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Hepatoprotective and *in vivo* antioxidant effects of aromatic acetals against CCl_4 - induced liver damage in rats

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ABSTRACT

Hepatoprotective activity of the p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal were investigated using rats with CCl_4-induced liver damage. The different groups of animals were administered with carbon tetrachloride (CCl_4). The acetals at the dose of 10 ml kg^{-1} and silymarin 25 mg kg^{-1} were administered to the CCl_4 treated rats. The effect of acetals and silymarin on antioxidant enzymes like superoxide dismutase [SOD], catalase [CAT], glutathione peroxidise [GPX] and thiobarbituric acid [TBARS] were assayed. The serum enzymes like aspartese aminotransferase [AST], alanine aspartese aminotransferase [ALT], alkaline phosphatase [ALP] and acid phosphatase [ACP] were determined in the rats induced hapatotoxicity by CCl_4 to measure liver damage. The acetals and silymarin produced significant ($p<0.001$) hepatoprotective effect by decreasing the activity of serum enzymes and bilirubin. Specific biochemical parameters were estimated in blood and in liver homogenate. Histopathological examinations of the liver were undertaken to monitor the liver status. From the results, it was suggested that acetals possess potent hepatoprotective and antioxidant properties.

Keywords: aromatic acetals, *in vivo* antioxidant, hepatoprotective effects.

INTRODUCTION

The experimental intoxication induced by carbon tetrachloride (CCl_4) is widely used for modelling liver injury in rats. Hepatotoxicity is connected with severe impairment of cell protection mechanisms. The location of liver injury is defined mainly by the biotransformation of CCl_4 which is cytochrome P-450 dependent [1, 2]. Free radicals initiate the process of lipid peroxidation, which is generally caused by the inhibition of enzyme activity [3].

Antioxidants play a crucial role in hepatoprotective ability and hence the search for crude drugs has become a cebtral focus of study of hepatoprotective today [4, 5]. Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats [6], which is the cause of rancidity. The possible mechanisms of actions of antioxidants were first explored when it was recognised that a substance with anti-oxidative activity is likely to be one that itself readily oxidised. Realizing the fact, this research was carried out to evaluate the hepatoprotective and antioxidant activity of p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal. Acetals find applications as new initiators for cationic polymerization [7] of isobutyl vinyl ether, in asymmetric heterocyclic addition reactions [8], act as sensitizers [9], precursor of acidic drugs [10] etc. The present investigation reports the antioxidant activity of such acetals. A general review on antioxidant and their applications is well established [11]. Recently the study of antioxidant activity of chemical compounds [12], and also the hepatoprotective activities of plants [13] are more pronounced, in order to achieve the compound to be useful for medicinal purposes.

EXPERIMENTAL SECTION

The substrates p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal were prepared in the laboratory and their purities were checked by usual methods [14-15]. Triply distilled water was used in preparing all aqueous solutions. All the other chemicals were of analytical grade.

Animal studies were carried out using male wistar albino rats (180-230 g). They were obtained from Sri Venkateswara Enterprises, Bangalore. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 ºC) with dark and light cycle (12/12 h). The animals were fed with normal pellet diet supplied by Hindustan Lever Ltd., Kolkata, India and fresh water *ad libitum*. The animals were maintained in an animal house with standard facilities. The animals were acclimatised for three days under laboratory conditions. Ethical clearance for handling the animals was obtained from ethic committee constituted for the purpose having CPCSEA approval (No: 265).

Drugs and chemicals

Silymarin was purchased from Microlabs, Holar, Tamil Nadu, India. Bovine serum albumin (Sigma Chemical, St. Louis, MO, USA), thiobarbituric acid, nitrobluetetrazolium chloride (NBT) (Loba Chemie, Bombay, India), carbon tetrachloride (Sicco Research Laboratory, Bombay). The solvent and / or reagent obtained were used as received.

Experimental design

Animals were divided into five groups of six rats each. Carbon tetrachloride (CCl_4) is used as a model for liver injury. CCl_4 (1 ml/kg i.p) was used as a positive control. Silymarin is the hepatoprotective drug, (25 mg/kg i.p) was used as a reference drug. All drugs were injected into animals.

