Hepatoprotective activity of whole Plant Extract of Catharanthus Pusillus (Murr.) G.Don (Apocynaceae)

Yokeswari Nithya, P Ananthi, S Mohan, V.R

Abstract**:** ***CCl_4 intoxicated rats showed significant elevation in serum enzymes, bilirubin and lipid per oxidation of the liver tissues and reduction in serum total protein, superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase activity. Treatment with ethanol extract of Catharanthus pusillus whole plant altered the above parameters to the levels of near normal. All the above results were comparable with the standard drug silymarin (100mg/kg) treated group. Thus the present study ascertains that the ethanol extract of Catharanthus pusillus whole plant possesses significant hepatoprotective activity.***

Keywords: Catharanthus pusillus, CCl4, silymarin, bilirubin

I. INTRODUCTION

Liver disease has become a global concern worldwide. Liver is often abused by environmental toxins, poor eating habits and alcohol, that damage and weaken the liver leading to important public health problems like hepatitis, cirrhosis and alcoholic liver diseases (Treadway, 1998). India, numerous medicinal plants and their formulations are used for treating liver disorders in traditional systems of medicine. Herbal drugs play an important role in treatment of various ailments including liver problems . Therefore, many folk remedies from plant sources are being tested for their potential hepatoprotective activity in experimental animal models (Ganga Rao *et al.,* 2012).

Catharanthus pusillus of Apocynaceae family is known with various names in India and all over the world. It is widely used as various treatments of diseases and traditionally used as herbal medicine (Don ,1999). The roots, leaves and latex of these plants are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumor and ear aches. The leaf powder of *C.pusillus* were mixed with coconut oil and used for treat the antidandruff activity and also used to kill the lice (Rajakaruna,2002). The main purpose of this study was to know about the hepatoprotective activity of whole plant extract of *C.pusillus* on CCl_4 induced liver toxicity in rats.

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II. MATERIALS AND METHODS

Plant Material:

The whole plant of *Catharanthus Pusillus* were agglomerated from Pechiparai, Kanayakumari District, Tamil Nadu. With the help of local flora, voucher specimens were identified and preserved in the Ethnopharmacology unit, Research department of Botany, V.O.Chidambaram College, Thoothukudi, Tamil Nadu for further references

Preparation of plant extract:

The whole plant was shade dried and then powdered to obtain a coarse powder, which was then subjected to extraction in a Soxhlet apparatus using ethanol. The extract was tested for the identification of various phytochemical constituents as per standard procedures (Brinda,1981&Lala, 1993.,).The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extract were used for study.

Experimental animal

Adult male wistar albino rats weighing about 180 – 240 g body weight were selected for this work. They were abide under standard environmental conditions at room temperature (25±2°C) in a well-ventilated animal house with constant 12 h of darkness and 12 h of light schedule. The rats were fed with standard pellet diet (Goldmohar brand, Hindustan Lever Ltd., Mumbai, India) and water *ad libitum.*

Acute Toxicity Studies

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class

method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study (OECD 2002.,)

III. EXPERIMENTAL PROTOCOL

Induction of hepatotoxicity

Carbon tetrachloride (CCl_4) 2.5 ml/kg body weight was dissolved in 7.5 ml of paraffin and administered intraperitoneally.

Grouping of animals

A total of 30 rats were taken and were divided into six groups of 5 rats each, of which, five groups contained CCl_4 hepatic toxicity induced rats and the remaining one group contained normal rats.

Group – I : Rats received normal saline (0.9%), by using an intragastric catheter tube (IGC)- Normal control

Group – II : Liver injured rats received 2.5 ml/kg body weight of normal saline (0.9%) for 14 days, by using IGC, -CCl_4 hepatic toxicity induced control.

Group – III : Liver injured rats received *C.pusillus* whole plant ethanol extract at the dose of 150 mg/kg body weight for 14 days, by using an IGC.

Group – IV : Liver injured rats received *C.pusillus* whole plant ethanol extract at the dose of 300 mg/kg body weight for 14 days, by using an IGC.

Group – V : Liver injured rats received *C.pusillus* whole plant ethanol extract at the dose of 500 mg/kg body weight for 14 days, by using an IGC.

Group – VI : Liver injured rats received silymarin orally at the dose of 100 mg/kg body weight for 14 days, by using an IGC.

