

Possible Combinational Effect of Silymarin with Hepatoprotective Plants in Amelioration of Hepatic Insufficiencies

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ABSTRACT

Background: Silymarin, a flavonoid, has been reported to have hepatoprotective, antioxidative, anti-inflammatory and immunomodulatory activities. *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Asparagus racemosus* and *Phyllanthus amarus* plants are well reported to possess anti-hepatotoxic ability. **Objective:** The present study was designed to investigate the effect of silymarin alone and in combination with the above mentioned hepatoprotective plants against experimentally induced liver injury. **Materials and Methods:** Wistar albino rats (180-220 g) of either sex (n=6) were employed in present study. Experimental hepatotoxicity was induced by the administration of paracetamol (3 g/kg, *p.o.*) or CCl₄ (1 ml/kg, *s.c.*). Silymarin in the dose of 12.5, 25, 50 mg/kg, and silymarin 25 mg/kg in combination with plant extract: *Picrorrhizakurroa*, *Tephrosiapurpurea*, *Phyllanthus amarus* and *Asparagus racemosus* were administered orally for 7 days. **Results:** Administration of PCM or CCl₄ caused significant increase in serum alanine transaminase, aspartate transaminase, alkaline phosphatase and bilirubin; tissue lipid peroxidation, nitrite/nitrate, and decrease in tissue GSH and Na⁺K⁺ATPase levels, characterizing experimental hepatotoxicity. Pretreatment with silymarin alone and its combination with each plant extract significantly attenuated the toxic effects of hepatotoxicants in liver. Histological study of liver was also carried out to estimate the extent of tissue injury. **Conclusion:** The present study provides pharmacological basis for the efficacy of silymarin in lower dosage, and its rational use in combination with hepatoprotective plants to ameliorate hepatic insufficiency.

Keywords: Hepatoprotective, Hepatotoxicity, Plant extracts, Silymarin, Paracetamol.

INTRODUCTION

Liver is a vital organ, responsible for the detoxification of drugs and xenobiotics used in the body. The drug-induced liver disease accounts for 5% of all hospital admissions and 50% of all acute liver failure.¹ It is the major cause of withdrawal of an approved drug from the market.² The traditional system of medicine has incorporated use of several hepatoprotective plants like *Silybum marianum*, *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus* and *Asparagus racemosus* to treat hepatic body ailments.

Silymarin, obtained from milk thistle (*Silybum marianum*) chiefly contains flavonoids: silybin, silybinin, silydianin and silychristin.³ It is used as a standard drug and exhibited potent hepatoprotective activity at the dose range from 25-200 mg/kg in various experimental and clinical studies.^{4,5,6} Moreover, it has been reported to possess antioxidative, anti-inflammatory and immunomodulatory properties.^{7,8,9} It is clinically approved for the use in drugs and alcohol induced hepatic insufficiencies.¹⁰ *Picrorrhiza kurroa* (Kutki) contains iridoid glycoside: picroside A & B, kutkoside, apocycynin; triterpenes, and has been reported to have hepatoprotective activity both *in-vivo* and *in-vitro*.^{11,12,13} *Tephrosia purpurea* (Sarpunkha) contains glycosides, flavonoids: rutin, quercetin; rotenoids: tephrosin; purpurin, purpurenone, purpuritenin and β -sitosterol,¹⁴ and acts as hepatoprotective by restoring antioxidant level.¹⁵ *Phyllanthus amarus* (Bhuiaamla) contains active constituents like tannins, phyllanthin, polyphenols, quercetin and ligitanins,¹⁶ and has been reported to have hepatoprotective,¹⁷ and antioxidant activities.¹⁸ *Asparagus racemosus* (Shatavari) contains steroidal saponins: shatavarins; isoflavones, asparagine and polysaccharides, and has been reported to possess potent hepatoprotective,

antioxidant,¹⁹ and immunomodulatory potentials.²⁰

So far, no experimental report available mentioning the dose response relationship of silymarin as hepatoprotective. Moreover, the combined effect of silymarin (a clinically approved drug) with the potential hepatoprotective plants on liver damage has also not been elucidated. Therefore, the present study was designed to investigate the dose response curve, if any of silymarin; and the effect of combination of silymarin with *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus* and *Asparagus racemosus* extracts on Paracetamol or CCl₄ induced hepatotoxicity in rats.