Group-I served as a control group and received liquid paraffin (LP) (10 mL/kg) body weight (i.p) of each animal. Group-II animals received CCl_4 + LP for three days, at the dose 1 mL CCl_4 / kg body weight in a suspension of double the volume of LP subcutaneously at the liver on every three days of the treatment. Group-III animals received subcutaneous administration of CCl_4 + LP. They also received silymarin at the dose of 25 mg/kg body weight of animals. Silymarin was used as a standard reference drug. Group-IV received the test drug-I (p-methoxy benzaldehyde di-n-butyl acetal) and Group-V received the test drug-II (p-nitro benzaldehyde di-n-butyl acetal).

The animals were kept starved overnight on third day of the experiment. On the next day, animals were sacrificed by decapitation and the blood was collected by cutting jugular vein. The liver in each case were dissected out, blotted of blood, washed in saline and stored in a freezer. Liver and serum were used for various biochemical estimations.

Hispathological Examinations

Animals were sacrificed on the day of blood sampling, the liver was removed, sliced and washed in saline. Liver pieces were preserved in 10% neutral buffered formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6µm in thickness were cut and stained with hematoxylin and eosin. In addition, portions of liver from the all the experimental groups were fixed in 10% formalin and then embedded in paraffin. The sections were examined for the pathological findings of hepatoxicity.

Statistical Analysis

Results are expressed as mean ± SD. Statistical differences between CCl_4 and test groups were determined using Student's t-test. The Student's t-test [16] was used to compare the means of specific groups, with $p < 0.001$ considered as significant.

Biochemical Estimations

The blood was obtained from all animals and the serum was separated by centrifugation at 2500 rpm at 30 °C for 15 min and utilized for the biochemical estimations of various biochemical parameters namely the liver function marker enzymes. The measurement of thiobarbituric acid reactive substances was done as an index of lipid peroxidation by using the method of Nichans and Samuelson [17], activities of superoxide dismutase [18], catalase [19], and glutathione peroxidise [20] were assayed. The liver function marker enzymes like aspartese aminotransferase [21], alanine aspartese aminotransferase, alkaline phosphatase [22] and glutamyl transpeptidase [23] were determined.

RESULTS

LD_{50} values of test compound is found to be 100 ml/kg for test drug-I and 110 ml/kg for test drug-II. The antioxidant enzyme activities of liver are presented in table-1. Glutamine peroxidise, superoxide dismutase, catalase,

thiobarbituric acid reactive substances activities were reduced significantly ($p < 0.001$) in the CCl_4 intoxicated rats, when compared with normal rats. In CCl_4 + acetal treated rats, the activities of these enzymes attained a near normalcy. The effect of samples seems to be dose dependent. The protection offered by silymarin was found to be higher. The concentrations of TBARS in liver were significantly ($p < 0.001$) higher in level of CCl_4 treated rats as compared to normal control animals (Table-1). These constituents were found to attain near normal levels in liver of CCl_4 + acetal treated groups.

The different biochemical parameters, registered a significant rise in serum of CCl_4 treated rats as compared to the normal control group. All these parameters were found recovered to near normal in CCl_4 + LP + acetal. Administration of silymarin exhibited significant improvement. p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal were compared with normal values and with silymarin. Test drug values some more coincide with the normal and silymarin values. The normalisation of serum marker enzymes by acetals suggests that they are able to condition the hepatocytes so as to protect from the liver damage. Data pertaining to the level of AST, ALT, ALP, ACP and bilurubin, registered a significant rise in serum of CCl_4 + LP + acetal (test drug-I and II) treated animals.

