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Protective Effect of Secoisolariciresinol Diglycoside in Carbon Tetrachloride Induced Hepatotoxicity in Rats

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Abstract

Aims: Effects of synthetic Secoisolariciresinol Diglycoside (SDG) on hepato-marker enzymes as well as on histomorphological changes in liver against carbon tetrachloride (CCl_4)-induced hepatotoxicity were evaluated.

Main Methods: Intoxication of animals by CCl4 significantly increased ($p<0.01$) Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Serum Alkaline Phosphatase (SALP) and Bilirubin level, and diminished thymus and spleen indices, which indicate acute hepatocellular damage and biliary obstruction. Intoxicated animals were treated orally with synthetic SDG at (12.5 and 25 mg/kg b.w) and Silymarin (25 mg/kg) for 14 consecutive days. The hepatocellular damage was determined by measuring the changes of these parameters using Wistar albino rats weighing 180-220 g.

Key Findings: *In vivo* results indicated oral administration of SDG at 12.5 and 25 mg/kg body weight could overcome CCl_4-induced immunosuppression and significantly ($p<0.05$) exhibited protective effect. High activity was found for synthetic SDG at 25 mg/kg body weight dose level and the reductions of SGOT, SGPT, SALP and total bilirubin in serum were 66.23%, 83.38%, 64.65% and 65.64%, respectively. In addition, SDG increased thymus and spleen indices by 2.7 and 2 folds, respectively when compared with CCl_4 treated rats. Histological examination of liver of treated animals also supported the hepatoprotective activity of SDG.

Significance: It is concluded that synthetic SDG have antihepatotoxic action against CCl4 induced hepatocellular injury and could effectively attenuate the alteration within the studied parameters in dose-dependent manner and prevent oxidative damage in immunological system.

Keywords: Synthetic SDG; Hepatotoxicity; SGOT; SGPT; SALP; Histopathology; CCl_4 induced hepatotoxicity

Introduction

Liver is constantly exposed to environment toxicants and abused by poor drug habits, alcohol and over the counter drugs which can eventually lead to various liver ailment [1,2]. Thus, disease of the liver continues to be a worldwide health problem. Scientists face a serious challenge to International Public Health through different ways to discover adequate synthetic drugs for the treatment of liver ailments with very little side effects.

Reactive Oxygen Species (ROS) can be made in different ways within living organisms, normal aerobic respiration stimulates polymorphonuclear leukocytes and peroxisomes appear to be the main endogenous sources of most of the oxidants formed by cells. Exogenous sources of ROS include tobacco smoke, solvents, certain pollutants, pesticides and organic [3-5].

Oxidative stress produced from the toxic effects of free radicals on the tissues plays a key role in the pathogenesis of various pathological conditions such as ageing process, asthma, inflammation, mongolism, anemia, arthritis, neurodegeneration, Parkinson's disease, ischemia, and perhaps dementia [6]. Antioxidants protect the human body by scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms thereby helping in preventing the free radical caused diseases [7].

Epidemiological studies have shown that diets rich in plants product are associated with a decreased risk of cardiovascular diseases [8] and certain types of cancers [9]. These beneficial health effects have been attributed in part to the presence of phenolic compounds in dietary plants, which may exert their effects as a result of their antioxidant properties [10,11]. The antioxidant effect of lignans varies considerably according to their backbone structures and kind of functional groups present in their chemical structure.

The abundant major lignan found in flaxseed is Secoisolariciresinol Diglucoside (SDG) which has been shown to exert *in vivo* and *in vitro* antioxidant activities [12] and is effective against development of hypercholesterolemic atherosclerosis [13] and Type I and II diabetes [14]. Moreover, flaxseed SDG has got chemoprotective potential in both colon [15] and mammary [16] cancers and displayed greater efficacy in reducing deoxyribose oxidation and DNA strand breakage with maximum protection offered at 100 Mm [17].

Based on *in vitro* and *in vivo* studies, many classical antioxidants have been appeared to protect various cells like hepatocytes and nephrocytes against lipid peroxidation or inflammation, thereby

preventing the occurrence of kidney damage, hepatic necrosis and other radical associated effects [18].

