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Effect of *Platycladus orientalis* on the serum biochemical markers of oxidative stress in liver cirrhosis with histopathological microscopic study

Alok Kumar Dash and Jhansee Mishra

Abstract

Objective: The aim of the present study evaluates the hepatoprotective effect of aqueous and petroleum ether extract of *Platycladus orientalis* leaf by paracetamol-induced liver damage in rats.

Materials and Methods: Hepatic damage, as revealed by histology and the increased activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities, and decreased levels of serum total protein (TP), albumin (Alb) were induced in rats by an administration of paracetamol (750 ± 5) mg/kg. Further, the effects of both extracts on serum thiobarbituric (TBAR), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were estimated to measure the degree of protection.

Results: petroleum ether extract of *Platycladus orientalis* at a dose level of (200 ± 5) mg/kg produce significant hepatoprotection by decreasing the activity of serum enzymes, while they significantly increased the levels of (GSH), (SOD) and (CAT) in a dose dependent manner. The effects *Platycladus orientalis* extract were comparable to that of standard drug, silymarin.

Conclusion: From this study, it can be concluded that the aqueous and petroleum ether extracts of *Platycladus orientalis* possesses both effective hepatoprotective as well as significant antioxidant activity.

Keywords: Chemopreventive, Silymarin, alp, aqueous, petroleum ether, hepatic damage

Introduction

Liver is the key organ of metabolism and excretion. It is often exposed to a variety xenobiotics and therapeutic agents. Until today, people have not yet found an actual curative therapeutic agent for liver disorder. In fact, most of the available remedies help the healing or regeneration of the liver (Subramoniam *et al.*, 1998) [26]. The hepatotoxin paracetamol is frequently used to induce liver fibrosis in animal models treatment with paracetamol generates free radicals that trigger a cascade of events that result in hepatic fibrosis, mimicking the oxidative stress that has a fibro genic effect on HSC. Although no successful therapeutic approach to this pathogenetic mechanism in liver disease has been developed, antioxidants therapies have shown to achieve some positive effects (Hallowell *et al.*, 1984; Hochstein *et al.*, 1988) [9, 11]. Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity. In view of this, the present study was undertaken to investigate the hepatoprotective activity of *Platycladus orientalis* extract against paracetamol induced hepatotoxicity in male Wistar rats. *Platycladus orientalis* has been used as a medicinal plant for thousands of years. *Platycladus orientalis* has held claim for therapeutic use for fevers, dyspepsia, gastric ulcers, sore throats, asthma, bronchitis, Addison's disease and rheumatoid arthritis and has been used as a laxative, antitussive and expectorant (Alok *et al.*, 2013). Among its most consistent uses are as a demulcent for the digestive system, to treat coughs, to soothe sore throats, and as a flavoring agent. The present study was undertaken to evaluate the protective effect of *Platycladus orientalis* aqueous and petroleum ether extract on paracetamol induced hepatotoxicity. In addition, the antioxidant property of *Platycladus orientalis* extracts in liver-injured rats was investigated.

Materials and Methods

Plant material

Leaves of *Platycladus orientalis* were collected in the month of November 2011 from its natural habitat from nearby Dasapalla forest division, Nayagarh district of Odisha, India. The plant was authenticated by Dr. A.K. SINGH (H.O.D) T.D.P.G. College, Jaunpur, U.P, India,

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and voucher specimens (PO/GU/2011-2012/01) were deposited in the herbarium Department. The leaves were cleaned and dried under the shade to avoid degradation of volatile oil. After qualitative photochemical screening of various extracts of *Platycladus orientalis* it was observed that the aqueous extract mainly contains carbohydrates, gums & mucilage, proteins & amino acids, fixed oils, fats, phytosterols, tannins, phenolic compounds but the petroleum ether extract contains Gums & mucilage, fixed oils, fats, phytosterols, tannins, saponins and phenolic compounds.

Acute toxicity study

Wistar rats (200 - 250 g) of either sex were maintained under standard environmental conditions and had free access to feed and water. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee. Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method). All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of PBRI, Bhopal (Reg No. - 1283/c/09/CPCSEA). The protocol approval reference number is PBRI/12/IAEC/PN-340.

