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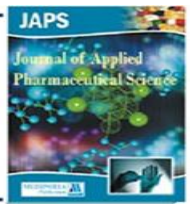

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Antioxidant mediated defense role of *Wedelia calendulacea* herbal extract against CCl_4 induced toxic hepatitis

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ABSTRACT

Liver,the most complex metabolic organ in human body is continuously exposed to various pathogenic oraganisms, xenobiotic agents,drugs and subjected to metabolic stresses. Being the organ of metabolism of xenobiotics and sources of free radicals, it becomes more vulnerable to toxic metabolites and free radical mediated disorders.The present study focuses on the antioxidant mediated protective effect of a popular medicinal herb *Wedelia calendulacea* against carbon tetrachloride induced oxidative stress in the liver of rats.Rats divided into four groups were administered with CCl_4 and CCl_4 along with methanol extract of leaves of *Wedelia* herb (200 and 400 mg/kg b.wt) for three weeks.At the end of treatment, rats were anaesthetized and blood samples were collected for serum separation.Biochemical analysis such as alanine aminotransferase(ALT),aspartate aminotransferase(AST),alkaline phosphatase(ALP),lactate dehydrogenase(LDH) and total bilirubin(TB) were done in serum.Liver tissue was used for glutathione(GSH),lipid peroxidation(LPO),gluatathione peroxidase (GPx),glutathione S-transferase(GST) ,superoxide dismutase (SOD)and catalase(CAT) analysis.CCl_4 administration caused significant elevation of the serum enzymes and total bilirubin content.Antioxidant enzymes were drastically inhibited with significant reduction of glutathione and increased lipid peroxidation in CCl_4 treated rats.*W.calendulacea* extracts administrated rats ,however did not show much changes in marker enzyme level.Antioxidant enzyme status was also not affected and found nearer to the control levels.Increased gluatathione level and reduced lipid peroxidation was also evident in *W.calendulacea* treated rats .The findings show that *W.calendulacea* extract offers better protection against the free radical toxicity of CCl_4 .

Keywords: CCl_4,free radical,antioxidant, liver, *Wedelia calendulacea*.

INTRODUCTION

Liver plays an important role in metabolism, detoxification and excretion of many xenobiotic compounds. Because of its anatomical location and its great capacity for xenobiotic metabolism, it is frequently a target for toxic chemicals. Although viral infection is one of the main causes of liver injury, xenobiotics, excessive drug therapy, environmental pollutants and chronic alcohol ingestion can also cause hepatic injury.Cancer chemotherapeutic drugs caused liver toxicity has been widely reported (King and Perry,2001). Most of these toxic chemicals have been reported to generate free radicals and reactive oxygen species which are the major culprits in liver pathogenesis (deBethizy and Hayes,2001). The improper balance between reactive metabolites production and antioxidant defense results in oxidative stress, which regulates the cellular functions leading to various pathological conditions. Free radical-mediated lipid peroxidation induced by these chemicals play a crucial role in various steps that initiate and regulate the progression of liver diseases independently of the agent in its origin (Loguercio and

Federico, 2003; Vitaglione *et al*., 2004).

The treatment for liver diseases has become a challenging problem of the modern medicine. A number of herbal preparations have been advocated for treating liver diseases. Research investigations conducted on several plant products as liver protective are well documented.(Handa *et al.*,1986;Subrata De,1994).Because free radicals and reactive oxygen species play a central role in liver diseases pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Hensley *et al*., 2000; Kaplowitz, 2002; Higuchi and Gores, 2003). Additionally, recent studies have suggested that natural antioxidants in complex mixtures ingested with the diet are more efficacious than pure compounds in preventing oxidative stress-related pathologies due to particular interactions and synergism (Vitaglione *et al.*,2004). It is evident that there is an increasing demand to evaluate the antioxidant properties of direct plant extract (McClements and Decker, 2000). And there is a need for screening more bioactive plant products with antioxidant properties.