Table-1: Effect of p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal on the activity of antioxidant enzymes in liver

Group	GPX	SOD	CAT	TBARS
I Normal	0.992 ± 0.05	75.81 ± 1.94	296.83 ± 10.05	1.29 ± 0.395
II CCl_4 1 mL/kg i.p	0.61 ± 0.03*	47.84 ± 0.50*	179.73 ± 5.78*	1.79 ± 0.14*
III Silymarin 25 mg/kg	0.95 ± 0.03	88.34 ± 2.54	268.27 ± 6.46	1.26 ± 0.14
IV p-methoxy benzaldehyde di-n-butyl acetal (10 mL/kg)	0.52 ± 0.04**	54.41 ± 2.22**	228.0 ± 6.8**	1.30 ± 0.14**
V p-nitro benzaldehyde di-n-butyl acetal (10 mL/kg)	0.56 ± 0.02**	68.32 ± 2.52**	234.12 ± 5.0**	1.24 ± 0.14**

*Values are mean ±SEM; n=6 animals in each group; *p < 0.001 is significant when compared with group-I; **p < 0.001 is considered significant when compared with group-II .*

Table-2: Effect of p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal on the activities of liver function marker enzymes in serum

Group	AST (U/I)	ALT (U/I)	ALP (IU/I)	ACP (U/I)	Bilirubin Total
I Normal	97.3 ± 1.18	35.08 ± 0.2	15.92 ± 0.72	10.5 ± 0.064	0.39 ± 0.04
II CCl_4 1 mL/kg i.p	186.7 ± 1.82*	136.9 ± 1.94*	98.2 ± 3.9*	38.6 ± 2.9*	0.89 ± 0.76*
III Silymarin (25 mg/kg)	105.3 ± 4.3	49.4 ± 3.6	34.8 ± 2.9	16.2 ± 1.2	0.24 ± 0.03
IV p-methoxy benzaldehyde di-n-butyl acetal (10 mL/kg)	138.8 ± 4.0**	112.0 ± 3.8**	50.2 ± 2.4**	28.0 ± 3.2**	0.40 ± 0.04**
V p-nitro benzaldehyede di-n-butyl acetal (10 mL/kg)	132.4 ± 3.2**	98.4 ± 4.2**	52.0 ± 3.6**	26.0 ± 4.0**	0.38± 0.06**

*Values are mean ±SEM; n=6 animals in each group; *p < 0.001 is significant when compared with group-I; **p < 0.001 is considered significant when compared with group-II.*

DISCUSSION

Carbon tetrachloride has been extensively studied as a liver toxicant and its metabolites such as trichloromethyl radical and trichloromethyl peroxyl radical are reported to be involved in the pathogenesis of liver and kidney damage. The massive generation of free radicals in the CCl_4 induced liver damage provokes a sharp increase of lipid peroxidation in liver [24]. CCl_4 induces fatty liver and cell necrosis and plays a significant role in inducing depletion of reduced gluthine, increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzyme activity.

Vivek [25] reported that CCl_4 caused significant increase in hepatic lipid peroxidation due to free radical injury in cirrhotic livers of rats. In the present study, elevated levels of TBARS observed in CCl_4 treated rats indicate excessive formation of free radicals and activation of lipid peroxidation system resulting in hepatic damage. The significant decline in the concentration of these constituents in the liver and kidney of CCl_4 + acetals treated rats indicate anti-lipid peroxidative effect of test drug-I and II.

The body has an effective mechanism to prevent and neutralise the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as SOD, CAT, GPX and TBARS. When the balance between ROS production and antioxidant defences is lost, oxidative stress result, which through a series of events deregulates the cellular functions leading to various pathological conditions [26]. Any compound, natural or synthetic, with antioxidant properties may contribute towards the partial or total alleviation of this type of damage. In the present study, decline in the level of antioxidant enzymes like SOD, CAT, GPX and TBARS observed in CCl_4 treated rat is a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation system

resulting in tissue damage. The significant increase ($p < 0.001$) in the concentration of these constituents in tissues of liver of CCl_4 + sample and silymarin treated animals indicate antioxidant effect of acetals.

Superoxide dismutase, one of the important intracellular antioxidant enzymes, present in all aerobic cells has an antitoxic effect against superoxide anion [27]. Catalase is a haemoprotein and it protects cells from the accumulation of H_2O_2 by dismutating it to form H_2O and O_2 or by using it as an oxidant in which it works as a peroxidise [28]. The site-specific oxidative damage of some of the susceptible aminoacids of protein is regarded as the major cause of metabolic dysfunction during pathogenesis [29]. The capacity of liver to synthesize albumin is adversely affected by hepatotoxins. The lower level of total protein recorded in the serum of CCl_4 treated rats can be attributed to the features. Attainment of near normalcy in protein content of serum in CCl_4 + silymarin + acetals (Groups IV and V) rats further confirmed the anti-hepatotoxic effect of acetal.

