PHARMACOLOGICAL POTENTIAL OF Silymarin IN COMBINATION WITH HEPATOPROTECTIVE PLANTS AGAINST EXPERIMENTAL HEPATOTOXICITY IN RATS

GAAMINEPREET SINGH*, ROHIT GOYAL, PYARE LAL SHARMA

Department of Pharmacology, ISF College of Pharmacy, G T Road, Moga, 142001, Punjab, India, Email: g psinghrohit@hotmail.com

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INTRODUCTION

Liver is a vital organ, responsible for the detoxification of various drugs and xenobiotics in the body. The drug-induced liver disease accounts for 5% of all hospital admissions and 50% of all acute liver failure (McNally, 2005). It is the major cause of withdrawal of an approved drug from the market (Ostapowicz et al., 2002). Currently, 25% of all modern medicines are directly or indirectly derived from higher plants. The traditional system of medicine has incorporated several hepatoprotective plants like Silybum marianum, Picrorhiza kurroa, Tephrosia purpurea, Phyllanthus amarus and Asparagus racemosus to treat hepatic body ailments.

Silymarin is a standardized extract of the milk thistle (Silybum marianum) chiefly contains flavonoids: silybin, silychristin, silydianin and silychristin (Flora et al., 1996). Seeds of this plant have been used for more than 2000 years to treat liver and gall bladder disorders, including hepatitis, cirrhosis and jaundice and to protect the liver against poisoning from chemicals, environmental toxins, snake bites, insect stings, mushroom poisoning and alcohol (Kren and Walterová, 2005). Moreover, 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 (Ramadan et al., 2002; Wills and Asha, 2006; Salam et al., 2007). Picrorhiza kurroa (Scrophulariaceae), commonly known as Kutki is a perennial herb, growing primarily in the northwest Himalayan mountains. Plant has been reported to possess iridoid glycosides: picroside A & B, kutkoside, apocynin; triterpenes (Dhawan, 1995; Saraswat et al., 1987; Lee et al., 2008). In Indian traditional system of medicine, plant has been used for antiinflammatory, antispasmodic, hepatoprotective, woundhealing, antioxidant and hepatoprotective activities (Indian Herbal Pharmacopoeia, 2002). Rhizomes and roots of this plant are widely used for the treatment of a range of liver diseases (Ansiari et al., 1991, Anandan et al., 1999 a,b).

Tephrosia purpurea (Sharapunka) contains glycosides, flavonoids: rutin, quercetin; retinoids: tephrosin; purpurenone, purpuritene, and B-sitosterol. (Gokhale and Sarsa, 2000). Aerial parts of plants are incorporated frequently in large number of commercial herbal formulations, such as Tephroli and Yakrifit, used to treat liver disorders (Khatree et al., 2009). In traditional system of medicine plant has been regarded as deobstruent, diuretic and claimed to provide relief from various biliary and splenic troubles (Kirtikar and Basu., 1956). Phyllanthus amarus (Bhuuma levels) is used in folk remedies in many countries around the world; therefore this plant is of great importance in traditional medicine as hepatoprotective (Foo, 1993). Phytochemical investigation of plant revealed presence of active constituent: tannins, phyllanthin, polyphenols, quercetin and ellagitannins (Foo, 1995). Asparagus racemosus(Shatavari) contains steroidal saponins: shatavarins; isoflavones, aspangamine, polysaccharides. Root juice of this plant has been advocated to cure various hepatic ailments and peptic ulcer (Kamit et al., 2000). It is a well-known Ayurvedic rasayana, which is used to impart immunity, improve mental function, and treatment of inflammation, neuropathy, hepatopathy (Sharma., 2001). Besides, various herbal treatments available today, the therapy for the liver disease remains unsatisfactory. Moreover, increasing incidences of xenobiotic induced hepatotoxicity requires search for more promising and higher grade of hepatoprotective therapy. One approach in this direction involves to test the effect of combination of herbal interventions in preventing liver injury. Therefore, the present study was designed to investigate the dose response curve, of silymarin alone and the synergistic effect if any, by combining low dose silymarin with above mentioned hepatoprotective plants extracts against experimentally-induced hepatotoxicity in rat.

MATERIAL AND METHODS

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. Biochemical enzymatic kits were purchased from Crest Biosystems, Goa, India.