In the present study, a popular medicinal plant in the Indian system of medicine *Wedelia calendulacea* was evaluated against carbon tetrachloride induced toxic hepatitis. The plant is widely used as cholagogues and deobstruents in hepatic enlargement, for jaundice and other ailments of the gall bladder(Bhargava and Seshadri,1974). Few studies on *W.calendulacea* proved to be effective in protecting liver from CCl_4 toxicity. However, antioxidant mediated protective effect of this herb on CCl_4 induced hepatopathogenesis have not been reported earlier. The present study primarily focuses on the antioxidant defense mechanisms of the plant extracts.

MATERIALS AND METHODS

Plant Extracts

Fresh *Wedelia calendulacea* herbs were collected from the wild and washed thoroughly.Fresh and infection free leaves were isolated and shade dried. The dried leaves were powdered mechanically and defattened using petroleum ether solvent.The defattened plant materials were extracted with methanol using soxhlet apparatus by hot percolation method.The extract(WDE) obtained was concentrated in a rotary vacuum evaporator and dissolved in DMSO for administration.

Reagents

All reagents used for the biochemical estimations in the study were procured from Qualigens Fine Chemicals and Himedia Laboratories Pvt Ltd,Mumbai and are analytical grade.

Animals

Male wistar rats weighing about 200±20 g were used for the study.They were housed in well conditioned room with 12 h light/12 h dark photoperiod.They were fed with standard animal feed(Lipton India,Bangalore,India) and water *ad libitum*.Experiments were conducted in accordance with the institutional ethical committee guidelines.

Experimental Design

Animals were divided into 4 groups with six animals each.

Group I received 0.2 ml of DMSO intraperitoneally and treated as experimental control.

Group II was administrated with 0.3 ml of CCl_4 intraperitoneally for five days for induction of toxic hepatitis.

Group III received CCl_4 similar to group II and 200 mg/ kg body weight of WDE orally.

Group IV rats received CCl_4 and 400 mg/kg body weight of WDE extracts.

After three weeks of extract administration rats were anesthetized and blood samples were collected by sino-orbital puncture. Serum separated was used for all biochemical estimations.Animals were autopsied and liver was excised carefully and washed in saline.Tissue homogenate was prepared in 0.1M Tris-HCl buffer(pH 7.4) and used for the determination of lipid peroxides (LPO), reduced glutathione (GSH), glutathione peroxidase(GPx),glutathione S-tranferase (GST), catalase (CAT) and superoxide dismutase(SOD).

Biochemical Estimations

Serum Aspartate and Alanine Transferase(AST and ALT)

The activities of these enzymes were estimated by the method of Reitman and Frankel (1957). 0.2 ml of serum was added to 1 ml of phosphate buffer containing substrate, mixed and incubated for 60 min for AST and 30 min for ALT at 37°C. Then 1 ml of dinitrophenylhydrazine was added and incubated for 20 min at room temperature and 10 ml of 0.4% sodium hydroxide was added, mixed well and after five minutes read at 550 nm. Blank and a series of standards were processed similarly.

Lactate Dyhydrogenase(LDH)

LDH was assayed according to the method of King(1965). To 1.0 ml of the buffered substrate (lithium lactate in 0.1M glycine buffer, pH 10), 0.1 ml of enzyme preparation was added and the tubes were incubated at 37.8^{o}C for 15 min. After adding 0.2 ml of NAD^{+} solution, the incubation was continued for another 15 min.

The reaction was arrested by adding 0.1 ml of DNPH (2, 4-dinitrophenyl hydrazine), and the tubes were incubated for a further period of 15 min at 37.8^{o}C after which 7.0 ml of 0.4N NaOH was added and the color developed was measured at 420 nm.

Alkaline Phosphatase(ALP)

Serum alkaline phosphatase activity was measured following the method of King and Armstrong(1934),using disodium phenyl phosphate as substrate.The colour developed was read at 510 nm.Activities are expressed as KAU/L.