All the treatments were given between 9.30 and 10.00 in the morning. After 24h of last treatment, the final body weight was noted and the animals were sacrificed by decapitation. Blood was collected from each group of rats. Serum from the blood was separated by centrifugation at 3000xg for 10 min and stored at -20°C until used for various biochemical assays. Estimation of GGT, Protein, Serum Glutamate Oxalo Transaminase (SGOT or AST), Serum Glutamate Pyruvate Transaminase (SGPT or ALT) and Alkaline Phosphate (ALP) was done as per the standard procedures.

Estimation of Total, Conjugated and Unconjugated Bilirubin

Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw (1987). The concentration of unconjugated bilirubin was the difference between total and conjugated bilirubin concentrations.

Estimation of Serum MDA, SOD, CAT, GPx and GRD

Quantitative estimation of MDA formation was done by the method proposed by Okhawa *et al.* (1979) and it was done by determining the concentration of thio barbituric acid reactive substance (TBARS) in serum. Enzymatic antioxidants, superoxide dismutase (SOD) (Mishra,1972), Catalase (Aebi, 1974), non enzymatic antioxidant glutathione peroxidase (GPx) (Paglia and Valentine, 1967), glutathione reductase (GRD) (Goldberg and Spooner, 1983) and reduced glutathione (GSH) were also assayed in serum.

Biochemical Analysis

The animals were betrayed at the end of experimental period of 14 days by decapitation. Blood was collected, sera separated by centrifugation at 3000g for 10 minutes. Serum protein and serum albumins was determined quantitatively by colorimetric method using bromocresol green.(Lowry et al., 1951). The total protein minus the albumin gives the globulin. Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (ALP), total, conjugated bilirubin, unconjugated bilirubin were determined as per the standard procedures (Shanmugasundaram et al.,2010 & Thangakrishnakumari et al., 2012) . Liver homogenates (10%W/V) were prepared in ice cold 10mM tris buffer (pH7.4). Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in 10% liver homogenates by the method of Pal et al (Pal et al.,2011). Antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD) were also assayed in liver homogenates as per the standard procedures (Anitha et al., 2010 & Suky et al., 2011)

Statistical Analysis

The data were expressed as the mean ± S.E.M. The difference among the means has been analyzed by one-way ANOVA. $p<0.05$and $p<0.01$ were considered as statistical significance using SPSS Software.

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IV. RESULTS

Body weight

The administration of CCl_4 caused a significant (*p*<0.01) decrease in the body weight of rats as compared with the control rats. The animals treated with whole plant extract of *C.pusillus* (150 mg/kg, 300 mg/kg and 500 mg/kg) also gained weight during the experimental period (Table 1).

Table 1: Effect of whole plant ethanol extract of *C.pusillus* on the body weight in the normal, liver damaged and drug treated rats.

Treatment	Dose	Initial Body weight (Gm)	Final Body weight (Gm)	Mean weight Gain (G↑) / loss (L↓) (Gm)	% of Difference
Group I	0.9% Saline	219.65±6.24	226.16±3.86	6.51	2.96
Group II	0.9% Saline	226.60±5.65	211.65±2.84**	15.15**	6.67
Group III	150(mg/Kg)	215.60±4.16	206.16±3.64**	9.44	4.37
Group IV	300(mg/Kg)	224.50±3.84	216.18±4.16*	8.32a	3.70
Group V	500(mg/Kg)	218.13±5.18	214.84±3.92ns	3.29aa	1.50
Groups VI	100(mg/Kg)	221.65±4.96	218.16±4.86ns	3.49aa	1.57

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. **P* <0.05; ***P* <0.01as compared with Normal Control to liver damaged control: a *P*<0.05; aa *P*<0.01; aaa*P*<0.001 as compared with liver damaged control to drug treated animal ns: not significant.

Biochemical parameters

The results of serum biochemical parameters are presented in Table 2. In the CCl_4 control group, significant (*p*<0.05; *p*<0.01) decrease in the levels of total protein, albumin and globulin was observed. But the group which received the drug of whole plant extract at the dose of 500 mg/kg body weight showed a significant (*p*<0.01) increase in the reduced levels of protein albumin and globulin. The elevation of SGOT, SGPT and ALP in CCl_4 intoxicated rat was significantly (*p*<0.01) high when compared to the normal. The elevated levels of SGOT, SGPT and ALP in groups IV and V rats (post treated with whole plant extract, 300 mg/kg and 500 mg/kg) were significantly (*p*<0.05; *p*<0.01) low as depicted in Table 2.

