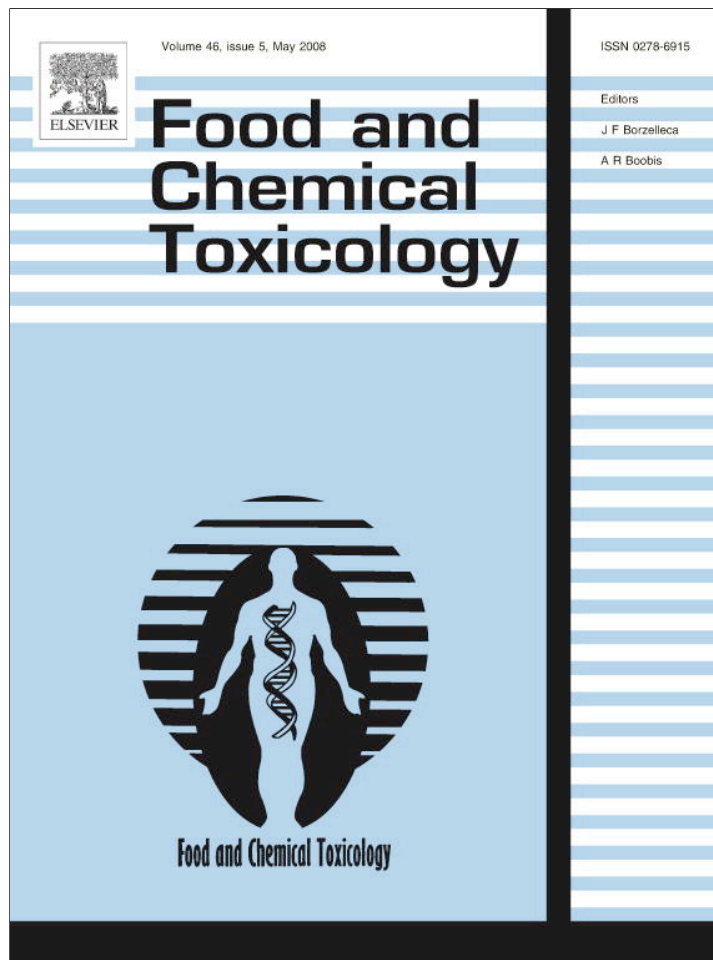


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Efficacy of a plant extract (*Chelidonium majus* L.) in combating induced hepatocarcinogenesis in mice

S.J. Biswas¹, N. Bhattacharjee, A.R. Khuda-Bukhsh*

Cytogenetics Laboratory, University of Kalyani, Kalyani, Nadia 741235, West Bengal, India

Received 24 March 2007; accepted 6 December 2007

Abstract

Ethanollic whole plant extract of *Chelidonium majus*, extensively used in traditional systems of medicine against various liver ailments, has been tested for its possible anti-tumor, hepato-protective and anti-genotoxic effects in *p*-dimethylaminoazobenzene (*p*-DAB) induced hepatocarcinogenesis in mice through multiple assays: cytogenetical, biochemical, histological and electron microscopical. Different sets of mice, 5 (for 7, 15 and 30 days' treatment) or 10 (for 60, 90 and 120 days) each, were chronically fed a diet suitably mixed with *p*-DAB and phenobarbital to develop liver tumors. One sub-group of carcinogen fed mice was also fed *C. majus* extract; 0.1 ml daily (drug-treated) while the other equal amount of dilute ethyl alcohol ("vehicle" of plant extract) (positive control). A separate group of mice was maintained with normal diet without any carcinogen treatment (negative control). Data of several cytogenetical endpoints and biochemical assay of some toxicity marker enzymes at all fixation intervals and histology of liver sections through ordinary, scanning and transmission electron microscopy at 60 and 120 days and that of spleen and kidney at 90 days were critically analyzed in the treated lots vis-a-vis controls. The results suggest anti-tumor, anti-genotoxic and hepato-protective effects of the plant extract, showing potentials for use in cancer therapy.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: *Chelidonium majus*; *p*-DAB; PB; Hepatocarcinogenesis; Anti-tumor; Toxicity marker enzymes; Anti-carcinogen; Mice

1. Introduction

The uses of plant and plant materials for curing various ailments have been known to the world since time immemorial (Suffiness and Douros, 1982) and gaining wide acceptance by scientific community, particularly in view of the toxic side-effects of most synthetic drugs. *Chelidonium majus* L. (Papaveraceae), which is widely distributed in Europe and Asia, is a plant of great interest for its wide use in various diseases in European countries and in Chinese herbal medicines. Extracts of *C. majus* are traditionally used in various complementary and alternative medicine

(CAM) systems including homeopathy mainly in combating diseases of the liver (Taborska et al., 1995), stomach (Kim et al., 1997) and various skin disorders. The crude ethanollic extract has already been claimed to exhibit, anti-inflammatory (Lenfeld et al., 1981), anti-viral (Kery et al., 1987), anti-microbial (Colombo and Bosisio, 1996) and anti-tumor effects (Panzer et al., 2000). On the other hand, the role of *Chelidonium* extract in cancer is controversial. Sokoloff, 1968 opined that their therapeutic use was rather limited due to its high cytotoxicity. However, to our knowledge, whether the homeopathically prepared crude extract (mother tincture) of *C. majus* in therapeutic (homeopathic) doses could really have any anti-tumor effects, or for that matter any anti-clastogenic or anti-genotoxic effects against *p*-DAB induced toxicity in mice, had not been tested earlier using multiple scientific assays, except for a brief report made by us covering some part of this work (Biswas and Khuda-Bukhsh, 2004). The present

* Corresponding author. Tel.: +91 33 25828750x315(O), +91 33 25828768(R).

E-mail addresses: khudabukhsh_48@rediffmail.com, prof_arkb@yahoo.co.in (A.R. Khuda-Bukhsh).

¹ Present address: Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad, Andhra Pradesh-500007.

communication is intended to evaluate critically if the ethanolic extract of whole plant (*C. majus*) could be effective in providing any anti-tumor, anti-hepatotoxic and anti-genotoxic activities in *p*-DAB induced hepatocarcinogenesis in mice *in vivo*, as revealed from the analysis of various assay systems, e.g. through cytogenetic endpoints like chromosome aberrations (CA), micronuclei (MN), and mitotic indices (MI) in bone marrow cells and sperm head anomaly (SHA), through biochemical studies on the activities of some marker enzymes like aspartate amino transferase (AST), alanine amino transferase (ALT), acid (ACP) and alkaline phosphatases (ALKP) and lipid peroxidation (LPO) in various tissues (liver, kidney and spleen), and through histological studies of liver sections under ordinary, scanning and transmission electron microscopies (TEM and SEM, respectively), during azo dye induced hepatocarcinogenesis in mice at different intervals of fixation.

2. Materials and methods

2.1. Materials

Adult, healthy mice, weighing between 18 and 22 g and maintained in hygienic conditions (under supervision of the Animal Welfare Committee, University of Kalyani, which also oversees ethical issues), with normal food (made up of powdered wheat, gram and milk) without any other animal/plant protein supplement and water *ad libitum*, were divided into various sets of 5–10 mice each for each fixation interval: (i) Set-I. Mice were maintained with normal diet without any kind of treatment (negative control), (ii) Set-II. Second set of mice were fed with (0.06 ml) of 0.05% PB (promoter) through gavage with the aid of a fine pipette, (iii) Set-III. Mice were chronically fed *p*-dimethylaminoazobenzene (*p*-DAB, SIGMA, D-6760) initiator @0.06% mixed with diet at a daily dose of 165 mg/kg bw per mouse following the method of Daoust and Molnar (1964) and Palekar and Sirsat (1966), (iv) Set-IV. Mice were chronically fed with *p*-DAB as in Set-III along with 0.05% PB as mentioned in (ii), (v) Set-V Mice were fed with *p*-DAB + PB as in previous sets along with 0.1 ml of dilute (1:20 plant extract to distilled water) extract of *C. majus* (C) fed 1 h after the feeding of PB daily, and (vi) Set-VI. Mice were fed chronically *p*-DAB and PB plus 0.06 ml dilute (1:20 alcohol to distilled water) ethyl alcohol (Alc, “vehicle” of the plant extract) (positive control). Different sets of mice were sacrificed at six different intervals, namely, 7, 15, 30, 60, 90 and 120 days after various treatments. Randomized “placebo” controlled method was adopted and the observers had been “blinded” during observation so that they did not know whether they were looking at “drug fed” or “placebo fed” mice till the codes were deciphered.

2.2. Preparation of the crude extract

Dried plant material (20 g) was macerated with 90% ethanol (450 ml) for 5 days, filtered and the mare was exhaustively percolated with the same solvent. Filtrate was evaporated to a thick residue at 50 °C. The yield of the extract was 40%. The extract was re-suspended in 90% ethanol (180 ml) and used for *in vivo* experiments.

2.3. Feeding procedure and dose

Each mouse was fed 0.06 ml of either 0.05% PB or double distilled water or drug with the aid of a fine pipette as per requirement of a particular series.

2.4. Cytogenetic assay

The standard and widely practiced cytogenetic protocols like assays of chromosome aberrations (CA), micronuclei (MN), mitotic index (MI)

and sperm head anomaly (SHA) have been adopted in the present study.

Mice were intra-peritoneally injected with 0.025% colchicine @ 1 ml/100 gm body weight 1 h and 30 min before sacrifice. Marrow of the femur was flushed in 1% sodium citrate solution at 37 °C and fixed in acetic acid/ethanol (1:3). Slides were prepared as described earlier (Biswas et al., 2004; Biswas and Khuda-Bukhsh, 2005) the conventional flame drying technique followed by Giemsa staining for scoring bone marrow chromosome aberrations. Chromosome aberrations of various nature have been pooled into two categories: the “major” type comprising aberrations like breaks, fragments, rings, etc. and the “other” types comprising less significant aberrations like gaps, erosion, precocious centromeric separation, pycnosis, stretching, etc.

For micronuclei (MN) preparation, a part of the suspension of bone marrow cells in 1% sodium citrate was smeared on clean grease free slides, briefly fixed in methanol and subsequently stained with May-Grunwald followed by Giemsa as previously described (Biswas et al., 2004; Biswas and Khuda-Bukhsh, 2005). Approximately 5000 bone marrow cells, comprising both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored and the ratios between PCE and NCE ascertained.

The mitotic indices (MI) was assessed from the same slide that was scanned for MN. The non-dividing and dividing cells were recorded and their ratios calculated.

For sperm head anomaly (SHA), the technique of Wyrobek et al. (1984) was adopted with minor modifications. The epididymis of testis of control and treated sets was dissected out separately and its inner content squeezed out into different vials with 10 ml of 0.87% normal saline, thoroughly shaken, filtered through a silken cloth and dropped on grease free clean slides. The slides were allowed to air dry and then stained by dilute Giemsa (1 ml Giemsa in 10 ml distilled water).