Total Bilirubin(TB)

Serum total bilirubin was estimated following the method of King and Coxon(1950).In brief, 1 ml of serum was mixed with

0.5 ml of diazo reagent,followed by 0.5 ml $(NH_4)_2SO_4$.The volume was made up to 10 ml with 85% ethanol.The contents were mixed well and allowed to stand for 30 min for even distribution of the precipitate.The precipitate was filtered and measured using colorimeter.

Lipid Peroxidation(LPO)

Heart tissue homogenate was used for the estimation of lipid peroxidation following the method described by Ohkawa *et al* .,(1979) in which malondialdehyde(MDA) released was used as the index for lipid peroxidation. In brief, to 0.2 ml of tissue homogenate,0.2 ml of 8.1% SDS,1.5 ml of 20% acetic acid and 1.5 ml of 0.8% thiobarbituric acid(TBA) were added.The volume was made upto 4 ml with distilled water and incubated in a water bath at 95.5^0 C for an hour.The contents were cooled and 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. The contents were centrifuged and the organic layer was separated for measurement of absorbance at 532 nm

Reduced Glutathione (GSH)

GSH was estimated by the method of Ellman(1959).Briefly, 0.5 ml of tissue homogenate was precipitated with 2 ml of 5% TCA. After centrifugation, 1 ml of supernatant was taken and added 0.5 ml of Ellman's reagent (19.8 mg of 5,5' dithio(*bis*)nitrobenzoic acid in 100 ml of 1% sodium citrate) and 3 ml of phosphate buffer. Standards were treated in a similar way and the color developed was read at 412 nm.

Glutathione Peroxidase (GPx)

GPx activity was measured by the method of Rotruck *et al*., (1973). To 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate were added. To that mixture, 0.2 ml of glutathione solution and 0.1 ml of hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 min along with the control tubes containing all the reagents but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 ml of 10% TCA. 0.2 ml of tissue homogenate was added to the control tubes. The tubes were centrifuged and supernatant was assayed for glutathione content by adding Ellman's reagent.

Glutathione-S-transferase(GST)

GST activity was measured by the method of Habig *et al*.,(1974). The reaction mixture containing 1 ml of buffer, 0.1 ml of 1-chloro-2, 4-dinitrobenzene (CDNB), 0.1 ml of homogenate and 1.7 ml of distilled water was incubated at 37°C for 5 min. The reaction was then started by the addition of 1 ml of glutathione. The increase in absorbance was followed for 3 minutes at 340 nm. The reaction mixture without the enzyme was used as blank.

Catalase(CAT)

CAT was assayed by the method of Takahara *et al*.,(1960). To 1.2 ml of 50 mM phosphate buffer pH 7.0, 0.2 ml of the tissue homogenate was added and reaction was started by the addition of 1.0 ml of 30 mM H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 30 s intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/min/mg protein.

Superoxide Dismutase (SOD)

SOD was assayed by the method of Misra and Fridovich(1972). 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured in spectrophotometer. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto-oxidation.

Statistical analysis

Statistical analysis of the results was done by one way analysis of variance (ANOVA) using GraphPad Prism 5 software followed by Dunnet's comparison test for significance. Significance was set at ($p<0.05$). Results are presented as Mean± S.E.

RESULTS

Serum marker enzymes

Rats administered intraperitoneally with CCl_4 (group II) showed significant elevation ($p<0.05$) of marker enzymes ALT,AST,ALP and LDH in the serum.Total bilirubin content in the serum of CCl_4 treated groups was also significantly increased when compared to the control group I.However,in groups II and III which received 200 and 400 mg/kg body weight of WDE respectively,didn't show drastic changes in the marker enzyme levels.The levels of AST,ALT,ALP and LDH in the serum of WDE treated groups were well within the range of control group I rats. Similarly total bilirubin content was also not significantly altered in groups III and IV administered with WDE when compared to the control group I (Table :1).

