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ORIGINAL PAPER

Chelidonium majus 30C and *200C* in induced hepato-toxicity in rats

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Introduction: **Homeopathy is a popular form of complementary and alternative medicine and is used to treat for certain liver ailments.**
Aim: **To analyze the efficacy of homeopathic *Chelidonium majus* (*Chel*) *30C* and *200C* in amelioration of experimentally induced hepato-toxicity in rats.**
Methods: **Rats were randomized into six sub-groups: negative control; negative control + EtOH; positive control; positive control + EtOH group; *Chel 30*; *Chel 200*. Rats were sacrificed at day 30, 60, 90 and 120; various toxicity biomarkers and pathological parameters were evaluated. Gelatin zymography for determination of metalloproteinases activity and Western blot of p53 and Bcl-2 proteins were also employed. All analyses were observer blind.**
Results: **Chronic feeding of p-dimethyl amino azo benzene (p-DAB) and phenobarbital (PB) elevated the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), triglyceride, cholesterol, creatinine and bilirubin and lowered the levels of glutathione (GSH), glucose-6-phosphate dehydrogenase (G-6-PD), catalase and HDL-cholesterol. There were statistically significant modulations of these parameters in the treated animals, compared to positive controls. In both treated groups, there was downregulation of metalloproteinases, p53 and Bcl-2 proteins compared to over-expression in the positive control groups.**
Conclusion: **Both the potencies of *Chel* exhibited anti-tumor and anti-oxidative stress potential against artificially induced hepatic tumors and hepato-toxicity in rats. More studies are warranted.** *Homeopathy* (2010) **99**, 167–176.

Keywords: Hepato-toxicity; Homeopathy; Amelioration; Toxicity biomarkers; Immunodetection

Introduction

Hepatocellular carcinoma (HCC), is one of the most prevalent malignancies world-wide. The prognosis of HCC is poor and depends on factors such as staging, tumor size, liver function and co-morbidities. Current treatments including chemotherapy and proton beam therapies are of limited effectiveness, and only a small percentage of patients are suitable for surgical resection and liver transplantation. New therapeutic approaches are under investigation and development, biological therapies such as gene therapy are in their experimental stages. Complementary and alternative medicine (CAM) therapies including Ayurveda, homeopathy etc are potentially useful palliative approaches that need more scientific investigation in animal models before application to human beings. Recently integrative medicine has achieved therapeutic importance, combining mainstream medical and CAM therapies for relief of symptom(s).

Homeopathy is a popular CAM treatment and has spread throughout the world as a complement to other systems of

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medicine.[1] But it is frequently challenged as there is no plausible mode of action for highly diluted remedies.[2] Nevertheless it is one of the most popular CAM treatments for cancer, seen to increase the body's ability to fight cancer, improve physical and emotional well-being, and alleviate symptoms resulting from the disease or conventional treatments.[3]

Chelidonium majus L, family Papaveraceae, commonly known as Greater celandine is an important medicinal plant. It contains alkaloids including sanguinarine, chelidonine, chelerythrine, berberine, coptisine and some non-alkaloid compounds like flavonoids and phenolic acids.[4] Extracts of *C. majus* exhibit anti-viral, anti-tumor and anti-microbial properties both *in vivo* and *in vitro*.[5–9] Anti-tumor effects of *C. majus* had been reported earlier.[4] Crude alcoholic extracts (mother tincture) of *C. majus* and homeopathic potencies are prescribed in the treatment of various liver, kidney, spleen and other disorders.[4,10–13]

In our laboratory Chelidonium has previously been studied in a mouse model, and reported to have protective effects in hepatic carcinogenesis.[14,15] In the present study rats were used with a view to replicate our previous results in mice, and deploying additionally a newer set of hepato-toxicity biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), reduced glutathione (GSH), catalase, glucose-6-phosphate dehydrogenase (G-6-PD), and lactate dehydrogenase (LDH), triglyceride, cholesterol, HDL-cholesterol, creatinine and bilirubin. The experimental animals were tested for the above at four time points: day 30, 60, 90 and 120. Chelidonium *30C* (*Chel 30C*) and Chelidonium *200C* (*Chel 200C*) were evaluated separately, against hepato-toxicity and tumor induction by the liver carcinogen p-dimethyl amino azo benzene (p-DAB) and the tumor promoter phenobarbital (PB).[16] p-DAB and PB are Group 2B carcinogens (possible human carcinogens),[17] capable of producing liver tumors when fed to experimental animals for a period exceeding two months. For this reason the main focus of our analysis was on the primary target organ, the liver. But these carcinogens have also been reported to affect other organs including spleen, kidney and testis. Therefore these were also included in this study. A combination of patho-physiological tests and widely accepted hepato-toxicity biomarkers were used to reflect hepato-toxicity and cytotoxicity in the experimental animals compared to controls.

Materials and methods

Maintenance of animals

White rats (*Rattus norvegicus*) were reared and maintained (acclimatized to standard laboratory conditions, temperature 25–35°C, and were housed identically under a 12 h light:dark cycle in standard cages) in the animal house of the Department of Zoology (with approval from the University Ethical Committee and under the supervision of the Animal Welfare Committee), University of Kalyani.