Experimental design

Rats were randomly divided into seven groups of six animals each and each group was kept in a separate cage. All the groups were treated orally for 7 days (Arjuman *et al.*, 2007)^[5]. Group I served as normal control and was treated with vehicle (0.5% carboxyl methyl cellulose). Group II served as toxin control and treated with vehicle paracetamol (750 ± 5) mg/kg. Group III served as standard and was treated with silymarin 25 mg/kg. Group IV was treated with 100 mg/kg aqueous extract of *Platycladus orientalis* by suspending in 0.5% carboxyl methyl cellulose. Group V was treated with 200 mg/kg aqueous extract of *Platycladus orientalis* by suspending in 0.5% carboxyl methyl cellulose, Group VI was treated with 100 mg/kg petroleum ether extract of *Platycladus orientalis* by suspending in 0.5% carboxyl methyl cellulose, Group VII was treated with 200 mg/kg petroleum ether extract of *Platycladus orientalis* by suspending in 0.5% carboxyl methyl cellulose. On the 6th day, rats of group II, III, IV, V, VI & VII were treated with a single dose of paracetamol (750 ± 5) mg/kg as 2 % w/v solution in double distilled water.

Measurement of biochemical parameters

For measurement of biochemical parameters the total experimental time was purposed for 28 days. The animals were handled carefully in these times and room temperature was maintained normal. At the end of experimental period i.e. on 28th days all the animals were sacrificed by decapitation. Initially Blood was collected, and then sera was separated by centrifugation. Serum lipid profile low density lipoprotein (LDL), very low density lipoprotein (VLDL), high density lipoprotein (HDL), atherogenic index and the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) was measured by the following method. The results of the measurement of biochemical parameters are expressed in Table-1.

Estimation of Serum SGPT /ALT (UV- Kinetic method) (Retimen *et al.* 1957)^[21]

Process: Standard and sample was prepared by considering 500 µl of working reagent and 50 µl each of distilled water, standard and sample respectively, later all the samples were incubated at 37°C, aspirated individually and absorbance was

recorded at 340 nm. Here I used SGPT kit (Erba Diagnostics, Mannheim GmbH, Germany)

Assessment of Serum SGOT /AST (UV- kinetic method) (Retimen *et al.*, 1957)^[21]

Process: As mentioned in the above SGPT, the same procedure had been followed. Here I used SGOT kit (Erba Diagnostics, Mannheim GmbH, Germany)

Assessment of Serum Alkaline phosphatase (ALP) (King *et al.*, 1934)

(SALP kit Erba Diagnostics, Mannheim GmbH, Germany)

Process: Standard and sample was prepared by considering 500 µl of working reagent and 10 µl each of distilled water, standard, sample respectively, later all the samples were incubated at 37 °C, aspirated individually and absorbance was recorded at 405 nm

Assessment of Serum Total Proteins (Gornall *et al.*, 1949)^[8]

Process: Standard and sample was prepared by considering 500 µl of working reagent and 10 µl each of distilled water, standard, sample respectively, later all the samples were incubated for 10 min at 37 °C, aspirated individually and absorbance was recorded at 546 nm.

Assessment of Serum Albumin (Saunders, 1986; 579)^[25]

Process: Standard and sample was prepared by considering 500 µl of working reagent and 5 µl each of distilled water, standard, sample respectively, later all the samples were incubated at 37°C, aspirated individually and absorbance was recorded at 630 nm

ALT, AST, ALP activities, TP and Alb were determined with (kit Erba Diagnostics, Mannheim GmbH, Germany)

Assessment of enzymatic antioxidants

Assay of Catalase (CAT) (Deepak *et al* 2007)^[6]

Catalase was assayed according to the method of Deepak *et al.* The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The liver tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-40 C and centrifuged at 5000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H₂O₂ and the enzyme extract. The specific activity of catalase was expressed in terms of units/gram of liver tissue. Absorbance values were compared with a standard curve generated from known CAT.

Assay of super oxide dismutase (SOD) (Kakkar *et al* 1984 and Deepak *et al* 2007)^[13, 6]

Superoxide dismutase (SOD) activity was determined by the method of Kakkar. The assay mixture contained 0.1ml of sample, 1.2ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulphate (186µm), 0.3 ml of nitro blue tetrazolium (300µ m), 0.2 ml of NADH (750 µm). Reaction was started by addition of NADH. After incubation at 300 C for 90 sec, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 ml of nbutanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. The colour intensity of the chromogen in butanol layer was measured at

560 nm against n-butanol and concentration of SOD was expressed as units/g of liver tissue. Absorbance values were compared with a standard curve generated from known SOD.

Determination of reduced glutathione (GSH) (Ellman *et al* 1959 and Deepak *et al* 2007) [7, 6]

Reduced glutathione (GSH) was determined by the method of Ellman. To 0.1 ml of different tissue homogenate 2.4 ml of 0.02M EDTA solution was added and kept on ice bath for 10 min. Then 2 ml of distilled water and 0.5 ml of 50 % TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 rpm for 15 min. 1 ml of supernatant was taken and 2ml of Tris-Hcl buffer was added. Then 0.05ml of DTNB solution (Ellman's reagent) was added and vortexed thoroughly. OD was read (within 2-3min after the addition of DTNB) at 412 nm against a reagent blank. Absorbance values were compared with a standard curve generated from known GSH.