Therefore, the current study was undertaken to explore the hepatoprotective effects of synthetic SDG by measuring the levels of hepatic diagnostic enzymes markers, as also bilirubin level along with thymus and spleen indices and total protein in Wistar albino rats.

Materials and Methods

Drugs and chemicals

All the chemical solvents used in the experiments were of analytical grade from Ranbaxy Chemicals Ltd. (Mumbai, India). The diagnosis hepatic enzymes (SGOT, SGPT, SALP), Bilirubin and total Protein kit were procured from Span Diagnostics Ltd. (Surat, India). Standard Silymarin was purchased from Micro labs, Tamilnadu. India.

Synthesis of SDG

SDG was chemically synthesized from commercially available compounds. In brief, a novel five-step synthesis sequence starting from bromination of commercially available 3,4-dimethoxy toluene with N-bromo succinimide in presence of carbon tetrachloride to achieve 1,2-dimethoxy-4-bromomethylbenzene (1). Further stirring of compound (1) with 1,4-butanediol in presence of n-butyl lithium and DMF 2,3-bis afforded (3,4-dimethoxy benzyl) butane-1,4-diol (2). Sequential condensation of compound (2) with 2,3,4,6 tetra-o-acetyl α-D glucopyranosyl bromide gave 2,3- bis(3,4-dimethoxybenzyl)butane-1,4-O-tetra acetyl glucose (3). Compound, 2,3-bis(3,4- dimethoxybenzyl)butane-1,4 -O- glucose (4) was achieved by deacetylation of compound (3) and finally SDG was recovered *via* regioselective partial demethylation of compound (4).

Animals

Healthy Wistar albino rats of either sex weighing 180-220 g (40 in total) were used in the study. The rats were procured from the animal house of the Department of Studies in Zoology, University of Mysore. Mysore, India. They were housed in well ventilated polypropylene cages with paddy husk as bedding at room temperature (24°C ± 2°C) and relative humidity of 40% in hygienic condition. A 12/12-h light–dark day cycle was followed. All the animals were allowed to have free access to water ad libitum and fed with standard commercial pelleted rat diet (Bangalore, India).

The hepatoprotective activity of the synthetic SDG was tested using CCl_4 model. The experiments were carried out based on the guidelines of 'Committee for Prevention and Control of Scientific Experimentation on Animals' (CPCSEA) New Delhi. All the experimental procedures and protocols used in this study were approved by the Institutional Animal Ethics Committee (IAEC), University of Mysore, Mysore, India, (Approval No :UOM/IAEC/01/2011). Prior to the start of experiments, the animals were allowed to acclimatize to laboratory conditions for a week.

The animals were randomly divided into eight groups, each consisting of five animals. Group-I (normal control) received normal saline solution orally (2 ml/kg); Group-II and III received olive oil with and without treatment of toxin, respectively. Group-IV (induction control) was given a single intraperitoneal dose of CCl_4 (2.0 g/ kg b.w, I.P.). Group-V served as positive control and received orally 25 mg/kg b.w. of Silymarin mixed with olive oil. Group-VI and VII served as pretreatment groups and received synthetic SDG at 12.5 and 25 mg/kg b.w, per orally, respectively. Group-VIII served as post treatment group and received synthetic SDG (25 mg/kg b.w, P.O.) after injection of CCl_4 (I.P.).

All groups were administered with their respective treatments for 14 consecutive days. The animals of Group-II, IV, V, VI and VII were given single dose of CCl_4 (2.0 g/ kg b.w, I.P.) 6 h after the last feeding on the fourteenth day.

Assessment of hepatoprotective activity

Hepatoprotective activity was evaluated biochemically and histopathologically. After 24 h of CCl_4 administration, the animals were anesthetized, weighed and dissected under ether anaesthesia. The liver, spleen and thymus were excised from the animal and weighed immediately. The thymus and spleen indices were calculated as for the following formula:

Thymus/spleen index=weight of thymus/spleen ÷ body weight × 100.