EXPERIMENTAL

Drugs and Chemicals

The paracetamol from SmithKline Pharma, Mumbai; Silymarin from Micro Labs Limited., Baddi, H.P., India; Carbon tetrachloride from Merck Specialities Pvt. Ltd., Mumbai were used. All other chemicals and biochemical reagents of analytical grade were used freshly. The standardized methanolic extracts of the four plants *Picrorrhiza kurroa* (Rhizome), *Tephrosia purpurea* (Aerial parts), *Phyllanthus amarus* (Aerial parts) and *Asparagus racemosus* (Roots) were procured from Ayush Herbs Pvt. Ltd., Kangra, Himachal Pradesh, India. Biochemical enzymatic kits were purchased from Crest Biosystems, Goa, India.

Animals

Wistar albino rats weighing 180-220 g of either sex were employed in present study. They were maintained at standard laboratory conditions of temperature (25±2 °C), humidity (45-55 %) and 12/12 h light and dark cycles, and were fed on standard chow diet procured from Aashirwad Industries Ltd,

Ropar, Punjab, and water *ad libitum*. All the experiments were conducted in approval with Institutional Animal Ethics Committee (IAEC) and carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA Approval No: ISFCP/IAEC/CPCSEA/01) for the use and care of experimental animals.

Experimentally-induced hepatotoxicity

The present study comprised two experimental models for hepatic damage using PCM and CCl₄ as hepatotoxicants respectively. Animals were divided into different groups each comprising six animals (n=6). Paracetamol (3 g/kg, *per oral* on 3rd and 5th days) or CCl₄ (1 ml/kg, subcutaneously on 4th and 5th days, diluted in olive oil 1:1 ratio,) was given 2 h after the test drug administration to induce experimental liver damage. Silymarin alone in three respective doses: 12.5, 25 and 50 mg/kg, and silymarin 25 mg/kg in combination with *P. kurroa*, *T. purpurea*, *P. amarus* and *A. racemosus* extracts (50 mg/kg each) were given orally for 7 consecutive days as test drugs.

The blood was collected, centrifuged at 3000 rpm and serum separated for biochemical estimations. The animal was sacrificed. Liver was isolated and surgically dissected out and then washed in cold saline and blotted dry. 10% liver homogenate of liver was prepared using tris-buffer and phosphate buffer (for glutathione only) for tissue biochemical estimations.

Pharmacological assessment of liver injury

Serum biochemical estimation

The serum biochemical markers of liver function tests like alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and bilirubin

were estimated using biochemical enzymatic kits purchased from Crest Bio Systems, Goa.

Tissue biochemical estimations

The tissue biochemical estimations: lipid peroxidation (TBARS),²¹ reduced glutathione (GSH),²² nitrite/nitrate using griess reagent,²³ and Na⁺K⁺ATPase²⁴ levels were assessed.

Histopathological studies

The liver tissue was preserved in 10 % formalin solution, sectioned (5 μM), stained with haematoxylin and eosin. Observed under microscope (10x) to estimate the histological changes²⁵.

Statistical Analysis

All the results obtained were expressed as mean ± SD (Standard deviation) and analyzed by One way ANOVA followed by Bonferroni's test as post hoc analysis. p<0.05 was considered as statistically significant.

RESULTS

Effect of silymarin and its combination with plant extracts (50mg/Kg each) on serum markers

Administration of PCM or CCl₄ produced significant (p<0.05) increase in serum ALT, AST, ALP and bilirubin levels as compared to normal untreated control rats. Pre-treatment with silymarin (12.5, 25 and 50 mg/kg), and the combination of silymarin (25 mg/kg) with each plant extract of *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus*, and *Asparagus racemosus* significantly attenuated the increase in these serum markers, in comparison to respective PCM or CCl₄ control rats. The effect of silymarin with each plant extract in lowering serum ALT level was significant in comparison to silymarin 25 mg/kg. Moreover, this effect was also significant (p<0.05) in comparison to the silymarin 50 mg/kg against

PCM induced hepatotoxicity. The effect of the combination of S-25 with each plant extract in lowering serum AST and ALP levels was significant ($p < 0.05$), as compared to S-25 against CCl_4 induced hepatotoxicity in rat (Table. 1).

