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IN VITRO AND *IN VIVO* ANTIHEPATOTOXIC ACTIVITY OF *OROXYLUM INDICUM* AGAINST CARBON-TETRACHLORIDE INDUCED HEPATIC DAMAGE

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ABSTRACT: Evaluation of the anti-hepatotoxic activity of methanol-dichloromethane (MDM) extract of *Oroxylum indicum* (OI) whole plant on carbon tetrachloride (CCl_4)- induced hepatotoxicity in rat liver explant cultures (*in-vitro*) and chronic liver damage in rats (*in-vivo*). In toxicant (CCl_4) treated explants culture set (*in-vitro*), the marker enzymes GOT, GPT and ALP level increased significantly ($p<0.001$) when compared to control set. Treatment of CCl_4-intoxicated liver explant culture sets with MDM extract of OI whole plant at a concentration of 3.3 mg/ml attenuated the marker enzymes GOT, GPT and ALP levels in culture supernatants significantly ($p<0.001$) as compared to CCl_4 treated explant culture sets. A significant hepatocellular damage as evident from significant elevation in serum activities of GOT, GPT, ALP, bilirubin concentration (total and direct) with decrease in albumin and total protein were found in CCl_4: liquid paraffin (1:1) treated animals (*in-vivo*) when compared to normal values. The MDM extract of OI whole plant (200 mg/kg/day, p.o.) exhibited a significant reduction ($p<0.001$) in CCl_4: liquid paraffin (1:1) induced increased serum levels of GOT, GPT, ALP and bilirubin concentration whereas the albumin and total protein content increased thus reversing hepatotoxicity causing significant liver recovery. Mild swelling of hepatic cells with minimal hepato-cellular injury is shown in T.S of liver tissue from OI extract treated group in contrasts to toxic group. The study suggested the hepatoprotective activity of MDM extract of *O. indicum* whole plant *in-vitro* and *in-vivo* against CCl_4 –induced hepatotoxicity.

INTRODUCTION: The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may injure the organ.

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Other chemical agents such as those used in laboratories and industries, natural chemicals (e.g. microcystins) and herbal remedies can also induce hepatotoxicity. In India, rural and tribal people use a varied range of plants of different genus as folk medicines for health problems related to liver [1].

In India about 20,000 deaths are found every year due to liver disorder. Hepatocellular carcinoma is one of the ten most common tumors in the world and the third leading cause of cancer-related death [2]. *Oroxylum indicum* Vent. (OI) (Family: Bignoniaceae; locally known as *Tatelo*), a small to medium sized deciduous tree, up to 12 m in height,

branched at top, bark light brown, soft and often with numerous corky lenticels, widely found throughout the Asian sub-continent including India [3]. *Oroxylum indicum* have been used as a single drug or as a component of certain poly-herbal drug preparations in Indian Ayurvedic system of medicine. It is active ingredient of well-known Ayurvedic formulations like Chyavanprash, Dashmularista etc [4].

Different parts of OI are used as astringent, tonic, anti-inflammatory, anti-rheumatic, anti-leucodermatic, anti-helminthic, expectorant, piles, bronchitis, asthma, stomachic [5], gastropathy and ulcers, diarrhoea, dysentery [6], jaundice [7], anti-cancer [8], free radical scavenging, immune-stimulant [9] etc. Phytochemical studies on leaves shown presence of baicalein-6-glucuronide, baicalein-7-glucuronide, scutellarein, scutellarein-7-glucuronide, alo-emodin and stem bark contains oroxylin-A, baicalein, scutellarein, ρ-coumaric acid [10]. Root bark contains oroxylin-A and ellagic acid [11]; heart wood contains prunetin and ß-sitosterol [12].

In view of excellent pharmacological activities and ethnomedicinal claim, it was decided to evaluate the anti-hepatotoxic activity of extract of whole plant on carbon tetrachloride-induced chronic liver damage in rats and the obtained results are presented here in.

MATERIALS AND METHODS:

Chemicals: Chemicals used in the study were of analytical grade and were procured from Merck Specialties Pvt., Ltd., Mumbai, India. All biochemical assay kits were purchased from Ecoline (Merck). Dulbeccos modified eagle's medium (DMEM) was procured from Sigma, USA. Silymarine (Silybon Suspension; Micro Labs Pvt. Ltd., India) and Phenobarbitone Sodium (Neon Labs., India) were procured from local chemist.

Animals: Healthy albino male rats of Wistar strain weighing between 150-200 g and Swiss albino mice weighing between 20-25 g (obtained from K.V. Virani Institute of Pharmacy and Research Centre, Badhada, Savarkundla, Gujarat, India) were used in the hepatoprotective activity study and oral acute toxicity study respectively. The animals were kept under standard laboratory condition (12 ± 1 h, day-night Schedule; temperature maintained at 25 ± 2^{o}C; housed in large spacious hygienic cages with access to food and water *ad libitum*). The Institutional Animal Ethics Committee approved the experimental protocol and the condition in the animal house was approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), vide registration no.: 1210/PO/ac/08/CPCSEA.