Blood samples were withdrawn from each rat by direct cardiac puncture under light ether anaesthesia. Blood samples were collected in previously labelled heparinized centrifuging tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min and was used for analysing SGPT, SGOT, SALP [19,20], bilirubin [21] and total protein [22].

Liver from each animal was removed after dissection and preserved in 10% formalin for histopathological study. Representative blocks of liver tissues from each lobe were taken and possessed for paraffin embedding as for the standard microtechnique [23]. Sections (5 µm) of livers stained with hemotoxylin and eosin were used for histopathological studies.

Statistical analysis

The values are expressed as mean ± SD. The statistical analysis was carried out by One Way Analysis of Variance (ANOVA) followed by the Student's t-test to determine the significant differences between treatments.

Results

Effect of synthetic SDG on thymus and spleen indices in CCl_4 induced rats

It is believed that thymus and spleen are important immune organs, and their indices could partially reflect the immune function of the organism. The effects of different concentrations of synthetic SDG on thymus and spleen indices in Wistar albino rats are summarized in Figure 1.

Administration of CCl_4 (2.0 g/ kg b.w, I.P.) to Group IV rats caused a significant decrease ($p<0.05$) in both spleen and thymus indices compared to PBS treated rats (Group I). This indicated that immune functions of spleen and thymus of rats were diminished with CCl_4 treatment. Treating rats with synthetic SDG at 12.5 mg/kg showed a significant increase ($p<0.01$) in spleen and thymus indices compared to CCl_4 treated group. In addition, rats pretreated with synthetic SDG at 25 mg/kg showed a marked increase ($p<0.05$) in thymus and spleen indices compared to CCl_4 treated group. Subsequently, posttreatment of synthetic SDG at 25 mg/kg showed significant effect ($p<0.01$) on

thymus and spleen indices compared to CCl_4 treated group. The restoration of thymus and spleen indices caused by synthetic SDG was dose dependent and comparable to that of silymarin treated animals (Group-V).

These results indicated that the immune function was reduced in animals treated with CCl_4. However, treating rats with synthetic SDG at different doses produced significant and dose dependent increase in thymus and spleen indices. Therefore, it is evident that administration of synthetic SDG overcame the immunosuppressed action of CCl_4.

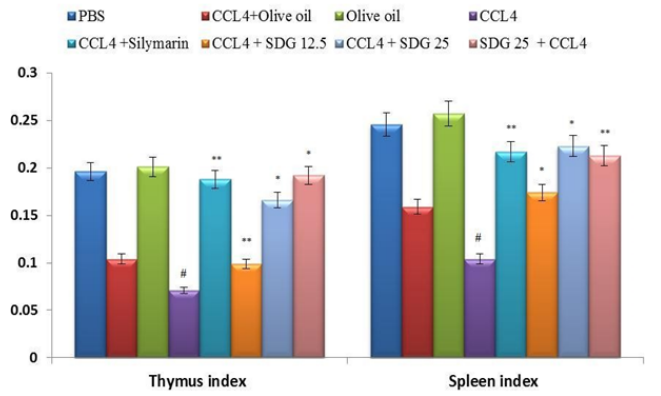

Figure 1: Effect of synthetic SDG on thymus and spleen indices in CCl_4 induced rats. Data were presented as means ± SD (n=5). #P<0.05, compared with normal group (PBS). *P<0.05, compared with CCl_4 treated group. **P<0.01, compared with CCl_4 treated group.

Effect of synthetic SDG on biochemical parameters in CCl_4 induced rats

The hepatocurative effect of synthetic SDG in CCl_4-induced hepatotoxicity in albino rats at different doses was carried out. The results showed that, treatment of rats with CCl_4 (Group-IV) caused a significant increase ($p<0.01$) in levels of SGOT, SGPT, SALP, bilirubin and also increase in liver weight and decrease in serum total protein when compared to normal control (Group-I). In contrast, pre-treatment of rats with Silymarin (Group-V) for 14 consecutive days lowered significantly ($p<0.05$) the levels of SGOT, SGPT, SALP and bilirubin and restored the serum total protein.