Liver slices of normal rats showed normal hepatic architecture and no fatty changes (Fig.1). Carbon tetrachloride treated groups showed fatty change and lobular ballooning degeneration of hepatocytes. The liver showed distorted architecture with nodule formation, distorted central vein and the portal triad showed fibrous portal expansion with moderate fibrosis and moderate inflammation (Fig.2). The liver tissues showed normal hepatocytes on treatment with silymarin (Fig.3). Administration of acetals (Test drug-I and II) corresponding to their LD_{50} levels and silymarin exhibited significant improvement (Fig.4 & 5).

The hepatocytes showed only mildly distorted architecture. Central vein showed no distinct changes. Portal triad exhibited mild fibrosis and mild inflammation.

Histophathological explanations of liver tissues

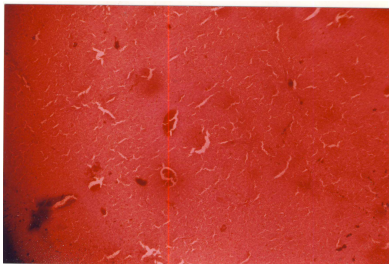

Fig.1 Liver tissue of control rats showing normal hepatic cells

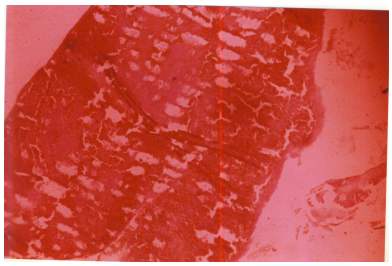

Fig.2 Liver tissues of rats treated with CCl_4 showing severe hepatotoxicity in rats

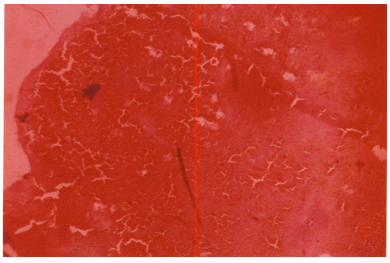

Fig.3 Liver tissues of rats treated with silymarin , showing normal hepatocytes.

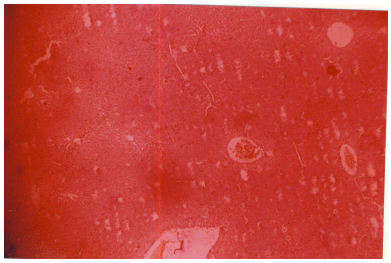

Fig.4 Liver tissue of rats treated with test drug-I (p-methoxy benzaldehyde di-n-butyl acetal) showing normal hepatocytes with regenerating hepatocytes.

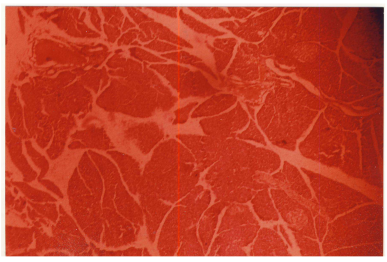

Fig.5 Liver tissue of rats treated with test drug-II (p-Nitrobenzaldehyde di-n-butyl acetal) showing some normal hepatocytes with mild inflammation.

CONCLUSION

The present study demonstrates the hepatoprotective and antioxidant properties of aromatic acetals.

Liver damage detected by the measurement of the activities of serum enzymes like AST, ALT, ALP, ACP and total bilirubin, which has been released into the blood from the damaged cells. They are also indicators of hepatic cell damage. The normalisation of the above enzyme levels in rats treated with the cyanobacterial formulation (100 mg/kg body weight) clearly establishes the hepatoprotective effect of benzaldehyde di-n-butyl acetal, which might be able to induce accelerated regeneration of liver cells, reducing the leakage of the above enzymes into the blood.
The above results showed that both p-methoxy benzaldehyde di-n-butyl acetal and p-nitro benzaldehyde di-n-butyl acetal have anti-hepatoprotective activity.

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