2.5. Enzymatic assay

Mice were sacrificed and their liver, kidney and spleen, were quickly harvested. The tissues were homogenized with cold 0.87% normal saline, followed by centrifugation at 3000g for 20 min in cooling centrifuge (C24, Remi Instruments). Before carrying out the enzymatic estimations the quantitative estimation of total protein was made by the method of Lowry et al. (1951). To 0.1 ml of the sample, 0.9 ml of 0.1(N) NaOH was added. Then 5 ml of alkaline solution was added to the sample solution followed by 0.5 ml of Folin-Phenol reagent and after 30 min the extinction was read at 750 nm against appropriate blank in spectrophotometer (Shimadzu, Double beam Spectrophotometer UV-180, Japan).

2.6. Estimation of mean activities of lipid peroxidation (LPO)

The LPO was estimated from the supernatant by the method of Buege and Aust (1984). One milliliter of sample (homogenate containing 0.1–0.2 mg of protein) was mixed thoroughly with 2 ml of TCA–TBA–HCl (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl). The absorbance of the sample was determined at 535 nm in a double beam spectrophotometer against a suitable blank. The malonaldehyde concentration of the sample was calculated by using extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

2.7. Estimation of mean activities of aspartate amino transferase (AST), alanine amino transferase (ALT)

For estimation of AST and ALT the methods of Bergmeyer and Brent (1974) was followed with some minor modifications. For AST 0.1 ml of tissue homogenate was made to react with 0.5 ml of the substrate solution L-aspartate and was incubated for 60 min at 37 °C. This was followed by addition of 0.5 ml of dinitrophenylhydrazine (DNPH) and then by 5.0 ml 0.4 N NaOH. The absorbance was measured at 510 nm.

For the analysis of ALT, 0.1 ml of tissue homogenate was made to react with 0.5 ml of the substrate solution (L-alanine) and incubated for

30 min at 37 °C. Rest of the procedure was same as that of ALT and the absorbance was measured at 510 nm.

2.8. Estimation of mean activities of acid (ACP) and alkaline phosphatases (ALKP)

For the study of ACP and ALKP, the method of Walter and Schutt (1974) was followed. For ACP, 0.2 ml tissue homogenate 1 ml of acid buffer was added to make the volume 1.2 ml. It was mixed and incubated at 37 °C for 30 min. Then 2 ml of 0.1(N) NaOH was added. The absorbance was measured at 405 nm against the standard.

For ALKP activity, 0.05 ml homogenate was mixed with 2 ml alkaline buffer so that the volume always stood at 2.05 ml. It was incubated at 37 °C for 30 min, then 10 ml of 0.05 N NaOH was added and the absorbance was measured at 405 nm against a blank.

2.9. Procedures for histology and electron microscopy

For preparation of histological slides, the routine technique of paraffin sectioning of liver being the major target organ of *p*-DAB + PB was followed up with haematoxylin–eosin staining at day 60 and day 120 only.

For SEM, liver samples (2–3 mm) were quickly isolated from the sacrificed animals and kept in 2.5% Karnovskys fixative (4% paraformaldehyde and 1% glutaraldehyde made in 0.1 M phosphate buffer pH 7.4) for 4–5 h. The samples were then washed in 0.1 M phosphate buffer thrice after which post fixation was carried out in 1% osmium tetra-oxide for about two hours. The samples were again washed with phosphate buffer and the dehydration steps followed. Further the samples were dried by a critical point drier (CPD-Biorad, Microscience Division, Warford England). Then the liver samples were mounted on aluminium stubs with the help of two side adhesive tapes. Gold coating of the samples was accomplished with the help of sputter coater (Agar Sputter Coater, Model 198, Stansted, United Kingdom). After gold coating the samples were observed under scanning electron microscope (LEO, 435VP, United Kingdom).

For TEM, 1–2 mm liver sections were isolated immediately after sacrifice of mice and fixed in Karnovsky fixative at 4 °C for 4–5 h, secondary fixation was followed in 1% v/v OsO₄ (E Merck) for about 1 h at 4 °C in dark. Dehydration and embedding were performed according to routine procedures. Semi-thin sections (60–90 nm) were cut in ultramicrotome (Reichert Jung-Ultracut E, United Kingdom) with the help of glass knives. Then ultra-thin serial sections were stained with uranyl acetate and lead citrate and observed under a Philips Microscope (TEM CM-10).

2.10. Statistical analysis

The significance test between different series of the data was conducted by students' *t*-test. Homogeneity of the different series was further analyzed by the Tukey one way ANOVA (Analysis of Variance) using SPSS 11.0 version. Groups indicated by *, ** or *** are significantly different. Though tests were conducted for significance values for normal vs. PB or normal vs. *p*-DAB, these were not included in the table as this would only make it more cumbersome (Supplemental materials for these data). Since we are more concerned with the differences between the drug fed series and *p*-DAB + PB + Alc fed control (positive), these were highlighted.

3. Results

Liver nodules appeared in the form of pale reddish multiple nodules in mice generally after chronic feeding of *p*-DAB + PB for 60 days or more. The number of mice in different series showing liver nodules on autopsy has been shown in Table 1.

Normal metaphase plates from untreated mice generally did not reveal many aberrations. However, various types of chromosome aberrations of both major (Figs. 1.1–1.3) and other types were encountered in some metaphase plates of mice that received *p*-DAB + PB, *p*-DAB + PB + C and *p*-DAB + PB + Alc. The total frequencies of aberration were found to be the maximum in *p*-DAB + PB + Alc fed mice as compared to normal controls followed by that of *p*-DAB + PB fed series at all fixation intervals. *p*-DAB + PB + C had comparatively less number of CA than that of *p*-DAB + PB + Alc and *p*-DAB + PB at all fixation intervals ($p < 0.01$, $p < 0.001$). However, in the mice fed alone *p*-DAB or PB the total CA were found to be less than *p*-DAB + PB and *p*-DAB + PB + Alc fed series at all fixation intervals. Therefore, while *Chelidonium* extract appeared to show protective effect on CA frequencies, Alc appeared to enhance them at all fixation intervals (Table 2).

Data on the occurrence of micronuclei (MN) in polychromatic PCE (Fig. 1.4) and normochromatic NCE (Fig. 1.5) erythrocytes have been provided. The percentages of micronuclei (MN) in *p*-DAB + PB + Alc were highest at all fixation intervals followed by *p*-DAB + PB, *p*-DAB, and PB fed series. However, the feeding of *Chelidonium* extract to the carcinogen fed mice appeared to decrease the frequency of MN appreciably at all fixation intervals ($p < 0.05$ – $p < 0.001$) (Table 2). The mitotic indices in *p*-DAB + PB + Alc, *p*-DAB + PB and *p*-DAB fed mice increased steadily from day 7 to day 60 but fell down at day 90 and then again rose at day 120. The treatment with *Chelidonium* extract alongside *p*-DAB + PB reduced the mitotic indices considerably ($p < 0.01$ – $p < 0.001$). However, in the only PB fed series the MI increased from day 7 to day 90 but decreased at day 120 (Table 3) as compared to normal control group.

There was an increase in the frequency of abnormal sperm (SHA) (Fig. 1.6–1.7) in the *p*-DAB + PB fed series as compared to normal controls. However, the frequency of sperm head abnormality in *p*-DAB + PB + Alc was

Table 1
Table showing the number of mice with tumor incidence at different fixation intervals and in different series

	No. of specimens	7 days	15 days	30 days	60 days	90 days	120 days
Normal	45	0/5	0/5	0/5	0/10	0/10	0/10
PB	45	0/5	0/5	0/5	0/10	1/10	2/10
<i>p</i> -DAB	45	0/5	0/5	0/5	5/10	6/10	7/10
<i>p</i> -DAB + PB	45	0/5	0/5	0/5	10/10	10/10	10/10
<i>p</i> -DAB + PB + C	45	0/5	0/5	0/5	5/10	4/10	4/10
<i>p</i> -DAB + PB + Alc	45	0/5	0/5	1/5	9/10	10/10	10/10

Ten mice were used per set for longer fixation intervals day 60, 90 and 120, and for shorter fixation intervals (day) 7, 15 and 30, 5 mice were used per set.

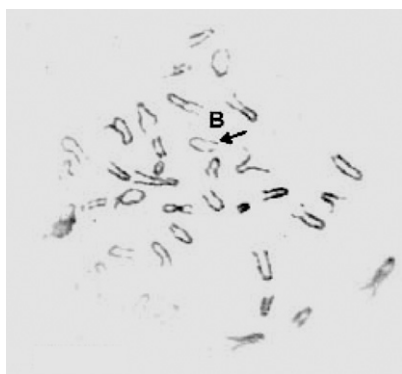


Fig. 1.1. Representative photomicrograph of metaphase plate showing break (B).

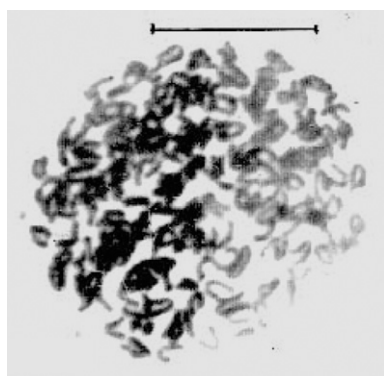


Fig. 1.2. Metaphase plate showing polyploidy.

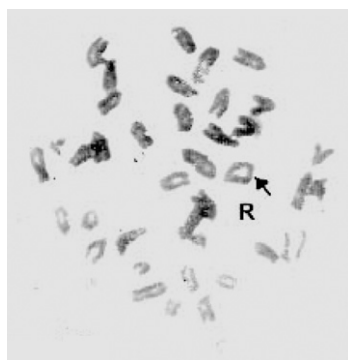


Fig. 1.3. Metaphase plate showing aberration ring (R).

considerably higher than in *p*-DAB + PB fed series. On the other hand, mice fed *p*-DAB + PB + C had considerably less number of abnormal sperm heads ($p < 0.001$) (Table 3).

Lipid peroxidation in liver steadily rose from day 7 to day 60 and fell down appreciably at day 90 and again rose at day 120 in the PB, *p*-DAB, *p*-DAB + PB and *p*-DAB + PB + Alc fed mice; however in the *p*-DAB + PB + C fed mice the activity was much less as compared to other series ($p < 0.001$), while in kidney of PB fed series the activity rose from day 7 to day 60 but fell down at day 90 and again rose at day 120. The LPO activity of *p*-DAB + PB + Alc fed series of kidney and spleen tissues

rose from 7 day to 60 day and fell down at day 90 and 120, respectively. However, the general trend suggests that lipid peroxidation was considerably reduced ($p < 0.05$ – $p < 0.001$) in *p*-DAB + PB + C fed mice at all fixation intervals in liver, kidney and spleen (see Table 4).

The AST activities in three tissues of *p*-DAB + P-B + Alc and *p*-DAB + PB fed mice were enhanced as compared to normal controls at all fixation intervals ($p < 0.001$). In contrast, in mice fed *Chelidonium* as well, the AST activities of liver were found to be generally decreased appreciably at different intervals of fixation ($p < 0.05$ – $p < 0.001$) except at day 60. However, in kidney the AST activity was found to increase at day 30, 90 and 120 while in spleen the activity was much lower when compared to *p*-DAB + PB + Alc and *p*-DAB + PB fed series. However, Alc appeared to trigger an increase in AST activity when fed to *p*-DAB + PB fed mice (Table 5).