Effect of silymarin and its combination with plant extracts (50mg/Kg each) on tissue biochemical estimations

Hepatointoxication with PCM or CCl_4 produced significant ($p < 0.05$) increase in TBARS, nitrite/nitrate; decrease in tissue GSH and $\text{Na}^+\text{K}^+\text{ATPase}$ levels in hepatotoxicant control groups, as compared to normal untreated control rats. Pretreatment with silymarin 12.5, 25 and 50 mg/kg, and the combination of silymarin 25 mg/kg with each plant extract (50mg/Kg each) significantly ($p < 0.05$) attenuated these changes in tissue markers, as compared to respective PCM or CCl_4 control rats. The effect of the combination of S-25 with each plant extract was significant ($p < 0.05$) as compared to the S-25 against CCl_4 induced acute hepatic damage in rat. The effect of these combinations in attenuating NO level was significant ($p < 0.05$) in comparison to S-25 against CCl_4 induced liver damage in rat. The plant extracts: *P. Amarus* and *A. Racemosus* in each combination with S-25 produced significant decrease in nitrite/nitrate level as compared to S-25 alone against PCM induced hepatotoxicity. The effect of these combinations of S-25 with each plant extract in restoring $\text{Na}^+\text{K}^+\text{ATPase}$ level was significant than that produced by S-25 and 50 against CCl_4 induced hepatic damage in rat (Table. 2).

Effect of silymarin and its combination with plant extracts (50mg/Kg each) on histological changes

In present study, intoxication with PCM (3 g/kg) or CCl_4 (1 ml/kg) caused marked inflammatory cell infiltration,

centrilobular necrosis, fatty infiltration, vacuolization and sinusoidal dilation respectively in PCM and CCl_4 control groups, as compared to liver of normal control group. Pre-treatment with three consecutive doses of silymarin, and the combination of silymarin (25 mg/kg) with extracts: *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus*, *Asparagus racemosus* each showed significant reduction in progression of these toxic cellular effects of hepatotoxicants, as compared to hepatotoxicant control groups (Fig. 1).

DISCUSSION

The present study investigated the protective effect of silymarin alone and in combination with the plant extracts of *Picrorrhiza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus* and *Asparagus racemosus* against experimental liver injury using PCM or CCl_4 induced hepatotoxicity models in rat. Silymarin is used as a standard drug in various experimental and clinical studies due to its proven hepatoprotective effects.⁹ PCM, on overdoses, results hepatotoxicity in men as well as in experimental animals.²⁶ The toxic changes associated with CCl_4 -induced liver damage are similar to that of acute viral hepatitis clinically.²⁷ Therefore, the PCM and CCl_4 -induced hepatotoxicity were selected as experimental models of liver injury in present study. PCM is metabolized to a toxic reactive metabolite: N-acetyl-p-bezoquinone imine (NAPQI), and CCl_4 to trichloromethyl free radicals by cytochrome P-450 which are further reported to cause massive oxidative stress,²⁸ and ultimately liver cell death.²⁹ Assessment of liver function can be made by the estimation of serum levels of metabolic enzymes like ALT, AST and ALP which are leaked out into systemic circulation during necrotic cell damage and hence are referred as sensitive indicators of liver injury.^{30,31} In present study, PCM and CCl_4 intoxications

caused significant increase in these hepatic enzymes and this was probably due to the consequences of increased oxidative stress and necrotic cell death.³² Pre-treatment with silymarin significantly attenuated the increased level of these serum markers, in a dose dependent manner, as compared to hepatotoxicant control. This ability of silymarin may be due to its free radical scavenger activity.³³ The combination of silymarin with each plant extract produced significant protection in correcting cellular damage, in comparison to silymarin alone. In an earlier report, the combination of silymarin (50 mg/kg) with *Phyllanthus amarus* extract offered significant hepatoprotection against CCl₄ induced liver damage,³⁴ as also observed in present study. This effect might be due to the combined effects of plant extracts with silymarin in improving the hepatic cell functioning upon experimental liver damage.