Table 2: Effect of whole plant ethanol extract of *C.pusillus* on the serum protein, albumin, globulin concentration and serum GOT, GPT and ALP enzyme activity in the normal, liver damaged and drug treated rats.

Groups	Parameters							
	Dose	T. Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
Group I	0.9% saline	9.36±0.36	5.11±0.26	4.25±0.54	1.20:1	22.96±1.34	27.16±1.56	162.65±5.67
Group II	0.9% saline	7.56±0.54**	4.16±0.36*	3.40±0.21*	1.20:1	86.92±1.69**	73.64±2.16**	248.65±9.27**
Group III	150 mg/kg	7.86±0.37*	4.54±0.16ns	3.32±0.16*	1.40:1	73.16±1.34**	61.67±2.24**	206.16±6.29**
Group IV	300mg/kg	8.48±0.37ns	5.06±0.54ns	3.92±0.12ns	1.30:1	56.22±1.29*a	43.16±0.88*a	188.2±5.96nsa
Group V	500 mg/kg	9.12±0.56aa	5.16±0.92a	3.96±0.27a	1.31:1	31.67±1.56ns aa	34.65±0.92aa	173.65±4.18aa
Groups VI	100 mg/kg	8.84±0.54a	4.96±0.55ns	3.88±0.36ns	1.27:1	29.66±1.26ns aa	31.84±0.73aa	184.46±5.18 a

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. **P* <0.05; ***P* <0.01as compared with Normal Control to liver damaged control: a *P*<0.05; aa *P*<0.01, aaa*P*<0.001 as compared with liver damaged control to drug treated animal ns: not significant.

Table 3 showed the levels of total bilirubin, conjugated and unconjugated bilirubins and GGTP levels. When compared to normal control rats (Group I), the serum of CCl_4 treated rats (Group II) showed a significant elevation in the total bilirubin, conjugated, unconjugated bilirubins and GGTP. In all the other groups treated with the whole plant ethanol extract of *C.pusillus* (Group III to V), the above said biochemical parameters were found to have decreased when compared to CCl_4 treated control rats (Group II). However, the decrease in the concentration of total bilirubin, conjugated, unconjugated bilirubins and GGTP levels were found to be greater in the liver damaged rat group IV, followed by group V, treated with whole plant extracts of *C.pusillus* respectively.

Table 3: Effect of whole plant ethanol extract of *C.pusillus* on the serum total, conjugated, unconjugated bilirubin and GGTP levels in the normal control, liver injured and drug treated rats.

Groups	Parameters				
	Dose	Total Bilirubin (Mg/dl)	Conjugated (Mg/dl)	Unconjugated (Mg/dl)	GGTP (U/L)
Group I	0.9% saline	0.61±0.011	0.20±0.031	0.41±0.31	8.06±0.36
Group II	0.9% saline	3.48±0.72**	2.31±0.26**	1.17±0.13**	27.93±0.27**
Group III	150mg/kg	3.04±0.59**	2.18±0.13**	0.86±0.031*	24.15±0.92**
Group IV	300mg/kg	2.65±0.21*	1.36±0.24*a	1.29±0.054a	11.36±0.84ns a
Group V	500mg/kg	1.36±0.63ns a	0.91±0.054ns aa	0.45±0.036aa	9.15±0.54aa
Group VI	100mg/kg	1.06±0.54ns aa	0.66±0.018ns aa	0.40±0.016aa	8.36±0.15aa

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. *P <0.05; **P <0.01as compared with Normal Control to liver damaged control: a *P*<0.05; aa *P*<0.01; aaa*P*<0.001 as compared with liver damaged control to drug treated animal ns: not significant.

a *P*<0.05; aa *P*<0.01; aaa *P*<0.001 as compared with liver damaged control to drug treated animal ns: not significant.

The effect of ethanol extract of whole plant of *C.pusillus* on lipid peroxidation (LPO), glutathione peroxide (GPx), glutathione reductive (GRD), superoxide dismutase (SOD), catalese (CAT) and reduced glutathione (GSH) activities are shown in Table 4. When compared to the normal control rats (Group I), the level of lipid peroxidation increased significantly ($p<0.01$) and the levels of glutathione peroxidase, superoxide dismutase, catalase and reduced glutathione decreased significantly ($p<0.01$) in CCl_4 intoxicated control rats (Group II). Treatment with the ethanol extract of whole plant of *C.pusillus,* at the dose of 500mg/kg decreased the elevated lipid peroxidation level significantly and restored the altered glutathione peroxidase, glutathione reductase, superoxide dismutase, cutalase and reduced glutathione levels towards normal in a dose dependent manner. The results were well camparable with that of silymarin, the standard drug treated rats.

