Protective potentials of a potentized homeopathic drug, Lycopodium-30, in ameliorating azo dye induced hepatocarcinogenesis in mice

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Abstract

The protective potentials of a potentized homoeopathic drug, Lycopodium-30, prepared from extract of spores of a plant, Lycopodium clavatum (Fam: Lycopodiaceae) and used as a remedy for various liver ailments, have been tested in mice chronically fed p-dimethyl amino azo benzene (p-DAB) - an initiator, and Phenobarbital (PB) - a promoter of hepatic cancer, by using some cytogenetic endpoints like chromosome aberrations (CA), micronuclei (MN), mitotic index (MI) and sperm head abnormality (SHA), and toxicity biomarkers like acid and alkaline phosphatases (AcP and AlkP, respectively), alanine and aspartate amino transferases (ALT and AST, respectively) and lipid peroxidation (LPO) and reduced glutathione (GSH) activities. The effects of chronic treatment of the carcinogens were assessed at different intervals of fixation, namely, at day 7, 15, 30, 60, 90 and day 120, and compared with that of mice fed conjointly with the carcinogens and the homeopathic remedy. Both the assay systems indicated considerable protective potentials of the homeopathic remedy against p-DAB induced hepatocarcinogenesis in mice. (Mol Cell Biochem 285: 121-131, 2006)

Key words: p-DAB, hepatocarcinogenesis, toxicity biomarkers, Lycopodium-30, hapatoprotection.

Abbreviations: DNA, deoxyribonucleic acid ;p-DAB, p-dimethyl amino azo benzene; PB, Phenobarbital; CA, chromosome aberrations; MN, micronuclei; MI, mitotic index; SHA, sperm head abnormality; AcP, acid phosphatases; AlkP, alkaline phosphatases; ALT, alanine amino transferases; AST, aspartate amino transferases; LPO, lipid peroxidation; GSH, reduced glutathione, ROS, reactive oxygen species; DTNB, 5,5'-Dithiobis (2 nitro benzoic acid); AAB, Aminoazo benzene; MAB, Mono aminoazo benzene; CAM, Complimentary & Alternative Medicine; DNPH, 2,4-Dinitrophenylhydrazine.

Introduction

The azo dye, p-dimethylaminoazobenzene(*p*-DAB), is widely used as a coloring agent of polishes and different dry foods. It has been listed as a group 2B carcinogen by IARC [1]. It was reported as a potent carcinogen of liver (initiator) by several researchers [2]. Phenobarbital (PB), generally used as an anti-epileptic drug, showed a great potential to promote liver cancer if used in combination with the azo-dye [3]. The success rate of orthodox mode of treatment for liver cancer is extremely poor. It generally involves one or the other method like surgery, radiation, liver transplantation and chemotherapy, all of which are quite expensive. Such treatments often have various side-effects and damage neighboring

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organs other than the target. Further, surgical removal of cancerous tissue in liver generally cannot totally rule out possibility of some metastasized cells remaining somewhere that many invade liver or some other organ(s) again. Therefore, homeopathic remedies which are affordable, easy to administer, and used in ultra-low doses and without any known side-effects but with experimentally proven ability to combat cancer development and growth, will be much sought after, particularly in developing countries. Even in some developed countries, there are serious efforts now to use medicines from the complementary and alternative regimens, generally in combination with other orthodox medicines [4-6]. With this perspective in view, we have been verifying efficacy of some homeopathic remedies through laboratory experiments [7-11] to combat artificially induced liver cancer in mice, a mammalian model close to human beings in respect of their genome, so that they can be used as a support therapy in human cancer, if needed.

In the present communication, we report the efficacy of another potentized homeopathic drug, Lycopodium-30 (Lyco-30) in ameliorating *p*-DAB induced hepatocarcinogenesis in mice, as evidenced from results obtained by using several cytogenetical protocols as well as testing some toxicity biomarkers of wide scientific acceptability.

