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Pharmacological influences of natural products as bioenhancers of silymarin against carbon tetrachloride-induced hepatotoxicity in rats

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Abstract

Background: Popularity of herbal remedies is increasing day by day despite the presence of synthetic drugs to treat the Liver Diseases owing to the adverse effects and high cost of synthetic drugs. Silymarin has tremendous potential for the treatment of various liver disorders because of its high antioxidant potential as liver diseases are associated with increased oxidative stress. The low oral bioavailability of Silymarin continues to be a major challenge in the development of its formulations having clinical efficacy. Our idea was to constitute a pharmaceutical composition of Silymarin with natural products as bioenhancers that might work positively and synergistically in the control of hepatotoxicity.

Methods: In this work, various combinations of Silymarin with natural bioenhancers such as Lysergol (L), Piperine (P) and Fulvic acid (FA) were prepared and their hepatoprotective activities were evaluated against carbon tetrachloride (CCl4) induced hepatotoxicity in animal model.

Results: Although, all the combinations decreased the liver enzymes and changed protein level significantly, group G (silymarin:FA (1:1) + P (10%) was found to be most significant as compared to the toxic control. It also displayed better protection when compared to the marketed tablet containing silymarin alone. None of the combinations showed any signs of cytotoxicity when screened on MCF-7 cells by MTT assay.

Conclusions: Group G (silymarin:FA (1:1) + P (10%) appeared to be the most effective combination in treating the liver diseases envisaging an industrially viable product of Silymarin as a contemporary therapeutic agent with enhanced bioavailability and medicinal value. Further this combination can be examined for safety and efficacy in clinical studies.

Keywords: Silymarin, Bioenhancers, Piperine, Fulvic acid, Lysergol, Bioavailability, Hepatoprotection, MTT assay

Background

Liver, a vital organ (organ of metabolism and excretion) in the human body, plays an astounding array of functions vital for the maintenance as well as performance of the body. Unfortunately, liver is exposed to a variety of xenobiotics, chemotherapeutic agents, drug-drug interactions and environmental pollutants which weaken and damage the liver leading to hazardous liver diseases such as Hepatitis, Cirrhosis and Cancer etc. [1]. LDs and their complications are often linked with imbalance between the production of free radicals (ROS) and body's antioxidant defense mechanism that result into increased oxidative stress. These ROS have an important role in the etiology of LDs andthe antioxidant therapy is expected to impart beneficial effects in treating these. Liver disease (LD), a multi-factorial disease remains one of the most serious health problems and millions of people world-wide are suffering from one form or the other. High cost of treatment and adverse effects are the disadvantages associated with synthetic drugs when used for prolonged periods [2]. Therefore, it is logical to think of herbal remedies for the treatment of LD. Silymarin, a known

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hepatoprotective drug has well defined hepatoprotective, free radical scavenging and antioxidant properties, that improves the antioxidant defense by preventing the glutathione depletion as well as antifibrotic activity. It has been investigated through in-vitro and in-vivo experimental studies by Radko and Cybulski 2007 [3]. Although, clinical trials suggested the safety of silymarin at higher doses (up to 1500 mg/day) in humans, but the pharmacokinetic studies have revealed poor absorption, rapid metabolism and excretion in bile and urine and all these ultimately results in poor oral bioavailability of silymarin [4].

Generally, all pharmacokinetic parameters of silymarin are referred to, and standardized as, silybin. According to Wu et al. 2007, silymarin (silybin), when administered orally, is rapidly absorbed with a T_{max} (2–4 h) and $t_{1/2}$ (6 h). Due to extensive enterohepatic circulation, only 20–50% of oral silymarin is absorbed from the gastrointestinal tract and 0.73% oral bioavailability of silymarin (silybin) in rat plasma was reported [5, 6].

