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Original Article

HEPATOPROTECTIVE EFFECT OF VIRGOLIV SYRUP AGAINST CCL_4 INDUCED HEPATIC INJURY IN RATS

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ABSTRACT

Objective: To evaluate the hepatoprotective effect of Virgoliv syrup on CCl_4 induced hepatic injury in rats.

Methods: The hepatic injury was induced by intra peritoneal injection of 0.3 % CCl_4 (10 ml/kg) diluted with olive oil (1:1). The biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB) and alkaline phosphatase (ALP) and antioxidant enzymes such as malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were estimated to assess the liver function. The histopathological changes of liver tissues were studied and compared with silymarin.

Results: The levels of biochemical parameters such as ALT, AST, ALP and TB were significantly increased in CCl_4 treated rats when compared with the normal rats but the Virgoliv syrup treated rats showed maximum reduction of ALT, AST, ALP and TB levels in significant manner. The CCl_4 treated rats showed significantly increase in MDA and decrease in GSH, CAT and SOD levels of liver tissue when compared to control rats. Treatments of rats with Virgoliv syrup and silymarin showed significant improvment in antioxidant enzyme levels in liver tissue. The hepatoprotective effect of Virgoliv syrup was confirmed by histopathological examination of liver tissues.

Conclusion: It can be concluded that Virgoliv syrup showed significant hepatoprotective activity against CCl_4 induced hepatic damage and this might be due to scavenging of free radicals as evident by recovery of antioxidant enzymes such as CAT, GSH and SOD towards normalization and decreased lipid peroxidation.

Keywords: Virgoliv syrup, Carbon-tetrachloride, Hepatoprotection, Silymarin, Histopathology.

INTRODUCTION

Virgoliv syrup (VLS) is an herbal liquid formulation useful for hepatoprotective, digestive, anthelmintics and blood purifier activity. The liver is a crucial organ in the human body that plays an essential role in detoxification and metabolism of various endogenous and exogenous injurious substances [1]. It is subjected to the variety of insults due to viral infections, hepatitis, cirrhosis metabolic disorders, diabetes, and alcoholism and drug abuse [2, 3]. The toxic substances in the environment are absorbed from the intestinal tract and gain entry to the liver resulting in a variety of liver illness. The various types of liver disorders are categorized into hepatitis, cirrhosis, tumors, liver cell necrosis, metabolic and degenerative lesions and many more. But most commonly liver disorders can arises due to excessive consumption of drugs, environmental pollution and alcohol intoxication. Thus, hepatic diseases are one of the severe health problems [4]. The synthetic drugs used in the treatment of liver diseases have numerous serious side effects on health [5]. Thus, management of hepatic disorders is a biggest challenge to the modern medicine.

Carbon tetrachloride (CCl_4) is the most extensively used toxin for the experimental induction of liver injury in laboratory animals [6]. The CCl_4 is metabolized by hepatic cytochrome P-450 enzyme, which results into formation of unstable trichloromethyl (CCl_3) and trichloromethyl peroxyl (CCl_3O_2) radicals [7]. Both trichloromethyl and its peroxy radical binds to proteins or lipids of the cell membrane, or removes a hydrogen atom from an unsaturated lipid, initiating the process of lipid peroxidation that results into hepatic damage [8]. The single dose administration of CCl_4 to laboratory animals produces a centrilobular necrosis and fatty changes in the liver.

The progress of hepatic necrosis is coupled with an outflow of hepatic enzyme into serum [9, 10]. Thus, hepatic necrosis results into biochemical changes of liver those are parallel to the clinical features observed in acute viral hepatitis [11-13]. The hepatic injury induced by CCl_4 toxin is characterized by two phases: First phase, a direct oxidative stress leading to hepatocyte death [14] and second phase, hepatic damage from tumor necrosis factor alpha (TNF-α) activated macrophages (Kupffer cells) [15, 16] and pro-inflammatory cytokines such as IL-1, IL-12 and IL-18 [17].