The chronic feeding of *p*-DAB + PB appeared to trigger an increase in ALT activities ($p < 0.001$) in all the three tissues. In contrast, *Chelidonium* extract feeding clearly brought down the level of ALT in the carcinogen fed mice in three tissues in most intervals of fixation (e.g. day 7, 15, 30, and 120) and some of them were highly significant ($p < 0.05$ – $p < 0.001$) (Table 6). However, feeding of Alc to *p*-DAB + PB fed mice appreciably elevated the ALT activities at day 7, 15 and 30, but were decreased at day 60 and 90 to rise again at day 120, especially in liver (see Table 6). Interestingly, in the *p*-DAB + PB + Alc fed series, the ALT activities were noticeably greater than in *p*-DAB + PB + C fed series. In most fixation intervals, the ALT activities were clearly less in *p*-DAB + PB + C fed series as compared to *p*-DAB + PB fed series.

There was an increase in ACP activities of liver, kidney and spleen in *p*-DAB + PB and DAB + PB + Alc fed mice when compared to normal, PB and *p*-DAB fed series at all fixation intervals. The simultaneous feeding of *Chelidonium* extract appeared to generally bring down this elevated activity to a considerable extent in most tissues at most intervals of fixation ($p < 0.01$ – $p < 0.001$), only with few exceptions where the change was non-significant (Table 7). Similarly, Alc feeding clearly elevated further the ACP activity in *p*-DAB + PB fed mice.

The same trend of elevation of ALKP activity in liver, kidney and spleen of *p*-DAB + PB and *p*-DAB + PB + Alc fed mice was noted at most fixation intervals when compared to normal, only PB and *p*-DAB + PB + C fed series (Table 8). The activity was appreciably reduced in *p*-DAB + PB + C fed series at most fixation intervals and in most tissues ($p < 0.01$ – $p < 0.001$).

The chronic feeding of *p*-DAB + PB for 60 and 120 days, respectively, induced various changes in the histological structure of liver in mice. There was indication of excessive fibrosis of the hepatic parenchyma (Fig. 2); cells were small largely proliferating and more than one nucleus was present, Kupffer cells were more in number, vacuolated cells were present in some cells and no cytoplasmic boundaries were evident. In the *p*-DAB + PB + C fed series,

Table 2

Mean distribution of major and other types of chromosome aberrations (CA), and micronuclei (MN) in different series of control and treated mice at shorter fixation intervals of day 7, 15 and 30 and longer fixation day 60, 90 and 120

Fixation intervals in days	7 days		15 days		30 days	
	CA	MN	CA	MN	CA	MN
<i>Series</i>						
Normal	2.21 ± 0.49	0.14 ± 0.05	2.21 ± 0.58	0.14 ± 0.05	2.2 ± 0.37	0.14 ± 0.05
PB	3.26 ± 0.37	0.24 ± 0.06	4.22 ± 0.97	0.40 ± 0.05	6.0 ± 0.32	0.48 ± 0.05
<i>p</i> -DAB	5.41 ± 1.21	0.40 ± 0.03	6.23 ± 1.28	0.62 ± 0.15	7.8 ± 0.21	0.56 ± 0.09
<i>p</i> -DAB + PB	8.62 ± 0.98**	0.68 ± 0.06*	9.81 ± 0.80**	0.82 ± 0.13*	10.8 ± 0.58***	1.06 ± 0.07*
<i>p</i> -DAB + PB + C	4.01 ± 1.30***	0.50 ± 0.01***	6.22 ± 0.20***	0.72 ± 0.04**	7.0 ± 0.32***	0.60 ± 0.01***
<i>p</i> -DAB + PB + Alc	10.40 ± 0.51	0.78 ± 0.10	10.8 ± 0.86	0.96 ± 0.14	12.4 ± 0.24	1.20 ± 0.20
	60 days		90 days		120 days	
Normal	2.82 ± 0.20	0.14 ± 0.02	2.8 ± 0.20	0.14 ± 0.02	2.8 ± 0.20	0.14 ± 0.02
PB	5.41 ± 0.25	0.24 ± 0.07	5.4 ± 0.40	0.62 ± 0.05	11.4 ± 0.25	0.64 ± 0.02
<i>p</i> -DAB	14.40 ± 3.72	0.74 ± 0.06	12.2 ± 0.58	1.04 ± 0.08	19.8 ± 0.66	0.60 ± 0.03
<i>p</i> -DAB + PB	15.81 ± 0.80	0.76 ± 0.04*	15.4 ± 0.68	1.20 ± 0.03*	21.6 ± 0.81***	1.01 ± 0.03
<i>p</i> -DAB + PB + C	8.21 ± 0.20***	0.70 ± 0.01*	9.0 ± 0.32***	1.06 ± 0.02*	17.6 ± 0.98***	0.54 ± 0.02***
<i>p</i> -DAB + PB + Alc	12.82 ± 0.37	0.84 ± 0.04	14.6 ± 0.40	1.40 ± 0.08	32.8 ± 3.13	1.04 ± 0.05

The different levels of statistical significances between any two given series have been designated by *, **, ***.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

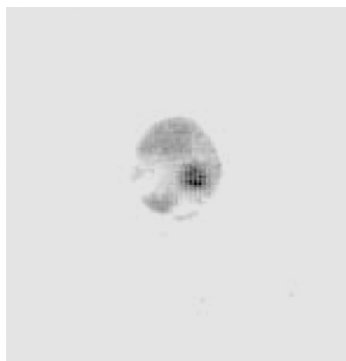


Fig. 1.4. Photomicrograph showing polychromatic erythrocytes with micronucleus.

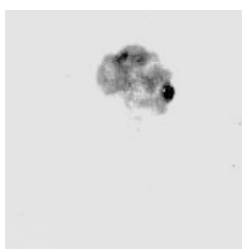


Fig. 1.5. Photomicrograph showing normochromatic erythrocytes with micronucleus.

however, the changes were to some extent less drastic. As for example, there was loss of cytoplasmic materials from almost all the cells but the nuclei were apparently intact; the newly proliferated blood capillaries were not seen, vacuolated cells were present but few in number, spaces between adjacent cells were distinct (Fig. 3).

Generally the spleen was much larger in the *p*-DAB + PB and also in *p*-DAB + PB + Alc fed series as compared to that of normal and *p*-DAB + PB + C fed series. The spleen of *p*-DAB + PB + Alc showed a capsular fibrosis at the periphery (Fig. 4) when compared to the normal control group. Further, there was an increase in number and size of giant cells in *p*-DAB + PB + Alc fed mice; on the other hand, there seemed to be a definite relationship of red pulp and white pulp at day 90 fixation interval in the spleen sections of *p*-DAB + PB + C fed series (Fig. 5).

Histological sections of kidneys from mice treated with *p*-DAB + PB + Alc revealed swelling in the epithelial cells of the proximal tubules, granular degeneration, blood vessels dilatation, tubular epithelium revealed varying degenerative changes including necrosis. Many foci appeared ruptured, with remnants in the lumen. Few areas showed swollen tubular epithelium; it obliterated the lumen, aggregation of mononuclear cells with hyperchromatic nuclei was seen around the blood vessels, glomeruli were swollen and revealed increased cellularity (Fig. 6). Kidney sections of mice treated with *p*-DAB + PB + C revealed the pattern of lesions was graded as being mild to moderate in the tubules and glomeruli; at certain foci, the changes were mild with less cellularity and less swelling in the epithelium showed normal histological picture (Fig. 7). The glomeruli were spared and the interstitium free from infiltration by chronic inflammatory cells in *p*-DAB + PB + C fed series when compared to *p*-DAB + PB and *p*-DAB + PB + Alc fed series.

In *p*-DAB + PB fed series at day 60 and day 120, various changes in structure of liver were noticed. As for example, fibrosis was evident at SEM level (Fig. 8). There was tissue necrosis causing appearances of holes, scattered RBCs were among the parenchymal tissue which suggest

Table 3

Mean distribution of mitotic indices (MI), and sperm head abnormality (SHA) in different series of control and treated mice at shorter fixation intervals of day 7, 15 and 30 and longer fixation intervals of day 60, 90 and 120

Fixation intervals in days	7 days		15 days		30 days	
	MI	SHA	MI	SHA	MI	SHA
<i>Series</i>						
Normal	1.40 ± 0.07	0.94 ± 0.04	1.40 ± 0.07	0.94 ± 0.16	1.40 ± 0.07	0.94 ± 0.16
PB	1.66 ± 0.28	1.08 ± 0.25	1.56 ± 0.27	1.28 ± 0.25	1.90 ± 0.27	1.26 ± 0.16
<i>p</i> -DAB	2.44 ± 0.35	1.24 ± 0.16	2.30 ± 0.34	1.52 ± 0.09	2.94 ± 0.26	1.88 ± 0.11
<i>p</i> -DAB + PB	2.50 ± 0.22 ^{***}	1.46 ± 0.16 [*]	2.66 ± 0.27 [*]	1.96 ± 0.07 ^{***}	3.56 ± 0.21 ^{***}	2.14 ± 0.15 ^{**}
<i>p</i> -DAB + PB + C	1.52 ± 0.05 ^{***}	1.06 ± 0.04 ^{***}	1.90 ± 0.05 ^{***}	1.58 ± 0.04 ^{***}	2.68 ± 0.09 ^{***}	1.72 ± 0.07 ^{***}
<i>p</i> -DAB + PB + Alc	3.76 ± 0.08	1.92 ± 0.08	2.70 ± 0.03	2.14 ± 0.15	4.14 ± 0.08	2.46 ± 0.15
	60 days		90 days		120 days	
Normal	1.32 ± 0.17	0.44 ± 0.09	1.32 ± 0.17	0.44 ± 0.09	1.32 ± 0.17	0.44 ± 0.09
PB	2.10 ± 0.26	1.08 ± 0.09	3.58 ± 0.12	1.84 ± 0.11	2.66 ± 0.29	2.46 ± 0.018
<i>p</i> -DAB	5.30 ± 0.23	3.18 ± 0.66	5.60 ± 0.01	2.98 ± 0.02	7.74 ± 0.37	2.02 ± 0.04
<i>p</i> -DAB + PB	8.70 ± 0.32 ^{***}	3.30 ± 0.01 ^{***}	7.60 ± 0.12 [*]	3.68 ± 0.12 ^{**}	9.50 ± 0.11	2.60 ± 0.06 ^{***}
<i>p</i> -DAB + PB + C	8.14 ± 0.19 ^{***}	2.40 ± 0.09 ^{***}	7.02 ± 0.16 ^{***}	3.14 ± 0.10 ^{***}	6.74 ± 0.09 ^{***}	1.90 ± 0.03 ^{***}
<i>p</i> -DAB + PB + Alc	9.10 ± 0.13	3.56 ± 0.19	7.90 ± 0.12	4.48 ± 0.17	9.48 ± 0.05	3.40 ± 0.12

The different levels of statistical significances between two given series have been designated by *, **, ***.

* $p < 0.05$.
 ** $p < 0.01$.
 *** $p < 0.001$.