Plant Materials: Fresh OI whole plant was collected in the month of September from Satkosia Forest (Dhenkanal, Odisha, India). Plant materials were identified at Regional Research Laboratory (Institute of Mineral and Material Technology, Bhubaneswar, Orissa, India) and a voucher specimen of OI (Voucher No.: 12500/ RRL (B)) was deposited therewith. After authentication fresh plant materials were collected, washed under running tap water, shade dried and pulverized in a mechanical grinder to get coarse plant materials.

Preparation of Plant extracts and Test doses: Pulverized plant materials were extracted [13] separately at 60^{o}C taking methanol-dichloromethane (MDM) [14] (1:1) as solvent over 24 h and extract was collected by removing excess solvent with the help of rotary evaporator (Heidolph Labrota-4000 Efficient) and preserved in air tight containers (yield: 15.87 %). Calculated amount of extract was suspended in 0.5% (v/v) of CMC to prepare the test doses.

Oral Acute Toxicity study: Oral acute toxicity study [15] of the extract was carried out on albino mice at a dose range of 100 – 4000 mg/kg of body weight and LD_{50} was calculated.

***In-vitro* Hepatoprotective activity:** For *in-vitro* hepatoprotective activity studies, rat liver was extracted and explant cultures were done by growing rat hepatocytes in Dulbeccos Modified Eagle's Medium (DMEM) with 5 % of new born calf serum (NBCS) [16]. The culture was maintained for 48 h prior to the experiment as per standard procedure [17]. Total 6 culture sets were taken for the study each set comprising six replicates (36 wells). The different experimental conditions for each culture sets are as follows, Normal: pure culture; Toxic: CCl_4 (83.3 µl/ml) was added to each plate; Standard: CCl_4 (83.3 µl/ml) + Silymarin (0.5 ml;

1.9 mg/ml) was added to each plate; Test sets: CCl_4 (83.3 µl/ml) + OI extract was added in an increasing order of concentration (0.825, 1.65 and 3.3 mg/ml) for each group. After the said treatments culture plates were incubated for an additional 2 h time period. Following the incubation, the explant supernatant from all culture sets were collected for the evaluation of various hepatic marker enzymes like GOT, GPT and ALP.

In-vivo **hepatoprotective activity:** Total 30 healthy albino rats were divided randomly into 5 groups each comprising 6 animals. Normal group received 0.5 % (v/v) of CMC in normal saline (5 ml/kg; p.o) once daily for 8 weeks. Toxic group received CCl_4 in liquid paraffin (1:1; 1 ml/kg of body weight; i.p) twice in a week for 8 weeks. Standard group received a standard drug Silymarin (5 ml/kg; p.o) once daily for 8 weeks along with CCl_4 in liquid paraffin (1:1; 1 ml/kg of body weight; i.p) twice in a week for 8 weeks. Test group received the extract at a dose of 200 mg/kg of body weight (once daily; p.o) respectively for 8 weeks along with CCl_4 as mentioned above.

The treatment duration was of 8 weeks [16] and the test animals were sacrificed 24 h after the last dosing at the end of 8th week. Rats were anaesthetized with Phenobarbitone sodium (40 mg/kg; i.p) before sacrificing and blood sample from each test animal was collected by cardiac puncture. The serum prepared from the collected blood samples were subjected to biochemical investigation of different parameters in serum including glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP), bilirubin (total and direct), albumin and total protein. The liver was dissected out immediately after sacrifice, washed in cold saline. Small pieces of liver tissue were collected and preserved in 10% formalin solution for histopathological studies.

Statistical analysis: The results obtained in biochemical assays were given in terms of mean ± SEM. The statistical significance of the data was assessed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test between different groups. Toxic, Standard and Test groups were compared with normal group. Standard and all Test groups were compared with the toxic group. $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Oral acute toxicity study: Oral acute toxicity study of OI whole plant extract was carried out on Swiss albino mice. Zero death / no death of test animals were observed after administration of the MDM extract of OI whole plant at the said dosage range (100-4000 mg/kg of body weight). Thus, 200 mg/kg was selected as a safe dose.