Determination of Thiobarbituric Acid Reactive Substances (TBARS) (Ohkawa *et al* 1979 and Deepak *et al* 2007) [18, 6]

Lipid peroxidations in liver tissues were estimated colorimetric ally by measuring thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa *et al*. To 0.2ml of sample, 0.2ml of 8.1% Sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. The volume of the mixture was Pyridine mixture was added and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min the upper organic layer was taken and its OD was read at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides were expressed as milimoles of thiobarbituric acid reactive substances (TBARS)/100gram of liver tissue using an extinction co-efficient of 1.56x10⁵M-1cm.

The results of *in vivo* antioxidant study are tabulated in the result section in Table-2

Histopathological examination: (Hamden K *et al*. 2009) [18]

For histopathological examination, the stomach was washed thoroughly with saline, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin. Sections (4–5 mm) were prepared and then stained with hematoxylin and eosin (H–E) dye for photo microscopic observation (magnification 100x).

Pieces of liver lobe were fixed in Bouin's fluid for 24 hr and washed in running tap water to remove the color of Bouin's fluid and dehydrated in alcohol in ascending and descending order, embedded in paraffin and cut at 5µm (Automatic Tissue Processor) in a rotary microtome. These sections were then deparaffinized in xylene, stained with hematoxylin-eosin dye and mounted with Canada balsam. The histopathological slides were examined and photographs were taken. Which was expressed in Photomicrograph No. 1

Analytical work**Isolation**

Bioactive molecules occur naturally in plant and animal

products and possess biological activity in addition to their nutritional value, normally at very low concentrations. In the past, drug discovery of bioactive compounds from plants was time consuming and the process of identifying the structures of active compounds from an extract could take weeks, months, or even years, depending on the complexity of the problem. Nowadays, the speed of bioassay-guided fractionation has been improved. By the process of phytochemical investigation it was observed that the aqueous extract of *Platycladus orientalis* consists of carbohydrates, protins & amino acids, fixed oils, fats, phytosterols, tannins, phenolic compounds and saponins So these were selected for purification of some of the secondary metabolites.

The HPLC of the isolated compound was recorded by Shriram murti Smarak college of engineering and technology, (pharmacy) Bareilly, U.P. All the figures are expressed in Fig-1, Fig-2 & Fig-3.

Spectroscopy

In the whole experiment in order to justify the research work two spectroscopy method carried out i.e- NMR & IR.

Nuclear magnetic resonance spectroscopy, most commonly known as NMR spectroscopy, is a research technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. 1H- NMR of the isolated compound was recorded on JEOL AL300 FTNMR Chemistry Department Banaras Hindu University (B.H.U), Varanasi, 22105. All the figures are expressed in Fig-4 & Fig-5

IR spectroscopy is the spectroscopy that deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. It covers a range of techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify and study chemicals. A common laboratory instrument that uses this technique is a Fourier transform infrared (FTIR) spectrometer. IR spectrum of isolates was recorded on Perkin Eimer Spectrum Version10.03.05 Chemistry Department Banaras Hindu University (B.H.U), Varanasi,22105. All the figures are expressed in Fig-6 & Fig-7

Statistical Analysis

Statistical analysis involved used of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Dunnet's multiple range tests at a level of P < 0.01

Results

Table 1: Effect of various extract of *Platycladus orientalis* on serum enzymes, Total bilirubin and total protein of paracetamol induced hepato toxic rats

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	BILD (mg/dl)	BILT (mg/dl)	ALB (mg/dl)	PRO (mg/dl)
Normal	45.06±1.68**	111.08±2.42**	113.11±2.67**	0.21±0.01**	0.25±0.02**	5.23±0.26**	15.18±0.59**
Toxicant Paracetamol	153.80±11.04	232.49±20.52	243.51±4.49	0.70±0.09	1.65±0.19	2.46±0.19	6.85±0.73
Std group Silymarin	61.32±2.06**	122.85±1.46**	121.81±3.33**	0.30±0.02**	0.31±0.02**	4.86±0.31**	14.26±0.65**
PCM+PO(PE) low dose 100 mg/kg	80.31±1.41**	187.88±5.28**	154.91±1.86**	0.44±0.02**	1.06±0.02**	3.81±.079**	11.33±0.48**
PCM+ PO(PE)high dose 200 mg/kg	63.11±0.36**	128.42±2.34**	128.95±0.83**	0.26±0.008**	0.36±0.01**	4.63±0.34**	13.67±0.44**