Silybum marianum (milk thistle, family: Asteraceae), is one of the oldest and thoroughly researched plants of ancient times used in the treatment of liver and gall bladder disorders, including jaundice, cirrhosis and hepatitis and Silymarin is the active constituent of this plant which is a 70–80% standardized extract consisting of silymarin flavonolignans (silybin A & B, isosilybin A & B, silydianin, and silychristin) and flavonoids (taxifolin and quercetin), and the remaining 20–30% consists of chemically undefined fraction comprising polymeric and oxidized polyphenolic compounds [7]. There are as many as 75 brands of silymarin available in market in different dosage forms such as tablets, capsules, syrups, etc. Some of the important brands are Legalon capsules, Carsil Tablets and Alrin-B syrup etc. An array of methods are available in the literature that can improve the bioavailability of silymarin like formation of microparticles, nanoparticles, self- emulsifying drug delivery systems, phytosomes, liposomes and micelles as summarized by Javed et al. 2012 [7]. But they suffer from disadvantages of using a large amount of surfactants, co-surfactants, exogenous compounds as these cause irritation to patients suffering from gastric disorders and ulcers and thus leading to abdominal discomfort [8]. The concept of using the bioenhancers to increase the drug bioavailability is one of the newest approaches. The discovery of first bioenhancer piperine in 1979 by scientists in RRL, Jammu, India introduced a new concept in science [9]. Non-toxicity, effectiveness at low concentrations, easy to formulate with the drug, enhanced uptake and absorption of drug and lastly, synergizing the activity of the drug are the advantages associated with the bioenhancers. Bioenhancers increase in the bioavailability of nutraceuticals by acting through several mechanisms, which include acting on gastrointestinal tract to enhance absorption, or by altering the drug metabolism process [10].

In our study, three natural products as bioenhancers were selected based on their mechanism of action: first, Fulvic acid (FA) – a water soluble carrier for increasing the solubility of silymarin by complex formation [11], second piperine (P) – a known inhibitor of hepatic and intestinal glucoronidation inhibitor [12] and third, lysergol (L) – a permeability enhancer of drugs across intestinal epithelial cells for better absorption and efficacy [13]. All the formulations no. 1–5 were subjected to accelerated stability studies as per ICH guidelines Q1A. The carbon tetrachloride induced hepatotoxicity study in rats was performed to evaluate the effect of silymarin alone and with bioenhancers in all the tablet formulations. Previously, researchers from all over the world have demonstrated the hepatoprotective activity of silymarin against various toxic models and partial hepatectomy models in experimental animals by using chemical toxins such as carbon tetrachloride (CCl_4), acetaminophen, D-galactosamine, ethanol, and *Amanita phalloides* toxin [14]. In cellular events that modulate hepatotoxicity, CCl_4 is metabolized by CYP450 enzymes in liver endoplasmic reticulum in reactive trichloromethyl free radicals which in turn react with oxygen and form trichloromethylperoxy radicals. These radicals attack lipids on endoplasmic reticulum of liver cells and leads to elevation of liver enzymes and ultimately cell death. CCl_4 interferes with the transport function of the liver cells, leading to leakage of SGOT and SGPT from the cell cytoplasm into the serum, thereby increasing their levels in serum and reduces the capacity of liver to synthesize albumin, leading to decreased serum levels [15].

Recent studies conducted in the past decade have shown the hepatoprotective potential of silymarin against CCl_4 induced liver injury. Silymarin and garlic oil were reported as highly promising compounds in protecting the hepatic tissue against oxidative damage and preventing hepatic dysfunction due to CCl_4 induced hepatotoxicity in rats [16]. In another study, the restoration of the CCl_4–induced hepatic fibrosis was reported due to high doses of silymarin in rats [17]. The biochemical parameters returned to normal values in CCl_4 intoxicated rats after treating with silymarin and/or ginger for one month [18]. A significant reduction in enzyme levels in silymarin lipid microspheres treated group was reported by Abrol et al. 2005 when compared to toxic control, normal control (plain lipid microspheres) as well as groups treated with silymarin solution [19]. In another study conducted by El-Samaligy et al. 2006, silymarin hybrid liposomes produced a significant decrease in both the transaminase levels (SGOT and SGPT) when challenged with intraperitonial CCl_4 () in comparison to the orally administered silymarin suspension [20]. Synergistic effects of silymarin and standardized extract of

Phyllanthus amarus against CCl_4 induced hepatotoxicity in rat model was also reported previously [21].

Results

MTT cytotoxicity studies of Silymarin, fulvic acid, Piperine and lysergol on MCF-7 cells