Preparation of homeopathic remedies

Chel 30C and *Chel 200C* were derived from plant extract of *C. majus* L. following the Indian Homoeopathic Pharmacopoeia, as previously described.[18,19] in 90% ethyl alcohol and were procured from HAPCO (165, BB Ganguly Street, Kolkata 700014). 1 ml each of the two drugs and Alc-*30* and Alc-*200* (potentized ethanol also procured from HAPCO, Kolkata) was diluted in 20 ml of Milli Q (ultra-pure) water to make the stock solutions of *Chel 30C*, *Chel 200C*, Alc *30C* and *200C*.

Carcinogens and homeopathic remedies

A group of 120 healthy rats, each weighing between 70–100 g were randomly selected. 30 were randomly used for each of the four fixation intervals. All the experimental animals were randomized by fixed random allocation. Feed was wheat, gram and milk powder.

Each group of 30 animals (both male and female) was further divided into 6 sub-groups, each of 5 rsts:

(1) Negative control group: fed normal diet.
(2) Negative control + EtOH group: succussed alcohol (0.06 ml orally by gavage) daily with normal diet.
(3) Positive control group: fed mixed with 0.06% p-DAB (Sigma, D-6760) and provided 0.05% aqueous solution of PB instead of water.
(4) Positive control + EtOH group: fed mixed with 0.06% p-DAB (Sigma, D-6760) and provided 0.05% aqueous solution of PB instead of water. From the sixteenth day onward, they were also treated with succussed alcohol (Alc – 0.06 ml orally through gavage).
(5) *Chel 30* and (6) *Chel 200* groups: fed mixed with 0.06% p-DAB (Sigma, D-6760) and provided 0.05% aqueous solution of PB instead of water and were also treated with 0.06 ml of stock solution of *Chel 30C* through gavage, twice daily (at 8 am and 8 pm) or *Chel 200C* once daily (at 8 am), respectively, starting from the sixteenth day and were sacrificed at day 30, 60, 90 and 120.

Unlike our earlier studies when the treatment was simultaneous with the carcinogen feeding, from day 0, in this study the medication or placebo administration started after 15 days of feeding of carcinogens to rats.

Animals were anaesthetized before being sacrificed at the specific fixation interval. Weights of whole body were recorded before sacrifice and that of liver, spleen, kidney and testes were recorded after sacrifice.

Selection of the parameters

A combination of widely accepted biomarkers of hepato-toxicity like ALT, AST, GSH, GGT, catalase, G-6-PD, and LDH and some pathological tests to indicate general physiological status were adopted. Since chronic dosing with p-DAB + PB (90 days or more) is known to induce liver tumors, some of which become malignant[20] the expression of matrix metalloproteinase (an indicator of metastatic process), p53 protein (tumor suppressor protein) and Bcl-2 protein (anti-apoptotic protein) were also analyzed.

Table 1 Number of rat showing tumors in different treated and control (positive and negative) series at different fixation intervals (30, 60, 90, 120 days)

Series	*Total number of rats used*	*Development of tumor at*				*Total rats with tumors*
		30 days	*60 days*	*90 days*	*120 days*	
Negative control	20	0/5	0/5	0/5	0/5	0/20
Negative control + EtOH	20	0/5	0/5	0/5	0/5	0/20
Positive control	20	0/5	2/5	4/5	5/5	11/20
Positive control + EtOH	20	0/5	2/5	3/5	5/5	10/20
Chel 30	20	0/5	1/5	4/5	2/5	7/20
Chel 200	20	0/5	0/5	2/5	3/5	5/20

5 rats were used per interval for each group.

Laboratory methodology

All analyses were conducted observer blind with respect to the animal belonging to treatment group.

Tumor incidence: On autopsy, the tumor incidence was recorded in control and treated rats.

Blood collection and tissue isolation: Blood was collected in two vials, one containing anti-coagulant (EDTA) and the other without EDTA. Serum was obtained from blood without EDTA by centrifugation for determination of cholesterol, HDL-cholesterol, triglyceride, creatinine, serum bilirubin, catalase, GGT and LDH activity. Blood with EDTA was used for determination of G-6-PD activity. Liver, spleen, kidney and testis tissue of sacrificed rats were quickly isolated in an ice tray and separately processed for the study of biochemical parameters and expression of metalloproteinases by gelatin zymography and also for immunodetection of p53 and Bcl-2 protein by Western blotting.

Biochemical assays: For estimation of ALT, AST, GSH standard methods were adopted.[21] LDH activity was assayed by the UV-Kinetic method of Gay *et al.*[22] Reagent kit was supplied by Reckon Diagnostics P. Ltd., Gorwa, Baroda, India (Code-64X014). For G-6-PD activity, 0.1 ml blood was estimated using diagnostic kit procured from Reckon Diagnostic P. Ltd., India.