Table: 1 Marker enzyme levels in the serum of different treatment group rats

Experimental Groups	AST (U/L)	ALT (U/L)	ALP (KAU/L)	LDH (U/L)	TB (mg/dl)
Group I	84.48±0.84	38.47±0.38	77.76±0.54	112.42±0.82	0.917±0.18
Group II	162.50±2.55**	88.65±0.27**	142.92±0.46**	146.91±1.31**	4.13±0.15**
Group III	87.17±0.50	40.32±0.43*	81.94±0.47*	116.62±0.80	1.42±0.94
Group IV	87.21±0.57	39.79±0.29	79.34±0.45	115.13±0.59	1.12±0.12

Values are Mean±S.E (n=5);significance **(p<0.05);*(p<0.01):Group I vs Groups II,III&IV.

Antioxidant enzymes

The oxidative stress caused by CCl_4 in the liver was assessed by measuring the levels of lipid peroxidation (LPO)

product MDA,reduced glutathione(GSH) and the antioxidant defense enzymes GPx,GST,SOD and CAT.CCl_4 administered Group II exhibited significant($p<0.05$) elevation of LPO and reduction of GSH in the liver. Similarly,antioxidant enzymes GPx,GST,SOD and CAT were also significantly reduced in liver of rats treated with CCl_4 when compared to the control Group I rats .On the other hand,administration of WDE(Group III and IV) reduced the severity of CCl_4 toxicity, as evident from the non-significant differences observed in the oxidative stress indicators and antioxidant enzyme levels in these groups.Normal level of LPO ,GSH and antioxidant enzymes within the range of control group I signifies the protection offered by WDE (Table:2)

Table: 2 Antioxidant enzymes,lipid peroxidation and gluatathione levels in the liver of experimental rats.

Experimental Groups	GPx	GST	SOD	CAT	GSH	LPO
Group I	116.12±0.87	13.79±0.38	12.11±0.37	112.21±0.79	5.32±0.184	4.67±0.12
Group II	82.09±0.69**	7.07±0.16**	7.84±0.24**	83.60±0.58**	2.52±0.13**	8.28±0.29**
Group III	112.25/0.71*	12.39/0.25	11.67/0.35	104.51/1.25*	4.87/0.93	5.22/0.27
Group IV	113.35/0.78	13.15/0.48	12.84/0.38	110.22/0.80	5.14/0.89	6.26/0.22

Values are expressed as GSH(nmole/g tissue),GPx(nmole GSH oxidized/ min/mg protein),GST(U/min/mg protein),SOD(U/g protein),CAT(nmol/min/mg protein),LPO(nmole/mg protein).Values are Mean±S.E(n=5);Significance **($p<0.05$),*($p<0.01$).Group I vs Groups II,III&IV.

DISCUSSION

Hepatotoxicity of CCl_4 is because of reductive halogenations catalysed by cytochrome P450 in the liver cell endoplasmic reticulum.This initial reductive halogenations yield CCl_3,with subsequent rapid formation of a variety of chemically reactive substances.This results in a series of secondary mechanism responsible for plasma membrane disruption and cell death(Recknagel and Glende,1988).Microsomal lipid peroxidation and covalent CCl_4 cleavage products to microsomal and mitochondrial lipids and proteins follow immediately(Rao and Recknagel,1968).These initiate a series of pathological events resulting in cell necrosis(Recknagel *et al*.,1989).Unsaturated lipids of the endoplasmic reticulum undergo peroxidation with resultant destruction of membrane structure.A chain reaction secondary to formation of free radicals from lipids produce further cellular damage.It is evident that free radical generation and increased lipid peroxidation account for more pathological damages of hepatic cells(Recknagel and Glende,1973).