The massive production of oxidative stress may lead to depletion of physiological antioxidant: glutathione, ensuing widespread propagation of alkylation as well as lipid peroxidation, causing damage to macromolecules.^{28,29} Administration of PCM and CCl₄ caused significant increase in lipid peroxidation reactions and decrease in tissue glutathione levels in present study. Pretreatment with silymarin significantly prevented lipid peroxidation and restored the reduced glutathione levels in a dose-dependent manner, as compared to hepatotoxicant control. Moreover, the combination of silymarin with each plant extract showed significant decrease in TBARS and increase in tissue GSH, as compared to silymarin alone. This suggests the synergistic action of plant extracts with silymarin in preventing oxidative stress and restoring reduced GSH upon experimental hepato-intoxication.

Hepatotoxicants induced oxidative stress produces release of proinflammatory mediators due to induction of inducible nitric

oxide synthase, resulting increased NO level and cellular dysfunction.³⁵ In present study, administration of PCM and CCl₄ significantly caused increase in nitrite/nitrate level, characterizing massive nitrosative stress due hepatointoxication. Pretreatment with silymarin decreased the tissue levels of NO and thereby prevented the recruitment of proinflammatory mediators and generation of free radicals, in this study. Silymarin is capable of reducing NO production and iNOS expression by inhibiting nuclear factor-kappaB/Rel activation.³⁶ Pretreatment with combined silymarin with each plant extracts decreased nitrite/nitrate level significantly as compared to silymarin alone, thus suggesting a similar mechanism for plant extracts in potentiating the effect of silymarin.

The sodium pump is ouabain-sensitive Na⁺K⁺ATPase, which acquires energy from ATP to extrude Na⁺ in exchange for K⁺ and plays pivotal mechanism in physiology of cell and membrane potential.^{37,38} As a consequence, inhibition of this pump may seriously cause disruption of mitochondrial energy and metabolism.³⁹ Present study also showed the inhibition of Na⁺K⁺ATPase activity due to experimental hepatic cell destabilization and dysfunction. Pretreatment with silymarin alone produced significant increase in Na⁺K⁺ATPase activity. Moreover, this protective effect was more pronounced on treatment with combination of silymarin and each plant extracts, suggesting the potential effect of these plant extracts in restoring membrane functions by improving Na⁺K⁺ATPase activity.

A massive centrilobular necrosis, central vein dilation, ballooning degeneration and inflammatory cellular infiltration of liver are associated with liver damage⁴⁰ as evidenced with histological findings in present study. However, silymarin alone and its combination with each plant extracts were effective in prevention of these toxic histological change, in present study.

In this way, the present study provides the evidences for the hepatoprotective efficacy of Silymarin in lower doses; and also demonstrates the potentiation of cellular protective mechanisms like anti-oxidative, anti-inflammatory, membrane stabilization effects of silymarin with inclusion of hepatoprotective plants in experimental hepatotoxicity in rat.

CONCLUSION

The findings from the present investigation may revealed the efficacy of silymarin in lower doses and its synergism with hepatoprotective plants. This may provide the pharmacological basis for the clinical efficacy of silymarin, and its rationalized therapeutic applications in combination with different plant extracts and their synergistic actions in amelioration of hepatic insufficiencies.