Animals

Wistar albino rats (180-220 g) of either sex, procured from Animal house, ISF College of Pharmacy, Moga, Punjab were employed in present study. They were maintained at standard conditions of temperature (25±2 °C), humidity (45-55 %) and 12/12 h light and dark cycles, and fed on standard chow diet purchased from Ashirwad 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) for the use and care of experimental animals.

Plant material

The dry, aerial parts of Picorrhiza kurroa, Phyllanthus amarus, Asparagus racemosus, and Tephrosia purpurea were procured from commercial herb dealer in Baddi, India. All plant materials were...
authenticated by museum and herbarium of the Department of Pharmacognosy, ISF College of Pharmacy and retained the voucher specimens of all plants in the department.

**Preparation of Plant Extract**

The whole plants were coarsely powdered and extracted using a soxhlet apparatus (at a proportion of 8 g of dried plant powder in 350 mL of methanol for 24 h). A rotary evaporator was used to remove the solvent from the filtrate. The yield of the methanolic extract Picrorhiza kurroa, Phyllanthus amarus, Asparagus racemosus and Tephrosia purpurea was obtained to be 11.2 % w/w, 10.5 % w/w, 13.4 % w/w and 14.1 % w/w respectively. Phytochemical constituents Phyllanthin (Phyllanthus amarus), Kutkin (Picrorhiza kurroa), Asparagamine (Asparagus racemosus) and Tephrosin (Tephrosia purpurea) were detected by preliminary TLC technique.

**Experimentally-induced hepatotoxicity**

The present study comprised two separate experimental models for hepatic injury using PCM and CCl4 as hepatotoxics 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 acute liver damage. Silymarin alone in three respective doses: 12.5, 25 and 50 mg/kg, and silymarin 25 mg/kg in combination with Picrorhiza kurroa, Tephrosia purpurea, Phyllanthus amarus and Aracomeous 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. The animal was sacrificed, liver was surgically dissected out, washed in cold saline and blotted dry. 10% liver homogenate of each plant extract significantly (p<0.05) attenuated these changes in respective PCM or CCl4 control rats.

**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 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. Pretreatment with silymarin (12.5, 25 and 50 mg/kg) and the combination of silymarin (25 mg/kg) with each plant extract: Picrorhiza 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 CCl4 induced hepatotoxicity in rat (Table 1).

**Tissue biochemical estimations**

The tissue biochemical estimations: lipid peroxidation (TBARS) (Ohkawa et al, 1979), reduced glutathione (GSH) (Ellman, 1959), Nitrite/nitrate using greiss reagent (Green et al, 1982), and NaK-ATPase (Bonting, 1970) levels were carried out.

**Histopathological studies**

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

**Table 1: Effect of silymarin and its combination with plant extracts on serum markers**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>ALT</th>
<th>AST</th>
<th>ALP</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µL/I</td>
<td>µL/I</td>
<td>µL/I</td>
<td>µL/I</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PCM control</td>
<td>225.5±16.6a</td>
<td>252.6±44.2a</td>
<td>256.6±49.8a</td>
<td>113±0.15a</td>
</tr>
<tr>
<td>2</td>
<td>PCM + S+P.K.</td>
<td>165±12.5a</td>
<td>103.9±12.4a</td>
<td>203.1±30.8a</td>
<td>80±2.06a</td>
</tr>
<tr>
<td>3</td>
<td>PCM + S+P.A.</td>
<td>128±13.3b</td>
<td>81.4±8.9b</td>
<td>177.9±31.7b</td>
<td>64±0.05b</td>
</tr>
<tr>
<td>4</td>
<td>PCM + S+P.K.</td>
<td>103.9±9.2c</td>
<td>49.9±8.3c</td>
<td>155.4±15.1c</td>
<td>43±0.04c</td>
</tr>
<tr>
<td>5</td>
<td>PCM + S+P.K.</td>
<td>79.3±11.2d</td>
<td>75.8±7.1d</td>
<td>158±10.6d</td>
<td>55±0.05d</td>
</tr>
<tr>
<td>6</td>
<td>PCM + S+P.A.</td>
<td>80.5±8.9b</td>
<td>65.7±6.7b</td>
<td>161.5±17.7b</td>
<td>55±0.05b</td>
</tr>
<tr>
<td>7</td>
<td>PCM + S+P.A.</td>
<td>106.5±12.5e</td>
<td>72±14.5e</td>
<td>143.5±15.9e</td>
<td>46±0.09e</td>
</tr>
<tr>
<td>8</td>
<td>PCM + S parch.</td>
<td>66.5±7.2e</td>
<td>63.2±9.5b</td>
<td>165±6.5e</td>
<td>56±0.03b</td>
</tr>
<tr>
<td>9</td>
<td>PCM + S parch.</td>
<td>253.5±16.5a</td>
<td>245.5±14.1a</td>
<td>244.9±19.1a</td>
<td>90±0.2a</td>
</tr>
</tbody>
</table>