PCM+ PO(AE) low dose 100 mg/kg	84.81±1.31**	189.74±10.99**	160.25±2.05**	0.56±0.02 ^{ns}	1.11±0.07**	3.37±0.14*	10.52±0.46**
PCM+PO(AE)high dose 200 mg/kg	64.94±1.37**	136.57±2.30**	129.70±1.20**	0.29±0.004**	0.62±0.01**	3.90±0.07**	12.59±0.50**

Values are Mean ± SEM; ** $P < 0.01$, * $P < 0.05$ & ^{ns} $P < 0.05$ is considered significant when compared with toxicant paracetamol-treated group by Dunnett's multiple comparison test

Serum lipid profile in different groups of treated rats. Group I: Normal, Group II: Toxicant paracetamol rats Group III: Std group Silymarine 25mg/kg IV PCM+ PO(Petroleum ether) low dose 100 mg/kg Group V: PCM+ PO(Petroleum ether) high dose 200 mg/kg Group VI PCM+ PO(Water) low dose 100 mg/kg Group VII: PCM+ PO(Water) high dose 200 mg/kg Group

Table 2: Result of the Estimation of *in vivo* antioxidant in *Platyclusus orientalis* treated Paracetamol induced hepatotoxic rats

Group	SOD (units/mg protein)	CATALASE (units/mg protein)	GSH (units/mg protein)	TBARS (units/mg protein)
Normal	17.69±0.11**	52.71±0.85**	115.6±0.50**	2.18±0.01**
Toxicant Paracetamol	6.20±0.31	39.66±0.30	75.81±0.16	5.74±0.22
Std group Silymarine	14.50±0.25**	47.01±0.32*	105.79±0.28**	3.13±0.08*
PCM+PO(PE) 100 mg/kg	8.55±0.13**	41.64±0.13**	87.09±0.44**	5.07±0.01**
PCM+ PO(PE)200 mg/kg	11.78±0.22**	43.76±0.08**	101.37±0.59**	4.03±0.06**
PCM+ PO(AQ) 100 mg/kg	7.42±0.16**	41.35±0.10**	83.06±0.35**	5.03±0.17**
PCM+PO(AQ) 200 mg/kg	10.70±0.12**	43.57±0.13**	100.39±0.51**	4.04±0.06**

Values are Mean ± SEM; ** $P < 0.01$, * $P < 0.05$ & ^{ns} $P < 0.05$ is considered significant when compared with toxicant paracetamol-treated group by Dunnett's multiple comparison test

Antioxidant profile in different groups of treated rats. Group I: Normal, Group II: Toxicant paracetamol rats Group III: Std group Silymarine 25mg/kg IV PCM+ PO(Petroleum ether) low dose 100 mg/kg Group V: PCM+ PO(Petroleum ether) high dose 200 mg/kg Group VI PCM+ PO(Water) low dose 100 mg/kg Group VII: PCM+ PO(Water) high dose 200 mg/kg Group