MTT cell viability assay is a versatile, quantitative, significantly advanced measurement of cell viability, proliferation and cell population's response to external factors. This test was based on the formation of water-insoluble purple formazan product from the yellow water- soluble tetrazolium dye by live cells. The amount of formazan generated is directly proportional to the number of viable cells [22]. This test was performed to evaluate the cytoxicity profile of silymarin and all the three bioenhancers on human breast adrenocarcinoma MCF-7 cell lines. Figure 1 shows the photomicrographs of control/untreated cells (a) and cells treated with silymarin, P, L and FA (b, c, d, e) respectively. No cell death, rupture, necrosis was visible in them and morphology and integrity remained intact. Figure 2 shows the Percentage cell death vs. Concentration (μg/mL) bar graph and it was found that neither the drug silymarin nor any of the bioenhancers appeared to be cytotoxic on MCF-7 cells over the concentration range of 25–500 μg/mL. Upto 12% and 18% cell death with P and L was observed at the concentration 500 μg/mL respectively. From this study, it was concluded that no cell death, rupture, necrosis was visible, morphology and integrity remained intact on MCF-7 cells. A higher cell viability throughout the experiment ensured non-cytotoxic behavior of drug and bioenhancers. The bioenhancers were considered non-cytotoxic and were carried forward for further studies.

Carbon tetrachloride (CCl_4) induced hepatotoxicity in rats

CCl_4 induces hepatotoxicity by interfering with the transport functions of the liver cells which leads to leakage of SGOT and SGPT from the cell cytoplasm into the serum. Also, enzymatic activation of CCl_4 by CYP P450 generates free radicals (ROS) which combine with proteins and cellular lipids in presence of oxygen resulting in liver necrosis [23, 24]. The results in this experiment showed marked increase in plasma SGOT and SGPT levels in toxic control group after CCl_4 treatment as compared to the normal control group signifying that the experiment was successful to induce liver injury in rats. Bilirubin is a metabolite of heme and is an important means to excrete the unwanted and toxic heme from body. It is found to be increased in a variety of liver disorders such as cirrhosis and jaundice.

Statistical analysis

The pharmacodynamic data analysis was carried out using the GraphPad Prism version 6.02 (Registered trademark of GraphPad software, Inc). All the numeric variables were expressed as Mean ± Standard Error of Mean (SEM) and results were statistically analyzed using One Way-Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test. For all tests a probability ($p < 0.0001$) was considered significant.

Biochemical estimation

Levels of SGOT, SGPT, ALKP and Serum Bilirubin were found to be significantly reduced in all treatment groups with most significant results obtained for Silymarin–FA–P formulation treated group when compared to toxic

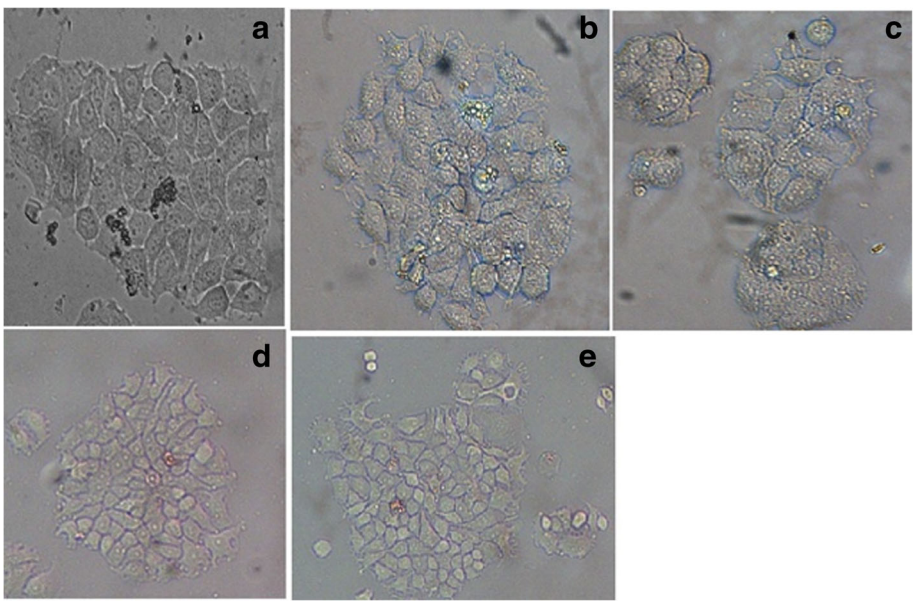

Fig. 1 Photomicrographs of control cells (**a**), silymarin (**b**), piperine (**c**), lysergol (**d**), and fulvic acid (**e**) treated MCF-7 cells respectively