Herbal drugs play a pivotal role in the management of the variety of liver disorders that speed up the natural healing processes of the liver. Numerous medicinal plants and their formulations are used for the liver disorders in ethanomedical practices as well as traditional system of medicine in India [18]. Natural antioxidants are known for scavenging of free radicals and enhancing the endogenous antioxidant enzyme levels such as superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT) and glutathione peroxidase (GPx) [19]. The herbs full of natural antioxidants can act as powerful hepatoprotective agent with antioxidant activity [20]. Plenty of research reports on medicinal plants pointed out that natural chemical constituents exhibited strong antioxidant activity that could protect the liver from CCl_4-induced damage [21], as they consists of lots of free radical scavenger such as phenolic acids and flavonoids as a key constituent.

The hepatoprotective activity of individual plants listed in VLS formulation has been already reported. VLS is multi herbal formulation used by ayurvedic practitioners for the treatment of liver dysfunction. In the present study, the VLS was examined for hepatoprotective effect in an animal model. Based on diversified pharmacological properties and its use in liver diseases in traditional Indian System of Medicine, an attempt has been made to validate the combinations of these plants for its hepatoprotective potential against most widely used hepatotoxin CCl_4 in experimental studies because drugs with multiple mechanisms of protective action are very limited, therefore the present study will be undertaken to investigate the hepatoprotective effect of formulation in experimentally induced hepatotoxicity model.

The present study was aimed to evaluate the *in vivo* hepatoprotective activity of VLS against CCl_4 induced hepatotoxicity in rats. The study consists of effects of VLS on the biochemical determinations of serum levels of alanine aminotransferase (ALT),

aspartate aminotransferase (AST), alkaline phosphates (ALP) and total bilirubin (TB) and the levels of hepatic antioxidants such as malondialdehyde (MDA), SOD, CAT and GSH in liver homogenate were also studied in combination with histopathological examination of liver tissue. In this study, the results were compared with silymarin (a polyphenolic flavonoid) isolated from milk thistle with clinically proven hepatoprotective effect [22].

MATERIALS AND METHODS

Experimental animals

Sprague–Dawley rats of either sex, weighing 200-250 g maintained under standard husbandry conditions (temperature 23±2 ℃ relative humidity 55±10 % and 12 h light: 12 h dark cycle) were used for all experiments. The animals were fed on a pelleted diet (Hindustan animal feeds, Gujarat) and water *ad libitum*. All the experiments described in the present study were conducted as per protocol number IPS/PCOL/CONS12-13/1008 dated 17-08-2012.

Drugs and chemicals

The VLS was obtained from Virgo UAP Pharma Pvt. Ltd. Ahmedabad, Gujarat. Carbon tetrachloride (CCl_4) and Silymarin were purchased from Merck India Ltd., Mumbai. Assay kits for serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TB) were purchased from Accurex Biomedical PVT. Ltd., Mumbai. All other chemicals and reagents used in the study were of analytical grade and obtained from Sigma Chemicals.

Polyherbal formulation

The VLS is obtained from Virgo UAP Pharma Pvt. Ltd. Ahmedabad, Gujarat.

Table 1: Virgoliv syrup polyherbal formulation

Plants used in the formulation	Part used	Indication	Dose
Eclipta alba	Whole Plant	20 mg	
Plumbago zeylanica	Root	20 mg	Children: ½ to 1 teaspoonful three times a day
Andrographis paniculata	Whole Plant	20 mg	
Boerhavia diffusa	Root	20 mg	
Solanum nigrum	Whole Plant	12 mg	Adult: 1 to 2 Teaspoonful three times a day or as advised by the Physician
Tecomella undulate	Stem bark	16 mg	
Picrorhiza kurroa	Rhizome	8 mg	
Cissampelos pareira	Root	12 mg	
Operculina turpethum	Root	12 mg	
Embelia ribes	Fruit	12 mg	
Cichorium intybus	Seed	16 mg	
Phyllanthus niruri	Whole Plant	16 mg	
Tinospora cordifolia	Stem	12 mg	
Tephrosia purpurea	Whole Plant	8 mg	
Piper longum	Fruit	8 mg	
Berberis aristata	Stem	10 mg	
Cassia occidentalis	Seed	12 mg	