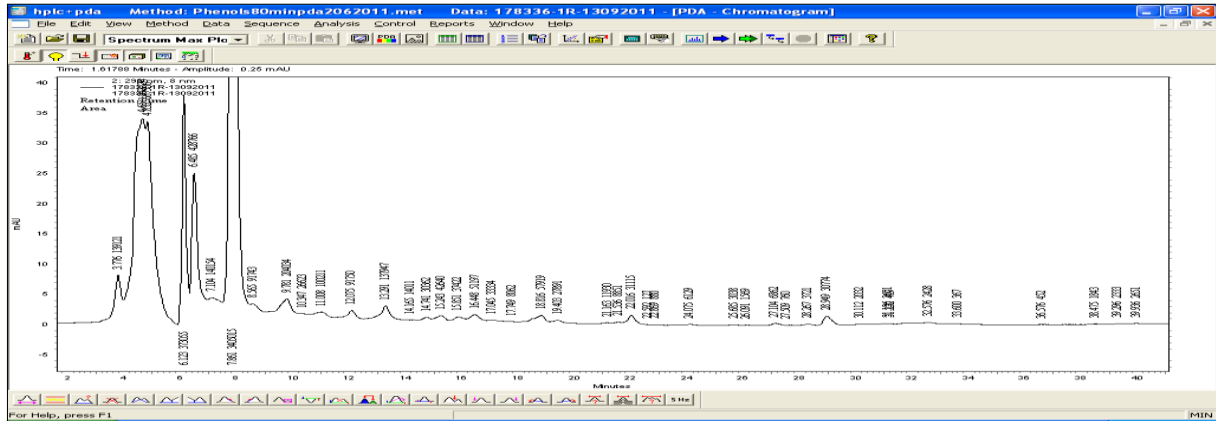


Fig 1: Aqueous extract of *Platyclusus orientalis* possess phytosterol

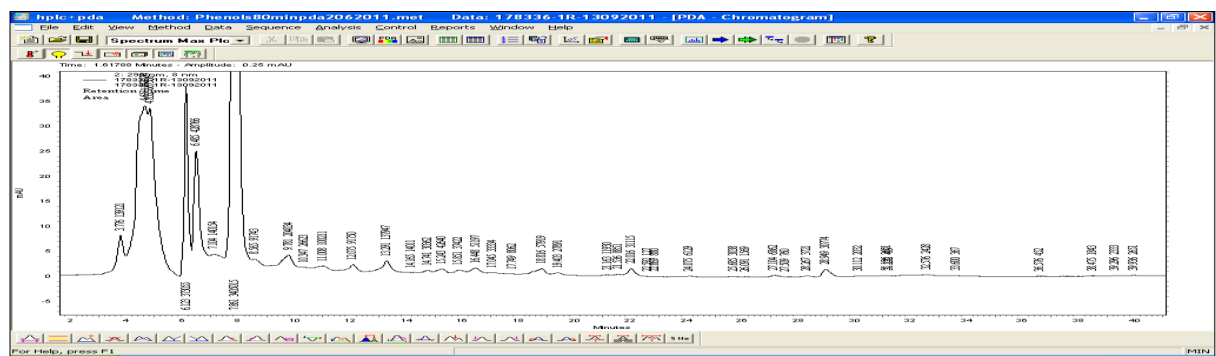


Fig 2: Petroleum ether extract of *Platyclusus orientalis* possess phytosterol

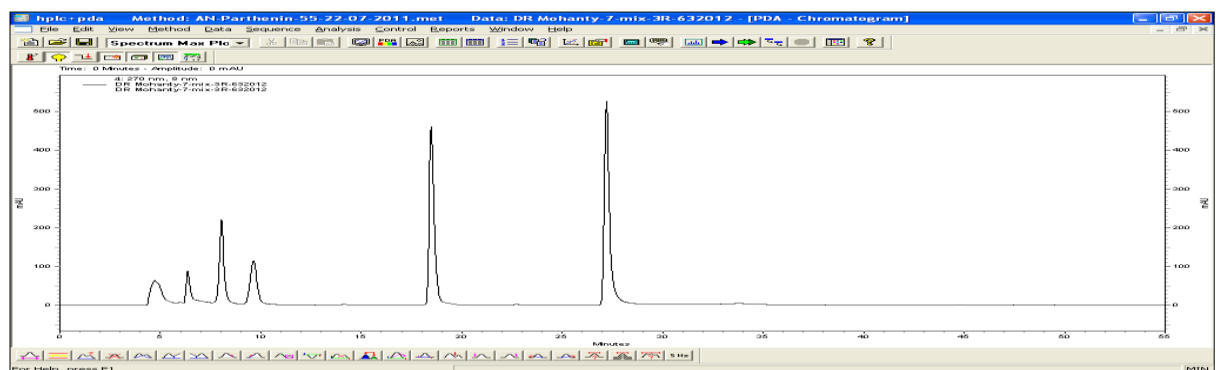


Fig 3: Standard data for detection of phytosterols

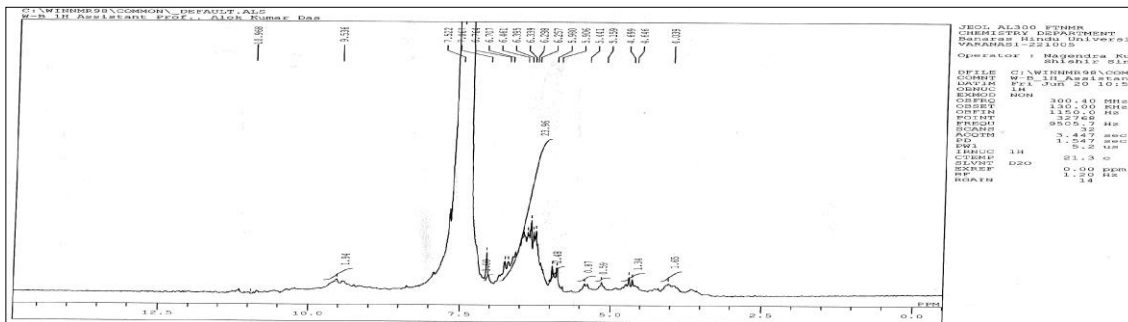


Fig 4: NMR Spectroscopy of aqueous extract of *Platycladus orientalis*

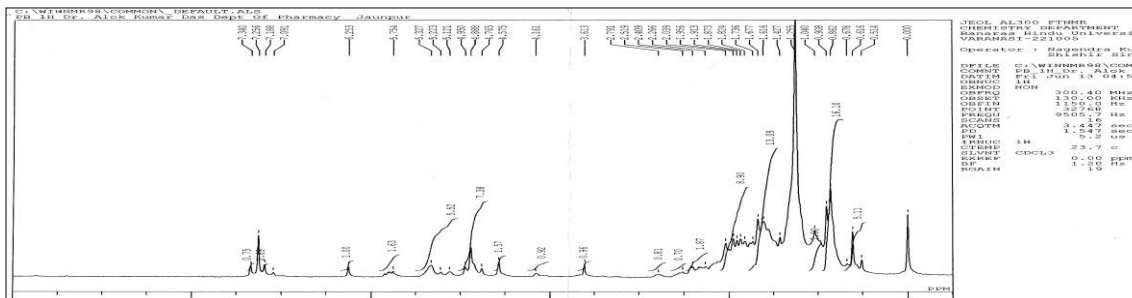


Fig 5: NMR Spectroscopy of petroleum ether extract of *Platycladus orientalis*

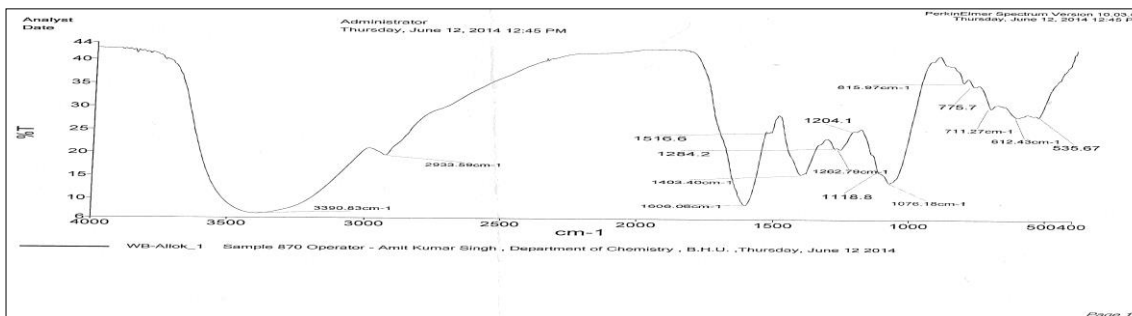


Fig 6: IR Spectroscopy of aqueous extract of *Platycladus orientalis*

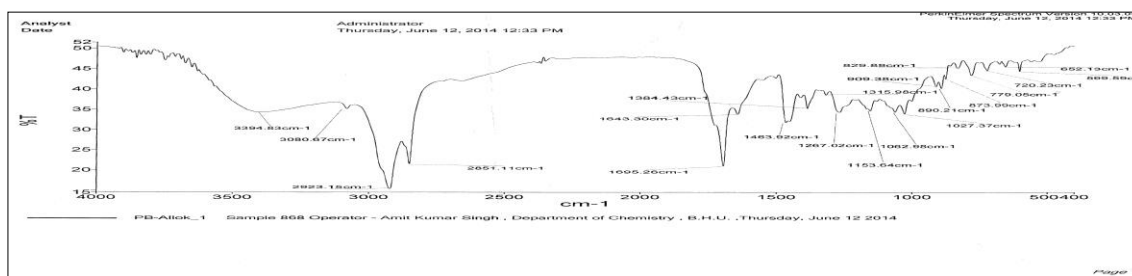


Fig 7: IR Spectroscopy of petroleum ether extract of *Platycladus orientalis*

Toxicity test showed that *Platycladus orientalis* treatment did not significantly affect rats.. None of experimental rats displayed toxic symptom and died. All rats had normal condition.

Histopathology

For histopathological study, animals from all groups were anaesthized with mild ether anaesthesia and dissected. Pancreas are excised out of the animal’s body and put immediately into 10% formalin solution in a stoppered container. These samples were then sent to diagnostic lab fixation (using Bouin’s solution), dehydration, embedding (in paraffin), sectioning (with standard microtome) and staining (Haematoxylin or eosin). The slides so prepared were than examined by pathologist and the pictures were clicked with the help of a binocular microscope fixed with a camera.