In-vitro **hepatoprotective activity:** The *in vitro* anti-hepatotoxicity study exhibited from attachment and proliferation of explant cultures within 48 h and the supernatant subjected to GOT, GPT and the ALP estimation exhibited significant variations in all experimental sets (**Table 1**). In the toxicant (CCl_4) treated explants culture set, GOT (42.34 ± 1.16 IU/l), GPT (20.47 ± 0.95 IU/l) and ALP (34.88 ± 2.07 IU/l) level increased significantly ($p<0.001$) when compared to control set. MDM extract of OI in higher dose (3.3 mg/ml) significantly lower the GOT (21.15 ± 0.82 IU/l; $p<0.001$), GPT (7.8 ± 0.92 IU/l; $p<0.001$) and ALP (22.5 ± 1.20 IU/l; $p<0.001$) level when compared to CCl_4 treated explant culture sets.

In-vivo **hepatoprotective activity:** Serum parameters like GOT, GPT, ALP, bilirubin (total and direct), albumin, total protein along with change in body weight of test animals were studied (*in-vivo*) and the data are tabulated in **Table 2**. Animals administered with CCl_4: liquid paraffin (1:1) dosage regimen developed significant hepatocellular damage as evident from significant elevation in serum activities of GOT, GPT, ALP, bilirubin concentration (total and direct) with decrease in albumin and total protein compared to normal values. The MDM extract of OI whole plant (200 mg/kg/day, p.o.) exhibited a significant reduction ($p<0.001$) in CCl_4: liquid paraffin (1:1) induced increased serum levels of GOT (235.25 ± 16.76 IU/l; $p<0.001$), GPT (156.21 ± 15.56 IU/l; $p<0.001$), ALP (194.1 ± 14.0 IU/l; $p<0.001$) and bilirubin concentration (Direct: 0.43 ± 0.12 mg/dl, $p<0.01$; Total: 1.51 ± 0.26 mg/dl, $p<0.05$) whereas the albumin (2.54 ± 0.54 g/dl) and total protein (5.35 ± 0.31 g/dl) content increased thus reversing hepatotoxicity causing significant liver recovery.

TABLE 1: SCREENING OF *IN-VITRO* HEPATOPROTECTIVE ACTIVITY OF *OROXYLUM INDICUM*

Explant Culture Sets	Dose	GOT (IU/l)	GPT (IU/l)	ALP(IU/l)
Normal	0.5 ml NS	13.91 ± 1.19	2.91 ± 0.56	15.57 ± 0.62
Toxic	CCl_4 (83.3 µl/ml)	42.34 ± 1.16 ***	20.47 ± 0.95 ***	34.88 ± 2.07***
Standard	0.5 ml (1.9 mg/ml of Silymarin)	16.28 ± 0.65 ns, c	4.22 ± 0.42 ns, c	17.76 ± 0.51 ns, c
OI	0.825 mg/ml	35.7 ± 1.08 ***, c	15.68 ± 1.14 ***, b	29.08 ± 0.7 ns , c
	1.65 mg/ml	28.8 ± 0.64 ***, c	10.23 ± 0.68 ***, c	25.7 ± 0.6 ***, c
	3.33 mg/ml	21.15 ± 0.82 ***, c	7.8 ± 0.92 **, c	22.5 ± 1.2 **, c
F value		139.67	69.034	40.832

Data are cited as mean ± SEM, for n=6. Statistical significance of the data was assessed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test between different groups. Toxic, Standard and Test groups were compared with Normal group: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. Standard and Test groups were compared with Toxic group: a: $P<0.05$, b: $P<0.01$, c: $P<0.001$. ns: non-significant. OI- *Oroxylum indicum* extract. ALP: alkaline phosphatase; DMSO: dimethyl-sulphoxide; GOT: glutamate oxaloacetate transaminase; GPT: glutamate pyruvate transaminase.

TABLE 2: PARAMETERS STUDIED FOR *IN-VIVO* HEPATOPROTECTIVE ACTIVITY OF *OROXYLUM INDICUM*

Group	GOT (IU/l)	GPT (IU/l)	ALP (IU/l)	Change in body weight (g)	Albumin (g/dl)	Total protein (g/dl)	Bilirubin (direct) (mg/dl)	Bilirubin (total) (mg/dl)
Normal	92.9 ± 6.52	71.63 ± 7.6	112.29 ± 0.42	2.65 ± 0.52	4.28 ± 0.59	7.4 ± 1.22	0.12 ± 0.03	0.77 ± 0.09
Toxic	474.25 ± 27.19 ***	262.62 ± 22.90 ***	330.71 ± 22.64 ***	-10.87 ± 1.61 ***	1.3 ± 0.33 **	3.24 ± 0.67 *	0.92 ± 0.12 ***	2.61 ± 0.45 ***
Standard	151.59 ± 28.45 ns, c	114.72 ± 14.45 ns, c	165.35 ± 16.62 ns, c	8.12 ± 0.79 **, c	3.53 ± 0.43 ns, a	6.6 ± 0.88 ns, a	0.34 ± 0.07 ns, b	1.20 ± 0.17 ns, b
OI-200	235.25 ± 16.76 ***, c	156.21 ± 15.56 **, c	194.1 ± 14.0 **, c	11.08 ± 1.0 ***, c	2.54 ± 0.54 ns, ns	5.35 ± 0.31 ns, ns	0.43 ± 0.12 ns, b	1.51 ± 0.18 ns, a
F value	60.069	25.944	33.887	86.377	7.128	4.684	13.190	8.045