Acute toxicity study

Healthy adult female albino mice (18-22g) were subjected to acute toxicity study as per guidelines (AOT 425) suggested by the organization for economic co-operation and development (OECD-2000). The mice were observed continuously for 2 h for behavioral, neurological and autonomic profiles, after 24 hours and seven days for any sign of toxicity or mortality [23]. According to OECD guidelines 2000 and AOT 425 the acute toxicity studied are only performed in female mice.

Carbon tetrachloride induced hepatotoxicity

The animals were divided into five groups comprising six mice in each. The group I (normal control) and group II (induced control) were received distilled water (10 ml/kg, p. o.) for 7 days. The animals of group III were treated with VLS (1 ml/kg, p. o.) respectively. The group IV animal was treated with standard drug silymarin (100 mg/kg, p. o.) In this protocol, all animals except group 1 received 0.3 % CCl_4 (10 ml/kg, i. p.) dilution with olive oil 1:1 after 1 h of their respective treatment on the 7th day.

The treatments were continued for 7 days and on the 8th day of the experiment all animals were sacrificed under light ether anesthesia and blood collected without the use of anti-coagulant for serum preparation. The blood samples were collected by direct cardiac puncture and allowed to stand for 10 min before centrifuged at 2,000 rpm for 10 min.

The dose of VLS was selected depending on the body surface area to body weight ratio the adult VLS dose has been converted to rat dose (1 ml/kg, p. o.). The silymarin standard is used in 50, 100, 200 mg/kg concentrations for hepatoprotective activity. So 100 mg/kg dose (the dose in between 50 to 200 mg/kg, p. o.) was selected for the present study.

Estimation of serum biochemical parameters (ALT, AST, ALP and TB level)

The liver damage was assessed by the estimation of serum activities of ALT, AST, ALP and TB using commercially available test kits (Span diagnostic Limited, India) and the results were expressed in IU/l.

Estimation of hepatic antioxidant enzymes activities (MDA, GSH, CAT and SOD)

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was estimated by its ability to react with thiobarbituric acid forming a 1:2 adduct [24]. Estimation of GSH content was performed spectrophotometrically, using Ellmans reagent [25]. Catalase activity was kinetically determined by monitoring the rate of decomposition of hydrogen peroxide [26]. SOD activity was measured by the degree of inhibition of the reduction of nitroblue tetrazolium dye [27].

Histopathological studies of liver tissues

The sample of liver tissue was collected and fixed in 10% formalin, dehydrated in graduated ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections 4–5 µm thick were prepared by microtome and then stained with hematoxylin and eosin (H & E) dye for photomicroscopic observation including cell necrosis, fatty change, hyaline regeneration, ballooning degeneration. Finally, the sample was analyzed by assessing the morphological changes under a light microscope.

Statistical analysis

The data are expressed as mean±SEM from 6 rats in each group. The difference among means has been analyzed by one-way analysis of variance (ANOVA) followed by *Tukey's* multiple comparison test. The minimum level of significance was fixed at $P<0.05$.

RESULTS

Acute toxicity study

Acute toxicity study shows that VLS was safe up to 2000 mg/kg, body weight. Animals were alive, active and healthy during the observation period.

Estimation of biochemical parameters

The hepatic injury induced by 0.3 % CCl_4 (10 ml/kg, i. p.) resulted in an increase in serum ALT, AST, ALP and TB levels as compared to the normal control group. The treatment of rats with VLS (1 ml/kg, p. o.) significantly reduced serum ALT, AST, ALP and TB levels as compared to the CCl_4 treated group. This observation was comparable to that of silymarin, a standard hepatoprotective drug.

Estimation of antioxidant enzymes activities

The treatment of rats with the VLS (1 mg/kg, i. p.) effectively restored the elevated levels of MDA, which was comparable to silymarin (100 mg/kg, i. p.).