GGT activity was assayed by the method of Szasz.[23] Reagent kit was supplied by Reckon Diagnostics P. Ltd. (Code-6LX010). Catalase activity was measured using the method of Chance and Maehly.[24] For creatinine, the standard modified Jaffe's Kinetic method was followed. For determination of cholesterol, HDL-cholesterol and triglycerides, kits provided by Span diagnostics, India, were used. For the estimation of total serum bilirubin reagent kit was supplied by Reckon Diagnostics (Code-64X014). For estimation of matrix metalloproteinases (MMPs) activity, the method of Billings *et al.*[25] was adopted. Metalloproteinase activity was analyzed by Image analyzer (Total Lab-2.01) from Ultra-Lum, 1D Image.

Immunodetection of p53, Bcl-2 proteins was done by the Western blotting method of Magi *et al.*[26] Expression of p53

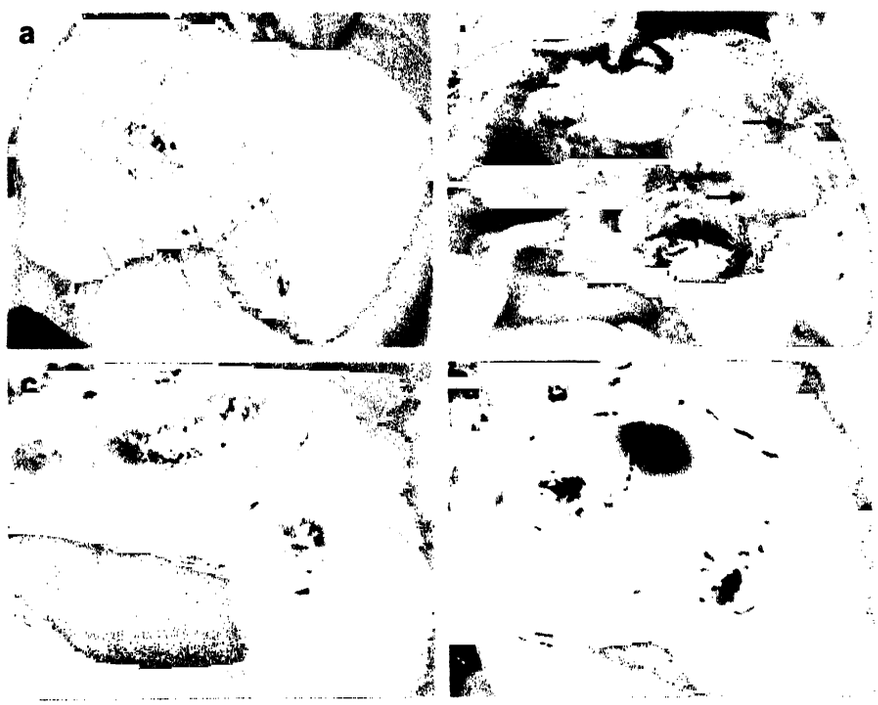


Figure 1 Representative photographs of liver of rats – (a) of negative control series without any nodules; (b) of positive control + EtOH series bearing numerous liver nodules (c) of *Chel 30* and (d) of *Chel 200* series with less number of liver nodules.

Table 2 (a) Mean alanine amino transferase (ALT), aspartate amino transferase (AST) activities (nM/100 mg protein/min), mean reduced glutathione content (GSH) (nM/mg tissue sample) in liver and (b) gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), glucose-6-phosphatase dehydrogenase (G-6-PDH) and catalase activities in serum and whole blood of rat of different treated and control (positive and negative) series at different fixation intervals (30, 60, 90, 120 days)