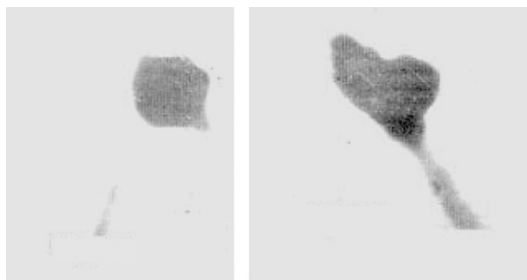


Fig. 1.6

Fig. 1.7

Fig. 1.6–1.7. Sperm with abnormal head morphology.

breakdown of blood liver barrier, although hepatic cell boundaries were recognizable. In the *p*-DAB + PB + C fed series though the hepatic cells appeared to be unhealthy there was an apparent decrease of necrosis. At day 120 in *p*-DAB + PB fed series the newly generated cells were in clusters and did not align in chords as seen in normal liver. They had a smooth and circular contour, blood liver barrier was not present and tissue necrosis was heavy and hepatic boundaries were not intact. On the other hand, the simultaneous feeding of *Chelidonium* extract apparently indicates less drastic damage and even some recovery in some structure. As for example, hepatic cells were arranged in chords as found in normal liver but their numbers were few; further, although sign of tissue necrosis was still evident, a few hepatic cells had intact boundaries indicative of a recovery process going on as well (Fig. 9).

At both day 60 and day 120, in *p*-DAB + PB fed series damage to intracellular organelles was evident under TEM (Fig. 10). The mitochondria were small and numerous, lipid droplets were present, nucleus round with dispersed nucleoplasm, endoplasmic reticulum broken at places;

few Kupffer cells were activated. Correspondingly, the damage appeared to be less drastic in the *Chelidonium* fed series (Fig. 11). As for example, mitochondria were fewer in number with distinct orientation of the cristae, Kupffer cells were less activated. The endoplasmic reticulum (ER) were broken at places, but these were not as conspicuous as in the *p*-DAB + PB fed series. Only a few glycogen granules were evident, lipid droplets were present but comparatively less in number than in the *p*-DAB + PB fed series. There were large number of mitochondrias which were small, condensed; loss of cristae was evident (particularly at day 120) which probably indicated decreased activity of some mitochondrial enzymes. In some cases the mitochondria were swollen with abnormal orientation of cristae; endoplasmic reticulum was largely broken, glycogen granules were absent, lipid droplets were numerous, Kupffer cells were activated which might be due to more secretions of lymphokines, lysosomes were numerous, nucleus round with dispersed nucleoplasm. In the *p*-DAB + PB + C fed series at day 120, endoplasmic reticulum was broken at places but they were much less when compared to *p*-DAB + PB fed series, few glycogen granules were evident, lipid droplets were present but comparatively less than in the *p*-DAB + PB fed group, Kupffer cells were less activated, mitochondria were few in number but some had orientation of cristae more or less like the normal.

4. Discussion

Chronic feeding of PB has been reported to have various toxic effects and has also been shown to promote development and growth of liver cancer in rodents when

Table 4
Mean lipid peroxidation in (nM MDA/gm of tissue) in different tissues of control and treated mice at shorter fixation intervals of day 7, 15 and 30 and longer fixation intervals of day 60, 90 and 120

Fixation intervals in days	7 days			15 days			30 days		
	Lipid peroxidation activity (Activity ± S.E.)			Lipid peroxidation activity (Activity ± S.E.)			Lipid peroxidation activity (Activity ± S.E.)		
Series	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	9.0 ± 0.5	6.1 ± 0.8	10.1 ± 1.0	7.8 ± 1.5	5.4 ± 0.7	9.5 ± 0.8	6.9 ± 1.0	7.4 ± 0.9	9.4 ± 0.6
PB	22.3 ± 6.3	7.2 ± 0.1	15.1 ± 0.8	35.8 ± 1.8	20.6 ± 1.5	19.6 ± 4.6	45.7 ± 1.4	46.1 ± 3.6	59.7 ± 1.2
p-DAB	35.1 ± 0.3	43.1 ± 0.9	32.7 ± 0.4	58.4 ± 2.2	41.6 ± 0.3	43.1 ± 1.6	87.7 ± 1.5	50.2 ± 1.2	68.9 ± 0.5
p-DAB + PB	35.3 ± 1.7***	39.5 ± 0.8***	36.2 ± 1.1***	57.7 ± 0.6***	34.5 ± 4.1***	33.8 ± 1.8***	87.9 ± 2.9***	54.4 ± 1.0***	68.2 ± 0.8***
p-DAB + PB + C	24.2 ± 0.3*	23.8 ± 0.5*	19.4 ± 1.6**	12.7 ± 1.1***	21.3 ± 5.0***	23.6 ± 0.2***	63.7 ± 1.2***	38.9 ± 0.8***	87.3 ± 1.7***
p-DAB + PB + Alc	43.1 ± 0.9	47.1 ± 0.5	41.6 ± 1.0	69.7 ± 0.2	31.5 ± 0.6	42.9 ± 6.4	69.0 ± 1.6	60.6 ± 1.3	90.0 ± 2.9
	60 days			90 days			120 days		
Normal	9.6 ± 0.2	7.9 ± 1.1	10.9 ± 1.2	9.1 ± 0.4	9.7 ± 1.2	10.7 ± 1.7	9.9 ± 0.84	10.2 ± 1.2	12.1 ± 1.0
PB	26.7 ± 3.7	47.8 ± 3.7	31.2 ± 3.1	47.9 ± 0.6	39.9 ± 3.2	64.9 ± 1.2	68.5 ± 2.87	68.4 ± 0.3	63.6 ± 5.5
p-DAB	80.4 ± 12.4	58.6 ± 0.9	71.5 ± 1.6	65.3 ± 2.3	38.8 ± 0.6	76.7 ± 1.2	78.9 ± 0.38	93.3 ± 1.2	63.3 ± 0.2
p-DAB + PB	101.1 ± 1.5***	70.3 ± 2.1***	74.5 ± 1.3***	67.8 ± 5.1***	45.2 ± 1.7***	71.0 ± 0.7***	97.5 ± 0.41***	92.4 ± 0.7***	63.8 ± 0.5***
p-DAB + PB + C	55.4 ± 3.2***	52.3 ± 1.0***	56.6 ± 8.9***	47.1 ± 0.8***	37.6 ± 7.5***	60.3 ± 2.0***	38.8 ± 2.64***	59.1 ± 0.7***	32.3 ± 0.2***
p-DAB + PB + Alc	82.3 ± 1.9	78.2 ± 0.6	90.1 ± 2.1	94.6 ± 5.6	51.9 ± 3.2	78.2 ± 0.4	69.3 ± 0.75	68.9 ± 1.4	43.6 ± 0.7

The different levels of statistical significances between two given series have been designated by *, **, ***.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 5
Mean activities of aspartate aminotransferase (AST) in mM/min/mg in different tissues of control and treated mice at shorter fixation intervals of day 7, 15, 30 and longer fixation intervals of day 60, 90 and 120

Fixation intervals in days	7 days			15 days			30 days		
	Aspartate aminotransferase (Activity ± S.E.)			Aspartate aminotransferase (Activity ± S.E.)			Aspartate aminotransferase (Activity ± S.E.)		
Series	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	0.08 ± 0.01	0.16 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.11 ± 0.03	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.04
PB	0.31 ± 0.01	0.56 ± 0.04	0.21 ± 0.02	0.51 ± 0.01	0.55 ± 0.03	0.22 ± 0.02	0.72 ± 0.01	0.47 ± 0.01	0.26 ± 0.04
p-DAB	0.64 ± 0.03	0.66 ± 0.002	0.16 ± 0.01	1.18 ± 0.02	0.76 ± 0.02	0.44 ± 0.01	1.04 ± 0.01	1.25 ± 0.01	0.84 ± 0.01
p-DAB + PB	0.53 ± 0.01***	0.41 ± 0.06**	0.44 ± 0.01***	0.69 ± 0.01***	0.97 ± 0.01***	0.44 ± 0.13***	1.20 ± 0.01***	3.21 ± 0.02***	0.41 ± 0.00***
p-DAB + PB + C	0.34 ± 0.002***	0.36 ± 0.05**	0.23 ± 0.004***	0.28 ± 0.01***	0.75 ± 0.002**	0.48 ± 0.01 ⁿ	0.84 ± 0.02***	1.55 ± 0.04***	0.21 ± 0.04***
p-DAB + PB + Alc	0.53 ± 0.01	0.38 ± 0.01	0.30 ± 0.004	0.78 ± 0.01	1.24 ± 0.01	0.45 ± 0.01	1.28 ± 0.003	1.07 ± 0.01	0.35 ± 0.00
	60 days			90 days			120 days		
Normal	0.07 ± 0.01	0.06 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.11 ± 0.03	0.05 ± 0.004	0.05 ± 0.01	0.03 ± 0.00
PB	0.70 ± 0.02	0.42 ± 0.04	0.19 ± 0.04	0.34 ± 0.01	0.33 ± 0.02	0.32 ± 0.004	0.37 ± 0.01	0.36 ± 0.01	0.26 ± 0.03
p-DAB	0.60 ± 0.01	0.54 ± 0.004	0.55 ± 0.01	1.72 ± 0.02	0.66 ± 0.01	0.42 ± 0.01	0.68 ± 0.01	0.80 ± 0.003	0.47 ± 0.003
p-DAB + PB	0.54 ± 0.01***	1.18 ± 0.01***	0.19 ± 0.004***	0.77 ± 0.01***	0.88 ± 0.02***	0.41 ± 0.03*	0.87 ± 0.001***	0.79 ± 0.001***	0.33 ± 0.002***
p-DAB + PB + C	1.09 ± 0.02***	0.68 ± 0.01***	0.23 ± 0.002 ⁿ	0.65 ± 0.01***	1.47 ± 0.02***	0.37 ± 0.01 [†]	0.49 ± 0.002***	0.71 ± 0.002 [‡]	0.26 ± 0.002 [‡]
p-DAB + PB + Alc	0.79 ± 0.03	1.06 ± 0.01	0.22 ± 0.003	0.67 ± 0.02	0.99 ± 0.03	0.54 ± 0.06	0.76 ± 0.003	0.68 ± 0.003	0.43 ± 0.004

The different levels of statistical significances between two given series have been designated by *, **, ***.