Table 4: Effect of whole plant ethanol extract of *C.pusillus* on serum LPO, GPX, GRD, SOD, CAT and GSH activity in the normal control, liver injured and drug treated rats.

Groups	Parameters						
	Dose	LPO (n mole of MDA/mg protien)	GPX (u/mg Protien)	GRD (u/mg)	SOD (u/mg)	CAT (u/mg)	GSH (u/mg)
Group I	0.9% saline	2.54±0.073	4.116±0.138	0.421±0.16	0.280±0.039	4.26±0.31	28.91±0.16
Group II	0.9% saline	5.98±0.094**	1.841±0.361**	0.265±0.32**	0.136±0.16**	2.35±0.076**	16.36±0.27*
Group III	150mg/kg	4.13±0.054**	2.18±0.549*	0.306±0.18*	0.196±0.027*	2.92±0.015*	21.46±0.34ns
Group IV	300mg/kg	3.24±0.074*a	3.84±0.063a	0.393±0.11a	0.216±0.013ns a	3.84±0.077ns a	26.16±0.76a
Group V	500mg/kg	3.01±0.056aa	4.36±0.072aa	0.436±0.56aa	0.248±0.036aa	4.56±0.054aa	32.16±0.036aa
Group VI	100mg/kg	2.94±0.086aa	3.98±0.18aa	0.441±0.39aa	0.251±0.035aa	4.13±0.036aa	31.84±0.16aa

Values are mean ± SD of 5 animals in each group. Statistical analysis ANOVA followed by Dunnett t-test. **P* <0.05; ***P* <0.01as compared with Normal Control to liver damaged control:

V. DISCUSSION

CCl_4 induced liver injuries are the best characterized system of xenobiotic – induced hepatotoxicity and commonly used models for the screening of antihepatotoxicity and or hepatoprotective activities of drug. (Clowson 1998; Lin etal 2002). Since the changes associated with liver damage induced by CCl_4 are similar to that of acute viral hepatitis. CCl_4 mediated hepatotoxicity was chosen as the experimental model. CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P_{450} dependent monooxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with simultaneous attack on polysaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage. Thus, antioxidant or free radical generation inhibition is important in production against CCl_4 induces liver lesions. Hepatotoxic compounds such as CCl_4 are known to cause marked elevation in serum enzymes and bilirubin levels. It causes marked reduction in total protein levels. Silymarin is used as standard hepatoprotective compound since it is reported to have a insulating effect on the plasma membrane of hepatocytes .

Normally, SGOT and SGPT are present in high concentration in liver mainly due to the hepatocyte necrosis or these enzymes are released from the cells and their levels in the blood increases. SGPT is a sensitive indicator of critical liver damage and upgrading of this enzyme in non hepatic diseases is unusal. SGPT is more selectively a liver parenchymal enzyme than SGOT (Shah *et al*; 2002). Assessment of liver function can be made by estimating the activities of serum SGPT, SGOT and ALP which are enzymes originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage (Nkosi *et al*; 2005).

In the present study, the elevated level of these marker enzymes are observed in the group II CCl_4 treated rats. The elevations of these enzymes are due to the extensive liver damage induced by toxin. The reduced concentrations of SGPT, SGOT and ALP as a result of ethanol extract of *C.pusillus* whole plant administration was observed during the present study. The normalization of serum markers by ethanol extract of *C.pusillus* whole plant suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against CCl_4 induced leakage of marker enzyme into the circulation. The above changes can be considered

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as an expression of the functional improvements of hepatocytes.

Alkaline phosphatase concentration is related to the functioning of hepatocytes, high level of alkaline phosphatase in the blood serum is related to the increased synthesis of its by cells lining bile canaliculi usually in response of cholestasis and increased bililary pressure (Graw *et al.,* 1999). Increased level was obtained after CCl_4 administration and it was brought to near normal level by *C.pusillus* whole plant extract treatment.