	ALT activity				*AST activity*			
(a) Fixation intervals in days ⟶	*30 days*	*60 days*	*90 days*	*120 days*	*30 days*	*60 days*	*90 days*	*120 days*
Series	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*	*Activity ± SE*
Negative control	0.009 ± 0.000	0.010 ± 0.001	0.009 ± 0.000	0.016 ± 0.004	0.005 ± 0.001	0.004 ± 0.001	0.003 ± 0.001	0.004 ± 0.001
Negative control + EtOH	0.009 ± 0.000	0.009 ± 0.000	0.011 ± 0.001	0.018 ± 0.003	0.004 ± 0.002	0.005 ± 0.001	0.006 ± 0.001	0.006 ± 0.001
Positive control	0.023 ± 0.001	0.019 ± 0.002	0.023 ± 0.001	0.033 ± 0.001	0.024 ± 0.003	0.020 ± 0.001	0.018 ± 0.001	0.019 ± 0.001
Positive control + EtOH	0.028 ± 0.001	0.029 ± 0.002	0.042 ± 0.001	0.035 ± 0.001	0.027 ± .0.004	0.028 ± 0.002	0.036 ± 0.003	0.038 ± 0.002
Chel 30	0.022 ± 0.003^{n}	0.020 ± 0.003*	0.026 ± 0.001***	0.011 ± 0.001***	0.017 ± 0.002^{n}	0.021 ± 0.001*	0.019 ± 0.001***	0.021 ± 0.001***
Chel 200	0.015 ± 0.001***	0.011 ± 0.002***	0.041 ± 0.005^{n}	0.017 ± 0.004**	0.019 ± 0.001^{n}	0.018 ± 0.001**	0.017 ± 0.001***	0.027 ± 0.003*
Fixation intervals in days ⟶	GSH activity							
Negative control	0.008 ± 0.001	0.004 ± 0.001	0.007 ± 0.000	0.009 ± 0.001				
Negative control + EtOH	0.006 ± 0.002	0.004 ± 0.001	0.006 ± 0.001	0.006 ± 0.001				
Positive control	0.001 ± 0.000	0.002 ± 0.001	0.002 ± 0.001	0.001 ± 0.000				
Positive control + EtOH	0.002 ± 0.001	0.001 ± 0.000	0.001 ± 0.000	0.002 ± 0.000				
Chel 30	0.005 ± 0.002^{n}	0.004 ± 0.002^{n}	0.005 ± 0.001**	0.007 ± 0.001**				
Chel 200	0.004 ± 0.001^{n}	0.003 ± 0.001^{n}	0.006 ± 0.001**	0.008 ± 0.001***				
(b) Fixation intervals in days ⟶	GGT activity (IU/L)				LDH activity (IU/L)			
Negative control	14.23 ± 0.756	14.294 ± 2.713	18.476 ± 0.376	16.377 ± 0.385	550.440 ± 15.892	567.664 ± 11.93	572.741 ± 4.096	534.378 ± 5.774
Negative control + EtOH	14.443 ± 0.616	14.443 ± 0.616	20.000 ± 0.252	17.167 ± 0.145	564.337 ± 7.839	571.555 ± 9.723	571.555 ± 9.723	545.667 ± 8.090
Positive control	34.211 ± 1.117	54.812 ± 1.732	50.299 ± 1.582	60.078 ± 0.848	645.168 ± 3.785	715.712 ± 2.865	677.296 ± 5.774	662.094 ± 4.333
Positive control + EtOH	41.034 ± 0.991	60.505 ± 1.006	45.663 ± 1.068	55.787 ± 2.124	654.944 ± 17.321	780.878 ± 3.508	551.101 ± 6.53	689.079 ± 5.774
Chel 30	27.406 ± 2.702**	46.233 ± 1.594***	28.593 ± 1.003***	28.837 ± 0.882***	485.018 ± 5.774***	612.931 ± 3.632***	587.271 ± 2.311***	465.647 ± 3.756***
Chel 200	18.507 ± 1.447***	62.899 ± 2.334^{n}	54.245 ± 0.972***	39.372 ± 1.155***	605.992 ± 8.660*	628.878 ± 3.924***	524.157 ± 2.033**	406.663 ± 4.096***
Fixation intervals in days ⟶	G-6-PDH activity (U/g Hb)				Catalase activity (Unit/min/mg protein)			
Negative control	18.238 ± 1.732	10.758 ± 1.245	15.304 ± 1.458	11.92 ± 1.660	3.395 ± 0.305	3.061 ± 0.041	4.574 ± 0.382	5.331 ± 1.278
Negative control + EtOH	18.046 ± 0.810	8.997 ± 0.494	14.067 ± 0.371	10.900 ± 0.173	3.037 ± 0.042	2.880 ± 0.055	4.270 ± 0.335	4.710 ± 0.300
Positive control	9.680 ± 0.692	7.716 ± 1.270	8.987 ± 1.458	6.973 ± 1.467	1.355 ± 0.245	0.702 ± 0.031	1.287 ± 0.102	1.859 ± 0.041
Positive control + EtOH	11.522 ± 0.809	7.007 ± 1.979	8.252 ± 0.577	11.522 ± 0.809	1.158 ± 0.286	0.938 ± 0.035	1.250 ± 0.228	1.210 ± 0.200
Chel 30	12.260 ± 0.439^{n}	9.336 ± 1.308^{n}	12.562 ± 0.882**	17.764 ± 0.898***	2.282 ± 0.721^{n}	1.518 ± 0.100***	2.073 ± 0.138*	2.458 ± 0.042***
Chel 200	14.522 ± 2.786^{n}	17.809 ± 1.851**	12.578 ± 1.155*	24.048 ± 1.932***	1.975 ± 0.025*	2.913 ± 0.256***	2.81 ± 0.332**	3.475 ± 0.169***

Values are expressed as mean ± SE for 5 rats each in each series. Comparisons are made between positive control + EtOH and carcinogen treated drug fed rats. Negative control = Normal diet fed; EtOH = Succussed Ethyl alcohol, positive control = p-DAB + PB. *Chel 30* = p-DAB + PB + Chelidonium 30 fed, *Chel 200* = p-DAB + PB + Chelidonium 200 fed rats; SE = Standard error. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ^{n} = non-significant.