The GSH, CAT and SOD concentrations of liver tissue were significantly reduced with the administration of 0.3 % CCl_4 (10 ml/kg, i. p.). A significant increase in GSH, CAT and SOD levels was observed with VLS (1 ml/kg, i. p.) dose and silymarin treatment.

Table 2: Effect of Virgoliv syrup on serum marker enzymes in CCl_4 induced hepatotoxicity in rats

Groups	Dose	ALT (IU/l)	AST (IU/l)	ALP (IU/l)	TB (g/dl)
Control	10 ml/kg, p. o	50.33±4.20	58.80±5.41	29.75±2.57	0.37±0.08
CCl_4	0.3% (10 ml/kg, i. p.)	108.8±11.78***	97.50±9.80***	55.68±5.50***	1.17±0.13***
VLS+CCl_4	1 ml/kg, p. o.	68.83±5.78###	67.60±5.38###	33.29±3.20###	0.64±0.16###
Silymarin+CCl_4	100 mg/kg, p. o.	62.28±6.16###	63.80±5.08###	30.12±2.90###	0.52±0.12###

Values are mean±SEM, n = 6, *** (P<0.001), # (P<0.05), ##(P<0.01), ###(P<0.001), CCl_4 data were analyzed by using one-way ANOVA followed by *Tukey's* multiple comparison test. AST, ALT, ALP and TB (Serum Alanine Transaminase, Serum Aspartate Transaminase, Serum Alkaline phosphatase and Serum Total bilirubin

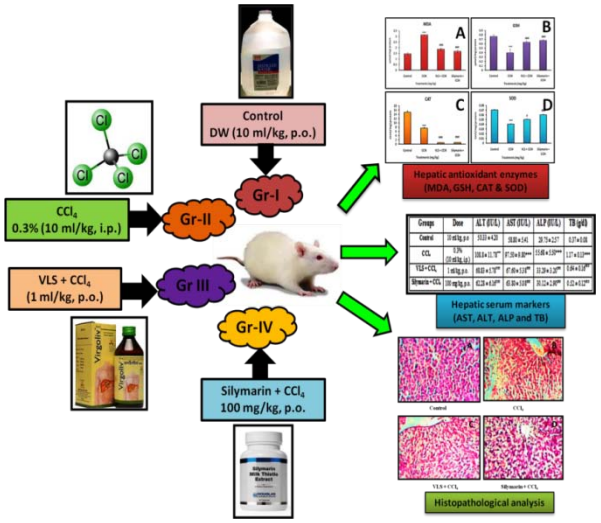

Fig. 1: Experimental procedure and findings of CCl_4 induced hepatotoxicity

Histopathological studies of liver tissues

The histological observations fundamentally supported the results obtained from serum biomarkers and hepatic antioxidant enzymes. The liver section in control animals had normal hepatic cells with well preserved cytoplasm, prominent nucleus and central vein (fig. 3A), whereas administration of carbon tetrachloride in animals showed severe centrilobular necrosis, inflammatory changes, lymphocyte infiltration, vacuolization and ballooning degeneration indicating severe damage of liver cytoarchitecture (fig. 3B). The administration of VLS (1 ml/kg, i. p.) showed recovery and protection from hepatocyte degradation, centrilobular necrosis, vacuolization (fig. 3C). All the fig. are compared with standard drug silymarin as shown in (fig. 3D).

DISCUSSION

The present study demonstrates the hepatoprotective effects of VLS against CCl_4 induced liver injury in rats. The vital organs present in vertebrates and some other animals is liver responsible for protein synthesis, and building of biochemical substances crucial for metabolism and detoxification of toxic chemicals and drugs. Hence, it is considered as one of the target organ for chemically induced liver injuries. CCl_4 is act as a direct hepatotoxic chemical responsible for production of liver centrilobular necrosis and steatosis [28-30]. CCl_4 is metabolized to trichloromethyl radical ($\bullet CCl_3$) is mediated by CYP2E1, as well as CYP2B1 and possibly by CYP3A enzyme [31].