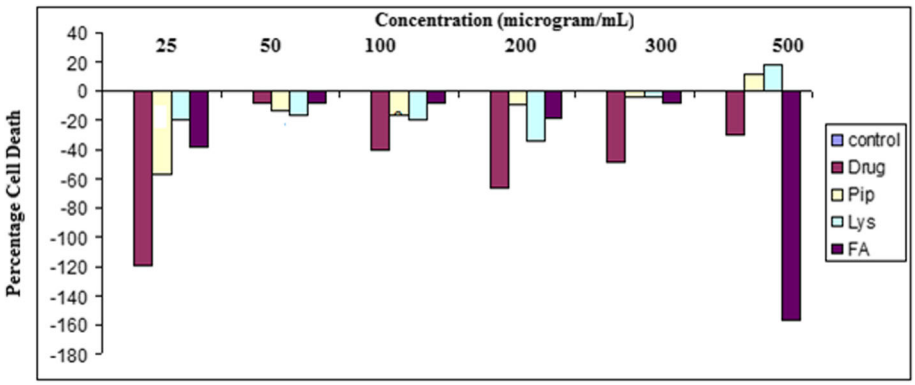

Fig. 2 Bar-graph between Percentage cell death vs. Concentration (μg/mL) shows that neither the drug silymarin nor any of the bioenhancers appeared to be cytotoxic on MCF-7 cells over the concentration range of 25–500 μg/mL

control. Table 1 indicates different degrees of hepatoprotection showed by these groups. The levels of total plasma protein were observed to be decreased in toxic control group which seconded the findings reported by Tanaka et al., 1998 in LDs [25]. This decrease in plasma proteins reflects decreased hepatic synthesis, which is often attributed to the hepatic impairment of albumin synthesis. The decrease may also be due to leakage in kidney function leading to the release of albumin in urine [18]. Hepatotoxin also decreases serum albumin levels by reducing the capacity of liver to synthesize albumin. Administration of Silymarin- FA- P (group G) formulation significantly counteracts CCl_4 induced changes suggesting that it provided better hepatoprotection by improving both synthetic and metabolic activities of the liver as compared to silymarin alone (group C).

The results of percent recovery of serum parameters showed that the combinations of silymarin and bioenhancers had a higher recovery of serum parameters in comparison to plain silymarin tablet (Table 2). Silymarin-L (10%) tablet formulation showed improved (50–70%) percent recovery of serum parameters when compared to plain silymarin formulation. Lysergol is an important constituent of *Ipomoea* sp. and two species of *Ipomoea* namely *Ipomoea hederacea* and *Ipomoea asarifolia* (Desr.) have been reported to have antioxidant and hepatoprotective potential against CCl_4 induced hepatotoxicity [26, 27]. It has been reported that lysergol modifies the drug transport across the cell membranes and has its own antioxidant and hepatoprotective activity.

Similarly, silymarin–P (10%) tablet formulation showed improved percent recovery of serum parameters (70–75%) in comparison to treatment by plain Silymarin tablet. Piperine is a known inhibitor of CYP 450 enzymes and thus inhibits the hepatic and intestinal glucoronidation thereby increasing the drug concentration. Furthermore, the antioxidant and hepatoprotective activity of *Piper longum* and *Piper nigrum* against the CCl_4 induced liver injury has been reported previously [28, 29]. Our findings are concordant with these findings and suggest that silymarin and P together in the formulation might have exhibited synergistic hepatoprotective and antioxidant activity.

Thirdly, silymarin – FA tablet formulation also showed improved percent recovery of serum parameters (upto 80%) in comparison to the treatment by our plain silymarin tablet. As, FA is a known enhancer of water

Table 1 Summary of biochemical parameters for all treatment groups

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
	Normal control	Toxic control	Plain Sily tab	Sily + Lys (10%) tab	Sily + Pip (10%) tab	Sily + FA (1:1) tab	Sily + FA (1:1) + Pip (10%) tab	Marketed Tab
SGOT (IU/L)	67.25 ± 4.29	303.3 ± 1.85[a]	225.3 ± 6.02[b]	175.2 ± 4.55[b]	125.4 ± 5.58[b]	106.2 ± 3.89[b]	83.03 ± 2.08[b]	199.2 ± 8.62[b]
SGPT (IU/L)	55.34 ± 4.27	195.6 ± 2.87[a]	123.5 ± 6.92[b]	99.14 ± 6.58[b]	82.89 ± 1.88[b]	78.90 ± 4.26 [b]	66.82 ± 3.14[b]	107.2 ± 6.56 [b]
ALKP (IU/L)	85.08 ± 5.17	353.3 ± 8.91[a]	183.5 ± 5.14[b]	163.8 ± 3.59[b]	145.0 ± 4.04[b]	133.3 ± 3.69[b]	109.8 ± 4.76[b]	172.2 ± 2.53[b]
Total Bilirubin (mg/100 mL)	0.38 ± 0.02	1.18 ± 0.04[a]	0.65 ± 0.03[b]	0.60 ± 0.01[b]	0.57 ± 0.02[b]	0.53 ± 0.03[b]	0.43 ± 0.04[b]	0.65 ± 0.03[b]
Total protein (g/dL)	7.37 ± 0.16	5.06 ± 0.33[a]	8.74 ± 0.05[b]	8.06 ± 0.11[b]	7.93 ± 0.13[b]	7.70 ± 0.13[b]	7.45 ± 0.33[b]	8.66 ± 0.08[b]