In the present study, drastic alterations in the level of serum marker enzymes AST,ALT,ALP and LDH were noted, indicating CCl_4 mediated hepatic damages.In addition,bilirubin, an endogenous substance and degradation product of hemoglobin was also found to be significantly elevated in CCl_4 treated rats,which is also a measure of hepatotoxicity(Plaa and Zimmerman,1997) and could be attributed to impaired hepatic clearance due to hepatic parenchymal damage and biliary obstruction(Blanckaert and Schmid,1982).The enzymes ALT and AST are localized in mitochondria and cytosol of hepatic cells,ALP usually found in sinusoidal and bile canaliculi membaranes(Hagerstrand,1975), and also in cytosol,in membranes of golgi apparatuses,endoplasmic reticulum and nucleus. LDH, an intracellular enzyme catalyses the readily reversible reaction involving oxidation of lactate to pyruvate.High concentrations of LDH are found in the liver.Elevation in total serum LDH activity is used as diagnostic indices for organ dysfunction(Cassidy and Reynolds,1994).The grave alterations noted in the present study following CCl_4 administration are a measure of the extent to which the liver has been damaged.Increased serum concentration of these enzymes indicates that they are leached out of the damaged cells in to the circulation.

Since the CCl_4 induced hepatotoxicity is due mostly to reactive free radical generation ,antioxidant mediated protective role of WDE has been assessed.CCl_4 treatment significantly elevated the lipid peroxidation as evident from the increased MDA level in the liver tissue.Antioxidant enzymes GPx,GST,SOD and CAT were significantly reduced by CCl_4 intoxication, besides reduction of GSH.GSH is a co-factor for several detoxifying enzymes of oxidative stresses such as glutathione peroxidase and glutathione transferase and scavenges hydroxyl radicals and singlet oxygen species directly and detoxifying hydroperoxides and lipid peroxides(Valko *et al*., 2007).Glutathione, synthesized from liver is the major source of plasma (Kaplowitz *et al*.,1985).It can also regenerate some of most important antioxidants vitamin C and E.And hence,it can be assumed that liver damage resulted in reduction in glutathione level and the antioxidant enzymes.Beddowes *et al*.,(2003)showed that CCl_4 increased lipid peroxidation and depletion of glutathione. Significant reduction of hepatic gluatathione,glutathione peroxidase and glutathione S-transferase in CCl_4 administrated rats was reported by Cabre *et al*.,(2000).

Adiminstration of WDE extract has exhibited considerable protection against oxidative damage of CCl_4 which is evident from the marker enzymes level and antioxidant enzymes status.The levels of these enzymes were nearer to the control values .Increased glutathione level with concomitant increase in the antioxidant enzymes and reduced lipid peroxidation product are the indications that WDE extract offered significant protection. The protective effect may be due to rich antioxidant phytochemicals such as phenolics and flavanoes(Meena *et al*.,2011).Kataki *et al*., (2012) reported free radical scavenging activity of *W.calendulacea* extracts and the presence of antioxidant phytochemicals such as phenolics,flavones and tannins. In addition ,Wagner *et al.*, (1986) reported the presence of coumastans,wedelolactones and dimethyl wedelolactones with significant hepatoprotective role primarily acting on lipid peroxidation process and cell regeneration.The hepatoprotective action may be attributed to scavenging of free radicals generated by microsomal reduction of CCl_4(Ferenci *et al.*, 1989) and prevention of free radical generation responsible for lipid peroxidation(Mehendale,1991).It is clear from the above that *W.calendulacea* can be an effective herbal protective agent against a wide variety of liver disorders.

CONCLUSION

In the present study,it has been observed that *W.calendulacea* herbal extract offered significant protection against the hepatotoxicant CCl_4.The marker enzyme levels and the important antioxidant enzymes activity were greatly protected,besides reducing lipid peroxidation by the herbal extract,showing that this plant is a better remedy for any diseases of the liver.The study also shows that plant extract has considerable antioxidant property .

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