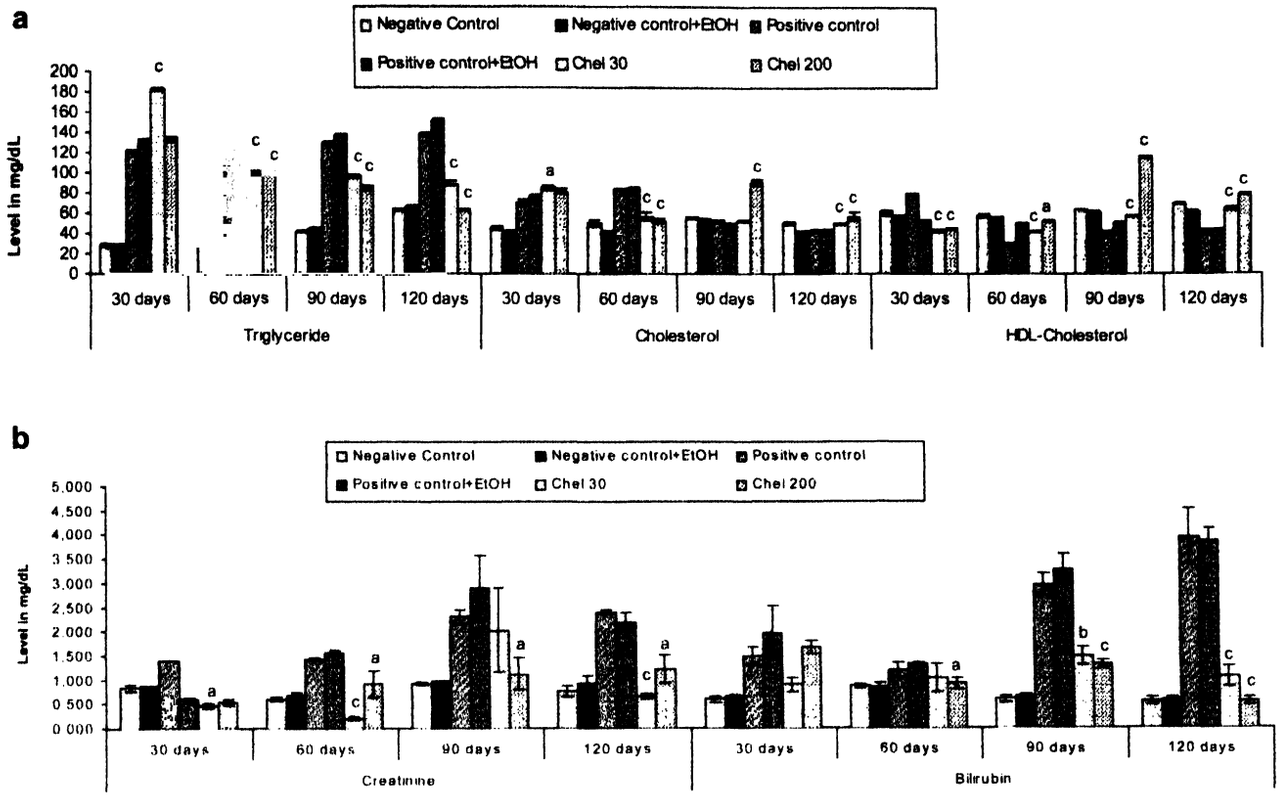


Figure 2 (a) Mean serum triglyceride, serum cholesterol and serum HDL-cholesterol levels and (b) serum creatinine content and serum bilirubin level in (mg/dL) of rat of different treated and control (positive and negative) series at different fixation intervals.

and Bcl-2 protein was analyzed by Image analyzer (Total Lab-2.01) from Ultra-Lum, 1D Image.

Statistical comparisons were made between the positive control + EtOH groups to that of verum administered groups (*Chel 30* and *Chel 200*). The significance of difference between data of the different series was calculated by Student's t-test.[27] ANOVA (SPSS 10.0 Software) was used to compare multiple groups and within the groups and these were also tested for multiple comparisons by Tukey's HSD.

Results

Tumor incidence

Out of 15 rats sacrificed at the three later time points (day 60, 90 and 120), 11 and 10 rats, respectively, showed liver tumors in the positive control and positive control + EtOH fed series, while 7 and 5 rats showed liver tumors in the *Chel 30* and *Chel 200* fed series, respectively (Table 1). In the carcinogen fed positive control group the tumors were present as multiple nodules, whereas some of treated animals presented solitary or a few individually distinguishable tumors. Both treated series showed lower incidence of tumors as compared to positive control at day 90 and day 120 (Figure 1).

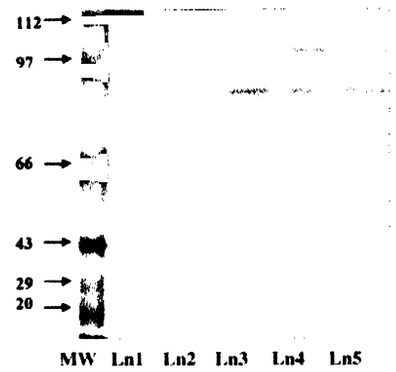
Activity of toxicity biomarkers in liver

ALT activity: ALT activity was higher in the positive control and positive control + EtOH groups at all fixation intervals compared to the negative controls. In rats treated with *Chel 30* the ALT activity was almost same as that of the positive control + EtOH series at day 30, but was significantly lower at all later fixation intervals. On the other hand, in *Chel 200* treated rats, the activity was significantly lower than positive control lower at day 30, 60 and 120, but rose at day 90 (Table 2a). In the *Chel 30* group the mean difference was significant compared to positive control + EtOH series at day 90 only ($p = 0.003$) and at day 30 and day 60 for *Chel 200* group ($p = 0.001$; $p = 0.001$ respectively, Tukey's HSD).