Photomicrograph No. 1. Photomicrograph of Liver section (A-Portal traid, B- portal inflammation, C- central vein, D- totally degenaretion, E- sinusoids, F- plates of hepatocytes, G- Pknosis)

Histology of liver revealed,

(PE) & (AQ) Extract Low dose (100 mg/kg) + Paracetamol treated group: The sections from the liver, showed Severe vacuolar degeneration, very mild inflammatory cell reaction, very mild perivascular edema and congested vessels.
 (PE) & (AQ) Extract High dose (200 mg/kg) + Paracetamol treated group: Sections from the liver, showed Mild perivascular edema, moderate vacuolar degeneration, very mild inflammatory cell reaction, congested vessels.

Histopathology

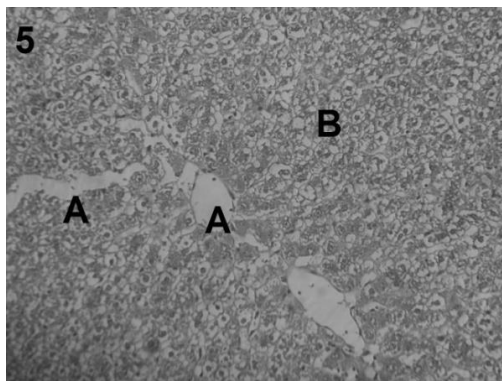


Fig 8: Photomicrograph of liver tissue of rats treated with (AQ) high dose 200 showing central vein, portal inflammation

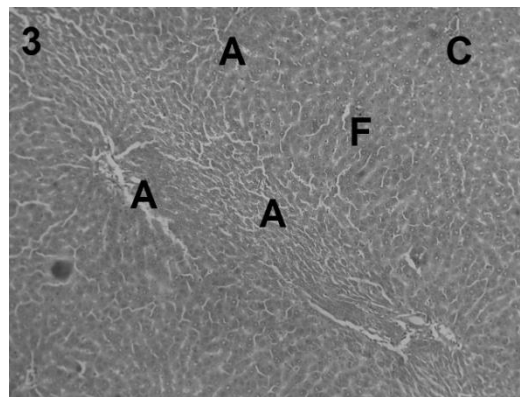


Fig 12: Photomicrograph of liver tissue of normal showing Portal traid, plates totally and central vein.

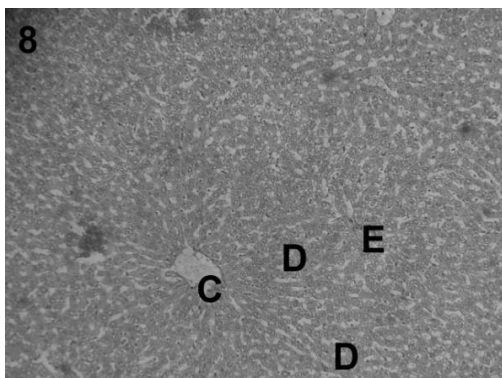


Fig 9: Photomicrograph of liver tissue of rats treated with (AQ) low dose 100mg/kg totally degenaretion & sinusoids

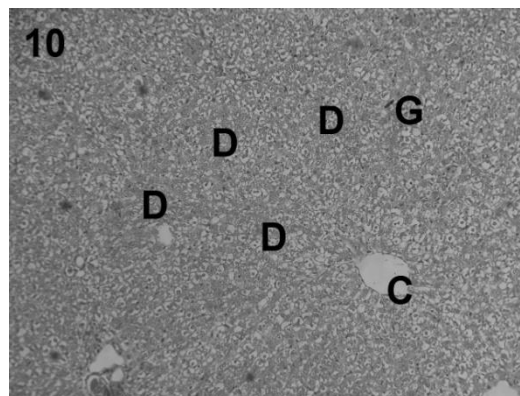


Fig 13: Photomicrograph of liver tissue of Toxicant paracetamol showing degenaretion and central vein

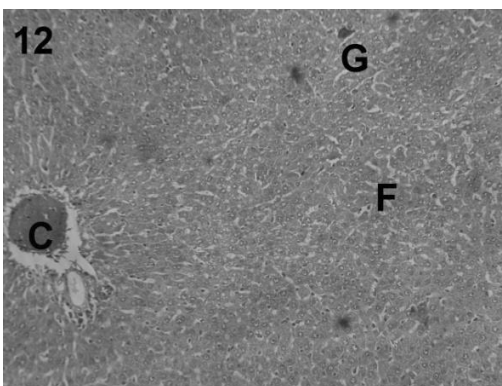


Fig 10: Photomicrograph of liver tissue of rats treated with (PE) high dose 200 mg/kg showing central vein & plates of hepatocytes & Pknosis