Materials and methods

Swiss albino mice (Mus musculus) were reared and maintained in the animal house of the Department of Zoology (with clearance from the University Ethical Committee and under the supervision of the Animal Welfare Committee), University of Kalyani, for the investigation. Mice under experiment were provided food and water and kept in hygienic condition. The food was made up of wheat, gram and powdered milk without any animal protein supplement. A group of 35 healthy mice weighing between 25-30g were used for each of the fixation intervals. Each group of 35 mice were divided into five different sets consisting of 7 mice each for normal, normal+alcohol, *p*-dimethylaminoazobenzene (*p*-DAB)+PB, *p*-DAB+PB+alcohol, and *p*-DAB+PB+Lyco-30 series. The first set of mice (group-I, normal negative control) was allowed normal low protein diet. The second set of mice (group II) was fed diluted Alcohol-30 with normal low protein diet. The third set of mice (group-III) was fed 0.06% *p*-DAB (Sigma, D-6760) and 0.05% aqueous solution of PB. The fourth set of mice (group IV, positive control) was fed 0.06% *p*-DAB along with 0.05% aqueous solution of PB and diluted alcohol-30 (as the "vehicle" of the drug was in ethyl alcohol), till they were sacrificed. The fifth set of mice (group V) was fed *p*-DAB plus PB as in group-III, but was also fed Lycopodium-30 twice a day till they were sacrificed at day 7, 15, 30, 60, 90 and 120.

Preparation of the potentized form of the drug

The potentized homeopathic drug, Lyco-30, was procured from "HAPCO", 165, Bipin Behari Ganguli Street, Kolkata. The potentized form was prepared as per the standard procedure of homeopathic principle of "succussions and dilution". The extraction from spores of Lycopodium clavatum was made in 44% ethyl alcohol (i.e. the "mother tincture"). The mother tincture (1 ml) was subsequently diluted with 99 ml HPI approved solvent (IP 96/HPI grade ethyl alcohol) and "succussed" 10 times to make the potency 1. The potency 2 was similarly made by diluting 1 ml of potency 1 with 99 ml of ethyl alcohol and giving 10 jerks, and the procedure was repeated to get the 30th potency of Lycopodium clavatum, which was used as the stock solution.

Feeding procedure and dose

Each mouse was fed 1 drop (0.06 ml) of stock solution of Lyco-30 or alcohol-30, as the case may be, at a time (one dose) with the aid of a fine pipette.

Laboratory methodology

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 injected (ip) with 0.03% colchicines at 1 ml/100 g body weight 90 min before sacrifice. Marrow of the femur was flushed in 1% sodium citrate solution at 37oC and fixed in acetic acid/ethanol (1:3). Slides were prepared by the conventional flame drying technique as previously described [12] 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 break, fragment, ring etc. and the "other" types comprising less significant aberrations like gap, erosion, etc. A total of 300 bone marrow cells were observed, 60 from each of 5 mice of a set.

For micronucleus 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 [13]. Approximately 3000 bone marrow cells, comprising both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored and the ratios between PCE and NCE were calculated.

The mitotic index (MI) was determined from the same slide which was scanned for MN, and a total of 5000 cells were examined from each series. The non-dividing and dividing cells were recorded and their ratios ascertained.

For sperm head anomaly, the technique of Wyrobek [14] was adopted with minor modifications. The epididymis of each side of mouse of both (control and treated) sets was dissected out and its inner content squeezed out into 10 ml of 0.87% normal saline separately. It was made free of fats, vas deferens and other tissues. The content was 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 10ml distilled water). 5000 sperm were examined in each series.

Biochemical assays

Mice were sacrificed and their liver and spleen tissues were quickly isolated. 50mg of liver tissue and 20mg of spleen tissue diluted in 20 ml of phosphate buffer saline were homogenized separately and centrifuged at 3000 x g for 20 min in a 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. [15]. 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 660 nm against appropriate blank in spectrophotometer (Shemadzu, Double beam Spectrophotometer UV-1700, Japan).

The lipid peroxidation activity was estimated from the supernatant by the method of Buege and Aust [16]. 1 ml homogenate 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 x 105 M-1 cm-1.

For the study of acid and alkaline phosphatases the method of Walter and Schutt [17] was followed. For acid phosphatase, to 0.2 ml tissue homogenate, 1 ml of acid buffer was added. It was thoroughly mixed and incubated at 37oC for 30 min. Then 2 ml of 0.1 N NaOH was added to the mixture. The absorbance was measured at 405 nm against the standard.