ⁿ denotes non significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 6
Mean activities of alanine aminotransferase (ALT) in mM/min/mg in different tissues of control and treated mice at shorter of day 7, 15, 30 and longer fixation intervals of 60, 90 and 120 days

Fixation intervals in days	7 days			15 days			30 days		
	Alanine aminotransferase (Activity ± S.E.)			Alanine aminotransferase (Activity ± S.E.)			Alanine aminotransferase (Activity ± S.E.)		
Series	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.003	0.03 ± 0.006	0.03 ± 0.004	0.04 ± 0.004	0.04 ± 0.01	0.04 ± 0.003	0.04 ± 0.01
PB	0.33 ± 0.13	0.03 ± 0.01	0.13 ± 0.02	0.26 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.41 ± 0.03	0.05 ± 0.01	0.06 ± 0.01
p-DAB	0.64 ± 0.03	0.66 ± 0.02	0.16 ± 0.005	0.93 ± 0.01	0.15 ± 0.002	0.11 ± 0.002	0.76 ± 0.01	0.17 ± 0.04	0.25 ± 0.003
p-DAB + PB	0.53 ± 0.01***	0.41 ± 0.06***	0.44 ± 0.01***	0.48 ± 0.002***	0.19 ± 0.004	0.09 ± 0.004†	0.81 ± 0.01***	0.42 ± 0.01***	0.15 ± 0.003***
p-DAB + PB + C	0.34 ± 0.002***	0.36 ± 0.05***	0.23 ± 0.004***	0.22 ± 0.004***	0.14 ± 0.06*	0.09 ± 0.003**	0.45 ± 0.003***	0.14 ± 0.004***	0.05 ± 0.002***
p-DAB + PB + Alc	0.53 ± 0.01	0.38 ± 0.01	0.30 ± 0.004	0.78 ± 0.01	1.24 ± 0.01	0.45 ± 0.01	0.84 ± 0.01	0.16 ± 0.002	0.15 ± 0.14
	60 days			90 days			120 days		
Normal	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.004	0.03 ± 0.002	0.04 ± 0.005	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.004
PB	0.47 ± 0.02	0.19 ± 0.03	0.08 ± 0.01	0.35 ± 0.01	0.13 ± 0.003	0.13 ± 0.001	0.29 ± 0.004	0.07 ± 0.003	0.07 ± 0.01
p-DAB	0.45 ± 0.01	0.09 ± 0.01	0.16 ± 0.01	1.33 ± 0.01	1.36 ± 0.01	0.12 ± 0.004	0.55 ± 0.002	0.17 ± 0.01	0.14 ± 0.003
p-DAB + PB	0.55 ± 0.04***	0.19 ± 0.01***	0.06 ± 0.002***	0.61 ± 0.01***	0.19 ± 0.01***	0.12 ± 0.003***	0.69 ± 0.01***	0.18 ± 0.004***	0.11 ± 0.003***
p-DAB + PB + C	0.70 ± 0.02***	0.10 ± 0.01*	0.05 ± 0.002	0.67 ± 0.01	0.14 ± 0.01	0.11 ± 0.004	0.39 ± 0.07***	0.13 ± 0.004*	0.07 ± 0.003***
p-DAB + PB + Alc	0.55 ± 0.01	0.19 ± 0.01	0.06 ± 0.003	0.49 ± 0.01	0.22 ± 0.02	0.17 ± 0.01	0.61 ± 0.002	0.16 ± 0.002	0.15 ± 0.004

The different levels of statistical significances between two given series have been designated by *, **, ***.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 7
Mean activities of acid phosphatase (ACP) in mM phenol liberated/100 mg protein after 30 min of incubation at 37 °C in different tissues of control and treated mice at shorter and longer fixation intervals of day 7, 15, 30, 60,90 and 120

Fixation intervals in days	7 days			15 days			30 days		
	Acid phosphatase (Activity ± S.E.)			Acid phosphatase (Activity ± S.E.)			Acid phosphatase (Activity ± S.E.)		
Series	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	0.04 ± 0.01	0.08 ± 0.004	0.10 ± 0.01	0.09 ± 0.003	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
PB	0.17 ± 0.05	0.12 ± 0.01	0.19 ± 0.06	0.29 ± 0.02	0.16 ± 0.03	0.35 ± 0.02	0.19 ± 0.02	0.43 ± 0.05	0.56 ± 0.06
p-DAB	0.42 ± 0.01	0.57 ± 0.002	0.65 ± 0.002	0.47 ± 0.02	0.63 ± 0.01	0.61 ± 0.004	0.66 ± 0.001	0.71 ± 0.01	0.99 ± 0.01
p-DAB + PB	0.74 ± 0.02***	0.76 ± 0.003***	0.86 ± 0.001***	0.73 ± 0.02***	0.78 ± 0.01***	0.78 ± 0.01***	0.85 ± 0.001***	0.72 ± 0.002***	0.78 ± 0.04***
p-DAB + PB + C	0.52 ± 0.001***	0.39 ± 0.00***	0.30 ± 0.004***	0.44 ± 0.03***	0.46 ± 0.01***	0.46 ± 0.004***	0.36 ± 0.02***	0.51 ± 0.01***	0.41 ± 0.03***
p-DAB + PB + Alc	0.74 ± 0.01	0.77 ± 0.01	0.85 ± 0.003	0.79 ± 0.002	0.79 ± 0.001	0.82 ± 0.002	0.74 ± 0.01	0.98 ± 0.01	0.93 ± 0.01
	60 days			90 days			120 days		
Normal	0.06 ± 0.01	0.08 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.95 ± 0.003	0.10 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
PB	0.58 ± 0.03	0.38 ± 0.02	0.98 ± 0.05	0.26 ± 0.02	0.22 ± 0.002	0.54 ± 0.004	0.44 ± 0.11	0.29 ± 0.04	0.38 ± 0.05
p-DAB	0.89 ± 0.01	1.05 ± 0.01	1.14 ± 0.01	0.68 ± 0.01	0.82 ± 0.03	1.14 ± 0.02	1.05 ± 0.002	0.71 ± 0.01	1.01 ± 0.01
p-DAB + PB	0.82 ± 0.01***	1.29 ± 0.01***	1.52 ± 0.01***	0.73 ± 0.18***	0.64 ± 0.01***	0.60 ± 0.01***	1.1 ± 0.001***	0.82 ± 0.002***	0.75 ± 0.01***
p-DAB + PB + C	0.71 ± 0.01**	0.65 ± 0.02***	0.66 ± 0.01***	0.49 ± 0.02***	0.48 ± 0.01***	0.65 ± 0.03***	0.40 ± 0.01***	0.60 ± 0.004***	0.56 ± 0.01***
p-DAB + PB + Alc	1.02 ± 0.01	1.09 ± 0.04	1.44 ± 0.02	1.44 ± 0.13	0.79 ± 0.04	1.58 ± 0.02	0.69 ± 0.002	0.64 ± 0.01	0.75 ± 0.01

The different levels of statistical significances between any two given series have been designated by **, ***.

** $p < 0.01$.
*** $p < 0.001$.

administered subsequent to feeding of an initiating carcinogen like *p*-DAB or DEN (Kitagawa and Sugano, 1977; Manjeswar et al., 1994; Aydinlik et al., 2001; IARC, 2001; Biswas and Khuda-Bukhsh, 2004; Pathak and Khuda-Bukhsh, 2007). However, while the chronic feeding of only *p*-DAB for a very long time 45–61 weeks (Akamatsu and Ikegami, 1968) has been reported to induce liver tumors, chronic feeding of PB alone has not been reported to produce such tumors. *p*-DAB fed alone for a long time in mice also produced elevated frequencies of CA and MN (Biswas et al., 2005; Biswas and Khuda-Bukhsh, 2005). However, while PB has been reported not to increase SCE significantly in mono therapy of human patients (Schaumann et al., 1989), Bellini et al. (2003) reported enhancement of SCE in vivo and CA and MN in mice (Biswas et al., 2004). But the chronic feeding of both *p*-DAB (initiator) and PB (promoter) has been successfully used to develop liver tumors (and subsequently cancer) by some earlier authors (Nesn et al., 1987; IARC, 2003; Biswas and Khuda-Bukhsh, 2005; Pathak and Khuda-Bukhsh, 2007) and a concomitant increase in both CA and MN has been reported presumably as a fall out of tissue damage and necrosis of liver tissue during the carcinogenetic process (Biswas and Khuda-Bukhsh, 2002, 2004; Pathak and Khuda-Bukhsh, 2007).

On the other hand, earlier cytogenetic studies conducted on chronic feeding of ethanol demonstrated only a non-significant increase in chromosomal aberration frequency and mitotic index in rat (Tavares et al., 2003). However, in some other studies (Biswas and Khuda-Bukhsh, 2002) it was shown that when alcohol was fed to mice alongside the carcinogens, *p*-DAB and PB, there was some degree of additional damage observed in all the cytogenetical and biochemical parameters of study, as has also been confirmed in the present study when the data of *p*-DAB + PB were compared with that of *p*-DAB + PB + Alc fed series. However, interestingly enough, when the homeopathic remedy prepared in ethyl alcohol vehicle was administered, the effect of the alcohol vehicle was not discernible, only the ameliorative effect of the remedy was demonstrable.

In the present study, chronic feeding of *p*-DAB + PB to mice for 60 days or more induced the formation of liver nodules in majority of mice (Table 1). It is well known that feeding of carcinogenic azo dye produces liver damage followed by regeneration of parenchymal cells, proliferation of bile ducts and connective tissue, and at later stages tumors develop from liver parenchyma that end up with neoplastic characteristics. The effects of azo dyes in early and advanced stages of hepatocarcinogenesis have been extensively studied earlier (Caballero et al., 2001). It is generally accepted that covalent binding of the metabolites of *p*-DAB (e.g. MAB, AAB, etc.) with DNA is a major carcinogenic factor (Ohnishi et al., 2001). This may be the main reason for the induction of various chromosome aberrations in the carcinogen fed series. Additionally, PB may also have cumulative effect by increasing reactive oxygen species and inducing oxidative stress (Klaunig et al.,

Table 8
Mean activities of alkaline phosphatase (ALKP) in mM phenol liberated/100 mg protein after 30 min of incubation at 37 °C in different tissues of control and treated mice at shorter fixation of day 7, 15, 30 and longer fixation intervals of day 60, 90 and 120