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Table 1. Effect of silymarin and its combination with plant extracts (50mg/Kg each) on serum markers

S.No.	Group	ALT	AST	ALP	Bilirubin
1	NC	50.6±10.1	65.8±13.6	107±12.7	0.57±0.16
PCM INDUCED HEPATOTOXICITY					
2	PCM control	223.5±16.6 ^a	252.6±44.2 ^a	256.6±49.8 ^a	1.13±0.15 ^a
3	PCM + S-12.5	165±12.5 ^b	103.9±12.4 ^b	203.1±30.8 ^b	0.82±0.06 ^b
4	PCM + S-25	128±13.3 ^b	81.4±8.9 ^b	177.9±31.7 ^b	0.64±0.05 ^b
5	PCM + S-50	103.9±9.2 ^b	49.9±8.3 ^b	155.4±15.1 ^b	0.43±0.06 ^b
6	PCM + S+P.K.	79.3±11.2 ^{bcd}	75.8±7.1 ^b	158.6±10.6 ^b	0.55±0.05 ^b
7	PCM + S+T.P.	80.5±8.8 ^{bcd}	66.5±7.6 ^b	161.5±17.7 ^b	0.55±0.05 ^b
8	PCM + S+P.A.	106.5±12.5 ^{bcd}	72.4±15.7 ^b	143.5±15.9 ^{bc}	0.46±0.09 ^b
9	PCM + S+A.R.	66.5±7.2 ^{bcd}	63.2±9.5 ^b	165.6±15.6 ^b	0.56±0.03 ^b
CCl₄ INDUCED HEPATOTOXICITY					
10	CCl ₄ control	230.6±25.4 ^a	245.5±14.1 ^a	244.9±19.1 ^a	0.9±0.02 ^a
11	CCl ₄ + S-12.5	195.5±24.1	200.9±11.6 ^{b'}	203.9±13.1 ^{b'}	0.81±0.03 ^{b'}
12	CCl ₄ + S-25	157.1±17.4 ^{b'}	174.2±11.8 ^{b'}	174.5±15.2 ^{b'}	0.65±0.05 ^{b'}
13	CCl ₄ + S-50	125.6±6.1 ^{b'}	133.2±5.8 ^{b'}	130.4±7.05 ^{b'}	0.62±0.04 ^{b'}
14	CCl ₄ + S+P.K.	109.1±10.9 ^{b'c'}	122±25.7 ^{b'c'}	117.8±13.2 ^{b'c'}	0.55±0.04 ^{b'}
15	CCl ₄ + S+T.P.	84.2±16.2 ^{b'c'd'}	111.5±19.5 ^{b'c'}	97.8±16.1 ^{b'c'd'}	0.54±0.04 ^{b'}
16	CCl ₄ + S+P.A.	106.6±12.5 ^{b'c'}	84.9±16.1 ^{b'c'd'}	116.8±30.4 ^{b'c'}	0.55±0.03 ^{b'}
17	CCl ₄ + S+A.R.	66.5±6.4 ^{b'c'}	109.2±23.4 ^{b'c'}	114.1±9.1 ^{b'c'}	0.54±0.03 ^{b'}

Results are expressed as mean ± SD; ^avs NC, ^bvs PCM control, ^{b'} vs CCl₄ control, ^cvs S-25 against PCM induced hepatotoxicity, ^{c'} vs S-25 against CCl₄ induced hepatotoxicity, ^dvs S-50 against PCM induced hepatotoxicity, and ^{d'} vs S-50 against CCl₄ induced hepatotoxicity. [NC: Normal control; PCM: Paracetamol; S-12.5, 25, and 50: Silymarin 12.5, 25 and 50 mg/kg; P.K.: *Picorrhiza kurroa*; T.P.: *Tephrosia purpurea*; P.A.: *Phyllanthus amarus*; A.R.: *Asparagus racemosus*.]