For alkaline phosphatase activity the 0.05ml homogenate was mixed with 2ml alkaline buffer. It was incubated at 37oC for 30 min, then 10ml of 0.05 N NaOH was added and the absorbance was measured at 405 nm against a blank.

For estimation of AST and ALT activities, the method of Bergmeyer and Brent [18] 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-asparte and alpha keto glutaric acid and was incubated for 60 min at 37oC. This was followed by addition of 0.5 ml of dintrophenolhydrazine (DNPH) and then by 5 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 substract

solution (L-alanine and alpha keto glutaric acid) incubated for 30 min at 37oC. Rest of the procedure was same as that of AST and the absorbance was also measured at 510 nm.

In case of estimation of reduced glutathione (GSH), the method of Ellman [19] was followed. For GSH 6.5 ml of phosphate buffer diluted tissue samples was made to react with 1% sulfosalicylic acid and then 4 ml of 0.1 M sodium phosphate buffer was added. Finally in test samples 0.02 ml DTNB was added. The absorbance was measured at 412 nm.

Statistical analysis and scoring of data

The significance test between different series of data was conducted by student's t-test. During preparation of slides for cytogenetical observation and biochemical estimation of the different enzymes, the "observer" was kept "blinded" of the animal belonged to "treated" or "control" group in order to remove any "bias" in observation and thereby uniformity was maintained in scoring data of both treated and control sets of mice.

Results

Tumor growth

Out of the total number of 210 mice under experiment, livers in some 54 (Table 1) mice showed distinct sign of tumor formation in the form of pale reddish multiple nodules. While all mice fed only p-DAB plus PB developed tumor nodules in liver at 60 days onward but one of the mice of p-DAB+PB+ Alcohol fed series developed tumor nodules in 30 days fixation intervals and had appreciably enlarged spleen, the group of mice that received Lyco-30 along with p-DAB+PB showed either no nodules or less number of nodules in their liver. However, at day 120, one mouse each in the normal diet group and normal plus alcohol group showed traces of tumor nodules in their liver, for reasons not known to us.

Table 1. Number of animals showing tumours at different fixation intervals after chronic feeding of *p*-DAB plus PB for 7, 15, 30, 60, 90 and 120 days, for each fixation interval 7 mice were used for each series

Series	No. of specimens used	Tumour Incidence					
		7 days	15 days	30 days	60 days	90 days	120 days
Normal	42	0/7	0/7	0/7	0/7	0/7	0/7
Normal+alcohol	42	0/7	0/7	0/7	0/7	0/7	0/7
DAB+PB	42	0/7	0/7	0/7	7/7	7/7	7/7
DAB+PB+AL	42	0/7	0/7	1/7	7/7	7/7	7/7
DAB+PB+Ly-30	42	0/7	0/7	0/7	3/7	4/7	4/7
Total	210	0/35	0/35	1/35	17/35	18/35	18/35

Cytogenetical studies

As compared to normal metaphase complement (Fig. 1a) which did not normally reveal any aberration, various types of chromosome aberrations of both major and minor nature (Fig. 1b-f) were encountered in certain metaphase plates of mice that received p-DAB+PB treatments (Fig. 2, Histogram-2a). The total frequencies of CA were found to be maximum in the p-DAB+PB+alcohol fed mice. There was, however, a much less incidence of CA in the Lyco-30 fed group of mice, indicating significant amount of suppression of CA in this series as compared to alcohol-30 fed controls, both at shorter and longer fixation intervals except at day 15. However, the protective effects in the shorter intervals were more prominent ($p < 0.05$-$p < 0.001$, Fig. 2, Histogram-2a). There were also some minor aberrations (spontaneous) found in the normal groups of mice.