The $\bullet CCl_3$ radical reacts with molecular oxygen to form a highly reactive trichloromethyl peroxy radicals ($CCl_3OO\bullet$) that are important mediators of CCl_4 induced liver injury. These trichloromethyl and trichloromethyl peroxy radicals then react with the sulfhydryl groups of glutathione and protein thiols of cell membrane. The covalent binding of these radicals to sulfhydryl-containing proteins in cells initiate the progression of membrane lipid peroxidation and cell necrosis [8]. This process results into the loss of cellular membrane integrity resulting in formation of pores in the cell membranes. A variety of biochemical enzymes such as, AST, ALT, ALP and bilirubin are usually present in the liver cells. In hepatic injuries, the leakage of these enzymes takes place from the hepatic cells into the blood stream leading to elevated levels of these enzymes in serum. [32] In the present study VLS a liquid herbal formulation containing many herbal plants rich in antioxidants restored the increased hepatic markers levels towards normal.

Several research studies have confirmed that the hepatic fibrosis can be attenuated if treated at a nearly stage and if not treated it is converted to irreversible cirrhosis and hepatocellular carcinoma [33-34]. Numerous studies have revealed that CCl_4 hepatotoxicity may be prevented by antioxidants supplementation which represents a rational use in the treatment of liver disorders [35-37]. Cellular antioxidant enzymes represent the innate defense system of the human body and these enzymes can be effectively scavenged by free radicals and limit their toxicity to human body [38]. Superoxide dismutase, catalase, reduced glutathione are the major enzymes have free radical scavenging property which involved in the protection of cells from oxidative stress [39]. SOD, CAT and GSH ameliorate the damaging effects of superoxide anion and hydrogen peroxide by converting them into nontoxic compounds [40]. SOD is a metalloenzyme that protects the cell from toxicity by the dismutation of superoxide radical into hydrogen peroxide and oxygen [41]. Hydrogen peroxide is a major product formed in normal cellular functioning which in extreme quantity can cause oxidative stress. Catalase, an enzyme with the prosthetic group as hematin is predominantly present in all aerobic cells in the cytochrome system. It is sufficiently available in the liver and responsible for normal functioning of hepatocytes [42]. It catalyses

the decomposition of hydrogen peroxide to form water and oxygen. Glutathione is a non-enzymatic antioxidant which is involved in the protection of cells from oxidative stress [43]. The reduced form of glutathione donates the electron to reactive species converting them into nonreactive species. The reactive glutathione thus formed get oxidized to form glutathione disulfide. Reduced glutathione has been recycled from glutathione disulfide by the enzyme glutathione reductase. Thus reduced glutathione is directly involved in the scavenging of reactive oxygen radicals formed in the cell due to oxidative stress [44]. All these antioxidant enzymes (SOD, CAT and GSH) are equally involved in the first line of defense mechanism against oxidative stress generated by free radicals. Natural antioxidants protect the cell from free radicals either by scavenging the free radical species or by inhibiting the oxidative reaction by self oxidation. Lipid peroxidation causes major cellular damage due to generation of reactive oxygen species by CCl_4 intoxication. These free radicals react with the phospholipids of the cell membrane, initiating a chain of reactions. This lipid peroxidation generates a number of end products that are injurious to the cell. MDA is a highly reactive end product which is considered as the indicator of lipid peroxidation [24].