AST activity: The AST activity gradually rose in rats chronically fed p-DAB + PB (positive control) and showed a further increase in the positive control + EtOH fed group up to 120 days, compared to the negative controls. On the other hand, the activity was significantly reduced at all fixation intervals in *Chel 30* treated rats. The same was true for *Chel 200* treated rats (Table 2a). Analysis revealed significant differences between treated groups and positive control + EtOH series only at day 60 ($p = 0.002$ for *Chel 30* group; $p < 0.001$ for *Chel 200* group) (Tukey's HSD).

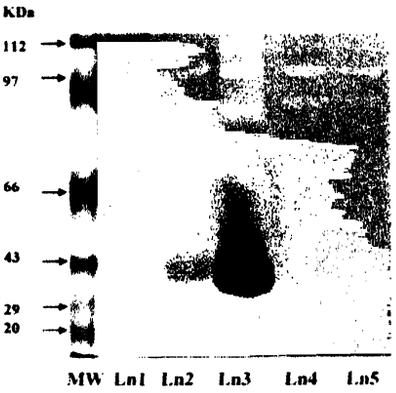
GSH: In the positive control and positive control + EtOH series, the GSH was decreased at all fixation intervals. In *Chel 30* treated rats, the GSH gradually increased. In the *Chel 200* treated group the increase in GSH appeared to be more pronounced at later time points (Table 2a). There were significant difference between the *Chel* groups and positive control + EtOH at day 90 ($p = 0.023$ for *Chel 30*; $p = 0.012$ for *Chel 200*) and at day 120 ($p = 0.004$ for *Chel 30*; $p = 0.001$ for *Chel 200*) (Tukey's HSD).

a



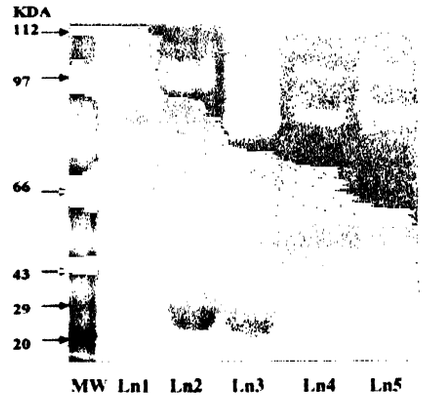
Band No	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5	
	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf
1	100.9	0.14	101.4	0.14	112	0.13	102.9	0.12	102.6	0.12
2	54.2	0.25	90.6	0.24	97	0.34	91	0.24	91.3	0.24
3			26.6	0.85	66	0.86				

b



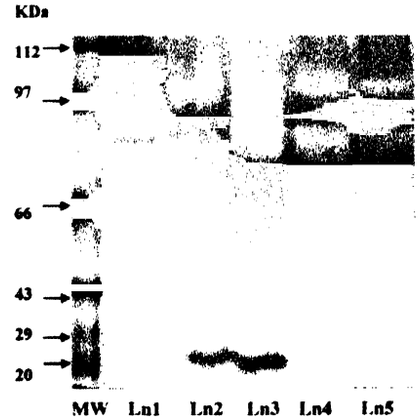
Band No	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5	
	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf
1	101.2	0.11	100.9	0.11	109.5	0.05	102.8	0.01	102.3	0.10
2			44.6	0.69	101	0.11	92.8	0.19	91.8	0.20
3			43	0.71	88.2	0.24				
4					42.7	0.71				

c



Band No	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5	
	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf
1	83.7	0.27	100.9	0.10	109.2	0.04	97.4	0.14	100.9	0.14
2			23.3	0.86	100	0.11	84.8	0.26	86.3	0.28
3					87.8	0.23				
4					24	0.85				

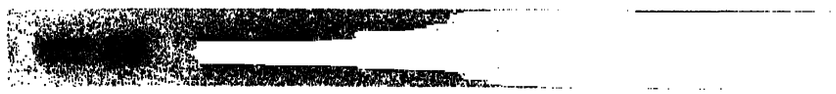
d



Band No	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5	
	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf
1	99.4	0.15	103.1	0.12	100.9	0.14	103.7	0.11	100.9	0.14
2	84.9	0.29	87.7	0.27	95.3	0.19	90.7	0.24	86.3	0.28
3			23.7	0.89	91.3	0.23				
4					87.2	0.27				

Figure 3 MW = Molecular weight marker. Ln1 = negative control, Ln2 = positive control, Ln3 = positive control + EtOH, Ln4 = *Chel 30* (p-DAB + PB + *Chel 30*), Ln5 = *Chel 200* (p-DAB + PB + *Chel 200*). The adjacent tables represent the Gelatin zymogram analysis of bands in liver samples of carcinogens and drug fed rats using Gel-documentation software (Total Lab-2.01) from Ultra-Lum, 1D Image.