Dunnett's Multiple Comparison Tests showed that all values of group A, C, D, E, F, G, H exhibited significant changes when compared to toxic control with 99.9% CI of difference. For each group $n = 5$, the values are expressed as mean ± SEM. 'a' exhibits significant ($p < 0.05$) changes from normal control, whereas, 'b' exhibits significant ($p < 0.05$) change when compared to toxic control

Table 2 Percent recovery of serum parameters

Parameters	Plain Sily tablet (No. 1)	Sily + L (10%) tablet (No. 2)	Sily + P (10%) tablet (No. 3)	Sily + FA (1:1) tablet (No. 4)	Sily + FA (1:1) + P (10%) tablet (No. 5)	Marketed tab
SGOT (IU/L)	33.17	54.26	75.36	83.49	93.31	44.10
SGPT (IU/L)	51.40	68.77	80.35	83.20	91.81	63.02
ALKP (IU/L)	63.30	70.65	77.60	82.02	90.78	67.51
Total Bilirubin (mg/100 mL)	66.25	72.51	76.25	81.25	93.75	66.21
Total Protein (g/dL)	45.84	72.72	77.86	86.95	96.83	49.01

% Recovery = (Toxin group − Treated group)/ (Toxin group − Control group) × 100

solubility by complexation, the increase in activity of silymarin can be attributed to this fact [30]. Recently, the antioxidant potential of FA was unearthed and researched by Rodriguez et al. 2011 who attributed the health benefits of FA to its antioxidant nature and categorized it as a good candidate in pharmaceutical and food industry [31].

Lastly, the administration of Silymarin-FA- P tablet formulation attenuated the increased levels of the serum SGOT, SGPT, ALKP and Total Bilirubin caused by CCl_4 and produced most subsequent recovery towards normalization (upto 90%).

Our findings suggest that FA and P exert bioenhancing effects on silymarin by dual mechanism. Firstly, FA might have improved the solubility of silymarin by its solubilizing nature [11] and P a known hepatic and intestinal glucoronidation inhibitor might have inhibited the metabolism of silymarin [12]. Secondly, as all the three components have antioxidant properties, the highest recovery conferred by this tablet formulation can be attributed to the antioxidant potential of their combination. Thus, it can be concluded that combination of silymarin with FA and P exhibited significant hepatoprotection as indicated by significant changes in various liver biochemical parameters.

Histopathology of liver

Histopathological examination of rat livers observed no alterations in normal control group, while necrosis and diffused kupffer cells proliferation among the hepatocytes of toxic group was seen (Fig. 3a and b). The liver sections of CCl_4 exposed rats showed major necrosis and degeneration of hepatocytes, and infiltration of inflammatory cells, when compared to the normal control which had normal lobular architecture with central vein and radiating hepatic cords. Fig. 3a shows normal hepatocytes where no alteration in the hepatocyte architecture was observed, while, in Fig. 3b enormous damage of the liver cells could be seen due to CCl_4 intoxication in between the hepatocytes because of focal necrosis and diffused kupffer cells proliferation. The results were concordant with those in the literature [32].

However, the CCl_4-induced destruction of liver architecture was not significantly improved in case of marketed tablet formulation and our plain silymarin tablet. A non-significant protection of hepatocytes against the hepatotoxin was seen as depicted in photomicrograph (Fig. 3c and d). Dilatation in the hepatic sinusoids associated with inflammatory cells infiltration and diffused kupffer cells proliferation in between the damaged hepatocytes was seen. This might be due to the incomplete or lesser bioavailability of plain silymarin to the liver cells from both these formulations.

Some improvements in results for silymarin in terms of partial protection against hepatotoxin were obtained in case of silymarin – L (Fig. 3e) and silymarin – P (Fig. 3f) formulation groups where lesser amount of necrosis was observed. The degree of vacuolation also decreased in these groups as compared to CCl_4 treated group showing better protection and improvement.