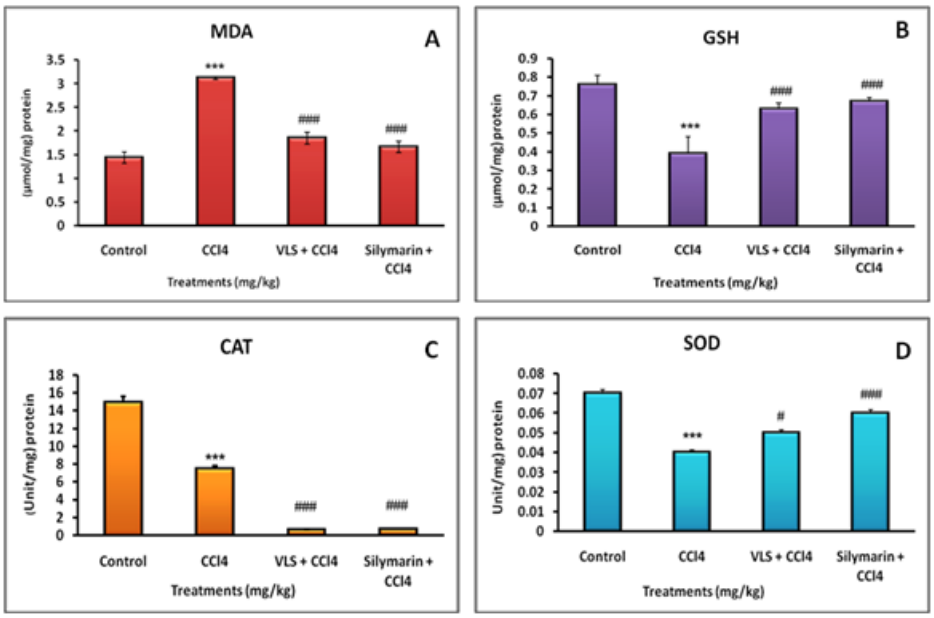

Fig. 2: Effect of VLS on hepatic antioxidant enzyme levels in CCl_4 induced hepatotoxicity in rats A) MDA B) GSH C) CAT D) SOD

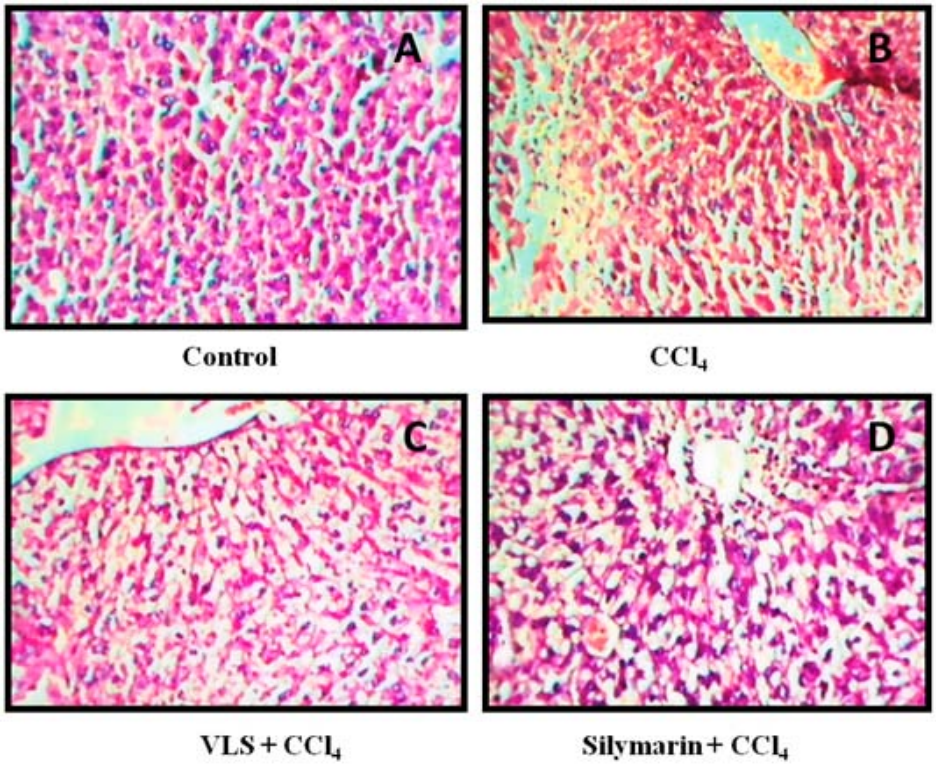

Fig. 3: Histopathological sections of liver with CCl_4 induced hepatotoxicity (A) Control rats (B) CCl_4 treated rats (C) VLS+CCl_4 treated rats (D) Silymarin+CCl_4 treated rats