Table 2. Effect of silymarin and its combination with plant extracts (50mg/Kg each) on tissue biochemicals

S.No.	Group	TBARS nM/mg tissue	GSH μM/mg tissue	Nitrite/nitrate μM/mg tissue	Na ⁺ K ⁺ ATPase μM of Pi/mg tissue
1	NC	0.48±0.2	0.82±0.07	0.89±0.04	3.5±0.32
PCM INDUCED HEPATOTOXICITY					
2	PCM control	2.3±0.6 ^a	0.39±0.1 ^a	1.78±0.06 ^a	1.3±0.08 ^a
3	PCM + S-12.5	1.4±0.2 ^b	0.64±0.08 ^b	1.35±0.04 ^b	2.1±0.07 ^b
4	PCM + S-25	0.69±0.3 ^b	0.74±0.06 ^b	1.22±0.03 ^b	2.6±0.06 ^b
5	PCM + S-50	0.68±0.16 ^b	0.78±0.06 ^b	1.2±0.05 ^b	2.9±0.05 ^b
6	PCM + S+P.K.	0.46±0.18 ^b	0.81±0.1 ^b	1.1±0.06 ^b	2.3±0.3 ^b
7	PCM + S+T.P.	0.51±0.24 ^b	0.78±0.11 ^b	1.12±0.06 ^b	2.5±0.3 ^b
8	PCM + S+P.A.	0.25±0.19 ^b	0.89±0.07 ^b	1.03±0.05 ^{bcd}	4.7±0.3 ^b
9	PCM + S+A.R.	0.36±0.22 ^b	63.2±9.5 ^b	0.85±0.15 ^{bcd}	3.5±0.22 ^b
CCl₄ INDUCED HEPATOTOXICITY					
10	CCl ₄ control	3.5±0.7 ^a	0.34±0.09 ^a	1.63±0.02 ^a	1.2±0.07 ^a
11	CCl ₄ + S-12.5	2.9±0.69 ^{b'}	0.66±0.11 ^{b'}	1.35±0.04 ^{b'}	2.7±0.03 ^{b'}
12	CCl ₄ + S-25	1.83±0.4 ^{b'}	0.8±0.05 ^{b'}	1.26±0.03 ^{b'}	2.8±0.08 ^{b'}
13	CCl ₄ + S-50	1.58±0.4 ^{b'}	0.9±0.04 ^{b'}	1.17±0.02 ^{b'}	3.1±0.15 ^{b'}
14	CCl ₄ + S+P.K.	0.85±0.21 ^{b'c'}	0.93±0.11 ^{b'}	1.13±0.01 ^{b'c'}	2.7±0.03 ^{b'c'd'}
15	CCl ₄ + S+T.P.	0.46±0.3 ^{b'c'd'}	0.97±0.1 ^{b'}	1.16±0.02 ^{b'c'}	2.4±0.2 ^{b'c'd'}
16	CCl ₄ + S+P.A.	0.75±0.15 ^{b'c'd'}	0.93±0.12 ^{b'}	1.11±0.01 ^{b'c'}	2.4±0.13 ^{b'c'd'}
17	CCl ₄ + S+A.R.	0.48±0.29 ^{b'c'd'}	0.87±0.08 ^{b'}	1.14±0.01 ^{b'c'}	2.5±0.3 ^{b'c'd'}

Results are expressed as mean ± SD; ^avs NC, ^bvs PCM control, ^{b'}vs CCl₄ control, ^cvs S-25 against PCM induced hepatotoxicity, ^{c'}vs S-25 against CCl₄ induced hepatotoxicity, ^dvs S-50 against PCM induced hepatotoxicity, and ^{d'}vs S-50 against CCl₄ induced hepatotoxicity. [NC: Normal control; PCM: Paracetamol; S-12.5, 25, and 50: Silymarin 12.5, 25 and 50 mg/kg; P.K.: *Picorrhiza kurroa*; T.P.: *Tephrosia purpurea*; P.A.: *Phyllanthus amarus*; A.R.: *Asparagus racemosus*]