Series	7 days			15 days			30 days			60 days			90 days			120 days		
	Alkaline Phosphatase (Activity ± S.E.)			Alkaline Phosphatase (Activity ± S.E.)			Alkaline Phosphatase (Activity ± S.E.)			Alkaline Phosphatase (Activity ± S.E.)			Alkaline Phosphatase (Activity ± S.E.)			Alkaline Phosphatase (Activity ± S.E.)		
	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen	Liver	Kidney	Spleen
Normal	0.06 ± 0.01	0.07 ± 0.004	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.04 ± 0.002	0.04 ± 0.004	0.07 ± 0.01
PB	0.53 ± 0.04	0.61 ± 0.16	0.96 ± 0.001	0.67 ± 0.03	0.05 ± 0.02	0.77 ± 0.03	0.43 ± 0.05	0.05 ± 0.02	0.47 ± 0.01	0.76 ± 0.07	0.46 ± 0.02	0.47 ± 0.01	0.43 ± 0.05	0.42 ± 0.07	0.36 ± 0.05	0.41 ± 0.04	0.42 ± 0.07	0.36 ± 0.05
<i>p</i> -DAB	0.53 ± 0.02	1.69 ± 0.12	0.12 ± 0.001	0.54 ± 0.01	1.18 ± 0.01	0.13 ± 0.03	1.03 ± 0.01	1.18 ± 0.01	0.38 ± 0.01	1.28 ± 0.02	0.79 ± 0.02	0.37 ± 0.01	0.86 ± 0.03	0.66 ± 0.001	0.29 ± 0.001	0.78 ± 0.001	0.66 ± 0.001	0.29 ± 0.001
<i>p</i> -DAB + PB	0.59 ± 0.01***	1.75 ± 0.02***	0.14 ± 0.01***	0.57 ± 0.01***	1.73 ± 0.04***	0.15 ± 0.004***	0.94 ± 0.07***	1.73 ± 0.04***	0.37 ± 0.01***	1.13 ± 0.01***	0.67 ± 0.02***	0.34 ± 0.01***	1.12 ± 0.03***	0.83 ± 0.00***	0.29 ± 0.00	0.94 ± 0.07***	1.12 ± 0.03***	0.83 ± 0.00***
<i>p</i> -DAB + PB + C	0.02 ± 0.00***	0.42 ± 0.17***	0.07 ± 0.00***	0.52 ± 0.01 ⁿ	0.99 ± 0.004**	0.11 ± 0.003*	0.40 ± 0.01***	0.99 ± 0.004**	0.34 ± 0.01***	0.89 ± 0.01***	0.45 ± 0.04***	0.37 ± 0.01***	0.42 ± 0.01***	0.54 ± 0.00***	0.25 ± 0.01	0.40 ± 0.01***	0.42 ± 0.01***	0.54 ± 0.00***
<i>p</i> -DAB + PB + Alc	0.59 ± 0.01	1.71 ± 0.12	0.14 ± 0.004	0.61 ± 0.01	1.83 ± 0.01	0.16 ± 0.01	0.98 ± 0.22	1.83 ± 0.01	0.41 ± 0.01	1.57 ± 0.01	0.41 ± 0.01	0.90 ± 0.002	1.28 ± 0.01	0.73 ± 0.03	0.26 ± 0.01	0.90 ± 0.002	0.73 ± 0.03	0.26 ± 0.01
Normal	0.05 ± 0.003	0.06 ± 0.002	0.07 ± 0.010	0.05 ± 0.01	0.05 ± 0.004	0.09 ± 0.01	0.04 ± 0.002	0.05 ± 0.004	0.09 ± 0.01	0.05 ± 0.01	0.05 ± 0.004	0.09 ± 0.01	0.05 ± 0.01	0.05 ± 0.004	0.07 ± 0.01	0.04 ± 0.002	0.04 ± 0.004	0.07 ± 0.01
PB	0.58 ± 0.01	0.54 ± 0.02	0.91 ± 0.04	0.46 ± 0.02	0.45 ± 0.04	0.47 ± 0.01	0.41 ± 0.04	0.45 ± 0.04	0.47 ± 0.01	0.46 ± 0.02	0.45 ± 0.04	0.47 ± 0.01	0.46 ± 0.02	0.42 ± 0.07	0.36 ± 0.05	0.41 ± 0.04	0.42 ± 0.07	0.36 ± 0.05
<i>p</i> -DAB	0.77 ± 0.01	0.76 ± 0.004	1.11 ± 0.05	0.71 ± 0.01	0.79 ± 0.02	0.38 ± 0.01	0.78 ± 0.001	0.79 ± 0.02	0.38 ± 0.01	0.79 ± 0.02	0.79 ± 0.02	0.38 ± 0.01	0.79 ± 0.02	0.66 ± 0.001	0.29 ± 0.001	0.78 ± 0.001	0.66 ± 0.001	0.29 ± 0.001
<i>p</i> -DAB + PB	0.49 ± 0.02***	0.54 ± 0.01***	1.14 ± 0.04***	0.71 ± 0.01	0.67 ± 0.02***	0.37 ± 0.01***	0.76 ± 0.001***	0.67 ± 0.02***	0.37 ± 0.01***	0.76 ± 0.001***	0.67 ± 0.02***	0.37 ± 0.01***	0.76 ± 0.001***	0.83 ± 0.00***	0.29 ± 0.00	0.76 ± 0.001***	0.83 ± 0.00***	0.29 ± 0.00
<i>p</i> -DAB + PB + C	0.36 ± 0.01**	0.37 ± 0.04***	1.09 ± 0.03***	0.39 ± 0.02***	0.45 ± 0.04***	0.34 ± 0.01***	0.60 ± 0.001***	0.45 ± 0.04***	0.34 ± 0.01***	0.60 ± 0.001***	0.45 ± 0.04***	0.34 ± 0.01***	0.60 ± 0.001***	0.54 ± 0.00***	0.25 ± 0.01	0.60 ± 0.001***	0.54 ± 0.00***	0.25 ± 0.01
<i>p</i> -DAB + PB + Alc	0.71 ± 0.03	0.48 ± 0.01	1.29 ± 0.07	0.75 ± 0.02	0.92 ± 0.001	0.41 ± 0.01	0.90 ± 0.002	0.92 ± 0.001	0.41 ± 0.01	1.57 ± 0.01	0.41 ± 0.01	0.90 ± 0.002	1.28 ± 0.01	0.73 ± 0.03	0.26 ± 0.01	0.90 ± 0.002	0.73 ± 0.03	0.26 ± 0.01

The different levels of statistical significances between two given series have been designated by *, **, ***,
ⁿ denotes non significant.
 * *p* < 0.05.
 ** *p* < 0.01.
 *** *p* < 0.001.

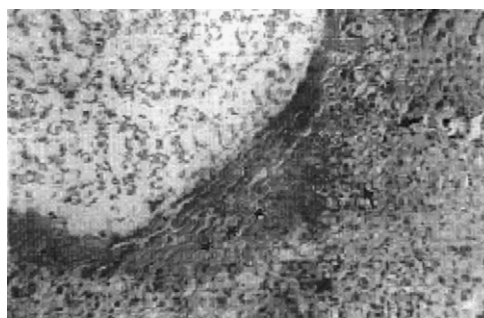


Fig-2

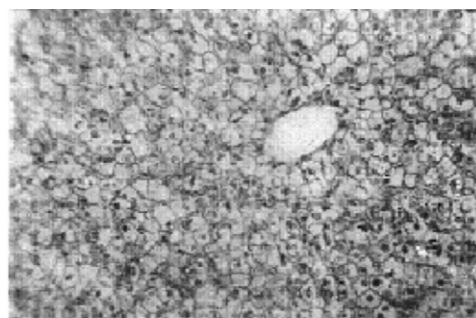


Fig-3

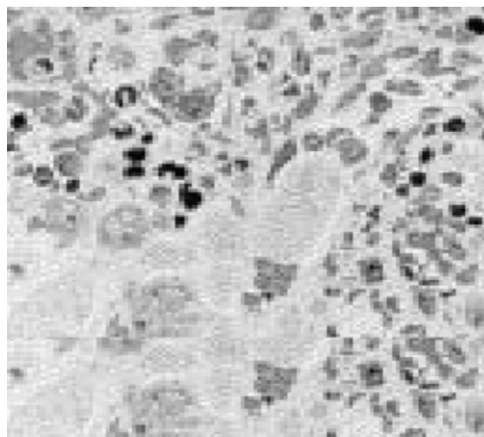


Fig-4

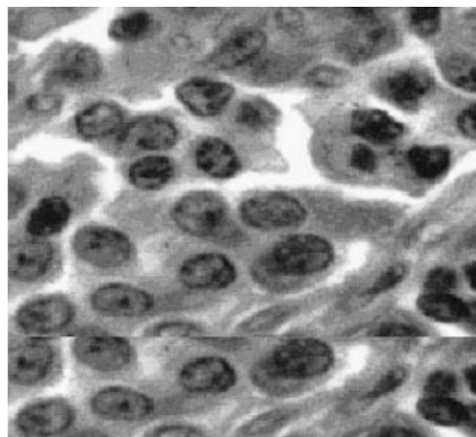


Fig-5

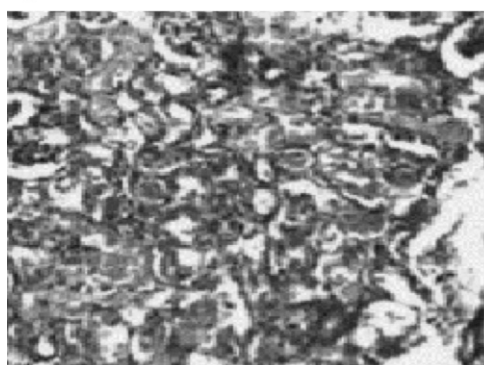


Fig-6

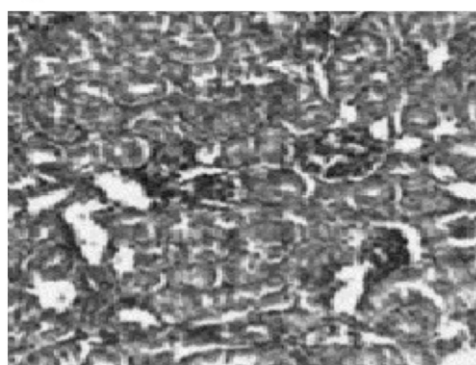


Fig-7

Fig. 2–7. Representative photomicrographs of histological sections: of liver of *p*-DAB + PB fed mice showing proliferating cells (*) and excessive fibrosis (Fig. 2), of *p*-DAB + PB + C fed mice (Fig. 3), of spleen of *p*-DAB + PB + Alc fed mice showing capsular fibrosis in periphery (Fig. 4) and that of *p*-DAB + PB + C fed mice (Fig. 5); of kidney of *p*-DAB + PB + Alc fed mice showing massive tubular necrosis (Fig. 6) and that of *p*-DAB + PB + C fed mice (Fig. 7); magnification 40×10 .

1998) in bringing about such DNA damage. The occurrence of several chromatid type breaks at longer intervals might indicate that the carcinogens possibly acted after reproduction of chromosomes and or at G₂ phases of the cell cycle. The other aberrations may be due to secondary toxic effect or due to the existence of some weaker spots on the chromosomes.

Micronucleus provides an indirect measurement of the induction of structural CA's. These are small chromatin containing bodies arising from the chromosomes that failed to incorporate into daughter nuclei following mitosis. The retention of MN could be attributed to the *p*-DAB + PB treatment during a recent cell cycle because

MN was normally extruded along with the main nucleus. The significant increase in the incidence of MN following *p*-DAB + PB administration could possibly be due to both spindle poisoning and clastogenic effects of these carcinogens. On the other hand, administration of *C. majus* extract in carcinogen fed mice showed a considerable decrease in the frequencies of both CA and MN. Incidentally, crude extracts of various parts like root, shoot and leaves have been reported to have several benzophenanthridine alkaloids, such as, sanguinarine, chelidone, chelerythrine, berberine, coptisine, etc. It was not known if a particular alkaloid or the conjoint action of a few or all the alkaloids were responsible for the ameliorating effect. Chelidonium