In our study it has been found that the drastic decrease in the levels of SOD and GSH oxidative enzyme has been reported in CCl_4 treated group. It may be due to inactivation of the antioxidant enzymes by reactive oxygen species [21]. Treatment with VLS (1 ml/kg, i. p.) Significantly ($p<0.05$, $p<0.001$) increased the enzymatic activities of SOD and GSH towards normal levels. The hepatic CAT activity was found to be decreased after CCl_4 administration. Animal groups administered with VLS (1 ml/kg, i. p.) significantly ($p<0.001$) increased the level of CAT activity. In this present study it has been found that the level of MDA was significantly higher in the CCl_4 treated group compared to the normal group. The treatment with VLS (1 ml/kg, i. p.) has significantly ($P<0.001$) decreased the levels of MDA. Many research studies have reported that scavenging of free radical is an important mechanism of hepatoprotective activity by inhibiting the binding of CCl_4 free radicals to the cell membrane and thus protecting the cell from lipid peroxidation and cellular damage [45, 46].

The serum and hepatic biochemical results of hepatoprotective activity of the VLS have been further confirmed by the histopathological analysis of liver tissues. In the CCl_4 model group, the severe hepatic injury including centrilobular necrosis, inflammatory changes, lymphocyte infiltration, vacuolization and ballooning degeneration indicating severe damage of liver cytoarchitecture was observed in the liver histopathological sections. The VLS treated groups showed lesser destruction in the cellular architecture which might be attributed to the rapid regenerative capacity of liver after damage [47]. The results of histopathological analysis were compared to standard hepatoprotective drug silymarin. Silymarin is a known hepatoprotective agent obtained from plant *Silybum marianum* is reported to have a protective effect on hepatocytes plasma membrane and possess multiple mechanisms of actions against different types of hepatotoxic agents.

VLS is a liquid herbal formulation and its major constituents are *Eclipta alba, Plumbago zeylanica, Andrographis paniculata, Boerhavia diffusa, Solanum nigrum, Tecomella undulate, Picrorhiza kurroa, Cissampelos pareira, Operculina turpethum, Embelia ribes, Cichorium intybus, Phyllanthus niruri, Tinospora cordifolia, Tephrosia purpurea, Piper longum, Berberis aristata, Cassia occidental* reported to have a wide range of antioxidant activity. It might postulated that the hepatoprotective effect of VLS is may be due to its inhibitory effect on free radical formation as evident by recovery of CAT, GSH and SOD contents towards normalization and decreased lipid peroxidation (MDA). Other biochemical and histopathological parameters indicate the structural and functional integrity of the hepatic cells and provide further support to the proposed mechanism of action. VLS appears to be safe and effective future therapy for treatment of liver fibrosis. However, the hepatoprotective and antioxidant properties of VLS need to be confirmed using the larger number of animals, by characterizing the active constituent(s) of these plants as well as its mechanism(s) of action.

CONCLUSION

VLS is liquid poly herbal formulation having hepato-spleno stimulant property. In the present study, VLS rich in antioxidant compounds reduced CCl_4 induced hepatotoxicity by increasing antioxidant enzyme activities, inhibiting lipid peroxidation and decreasing the levels of serum hepatic marker enzymes. The restoration of hepatic enzyme activities indicated the improvement in functional status of liver and the improved excretory and secretary capacity of hepatocytes. The serum and hepatic antioxidant results of VLS have been further confirmed by the histopathological analysis of liver tissues. The results also suggest that its mechanism of action might be associated with the antioxidative activity. Our work substantiated the well known correlation between the hepatoprotective activity and antioxidant activities. Further studies are in progress to identify the active constituent responsible for the hepatoprotective and antioxidant activity of Virgoliv syrup. Thus, the results provide a basis for usefulness of VLS in the treatment of liver fibrosis.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

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