**Results are expressed as mean ± SD; vs NC, vs PCM control, vs CCl4 control, vs S-25 against PCM induced hepatotoxicity, vs S-25 against CCl4 induced hepatotoxicity, vs S-25 against CCl4 induced hepatotoxicity, vs S-50 against PCM induced hepatotoxicity, and 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.: Picrorhiza kurroa; T.P.: Tephrosia purpurea; P.A.: Phyllanthus amarus; A.R.: Asparagus racemosus.]**

**Effect of silymarin and its combination with plant extracts on tissue biochemical estimations**

Hepatotoxicity with PCM or CCl4 produced significant (p<0.05) increase in TBARS, nitrite/nitrate level; decrease in tissue GSH and NaK-ATPase levels in hepatotoxic 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 significantly (p<0.05) attenuated these changes in tissue markers, as compared to respective PCM or CCl4 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 CCl4 induced acute hepatic damage in rat. The effect of these combinations in diminishing NO level was significant (p<0.05) in comparison to S-25
against CCl₄ induced liver damage in rat. The plant extracts: *Parnas* and *Aracemosus* in 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 Na⁺K-ATPase level was significant than that produced by S-25 and S-50 mg/kg alone against CCl₄ induced hepatic damage in rat (Table. 2).

Effect of silymarin and its combination with plant extracts on histological changes

In present study, intoxication with PCM (3 g/kg) or CCl₄ (1 ml/kg) caused marked inflammatory cell infiltration, centrilobular necrosis; fatty infiltration, vacuolization and sinusoidal dilatation respectively in PCM and CCl₄ control groups, as compared to normal control in liver. Pretreatment with three consecutive doses of silymarin, and the combination of silymarin (25 mg/kg) with extracts: *Piericrhoriza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus*, *Asparagus racemosus* showed significant reduction in progression of these toxic cellular effects of hepatotoxicants, as compared to hepatotoxicant control groups (Fig. 1).

**Table 2: Effect of silymarin and its combination with plant extracts on tissue biochemicals:**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>TBARS nM/mg tissue</th>
<th>GSH µM/mg tissue</th>
<th>Nitrite/nitrate µM/mg tissue</th>
<th>Na⁺K-ATPase µM of Pi/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC</td>
<td>4.49±0.2</td>
<td>0.49±0.7</td>
<td>0.89±0.4</td>
<td>3.55±0.2</td>
</tr>
<tr>
<td>2</td>
<td>PCM control</td>
<td>2.3±0.6</td>
<td>0.39±0.1</td>
<td>1.78±0.6</td>
<td>1.3±0.8</td>
</tr>
<tr>
<td>3</td>
<td>PCM + S-12.5</td>
<td>1.4±0.2</td>
<td>0.64±0.08</td>
<td>1.35±0.04</td>
<td>2.1±0.07</td>
</tr>
<tr>
<td>4</td>
<td>PCM + S-25</td>
<td>0.69±0.3</td>
<td>0.74±0.06</td>
<td>1.22±0.03</td>
<td>2.6±0.06</td>
</tr>
<tr>
<td>5</td>
<td>PCM + S-50</td>
<td>0.68±0.16</td>
<td>0.78±0.06</td>
<td>1.2±0.05</td>
<td>2.9±0.05</td>
</tr>
<tr>
<td>6</td>
<td>PCM + S+P.K.</td>
<td>0.46±0.18</td>
<td>0.81±1.1</td>
<td>1.15±0.06</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>7</td>
<td>PCM + S+T.P.</td>
<td>0.51±0.24</td>
<td>0.78±0.11</td>
<td>1.12±0.06</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>8</td>
<td>PCM + S+P.A.</td>
<td>0.25±0.19</td>
<td>0.89±0.07</td>
<td>1.03±0.05</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>9</td>
<td>PCM + S+A.R.</td>
<td>0.36±0.22</td>
<td>63.2±9.5</td>
<td>0.85±0.15</td>
<td>3.5±0.22</td>
</tr>
</tbody>
</table>