It is worthy to state that as the antioxidant potential of silymarin increased the most when used in combination with both FA and P and even better hepatoprotective results were observed with least liver damage. It suggested the superior hepatoprotective activity of this formulation over rest of the drug-bioenhancer combinations. The antioxidant property might have helped the hepatocytes counteract the oxidative stress and this might have contributed to blocking the progression of LD (Fig. 3g and h).

Discussion

In recent times, several studies have been carried out to demonstrate the efficacy of herbal drugs and nutraceuticals in LDs and most of these studies showed significant hepatoprotectivity with lesser side effects and good efficacy [33].

By now, it is well understood that silymarin has significant antioxidant and hepatoprotective potential if it is bioavailable [34]. The only limitation in its use is its poor bioavailability that leads to higher daily doses in order to observe some of its pharmacological activity. If by some approach, the bioavailability increases, it would lead to lesser amount and frequency of dosing and better pharmacological activity of silymarin. The aim of our work was to use natural products as bioenhancers along

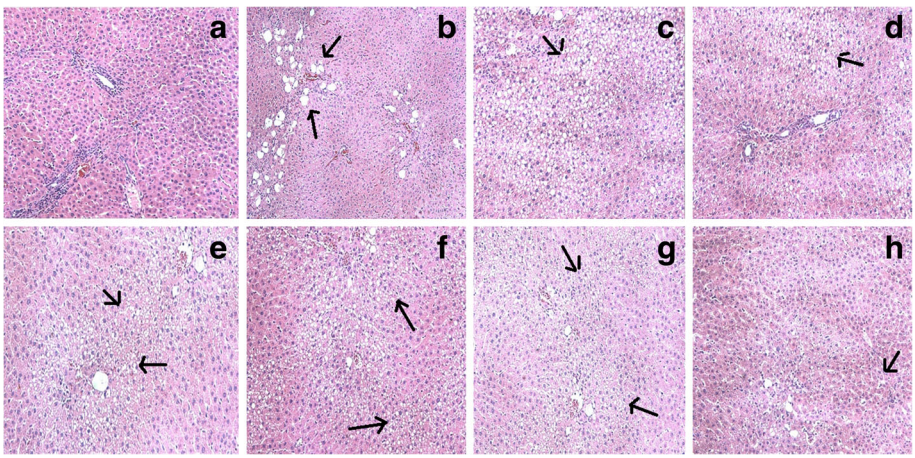

Fig. 3 Liver histological structure of rats in normal control (**a**), toxic control (**b**), plain silymarin tablet (**c**), marketed formulation (**d**), Silymarin – Lysergol (**e**), Silymarin – Piperine (**f**), Silymarin – Fulvic acid (**g**) and Silymarin – Fulvic acid- Piperine (**h**) (H + E × 100). The small arrows are used to show the extent of necrosis and presence of vacuoles

with silymarin in order to increase its bioavailability either by increasing its water solubility, increasing its permeability or by inhibiting its metabolism. Silymarin can also modify the plasma membrane phospholipid content therefore, protects against the CCl_4 induced alterations of the liver plasma membrane through its antioxidant properties [35].

Our systematic study brought up the results that if the bioavailability of silymarin is increased with the help of bioenhancers like FA and P, together these three compounds may act as strong antioxidants and provide synergistic and additive hepatoprotective effects. So we suggest, a formulation with good anti–inflammatory and antioxidant potential and is anticipated to show good hepatoprotective activity if used properly. We hypothesized that silymarin along with FA and P in a definite concentration in a pharmaceutical dosage form would provide much better hepatoprotection because of two reasons: Firstly, with their bioenhancing effects on silymarin and secondly, together with silymarin they proved to be a good antioxidant combination which is important for the protection against the injury caused by CCl_4.

The results obtained from the present study indicated that SGOT, SGPT, ALKP and Total Bilirubin levels were markedly increased in toxic group after CCl_4 treatment as compared to the normal group signifying the induction of liver injury in rats ($p < 0.05$). Silymarin along with bioenhancers ameliorated the hepatotoxic effect of CCl_4 and exhibited significant hepatoprotective activity against CCl_4-induced liver injury in the following order: silymarin- FA- P formulation, > silymarin – FA > silymarin – P > silymarin – Lformulation by normalizing the elevated levels of hepatic enzymes when compared to plain silymarin formulation. A novel treatment of LDs by the use of a strong antioxidant silymarin in combination with FA and P in a tablet dosage form is anticipated. To further prove this point and idea, the safety as well as the efficacy must be evaluated in pre-clinical and clinical studies.