The rats pretreated with synthetic SDG (Group-VI & VII) demonstrated dose dependent control of elevation of the biochemical parameters. Pretreatment of rats with synthetic SDG at 12.5 and 25 mg/kg for 14 consecutive days significantly ($p<0.05$) controlled the elevation of biochemical parameters which is comparable with Silymarin (Group-V). Post treatment with synthetic SDG of rats intoxicated with carbon tetrachloride, illustrated significant ($p<0.05$) recovery of biochemical parameters when compared to CCl_4 treated rats (Group-IV). Table also shows the comparison of effects among the untreated (normal control), CCl_4 treated (induction control) and silymarin treated (standard) group with different doses of synthetic SDG treated group of rats. The synthetic SDG exhibited significant protection against CCl_4-induced liver injury as manifested by the reduction in toxin mediated rise in SGPT, SGOT and SALP level of rats.

Effect of synthetic SDG on histology of CCl_4 induced rats liver

Histopathological results provided supportive evidence for biochemical findings. Histology of liver section of one animal from each group is presented in Figure 2. As shown in Figure 2(A), liver section of normal control rat (Group-I) exhibited distinct hepatic cells, the central vein is seen and there is no visible lesion, the sinusoids are normal and the epithelium lining is also seen. CCl_4 induced liver damage can be observed directly in Figure 2(D), where the section shows total loss of hepatic architecture, massive fatty change, necrosis, lymphocyte infiltration, loss of cellular boundaries, and joining together of nucleus. In addition congestion of sinusoids, Kupffer cell hyperplasia, crowding of central vein and apoptosis are also evident.

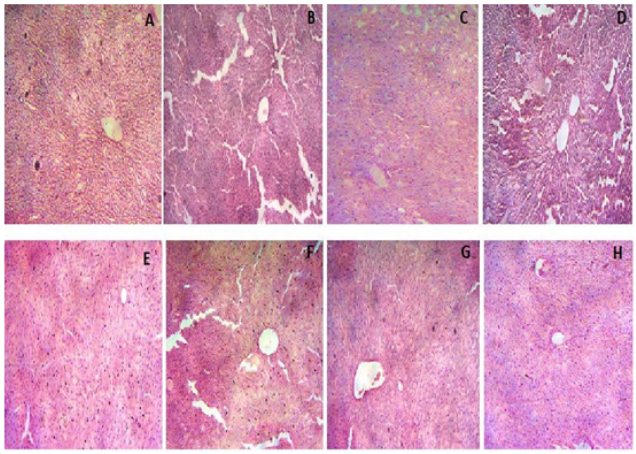

Figure 2: Photomicrographs showing the effect of Synthetic SDG (12.5 and 25 mg/kg) and Silymarin on liver histopathology of CCl_4 treated rats. (A) Liver section of normal group; (B) Olive oil +intoxicated with CCl_4 group; (C) Olive oil group ; (D) CCl_4 control group; (E) Silymarin (25 mg/kg)+intoxicated with CCl_4 group ;(F) SDG (12.5 mg/kg)+intoxicated with CCl_4 group; (G) SDG (25 mg/kg)+intoxicated with CCl_4 group; (H) intoxicated with CCl_4+SDG (25 mg/kg) group. (Magnification 10X).

Rats treated with Silymarin (Figure 2(E)) show less necrosis and almost normal liver architecture with no obvious necrosis as compared to CCl_4 treated rats section (2(D)). Sections of Figure 2 (F and G) pretreated groups with SDG (12.5 and 25 mg/kg) show gradual recovery of hepatic architecture and fatty changes and the central vein is seen and the sinusoids are having hepatitis alteration. Only very slight lymphocyte infiltration is observed in Figure 2G. These sections are nearly comparable to the silymarin treated group. In the posttreatment with 25 mg/kg SDG of intoxic rats for 14 consecutive days, section suggests moderate degree of damage, with some fatty change, necrosis, and lymphocyte infiltration (Figure 2H). The overall histopathological examination reveals hepatic degeneration, necrosis and fatty infiltration in CCl_4 treated rats indicating liver damage and the histopathology results suggest that this damage could be overcome by utilizing SDG up to 25 mg/kg as this concentration will not have any adverse effects on human liver.