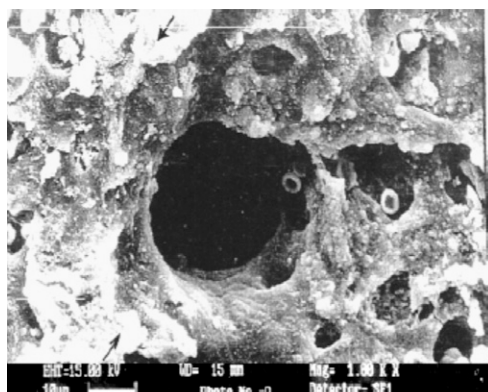


Fig-8

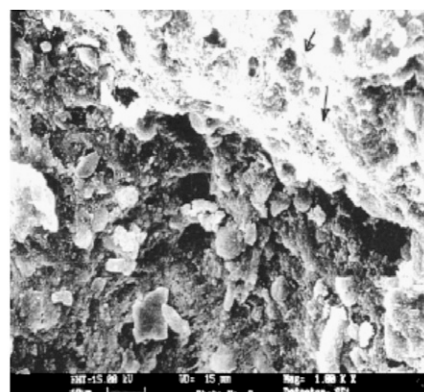


Fig-9

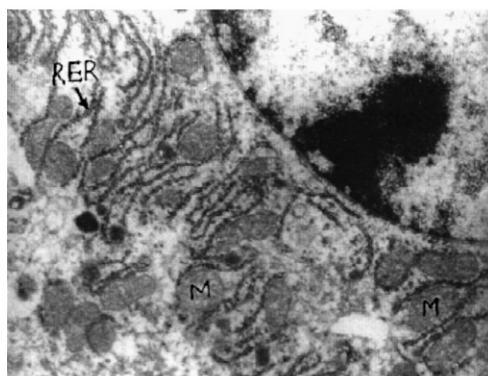


Fig-10

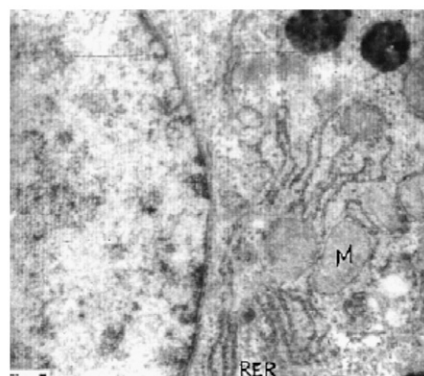


Fig-11

Fig. 8–11. Representative photomicrographs of liver sections of *p*-DAB + PB fed mice under SEM showing scattered RBCs and tissue necrosis causing appearances of holes. Magnification: 1.00 KX (Fig. 8) and that of *p*-DAB + PB + C fed mice showing less drastic damage though the hepatic cells looked unhealthy and had intact boundary (Fig. 9). Magnification: 1.00 KX. Representative photomicrographs of sections liver of *p*-DAB + PB fed mice under TEM showing broken endoplasmic reticulum (ER), numerous mitochondria (M) (Fig. 10) and that of *p*-DAB + PB + C fed mice showing less drastic damage to liver and number of mitochondria was less and ER were more or less continuous (Fig. 11). Bar represents 40 μ M.

extract showed some protective ability on spindle poisoning as well. However, the mechanism of action remains unknown. Wolff and Knipling (1993) suggested that chelidionine, one of the alkaloids present in *C. majus*, might inhibit microtubule polymerization and cause a mitotic block. This may be one of the reasons why the mitotic index was found to decrease in the *p*-DAB + PB + C fed series. Interestingly, even micro-doses of two ultra-high dilutions of this homeopathic drug, namely, *Chelidonium* 30 and *Chelidonium* 200, were also found to have ameliorating effect on *p*-DAB induced hepatocarcinogenesis in mice (Biswas and Khuda-Bukhsh, 2002).

An increasing number of sperm head abnormalities were also observed in the *p*-DAB + PB fed mice along with lapse of time which apparently indicates cytotoxic effect of the carcinogens manifested in the germinal cells as well. In general, mutagens that affect germ cells are also known to have spermatotoxic effects in mice (Topham, 1980). Evenson et al. (1986) demonstrated that such spermatotoxic effect might be due to alteration of testicular DNA and sperm chromatin structure. The feeding of *C. majus* extract showed that it could combat the spermatotoxic effects to some extent. As benzophenanthridine alkaloids have marked nucleophilic properties, they might intercept the reactive metabolites; thereby preventing their attack on

nucleophilic sites on DNA, and hence blocking adduct formation (Vavreckova et al., 1996a,b). Further it has been suggested that many enzymatic functions are essential for the normal integrity and function of testis i.e. synthesis, development and maintenance of normal sperm. Therefore, the protective role of *C. majus* on sperm head observed in the present study could also be attributed to its regulatory effect on protein metabolism and repair activities in the germinal cells.

In cellular peroxidation states the intracellular concentration of activated forms of oxygen is increased, presumably because cells either overproduce reactive substances or are deficient in their ability to destroy them and the biological consequences are mutations. It is well known that active oxygen species generated by xenobiotics interact with unsaturated fatty acids to initiate LPO, which is a major factor influencing the breakdown and turnover of bio-membrane. The elevated LPO is an indication of increased mitochondrial respiration which is the important source of active oxygen. So with the increase in time after *p*-DAB + PB insult, there was an increase in LPO activity. This would indicate the possibility of free electron leakage which, in turn, probably caused enhanced toxicity.

However, there was somewhat erratic response in the activity of some values of lipid peroxidation, and to some

extent in regard to other biomarkers as well, in the different tissues like liver, kidney and spleen of chronic carcinogen plus ethanol treated mice at some of the fixation intervals, particularly noticeable at day 120, the reason for which could not be properly understood. One hypothesis to explain this could be that the chronic feeding of ethanol led to its conversion to aldehyde after intake, which might interfere with the transfer of methyl groups (affecting function of ALT and AST) and thereby might contribute to alteration of gene expression (Seitz and Stickel, 2006). Alternatively, the metabolic products of *p*-DAB (e.g. MAB, AAB) and also of PB, might form adducts to parts of DNA, thereby obstructing/modifying activities of such genes that regulate the synthesis of the different enzymes studied. The plant extract of *Chelidonium* could in some way either interfere with the adduct formation or else had further modifying effects to clear these DNA of the products of the carcinogens by some unknown way. However, there were changes in the toxicity marker enzymes in liver, spleen and kidney during the carcinogenic process, though not at the same scale, a fact which is somewhat difficult to explain. However, one hypothesis to explain it could be that the release of the transaminases in serum of liver by the tissue destruction and necrosis, the primary target organ of the carcinogens being liver, could also affect AST and ALT levels in spleen (hemopoietic tissue) and kidney (excretory tissue always in contact with blood). Changes in these enzymes in different organs have also been reported by some earlier workers in some experimentally treated birds (Fowler, 1970) and in small mammals (Coles et al., 1980) although high ALT is generally considered specific for liver damage. Interestingly enough, although the carcinogens caused greater damage to liver, they also caused different degrees of tissue damage to all three organs as observed in the histological preparations, presumably as a secondary effect of the carcinogens.

Another possibility can not also be ruled out. It is known that ethanol-induced cytochrome P-450 2E1 produces various reactive oxygen species, leading to the formation of lipid peroxides such as 4-hydroxy-nonenal. Furthermore, alcohol impairs the antioxidant defense system, resulting in mitochondrial damage (as also found in the present study) and apoptosis. Chronic alcohol exposure is also known to elicit hepatocyte hyper-regeneration due to the activation of survival factors and interference with retinoid metabolism (Seitz and Stickel, 2006).

Alternatively, there could be some other possible explanations. As for example, anti-inflammatory effect of *Chelidonium* has been reported by Kim et al. (1997). This effect could be due to its influence on the immune system, oxygen radicals and tissue turn over. Earlier, Kim et al. (1993) suggested that the main mechanism of benzophenanthridine alkaloids for interaction with various cellular targets could be due to the reaction of the minimum bond with nucleophiles. It might be possible that the inhibitory action of the *Chelidonium* extract may be due to nucleophilic attack by SH groups of the enzymes on the minimum

moiety of the benzophenanthridine alkaloids. A similar inhibition has been reported on ALT by sanguinarine (Walterova et al., 1981). Alternatively, the liver dysfunction was thought to be due to a direct toxic action of one of the metabolites of *p*-DAB on liver (Timbrell, 1991) and inhibitory action of the extract is believed to be due to scavenging of the intermediate radicals that are formed within the active site of the enzyme. Stimulatory effect of *Chelidonium* has been reported on bile acid independent flow in isolated perfused rat liver (Vahlensieck et al., 1995). However, further in-depth studies are warranted to understand some other aspects of the mechanism of action of the plant extract in showing positive amelioration of *p*-DAB induced hepatocarcinogenesis. Our present observation of decreased ALT and AST in different tissues of *p*-DAB + PB + C fed mice corroborated well with those of Kosina et al. (2004) whose experiments were on pigs. Histological studies of liver tissues also revealed some preventive/recovery effects of *Chelidonium* extract against *p*-DAB induced liver damage. Incidentally, Panzer et al. (2000) reported that chelidonine and protopine, the principal alkaloids of the *Chelidonium* plant extract had immense therapeutic values in cancer, a view which is also validated in the present study.

The obtained results provide ample evidences for the potential of this plant extract in combating liver tumors and ameliorating hepato-toxicity in mice, a mammalian model. Therefore, the ability of this drug in producing similar anti-tumor, anti-clastogenic and hepato-protective effects in human, which has a similar genome, is a possibility and it has a potential for use as a supporting palliative medicine in cancer therapy. More in-depth research on this plant towards development of specific drug against specific liver ailments may be rewarding, particularly in view of certain plant extracts showing potentials of CAM for use in cancer therapy (Bruss, 2000; Balzarini et al., 2000; Richardson and Straus, 2002; Khuda-Bukhsh, 2003, 2006; Pathak et al., 2006, 2007).

Acknowledgements

Grateful acknowledgements are made to the University of Kalyani, for providing a Senior Research Fellowship to SJB and to the Department of AYUSH, Government of India, New Delhi, for providing major financial support of this work including an SRF to NB; to Dr. Taposh Das and Dr. Tapas Nag, Assistant Professor, AIIMS, New Delhi for their help in electron microscopy, and to Dr. S.P. De, Kolkata, and Dr. Philippe Belon, Boiron Lab, Lyon for encouragements.