Conclusions

In addressing the current status of the treatment of LDs, there is a need for development of new hepatoprotective formulation with higher efficacy and safety. We intended to focus on a novel approach for the treatment of LD by increasing the bioavailability of silymarin with the help of natural products as bioenhancers. Bioenhancers when combined together proved to be a potential antioxidant combination and are proposed to have synergistic and additive effects with silymarin. A number of LDs are commonly associated with oxidative stress which plays a vital role in the pathogenesis of ailments such as alcoholic liver disease, chronic hepatitis C, non-alcoholic steatohepatitis (NASH), haemochromatosis, and Wilson's disease. Thus, antioxidant therapy has been believed to have beneficial effects in managing these diseases. Both FA and P work by different mechanisms in order to increase the bioavailability of silymarin and a pilot scale study is required to determine the optimal dose of the combination that shows highest safety and efficacy, and if worthwhile effects are revealed in preclinical studies clinical studies can also be designed.

Methods

Tablet manufacturing techniques

Silymarin tablets were made by the three techniques viz. Direct Compression Technique, Foam Granulation Technique and Solid Dispersion Technique as shown in Fig. 4. Direct compression plain silymarin tablet formulation (no.1) was used as control. Foam granulation technique was used to make silymarin – L (no. 2) and

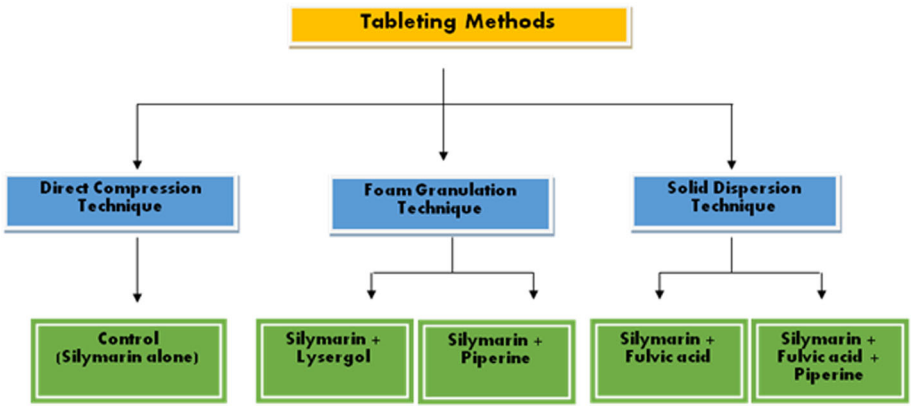

Fig. 4 Various tablet manufacturing techniques employed for the formulation of different combinations of silymarin and bioenhancers

silymarin-P (no. 3) tablets. Methocel E6 PLV was used as foam binder that aided in improved dissolution profile of silymarin because of its surfactant properties [36]. Solid dispersion technique was used to make silymarin – FA (no. 4) and silymarin-FA-P (no. 5) tablets. FA used as carrier moiety for silymarin, aided in improved solubility and enhanced dissolution profile because of its water soluble nature [30].

MTT cytotoxicity studies of Silymarin, fulvic acid, Piperine and lysergol on MCF-7 cells

MTT assay was employed to evaluate the cytotoxic effect of the free drug silymarin and the bioenhancers P, FA and L on MCF-7 cells. For MTT assay, MCF-7 cells were grown using DMEM media mixed with 10% Fetal Bovine Serum (FBS), seeded on 96 well plate and allowed to adhere. Concentrations of free drug and the bioenhancers amounting to 25–500 μg/mL respectively were added to the 96 well tissue culture plate (Falcon Plate) in duplicates. MTT assay was performed after 24 h of treatment to assess cell viability. The respective media was removed from all the wells and 10 μL of MTT reagent (Chemicon International, from Millipore) was added in each well from a working stock (5 mg/mL) solution and the plates were kept in incubator for 2–3 h. The reagent was then removed and the remaining crystals were solubilized in DMSO. Formazan gets dissolved to give homogeneous purple solution and its absorbance was measured at a test wavelength of 570 nm and reference wavelength 630 nm using ELISA plate reader. The absorbance value is a direct measure of the number of live cells. The corresponding values for O.D. for different drug and bioenhancer concentrations were recorded [24].