Group	Treatment	Total Bilirubin mg/dL	direct Bilirubin mg/dL	Indirect Bilirubin mg/dL	SGOT U/L	SGPT U/L	SALP U/L	Total Protein g/dL
I	PBS (Control)	0.882 ± 0.04	0.164 ± 0.02	0.402 ± 0.02	389.3 ± 5.6	77.38 ± 4.74	199.72 ± 6.1	7.1 ± 0.62
II	CCl_4+Olive oil	3.15 ± 0.05	1.56 ± 0.278	2.09 ± 0.24	920.2 ± 22.3	401.6 ± 14.15	493.4 ± 11.78	4.81 ± 0.72
III	Olive oil	0.788 ± 0.05	0.152 ± 0.028	0.268 ± 0.04	340.2 ± 5.4	67.64 ± 3.67	198.64 ± 11.14	6.98 ± 0.29
IV	CCL_4	3.44 ± 0.42^d	1.72 ± 0.319^d	2.22 ± 0.37^d	1120.6 ± 242^d	569.1 ± 7.38^d	526.2 ± 22.77^d	4.44 ± 0.45^d
V	CCl_4+Silymarin 25 mg/kg	1.67 ± 0.05^c	0.157 ± 0.03^c	0.202 ± 0.9^a	360.6 ± 9.44^b	78.6 ± 3.0^a	190.2 ± 3.83^a	7.82 ± 0.645^b
VI	CCl_4+SDG 12.5 mg/kg	1.786 ± 0.04^a	0.276 ± 0.05^b	0.396 ± 0.2^b	454.24 ± 6.3^c	115.2 ± 2.46^c	259. 4 ± 3.75^b	5.96 ± 0.24^b
VII	CCl_4+SDG 25 mg/kg	1.284 ± 0.056^c	0.142 ± 0.09^c	0.198 ± 0.1^a	389.8 ± 3.96^a	88.4 ± 3.70^a	197.9 ± 3.6^c	8.85 ± 0.35^a
VIII	*SDG 25 mg/kg+CCl_4	1.182 ± 0.046^a	0.136 ± 0.07^a	0.218 ± 0.09^c	378.4 ± 3.05^a	94.6 ± 3.1^a	186 ± 2.74^a	6.62 ± 0.39^c

Values are mean ± SD, (n=5); number of rats used in each group=5; ^{a}P<0.001 compared to respective CCl4 treated group (IV). ^{b}P <0.05 compared to respective CCl_4 treated group (IV). ^{c}P<0.01 compared to respective CCl4 treated group (IV). ^{d}P<0.01 compared to respective control group (I). *Pre-treatment group.

Table 1: Effects of synthetic SDG administration on the levels of SGOT, SGPT, SALP, Bilirubin and Protein in serum of CCl4 induced rats.

Discussion

The possible role of hepatoprotective activity of synthetic SDG in carbon tetrachloride induced hepatotoxicity has been demonstrated. CCl_4 is commonly used hepatotoxin in the experimental study of liver ailments. The CCl_4 hepatotoxic effects are primarily due to production of free radicals [24]. Thus, it was extensively used as a liver toxicant, and its metabolites such as trichloromethyl radical (CCl_3S) and trichloromethyl peroxyl radical (CCl_3O_2S) are found to be involved in the liver pathogenesis [25]. The biotransformation of CCl_4 by the cytochrome P_45_0 system produces free radicals (CCl_3S, CCl_3O_2S), which in turn bind to cell membranes and organelles to elicit lipid peroxidation [26]. The massive production of free radicals in CCl_4 caused liver damage provokes a sharp increase in lipid peroxidation in liver due to the increase in interaction of these free radicals with phospholipids structure and finally destroying the organ structure [27].