References

- Akamatsu, Y., Ikegami, R., 1968. Induction of hepatoma and systemic amyloidosis in mice by 4-dimethylaminoazobenzene feeding. *Gann* 59, 201–206.
- Aydinlik, H., Nguyen, T.D., Moennikes, O., Buchmann, A., Schwarz, M., 2001. Selective pressure during tumor promotion by phenobarbital

- leads to clonal outgrowth of beta-catenin-mutated mouse liver tumors. *Oncogene* 20, 7812–7816.
- Balzarini, A., Felisi, E., Marini, A., De Conno, F., 2000. Efficacy of homeopathic treatment of skin reactions during radiotherapy for breast cancer: a randomized, double blind clinical trial. *British Homeopathic Journal* 89, 8–12.
- Bellini, C., Citana, A., Rosa, C.L., Bonioli, E., 2003. Phenobarbital enhances SCE in vivo. *Epilepsia* 44, 621–622.
- Bergmeyer, H.U., Brent, E., 1974. Methods of Enzymatic analysis. In: Bergmeyer, H.U. (Eds.), Verlag Chemie Weinheim, vol. 2, Academic Press, New York, pp. 735–760.
- Biswas, S.J., Khuda-Bukhsh, A.R., 2002. Effect of homeopathic drug, *Chelidonium*, in amelioration of *p*-DAB induced hepatocarcinogenesis in mice. *BMC Complementary and Alternative Medicine* 2, 4–12.
- Biswas, S.J., Khuda-Bukhsh, A.R., 2004. Evaluation of protective potential of a crude plant extract (*Chelidonium majus*) against *p*-dimethylaminoazobenzene induced hepatocarcinogenesis in mice. In: Proc. Predictive Oncology & Intervention Strategies: Molecular Basis of Oncogenesis & Cancer Control, 7–10 February, Nice, France. Presentation No. 394, ISPO Symposium.
- Biswas, S.J., Khuda-Bukhsh, A.R., 2005. Cytotoxic and genotoxic effects of the azodye *p*-dimethylaminoazobenzene in mice: A time course study. *Mutation Research* 587, 1–8.
- Biswas, S.J., Pathak, S., Bhattacharjee, N., Das, J.K., Khuda-Bukhsh, A.R., 2005. Efficacy of the potentized homeopathic drug, *Carcinosin* 200, fed alone and in combination with another drug, *Chelidonium* 200, in amelioration of *p*-dimethylaminoazobenzene-induced hepatocarcinogenesis in mice. *Journal of Alternative and Complementary Medicine* 11, 839–854.
- Biswas, S.J., Pathak, S., Khuda-Bukhsh, A.R., 2004. Assessment of the genotoxic and cytotoxic potentials of an anti-epileptic drug, phenobarbital, in mice. *Mutation Research* 563, 1–11.
- Bruss, Ke., 2000. Complementary and Alternative Cancer Methods. Am Cancer Soc, Atlanta, GA, USA.
- Buege, J.A., Aust, S., 1984. Microsomal lipid peroxidation. *Methods in Enzymology* 105, 302–310.
- Caballero, F., Gerez, E., Oliveri, L., Falcoff, N., Battle, A., Vazquez, E., 2001. On the promoting action of tamoxifen in a model of hepatocarcinogenesis induced by *p*-dimethylaminoazobenzene in CF1 mice. *International Journal of Biochemistry and Cell Biology* 33, 681–690.
- Coles, L.S., Brown, B.W., Engelhard, C., Halpern, J., Fries, J.F., 1980. Determining the most valuable clinical variables: a stepwise multiple logistic regression program. *Methods of Information in Medicine* 19, 42–49.
- Colombo, M.L., Bosisio, E., 1996. Pharmacological activities of *Chelidonium majus* L.. *Pharmacology. Research* 33, 127–134.
- Daoust, R., Molnar, F., 1964. Cellular populations and mitotic activity in rat liver parenchyma during azodye carcinogenesis. *Cancer Research* 24, 1909–1989.
- Evenson, D.P., Baer, R.K., Jost, L.K., Gesch, R.W., 1986. Toxicity of thiotepa on mouse spermatogenesis as determined by dual-parameter flow cytometry. *Toxicology and Applied Pharmacology* 82, 151–163.
- Fowler, J., 1970. Chlorinated hydrocarbon toxicity in the fowl and duck. *Journal of Comparative Pathology* 80 (3), 465–471.
- International Agency for Research on Cancer (IARC), 2001. Summaries and Evaluations, Phenobarbital and its Sodium Salt, Group 2B, 79, pp. 161–162.
- International Agency for Research on Cancer (IARC), 2003. IARC monographs program on the evaluation of carcinogenic risks to human. Lyons. Available at: <<http://193.51.164.11/default/html>>.
- Kery, R.Y., Horvath, J., Nasz, L., Verzar-Petri, G., Kulcsar, G., Dan, P., 1987. Antiviral alkaloid in *Chelidonium majus* L.. *Acta Pharmaceutica Hungarica* 57, 19–25.
- Khuda-Bukhsh, A.R., 2003. Towards understanding molecular mechanisms of action of homeopathic drugs. *Molecular and Cellular Biochemistry* 253, 339–345.
- Khuda-Bukhsh, A.R., 2006. Laboratory Research in homeopathy: pro. *Integrative Cancer Therapies* 5 (4), 320–332.
- Kim, D.J., Ahn, B., Han, B.S., Tsuda, H., 1997. Potential preventive effects of *Chelidonium majus* L. (Papaveraceae) herb extract on glandular stomach tumor development in rats treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and hypertonic sodium chloride. *Cancer Letters* 112, 203–208.
- Kim, O.H., Yang, J.S., Jung, E.J., Kim, H.S., Kang, S.Y., Rho, Y.N., Yi, S.Y., Park, J.G., Rheu, H.M., 1993. In vitro anticancer screening and evaluation of natural products in human tumor cell lines. Reports of National Institute of Safety Research 6, 201–207 (in Korean).
- Kitagawa, T., Sugano, H., 1977. Enhancement of azo-dye induced hepatocarcinogenesis with dietary phenobarbital in rats. *Gann* 68, 255–256.
- Klaunig, J.E., Xu, Y., Isenberg, J.S., Bachowski, S., Kolaja, K.L., Jiang, J., Stevenson, D.E., Walborg Jr., E.F., 1998. The role of oxidative stress in chemical carcinogenesis. *Environmental Health Perspectives* 106 (1), 289–295.
- Kosina, P., Walterova, D., Ulrichove, J., Lichnovsky, V., Stiborova, M., Rydlova, H., Vicar, J., Krecman, V., Brabec, M.J., Simanek, V., 2004. Sanguinarine and Chelerythrine: assessment of safety on pigs in ninety days feeding experiment. *Food and Chemical Toxicology* 42, 85–91.
- Lenfeld, J., Kroutil, M., Marsalek, E., Slavik, J., Preininger, V., Simanek, V., 1981. Anti-inflammatory activity of quaternary benzophenanthridine alkaloids from *Chelidonium majus*. *Planta Medica* 43, 161–165.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin-Phenol reagent. *Journal of Biological Chemistry* 193, 265–275.
- Manjeswar, S., Laconi, E., Sheik, A., Rao, P.M., Rajalakshmi, S., Sharma, D.S., 1994. *In vitro* and *in vivo* response of hepatocytes from hepatic nodules to the mitoinhibitory effects of Phenobarbital. *Carcinogenesis* 15, 1963–1968.
- Nesn, S., Argus, M., Bergman, H., Chu, K., Frith, C., Helmes, T., Mcgaughy, R., Ray, V., Slaga, Tj., Tennant, R.E., Weisburger, E., 1987. Chemical carcinogenesis: A review and analysis of the literature of selected chemicals and the establishment of the gene-tox carcinogen data base: A report of the US. Environmental Protection Agency Gen-Tox Program. *Mutation Research* 185, 1–195.
- Ohnishi, S., Murata, M., Degawa, M., Kawanishi, S., 2001. Oxidative DNA damage induced by an *N*-hydroxy metabolite of carcinogenic 4-dimethylaminoazobenzene. *Japanese Journal of Cancer Research* 92, 23–29.
- Palekar, S.D., Sirsat, S.M., 1966. Studies on the hepatocyte in azodye carcinogenesis. *Indian Journal of Experimental Biology* 4, 73–78.
- Panzer, A., Joubert, A.M., Bianchi, P.C., Seegers, J.C., 2000. The antimitotic effects of Ukrain, a *Chelidonium majus* alkaloid derivative, are reversible *in vitro*. *Cancer Letter* 150, 85–92.
- Pathak, S., Bhattacharjee, N., Das, J.K., Choudhury, S.C., Karmakar, S.R., Banerjee, P., Paul, S., Banerjee, A., Khuda-Bukhsh, A.R., 2007. Supportive evidence for the anticancerous potential of alternative medicine against hepatocarcinogenesis in mice. *Forsch Komplementarmed* 14 (3), 148–156.
- Pathak, S., Das, J.K., Biswas, S.J., Khuda-Bukhsh, A.R., 2006. Protective potentials of a potentized homeopathic drug, *Lycopodium-30*, in ameliorating azo dye induced hepatocarcinogenesis in mice. *Molecular and Cellular Biochemistry* 258, 121–131.
- Pathak, S., Khuda-Bukhsh, A.R., 2007. Assessment of hepatocellular damage and hematological alterations in mice chronically fed *p*-dimethyl aminoazobenzene and phenobarbital. *Experimental and Molecular Pathology* 83 (1), 104–111.
- Richardson, M.A., Straus, S.E., 2002. Complementary and alternative medicine: opportunities and challenges for cancer management and research. *Seminars in Oncology* 29, 531–545.
- Seitz, H.K., Stickel, F., 2006. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biological Chemistry* 387 (4), 349–360.
- Schaumann, B.A., Winge, V.B., Pederson, M.J., 1989. Genotoxicity evaluation in patients on phenobarbital monotherapy by sister chromatid exchange. *Toxicology and Environmental Health* 28, 277–284.

- Sokoloff, B., 1968. The oncostatic and oncolytic factors present in certain plants. *Oncology* 22, 49–60.
- Suffness, M., Douros, J., 1982. Current status of NCI plant and animal product program. *Journal of National Products* 45, 1–14.
- Taborska, E., Bochorakova, H., Dostal, J., Paulova, H., 1995. The greater celandine (*Chelidonium majus* L.) – A review of present knowledge. *Ceska a Slovenska Farmacie* 44 (2), 71–75.
- Tavares, D.C., Cecchi, A.O., Jordao Jr., A.A., Vannuchi, H., Takahashi, C.S., 2003. Cytogenetic study of chronic ethanol consumption in rats. *Teratogenesis, Carcinogenesis and Mutagenesis* 21, 361–368.
- Timbrell, J.A., 1991. *Principles of Biochemical Toxicology*. Taylor and Francis, London.
- Topham, J.C., 1980. Do induced sperm head abnormalities in mice specially identify mammalian mutagens rather than carcinogenesis? *Mutation Research* 74, 379–387.
- Vahlensieck, U., Hahn, R., Winterhoff, H., Gumbinger, H.G., Nahrstedt, A., Kemper, F.H., 1995. The effect of *Chelidonium majus* herb extract on choleresis in the isolated perfused rat liver. *Planta Medica* 61 (3), 267–271.
- Vavreckova, C., Gawlik, I., Muller, K., 1996a. Benzophenanthridine alkaloids of *Chelidonium majus*; I. Inhibition of 5- and 12-lipoxygenase by a non redox mechanism. *Planta Medica* 62, 397–401.
- Vavreckova, C., Gawlik, I., Muller, K., 1996b. Benzophenanthridine alkaloids of *Chelidonium majus*; II Potent inhibitory action against the growth of human keratinocytes. *Planta Medica* 62, 491–494.
- Walter, K., Schutt, C., 1974. Acid and alkaline phosphatase in serum (two point method). In: Bergmeyer, H.U. (Ed.), . In: *Methods in Enzymatic Analysis*, vol. 2. Academic Press, New York, USA, pp. 856–860.
- Walterova, D., Ulrichova, J., Preininger, V., Simanek, V., Lenfeld, J., Losovsky, J., 1981. Inhibition of liver alanine aminotransferase activity by some benzophenanthridine alkaloids. *Journal Medicinal Chemistry* 24, 1100–1103.
- Wolff, J., Knipling, L., 1993. Antimicrotubule properties of benzophenanthridine alkaloids. *Biochemistry* 32, 13334–13339.
- Wyrobek, A.J., Watchmaker, G., Gordon, L., 1984. In: Kilbey, B.J., Legator, M., Nichols, W., Ramel, C. (Eds.), *Handbook of Mutagenicity Testing Protocols*. Elsevier Science, Netherlands, pp. 733–750.