The liver failure results the drastic come down of total protein and albumin levels of serum because of the hepatotoxicity. The normalized values of total protein and albumin were seen in the group III, IV and V which was because of the rejuvenating or repairing of liver. Ethanol extract of *C.pusillus* whole plant may have the capability to reform the liver cells by increasing protein values of serum.

The most sensitive tests employed in the diagnosis of hepatic diseases is serum bilirubin. Excessive heme destruction and blockage of bilary tract leads to Hyperbilirubinemia. Due blockage of the bilary tract, there is a mass inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged and dead hepatocytes (Wolf *et al*., 1997). Ethanol extract of *C.pusillus* whole plant decreased the level of bilirubin and increased the level of protein suggesting that it offered protection.

The activity of serum γ-glutamyl transferase is generally increased as a result of liver disease, since γ-glutamyl transferase is a hepatic microsomal enzyme. Serum γ-glutamyl transferase is the most useful enzyme in the diagnosis of liver disease. Change in γ-glutamyl transferase is parallel to those of amino transferase. The critical damage induced by CCl_4 elevated the γ-glutamyl transferase level but, the same attains the normal after treatment with the whole plant of *C.pusillus* due to its antioxidant potential.

Lipid peroxidation has been identified as the cause for the destructive process of liver injury due to CCl_4 administration. In the present study, increased MDA levels in liver indicated increased lipid peroxidation induced by CCl_4 (GroupII). This enhanced lipid peroxidation finally triggered hepatic tissue damage and failure of antioxidant defense mechanisms to prevent formation of extravagant free radicals. Treatment with ethanol extract of whole plant of *C.pusillus* significantly reversed these changes. Hence, it may be possible that the mechanism of hepatoprotection by ethanol extract of whole plant of *C.pusillus* is due to its antioxidant effect.

Pivotal components of the antioxidant defense system in the body are cellular antioxidant enzymes (SOD and glutathione), which are involved in the reduction of reactive oxygen species (ROS) and peroxides produced in the living organisms as well as in the detoxification of certain compounds of exogenous origin. Thus are cellular antioxidant enzymes playing a primary role in the maintenance of a balanced redox status (Michiels *et al.,* 1994). Hence, they can serve as a potential marker of susceptibility, early and reversible tissue damage, and of decrease in antioxidant defense. (Packer *et al.,* 1994). SOD plays an important role in the elimination of ROS and protects cells against the deleterions effects of super oxide anion derived from the peroxidative process in liver and kidney tissues (Fridovich *et al*., 1975). In the present study, it was observed that the ethanol extract of *C.pusillus* whole plant significantly increased the SOD activity in CCl_4 intoxicated rats there by diminished CCl_4 induced oxidative damage.

CAT is considered as the most important H_2O_2 removing enzyme and also a key component of antioxidative defense system. Therefore, the reduction in the activity of catalase may result in a number of deleterious effects due to accumulation of hydrogen peroxide (Chance *et al.,* 1952). In the present study, treatment with ethanol extract of whole plant of *C.pusillus,* increased the level of catalase significantly in dose dependent manner and protected the liver from CCl_4 intoxication.

Glutathione peroxide (GPX) is a selenoenzyme and it protects the cells from damage due to free radicals like hydrogen and liplid peroxides (Zaltzber *et al.,* **1**999).It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphides and reaction product of the hydroperoxides .Glutathione (GSH) extensively found in cells, protects cells from electrophilic attacks provided by xenobiotics such as free radicals and peroxides. GSH deficiency leads to cellular damage in kidney, muscle, lungs, colon, liver, lymphocytes and brain. (Orhan *et al*., 2007). In the present study, treatment with the whole plant ethanol extract of *C.pusillus* increased the activities of GPx and GSH in CCl_4 induced liver damaged rats.

VI. CONCLUSION

This study finally confirms *C.pusillus* having hepatoprotective effect on hepatic damage induced by CCl_4. Thus it can be finalized that mechanism of hepatoprotective activity of *C.pusillus* whole plant may be due to its free radical scavenging, antioxidant activity and synergistic effect of the phenolics and flavonoids present in the whole plant. The GC-MS analysis of the whole

plant of *C.pusillus*, confirmed the presence of 9, 10-Secocholesta-5,7,10 (19)- triene-3,21,25-triol, (3β,5Z,7E)-, 4-Chloro-17-hydroxy-(17β) Androst-4-en-3-one, α-Tocopherol and Campersterol. The presence of these compounds might be responsible for the hepatoprotective activity.

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