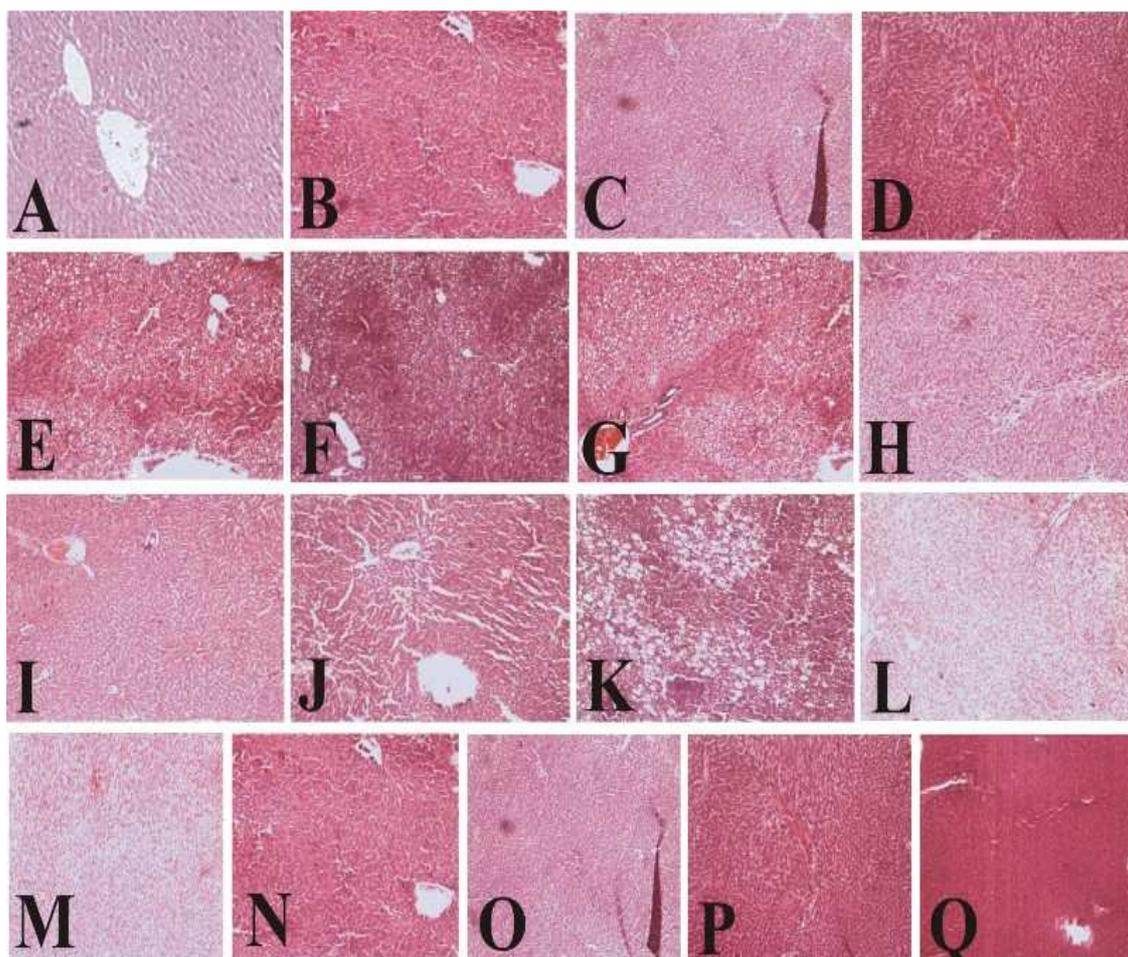


Figure.1. Effect of silymarin and its combination with plant extracts (50mg/Kg each) on histological changes against experimental hepatotoxicity (10x)

(A) Normal Control, (B) PCM Control, (C) Silymarin 12.5 + PCM, (D) Silymarin 25 + PCM, (E) Silymarin 50 + PCM, (F) Silymarin+ *Picrorrhiza kurroa* + PCM, (G) Silymarin + *Tephrosia purpurea* + PCM, (H) Silymarin + *Phyllanthus amarus* + PCM, (I) Silymarin + *Asparagus racemosus* + PCM, (J) CCl₄ Control, (K) Silymarin 12.5 + CCl₄, (L) Silymarin 25 + CCl₄, (M) Silymarin 50 + CCl₄, (N) Silymarin + *Picrorrhiza kurroa* + CCl₄, (O) Silymarin + *Tephrosia purpurea*+ CCl₄, (P) Silymarin + *Phyllanthus amarus*+ CCl₄, (Q) Silymarin + *Asparagus racemosus*+ CCl₄.