Data are represented as mean ± SEM, for n=6. statistical significance of the data was assessed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test between different groups. Toxic, Standard and Test groups were compared with Normal group: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. Standard and Test groups were compared with Toxic group: a: $P<0.05$, b: $P<0.01$, c: $P<0.001$. ns: non-significant. ALP: alkaline phosphatase; GOT: glutamate oxaloacetate transaminase; GPT: glutamate pyruvate transaminase; OI-200: MDM extract of OI at a dose of 200 mg/kg of body weight.

Photomicrographs of liver tissue sections (T.S) of different groups are presented in **Figure 1 (a-d)**. Plate (a) represents the tissue section of normal group rat, with normal hepato-cellular architecture and hepatic portal vein. Plate (b) is the liver section of toxic group. In plate (b) hepatic architecture is completely disarranged, hepatic blood vessels are severely congested and inflammatory cell infiltration is prevalent. Plate (c) represents the tissue section of group treated with standard drug (Silymarin), showing hepato-cellular regeneration with normal hepatic arrangement. T.S of liver tissue from test extract (OI extract, 200 mg/kg) treated group (plate (d)) showing mild swelling of hepatic cells with minimal hepato-cellular injury in contrasts to toxic group.

The results obtained in toxic group showed that, CCl_4 treated rats encountered vast hepato-cellular injury as marker hepatic enzymes in serum like GOT, GPT and ALP levels were elevated abnormally when compared to the normal rats. Changes in serum bilirubin (total and direct) level explains a deterrent hepato-biliary functions and reduced serum protein level with decreased net body weight supports massive lipid peroxidation and cell death in test rats [18].

It is a known fact that, CCl_4 is a potent hepato-toxin which produces free radicals to induce oxidative stress and lipid peroxidation in hepatic cells and consequently promotes acute liver damage [19].

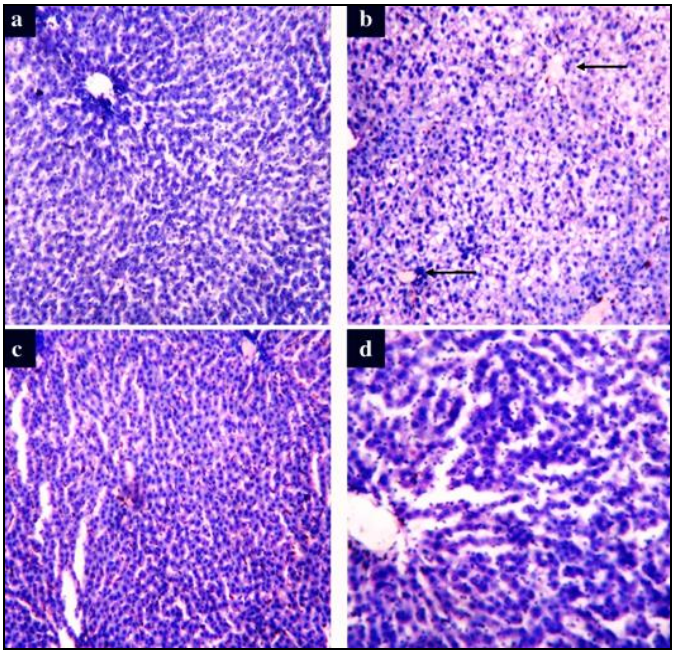

FIGURE 1 (a-d): TRANSVERSE SECTION (T.S.) OF LIVER TISSUE SAMPLES FROM DIFFERENT GROUPS: (a) Normal group, (b) Toxic group, (c) Standard group and (d) Test group

The results obtained in our study are comparable and in accordance with the previously reported hepatoprotective activity of *Pterocarpus santalinus* [20] and *V. negundo* leaves [21] when evaluated *in-vivo* and *Ficus gnaphalocarpa* [22], *Bacopa Monnieri* [17] and *V. negundo* leaves [21] *in-vitro.*

The previous reported findings evidenced the presence of many active phytochemicals including flavonoids, triterpinoids and sterols with potential hepatic restoration capacity [23] which may be the reason behind anti-hepatotoxicity potential of the plant.

CONCLUSION: The results obtained from the study suggested the hepatoprotective activity of MDM extract of *O. indicum* whole plant *in-vitro* and *in-vivo* against CCl_4 –induced hepatotoxicity. Further studies aimed at elucidation of exact mechanism, isolation and purification of active phytoconstituents with potent hepatoprotective activity.

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