The several doses influence of synthetic SDG has been investigated for their efficacy in controlling the CCl_4 induced liver damage. The findings of the current study proved that the treatment of CCl_4 intoxicated rats with synthetic SDG restored the depleted thymus and spleen indices. The increase in thymus and spleen indices suggested that SDG effectively stimulated the immune function of treated rats. SDG showed significant effects on both thymus and spleen indices as shown in Figure 1. Thymus is the organ in which T-lymphocytes develop, differentiate, and mature, while spleen contains T and B-cells. The SDG acts on T and B-lymphocytes resulting in different effects on spleen and thymus indices in treated albino rats. However, to understand the exact mechanism requires further and in-depth investigations.

Liver function can be determined by estimating the activities of SGPT, SGOT, and SALP which are enzymes originally present in cytoplasm with high concentration. These enzymes are leaked into the blood stream in conformity with the extent of liver damage when there is hepatopathy [23]. These enzymes are used as diagnostic indicators of hepatic injury. SGOT, SGPT, SALP and serum bilirubin are the most sensitive tests employed in the diagnosis of hepatic diseases [28,29]. The major release of these enzymes from the cells are indicative of cellular leakage and loss of functional integrity of the cell membrane in liver [30,31] and this could be as a result of hepatocyte necrosis or abnormal membrane permeability. SGOT is a sensitive indicator of acute damage of liver and elevation of this enzyme in non-hepatic diseases is unusual. SGPT is more selectively a liver parenchymal enzyme than SGOT [32].

It is believed that liver damage is reflected by an increase in the levels of hepatospecific enzymes; these are cytoplasmic and are released into circulation when cellular damage occurs [33].

CCl_4-treated rats experiment extensive liver damage induced by toxin. The elevated levels of these marker enzymes in the current study were observed in Group IV rats. However, during the study, reduction of SGOT, SGPT and SALP concentrations were observed due to the influence of SDG (Table 1).

Bilirubin is considered as one of the most useful clinical clues to the severity of necrosis and its accumulation is a measure of binding, conjugation and excretory capacity of hepatocyte. SDG treatment decreased serum bilirubin level in liver damage induced by CCl_4, indicating the effect of SDG in normalizing the liver functions.

In this study, significant increases in total bilirubin content and SGOT, SGPT and SALP activities in the CCl_4 treated group are the indicatives of liver damage. SDG treatment attenuated the CCl_4 induced increase in total bilirubin and SGOT, SGPT and SALP activities. CCl_4 induces dramatically increase in liver weight due to blocking the secretion of hepatic triglycerides in plasma [34]. The increase in liver weight in rats was overcome with the administration of either Silymarin or SDG in dose dependent manner. The histological observations of liver of studied animals induced by CCl_4 administration provided complementary evidence to prove that pre and post treatment with synthetic SDG attenuated the cytoplasmatic changes. This effect could be attributable to the antioxidant activity of SDG, which normalizes the oxidative threat caused by CCl_4 and restored normal physiological functions.

The mechanism by which synthetic SDG offers protection towards CCl_4-induced hepatocellular metabolic alterations could be due to inducing microsomal enzymes either by accelerating the detoxification and excretion of CCl_4 or by inhibition of lipid peroxidation through inhibition of cytochrome P-450 aromatase favoring liver regeneration.

Conclusion

The present study was aimed to evaluate the efficacy of synthetic SDG on the liver functions in CCl_4 induced injuries. Enzymatic activities, bilirubin and microscopic appearance of liver were used as parameters and hepatocurative studies were performed. Activities of SGPT, SGOT and SALP in serum were increased in CCl_4-intoxicated rats. A marked elevation in the concentration of bilirubin was observed in the hepatotoxin-treated rats. The findings of the study have shown the ability of SDG to recover from the CCl_4 damage hepatic enzymes, bilirubin and thymus and spleen indices levels to almost the normal levels and restore the normal functioning of the liver. The severe necrosis and disappearance of nuclei in liver histology of CCl_4-treated rats which could be due to the formation of highly reactive radicals were very much reduced in rats treated with synthetic SDG. The current study results provide scientific bases to the use of SDG in liver disease and can be used to compensate the declining activities of antioxidant enzymes and thereby reduce the risks of lipid peroxide.

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