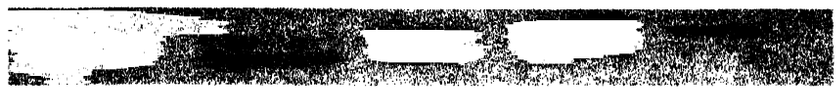
e



p53 at day 90

Ln5 Ln4 Ln3 Ln2 Ln1

Lane 5		Lane 4		Lane 3		Lane 2	Rf	Lane 1	
MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)		MW (kd)	Rf
46.9	0.54	46.9	0.54	46.9	0.55	46.6	0.56	47.5	0.54
45.4	0.59	45.4	0.59	45.4	0.59	45.6	0.59		



p53 at day 120

Ln5 Ln4 Ln3 Ln2 Ln1

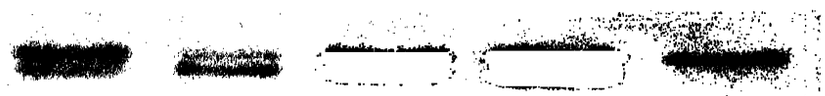
Lane 5		Lane 4		Lane 3		Lane 2	Rf	Lane 1	
MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)		MW (kd)	Rf
47.6	0.53	47.9	0.53	48.2	0.52	48.8	0.51	48.8	0.51
46.4	0.56	47.0	0.56	46.7	0.56	47.0	0.55		

f

Bcl-2 at day 90

Ln5 Ln4 Ln3 Ln2 Ln1

Lane 5		Lane 4		Lane 3		Lane 2	Rf	Lane 1	
MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)		MW (kd)	Rf
32.7	0.57	33.4	0.57	32.3	0.58	30.2	0.60	30.9	0.59
28.5	0.61	26.6	0.63	27.0	0.63	23.9	0.65		



Bcl-2 at day 120

Ln5 Ln4 Ln3 Ln2 Ln1

Lane 5		Lane 4		Lane 3		Lane 2	Rf	Lane 1	
MW (kd)	Rf	MW (kd)	Rf	MW (kd)	Rf	MW (kd)		MW (kd)	Rf
28	0.68	27.6	0.69	27.4	0.69	27.4	0.69	26.9	0.71
253.9	0.73	26.2	0.72	25.7	0.74	25.7	0.74		

Figure 3 (*continued*).

Activity of toxicity biomarkers in secondary target organs

In spleen, kidney and testis the ALT, AST and GSH activities were elevated in rats chronically fed p-DAB + PB and p-DAB + PB + EtOH. The administration of *Chel* in either dilution only marginally reduced these parameters in spleen mostly at day 90 and day 120, this was not statistically significant (data not shown).

GGT activity: The GGT activity in the carcinogen intoxicated rats was higher than controls and increased with duration of feeding. But in rats treated with *Chel 30*, an appreciable lowering was noted at all fixation intervals compared to positive control and positive control + EtOH groups. In *Chel 200* treated rats the GGT activity showed a significant lower activity at day 30 and 120. The effects in the *Chel 30* group was somewhat more pronounced than the *Chel 200* (Table 2b). The mean difference was significant at all the intervals in the *Chel 30* series ($p \leq 0.001$ at day 30, $p = 0.023$ at day 60, $p < 0.001$ at day 90, $p = 0.0$ at day 120) but was not significant at all other intervals except

Table 3 Total liver weight/body weight ratio

Fixation intervals ⟶	*30 days*	*60 days*	*90 days*	*120 days*
Series	*LW/BW*	*LW/BW*	*LW/BW*	*LW/BW*
Negative control	0.024 ± 0.002	0.028 ± 0.002	0.025 ± 0.001	0.033 ± 0.005
Negative control + EtOH	0.025 ± 0.002	0.031 ± 0.003	0.027 ± 0.001	0.036 ± 0.006
Positive control	0.051 ± 0.002	0.050 ± 0.002	0.047 ± 0.001	0.052 ± 0.002
Positive control + EtOH	0.055 ± 0.003	0.054 ± 0.014	0.050 ± 0.001	0.066 ± 0.013
Chel 30	0.040 ± 0.002^{n}	0.037 ± 0.001^{n}	0.036 ± 0.004**	0.046 ± 0.004^{n}
Chel 200	0.053 ± 0.004^{n}	0.048 ± 0.007^{n}	0.036 ± 0.002***	0.043 ± 0.001^{n}

Values are expressed as mean ± SE for 5 rats each in each series. Comparisons are made between positive control + EtOH and carcinogen treated drug fed rats. Negative control = Normal diet fed; EtOH = Succussed Ethyl alcohol, positive control = p-DAB + PB, *Chel 30* = p-DAB + PB + Chelidonium 30 fed, *Chel 200* = p-DAB + PB + Chelidonium 200 fed rats; SE = Standard error. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ^{n} = non-significant.

at day 60 only in *Chel 200* series ($p < 0.001$ at day 30, $p = 0.001$ at day 90, $p < 0.001$ at day 120) (Tukey's HSD).