Carbon tetrachloride induced hepatotoxicity in rats

Animal protocol

For the experimental purpose, male wistar rats, weighing approximately 250 ± 10 g, fasted over night with free access to water were used. The protocol of the study was approved by Jamia Hamdard Institutional Animal Ethics Committee (Registration No – 173/CPCSEA). The guidelines provided by the institutional ethics committee for the usage of animals in scientific research were strictly followed [37]. All the painful procedures were performed under anesthesia and the animals which cannot be relieved or repaired at the end of the study were sacrificed ethically under anesthesia. Throughout the study, animals were hygienically kept in a controlled environment in large polypropylene cages at air conditioned temperature (25 ± 2 °C) with a 12 h light/dark cycle in Central Animal House, Jamia Hamdard, New Delhi (India). Principle of 4 Rs (replacement, reduction, refinement, and rehabilitation) given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India was followed in this study.

In-vivo hepatoprotective study protocol

The CCl_4 induced hepatotoxicity model as described by Yadav et al. 2008, was employed with some modifications in order to assess the hepatoprotective potential of our various formulation groups [23]. For this purpose, the rats were divided into eight groups (A, B, C, D, E, F, G, H) with five animals each ($n = 5$). The rats were assigned treatment as follows:

I. Group A assigned as normal control group and was fed with light liquid paraffin (1 mL/kg b. w.) orally for six days.
II. Group B marked as toxic control group and toxicity was induced using CCl_4 (1 mL/kg b. w.), in light liquid paraffin orally on day 3rd and 4th and with plain vehicle on rest of the days.
III. Group C received plain silymarin formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.

IV. Group D received silymarin + L formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.
V. Group E received silymarin + P formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.
VI. Group F received silymarin + FA formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.
VII. Group G received silymarin + FA + P formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.
VIII. Group H received silymarin marketed formulation (equivalent to 100 mg/kg/mL) for all six days and CCl_4 (1 mL/kg b.w.) orally on day 3rd and 4th.

On 7th day, blood was collected in separator tubes from retro orbital plexus of each animal, allowed to clot and centrifuged at 3000 rpm for 15–20 min. Serum was separated and stored at − 20 °C till further analysis. All the samples were analyzed for various biochemical parameters namely SGOT, SGPT, ALKP, Total Bilirubin and Total Proteins by using diagnostic kits and manufacturers' protocol present therein.

Biochemical analysis

In vitro determination of SGOT and SGPT in rat plasma/serum was carried out by 2, 4- DNPH Reitman and Frankel Method – an end point colorimetric method for the estimation of enzyme activity. Alkaline phosphatase determination in rat serum was carried out by King and King's method. Bilirubin analysis in rat serum was carried out by Malloy and Evelyn method and Total Protein Analysis in rat serum/plasma was carried out by Modified Biuret End point Assay by using diagnostic kits and manufacturers' protocol.

Histopathological assessment

The liver tissue specimens taken from rats of all groups were placed in 10% formalin solution for 24 h, blocked in paraffin and sectioned at 5 μm thickness with a microtome followed by staining with hematoxylin-eosin dye stains. Microscopic images were taken through the light microscope.

Abbreviations

ALKP: Alkaline Phosphatase; ANOVA: Analysis of Variance; BW: Body Weight; CCl_4: Carbon tetrachloride; CYP450: Cytochrome P450; DMEM: Dulbecco's Modified Eagle Medium; DMSO: Dimethylsulfoxide; DNPH: Dinitrophenylhydrazine; ELISA: Enzyme-linked Immune Sorbent Assay; FBS: Fetal Bovine Serum; lCH: International Council for Harmonization; LD: Liver Disease; MCF-7: Michigan Cancer Foundation-7; MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; OD: Optical Density; RRL: Regional Research Laboratories; SEM: Standard Error of Mean; SGOT: Serum Glutamic Oxaloacetic Transaminase; SGPT: Serum Glutamic Pyruvic Transaminase; $T_{1/2}$: Terminal Half Life; T_{max}: Time to reach Peak Concentration

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Authors' contributions

SJ: Carried out the experimental work. Also involved in writing the manuscript. WA: Worked as consultant to the work, helped SJ in writing the manuscript. KK: Designed the work, guided the whole project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was submitted to and approved by Institutional Animal Ethics Committee and the animals were provided by the Central Animal House Facility, Jamia Hamdard (CPCSEA Registration No – 173/CPCSEA). All the studies were performed at the Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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