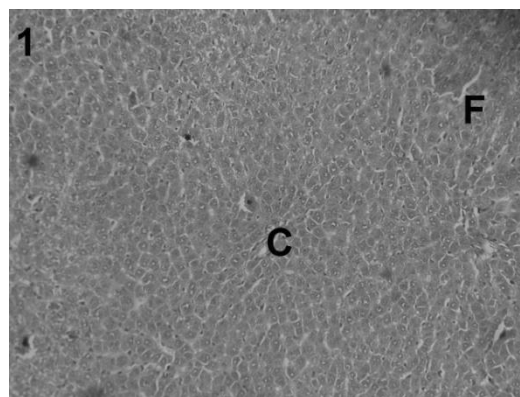


Fig 14: Photomicrograph of liver tissue of Std group Silymarine showing plates of hepatocytes & central vein.

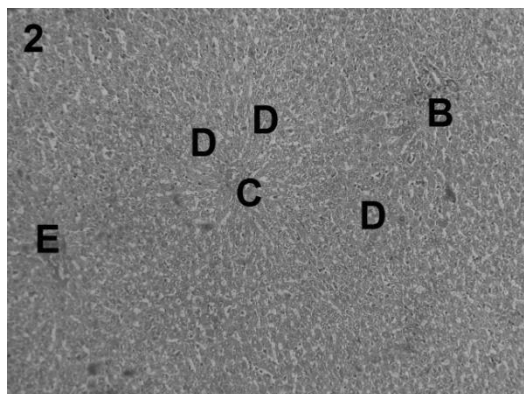


Fig 11: Photomicrograph of liver tissue of rats treated with (PE) low dose 100 mg/kg showing central vein, totally degenaretion & sinusoids

Discussion

Clinical benefits of *Platycladus orientalis* have been reported. *Platycladus orientalis* extracts have been used in the clinical treatment of numerous illnesses with considerable success in world (Alok *et al.*, 2014) [2]. It has a wide range of functions, including detoxification, protein synthesis, and production of biochemicals necessary for digestion. A large number of plants and formulations have been claimed to have hepatoprotective activity. In India, many plant medicines possessed hepatoprotective activity. Paracetamol-induced hepatic destruction, as evidenced by decreased serum AST, ALT, and ALP levels (Vaishwanar *et al.*, 1976) [28]. Paracetamol is a well-known hepatotoxic agent. The basis of its hepatotoxicity lies in its biotransformation by the cytochrome P450 system to free radicals. Since free

radicals play such an important role in paracetamol-induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have a hepatoprotective effect. Indeed, various natural products have been reported to protect against paracetamol-induced hepatotoxicity. The medicinal herb Antioxidant enzymes (SOD, TBAR, GST and CAT) represent one protection against oxidative tissue-damage (Scott *et al.*, 1991). SOD converted O₂ into H₂O₂. GPx and catalase metabolize H₂O₂ to non-toxic products. The GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. This system consists of GSH and an array of functionally related enzymes, of which GR is responsible for the regeneration of GSH (Ellman *et al.*, 1959) [7], whereas GPx and GST work together with GSH in the decomposition of hydrogen peroxide other organic hydroperoxides. Paracetamol also caused an increased in SOD, GR, GST and catalase activities in the liver over those of the control group. Under oxidative stress, some endogenous protective factors such as GPx and catalase are activated in the defense against oxidative injury. Antioxidant enzymes levels back to their control levels, implying that *Platycladus orientalis* leaf extract may prevent the peroxidation of lipids by paracetamol.

Conclusion

The present investigation indicates that *PO* (AQ) and *PO* (PE) extract shows significant protection against paracetamol induced toxicity by its ability to ameliorate the lipid peroxidation through the free radicals scavenging activity, which enhanced the levels of antioxidant defense system. Our study also showed that petroleum ether extract of *Platycladus orientalis* in the dose of 100mg/kg and 200mg/kg has greater effect than aqueous extract at the same dose level due to the presence of tannins, saponins and phenolic compounds. Therefore petroleum ether extract appears to be useful in the attenuation of paracetamol induced lipid peroxidation and showed more prominent effect than petroleum extract. Both the extract showed significant activity against paracetamol induced liver damage in rats when compared with that of standard drug silymarin. Further investigation is underway to determine the exact phytoconstituents in the extracts that are responsible for its hepatoprotective effect. In the present analytical study it was justify that due to the present of phytosterol hepatoprotective activity, in addition to antioxidant activity expressed in *platycladus orientalis*.

Declaration of Interest

We the authors have no conflict in this paper.

Acknowledgments

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