**Discussion**

The present study investigated the protective effect of silymarin alone and in combination with the plant extracts: *Piericrhoriza kurroa*, *Tephrosia purpurea*, *Phyllanthus amarus* and *Asparagus racemosus* on experimental liver injury due to CCl₄ induced hepatotoxicity models in rat. Silymarin is used as a standard drug in various experimental and clinical studies due to its proven hepatoprotective effects (Dhiman and Chawla, 2005). The toxic changes associated with CCl₄-induced liver damage are similar to that of acute viral hepatitis clinically (Rubinstein, 1962). Therefore, the PCM and CCl₄-induced hepatotoxicity were selected as experimental models of liver injury in present study. PCM is metabolized to a reactive metabolite: N-acetyl-p-benzoquinone imine (NAPQI), and CCl₄ to trichloromethyl(CCL₃-) free radicals by cytochrome P-450 which are further reported to cause massive oxidative stress, (Aldridge, 1981) and ultimately liver cell death (Eissi et al, 1993). 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 (Molander et al, 1955; Nkosi et al, 2005). In present study, PCM and CCl₄ intoxications caused significant increase in these hepatic enzymes and this was probably due to the consequences of increased oxidative stress and necrotic cell death (Kyle et al, 1987). Pretreatment 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 (Song et al, 2006). In an earlier report, the combination of silymarin (50 mg/kg) with *Phyllanthus amarus* extract offered significant hepatoprotection against CCl₄ induced liver damage (Yadav et al, 2008) this was also confirmed by findings from present study. The observed effect in our study is due to the combined action 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 antioxidants: glutathione, ensuing widespread propagation of allogulation, as well as lipid peroxidation, causing damage to macromolecules (Aldridge, 1981; Eissi et al, 1993). 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 hepatotoxication. These results from our study indicate the potential role of combination pretreatment in preventing oxidative stress mediated damage and strengthening antioxidant defense mechanism.

Hepatotoxicants induced oxidative stress produces release of proinflammatory mediators due to induction of inducible nitric oxide synthase, resulting increased NO level and cellular dysfunction (Beckman et al, 1990). In present study, administration of PCM and CCl₄ significantly caused increase in nitrite/nitrate level, characterizing massive nitrosative stress due hepatotoxication. 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 (Kang et al, 2002). In present study combined pretreatment by Silymarin and each plant extracts resulted in significant decrease in nitrite/nitrate level as compared to silymarin alone, thus suggesting a similar mechanism for these plant extracts in synergising 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 (Rossier et al., 1987; Lamb, 1990). As a consequence, inhibition of this pump may seriously cause disruption of mitochondrial energy and metabolism (Vaskelis and Schwarz., 1993). 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.

CONCLUSION
The findings from the present investigation demonstrate the efficacy of silymarin in lower doses and its synergism with hepatoprotective plants against experimental hepatic damage. Thus hereby provides preclinical evidence and therapeutic rationale requiring further studies combining low dose Silymarin with above mentioned hepatoprotective plants in attenuation of hepatic insufficiencies, in men as well.

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A massive centrilobular necrosis, central vein dilation, ballooning degeneration and inflammatory cellular infiltration of liver are associated with liver damage (Pah and Hewitt, 1982) 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 changes associated with liver damage.

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.

Fig. 1: Effect of silymarin and its combination with plant extracts on histological changes against experimental hepatotoxicity (10x): (A) Normal Control, (B) PCM Control, (C) Silymarin 1.25 + PCM, (D) Silymarin 25 + PCM, (E) Silymarin 50 + PCM, (F) Silymarin + Picrorhiza 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 + Picrorhiza kurroa + CCl₄, (O) Silymarin + Tephrosia purpurea + CCl₄, (P) Silymarin + Phyllanthus amarus + CCl₄, (Q) Silymarin + Asparagus racemosus + CCl₄.

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