LDH activity: The activity of LDH was substantially higher in positive control and positive control + EtOH groups than in the negative control group. The LDH activity was lower in *Chel 30* treated rats at all fixation intervals except at day 90, but the effect was more significant in *Chel 200* treated rats (Table 2b). The mean of both the *Chel 30* and *Chel 200* groups was significant against the positive control + EtOH group at all the fixation intervals except at day 30 in *Chel 200* group.

G-6-PD activity: A significant elevation in G-6-PD activity was observed in *Chel 30* and *Chel 200* treated rats compared to positive control and positive control + EtOH series at several time points (Table 2b).

Catalase activity: The catalase activity in positive control rats was reduced at all fixation intervals. There was significant recuperation in both the treated groups at all fixation intervals of which, *Chel 200* appeared to have slightly greater effect (Table 2b).

Pathological parameters

In the positive control and positive control + EtOH series, there was elevation in serum triglyceride, cholesterol, creatinine and bilirubin levels and reduction in HDL-cholesterol level at most of the later time points compared to negative control. There was significant reduction in serum triglyceride, creatinine and bilirubin levels in the *Chel 30* and *200* groups compared to positive control. A slight increase in serum cholesterol and a marked increase in HDL-cholesterol level in the *Chel 30* and *200* series suggest detoxifying effects (Figures 2a, b).

Zymogram profile for protease activity

The expression of MMP was slightly lower in *Chel 200* treated rats than in *Chel 30* treated series (according to band width), compared to positive control (Figure 3a–d).

Immunodetection of p53 and Bcl-2 proteins by Western blotting

The expression of p53 protein in normal rat (negative control) at day 90 and 120 is shown in Figure 3e. In both the treated series the expression of p53 protein was significantly reduced at day 90 and 120 as compared to positive control + EtOH group, the decrease in expression was more prominent in *Chel 200* treated series than *Chel 30*.

The Bcl-2 protein expression at day 90 and 120 (Figure 3f) can be seen in normal rat in Lane-1 with a fairly thin band. There was an increase in the expression of Bcl-2 protein in the positive control as well as positive control + EtOH groups, Lane-2 and Lane-3, respectively. In *Chel 200* treated rats, there was a significant decrease in expression of this protein at day 90 as compared to *Chel 30* treated one. But at day 120 both showed similar results in reduction of Bcl-2 expression.

Tissue weight/body weight ratio

Tissue weight/body weight ratio in carcinogen fed animals was significantly increased compared to negative controls (Table 3).

Discussion

From earlier studies and the present study[16,18,28] it is evident that chronic exposure to p-DAB and PB alone or in combination induce hepato-toxicity and oxidative stress. It is generally accepted that covalent binding of the metabolites of p-DAB with DNA is a major carcinogenic factor. Although it was not possible to record the data on day 0 for obvious reasons (the animals had to be killed for recording data of most parameters studied) an analysis of the results of the toxicity biomarkers at subsequent fixation intervals reveals that an increase in activities of ALT, AST, GGT, and LDH and decrease in activity of catalase, G-6-PD and in GSH content, denoting an elevated level of hepato-toxicity in the carcinogen fed rats. In both the treated series (*Chel 30* and *Chel 200*) this trend was largely reversed. This indicates a detoxifying effect and reduced oxidative stress. The expression of metalloproteinases and p53 and Bcl-2 proteins, which were over-expressed in the positive control and positive control + EtOH series, as compared to drug treated groups, leads to the same inference.

The activity of enzymes such as aspartate aminotransferases and alanine aminotransferases has been linked to hepatocellular injury or necrosis in liver and striated muscle tissue[20,29–33] and release of ALT from the cytosol may be secondary to cellular necrosis or a result of cellular injury with membrane damage.[34] The levels of these

enzymes were greatly elevated in the positive control groups but significantly less in the treated groups.

The presence of fewer tumor nodules in the treated rats suggests the possibility of the practical use of *Chel* in liver disorders including carcinoma, at least as a supportive therapy. The favorable modulations in most endpoints in the Chelidonium treated rats suggests that *Chel 30C* and *Chel 200 C* had similar protective effects against p-DAB + PB induced hepatocarcinogenesis, although *Chel 200C* appeared to have slightly better effects at the later time points. The mechanism by which ultra-low homeopathic doses could bring about such alterations remains unclear at the present state of our knowledge. We[18,19,35] have elsewhere hypothesized that they can act as a 'molecular switch', regulating expression of relevant genes (e.g. tumor suppressor genes like p53, Proliferating Cell Nuclear Antigen (PCNA) and pro-apoptotic genes like Bax, Bad, caspase 3, 9 etc.) through a cascade of actions after the first gene is activated or inactivated. Recently, DeOliviera *et al.*[36] reported that a homeopathic drug provoked decreased interleukin-2 and interleukin-4 production, resulting in a chain of differential expressions in some 147 genes, in line with our contention.

Conclusion

The results indicate that chronic feeding of p-DAB and PB induced many cytotoxic changes, and that these were positively modulated by administration of homeopathic medicines. It is very important that such studies are replicated independently and in various animal models to verify and confirm (or refute) our results, particularly in the light of recent reports of beneficial use of homeopathic drugs in breast cancer patients[37] and animal cancer models.[38]

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