + *P. amarus* aq. ext.	Sily + *P. amarus* eth. ext.	Marketed formulation
SGOT	79.44 ± 4.34	63.60 ± 6.26	73.30 ± 16.80	80.50 ± 6.80	94.57 ± 12.88	82.83 ± 16.01
SGPT	76.46 ± 8.74	33.38 ± 14.95	62.3 ± 14.83	82.77 ± 6.95	84.46 ± 7.5	95.71 ± 6.95
ALKP	74.08 ± 18.56	29.64 ± 14.31	89.29 ± 12.96	40.58 ± 11.6	70.58 ± 12.1	94.03 ± 18.43
Total bilirubin	78.68 ± 4.09	86.2 ± 12.28	45.68 ± 3.79	96.36 ± 4.17	99.66 ± 7.58	98.11 ± 4.55
Total protein	82.90 ± 8.43	14.04 ± 4.97	65.07 ± 14.8	130.07 ± 15.98	145.96 ± 9.5	141.11 ± 14.69
Creatinine	86.57 ± 4.1	83.21 ± 4.94	91.15 ± 12.31	95.57 ± 7.9	98.01 ± 4.97	81.04 ± 9.89

For each group $n = 5$, the values are mean ± SEM.
Formula for calculation of liver protection (denoted by percent recovery).

$$\text{Percent recovery} = \frac{\text{toxin group} - \text{treated group}}{\text{toxin group} - \text{control group}} \times 100.$$

serum. The present study revealed a significant increase in the activities of AST, ALT, SALKP and SBLN after exposure to CCl_4, indicating considerable hepatocellular injury. Administration of silymarin and *P. amarus* extracts attenuated the increased levels of the serum enzymes, produced by CCl_4, and caused a subsequent recovery towards normalization. These recoveries have been presented in Table 3 in the form of percent recovery (Yadav and Dixit, 2003) of serum parameters by different test samples, where it was found that the combination of silymarin and *P. amarus* extracts are showing higher recovery of serum parameters in comparison to individual drug treatment. Silymarin with *P. amarus* eth. ext. is showing highest recovery of SGOT, SGPT, total bilirubin and creatinine (94.57 ± 12.88, 84.46 ± 7.5, 99.66 ± 7.58 and 98.01 ± 4.97, respectively). This suggested that *P. amarus* extracts (aqueous and ethanolic) in combination with silymarin are able to condition the hepatocytes, so as to cause accelerated regeneration of parenchyma cells, thus protecting against membrane fragility and decrease of leakage of the marker enzymes into the circulation. Silymarin is a known hepatoprotective compound. It is reported to have a protective effect on the plasma membrane of hepatocytes (Ramellini and Meldolesi, 1976).

In the present study, the level of SGOT, SGPT, ALKP and serum bilirubin have been raised in toxin control group and these enzyme levels have been decreased in silymarin + *P. amarus* eth. ext. group, silymarin group and marketed formulation group, which is a indication of hepatoprotection by these groups.

Increased level of serum creatinine is the indication of malfunctioning of kidney. It has been maintained in silymarin + *P. amarus* group significantly.

The primary function of liver is to store the energy in the form of glycogen. If the liver is functioning well, it will have the high glycogen storage but if the liver is injured, the glycogen storage capacity will be decreased. In our studies, the silymarin in combination with ethanolic extract of *P. amarus* is maintaining the glycogen level significantly higher than the toxin control group. Silymarin and marketed formulation (M.F.) are also maintaining the glycogen level up to some extent.

Level of triglyceride in the liver also has been estimated to explain the status of liver. High level of triglyceride in the liver is the indication of the liver injury. Triglyceride level has been increased in the toxin group, and has been decreased by silymarin + *P. amarus* aq. ext., silymarin + *P. amarus* eth. ext. and marketed formulation (M.F.) group.

In conclusion, we have demonstrated that acute CCl_4 administration caused significant liver injury as indicated by the elevation of enzymes levels in the serum viz., SGOT, SGPT, ALKP and SBLN. In our experiment, treatment of animals with different extracts attenuated the liver injury. Ethanolic and aqueous extracts of *Phyllanthus amarus* showed the partial hepatoprotection, where as these extracts in combination with silymarin exhibited hepatoprotection as indicated by significant changes in various liver parameters in these groups of animals. The combination therapy is exhibiting synergistic activity because the drugs in combination are showing higher liver protection (Table 3, denoted by percent recovery) when compared with either drug alone as evidenced by the results (Chambers, 2001). Further, it was found that *Phyllanthus amarus* ethanolic extract in combination with silymarin was showing better hepatoprotective activity in comparison to that of aqueous extract which may be due to higher availability of phyllanthus lignans particularly phyllanthin in the ethanolic extract of *Phyllanthus amarus* as evidenced by chromatographic analysis.

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