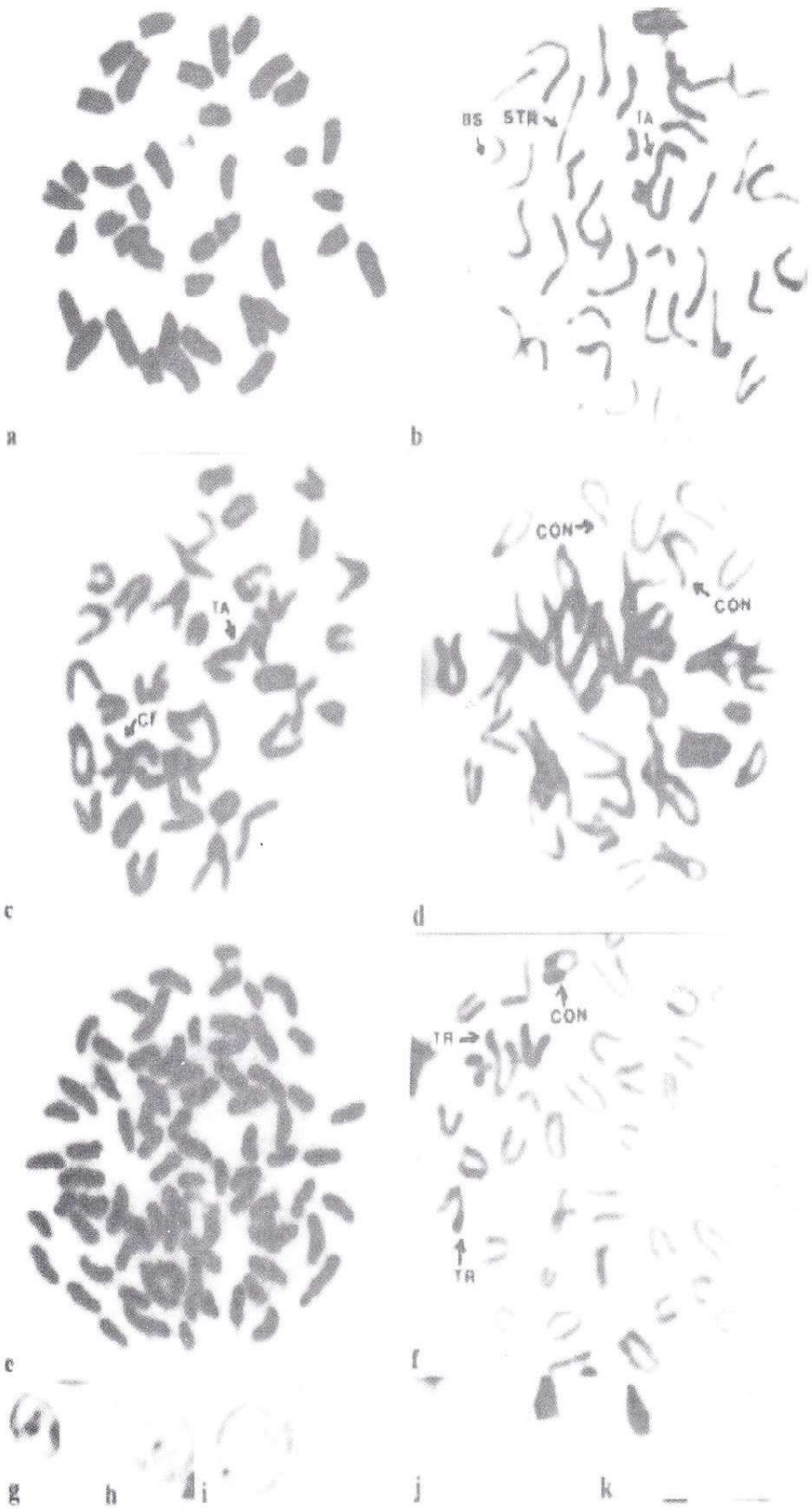

Fig. 1. (a–k) Photomicrographs of metaphase complements: (a) a typical normal spread, (b) spread with Break (BS), stretching (STR), (b, c) terminal association (TA), (c) centric fusion (CF), (d, f) constriction (CON), (e) c-mitotic effect, polyploidy, (f) translocation (TR). Bone marrow smears showing micronucleated erythrocytes: (g) polychromatic and (h, i) normochromatic erythrocytes. (j, k) Photomicrographs of sperm showing various abnormal head shapes.

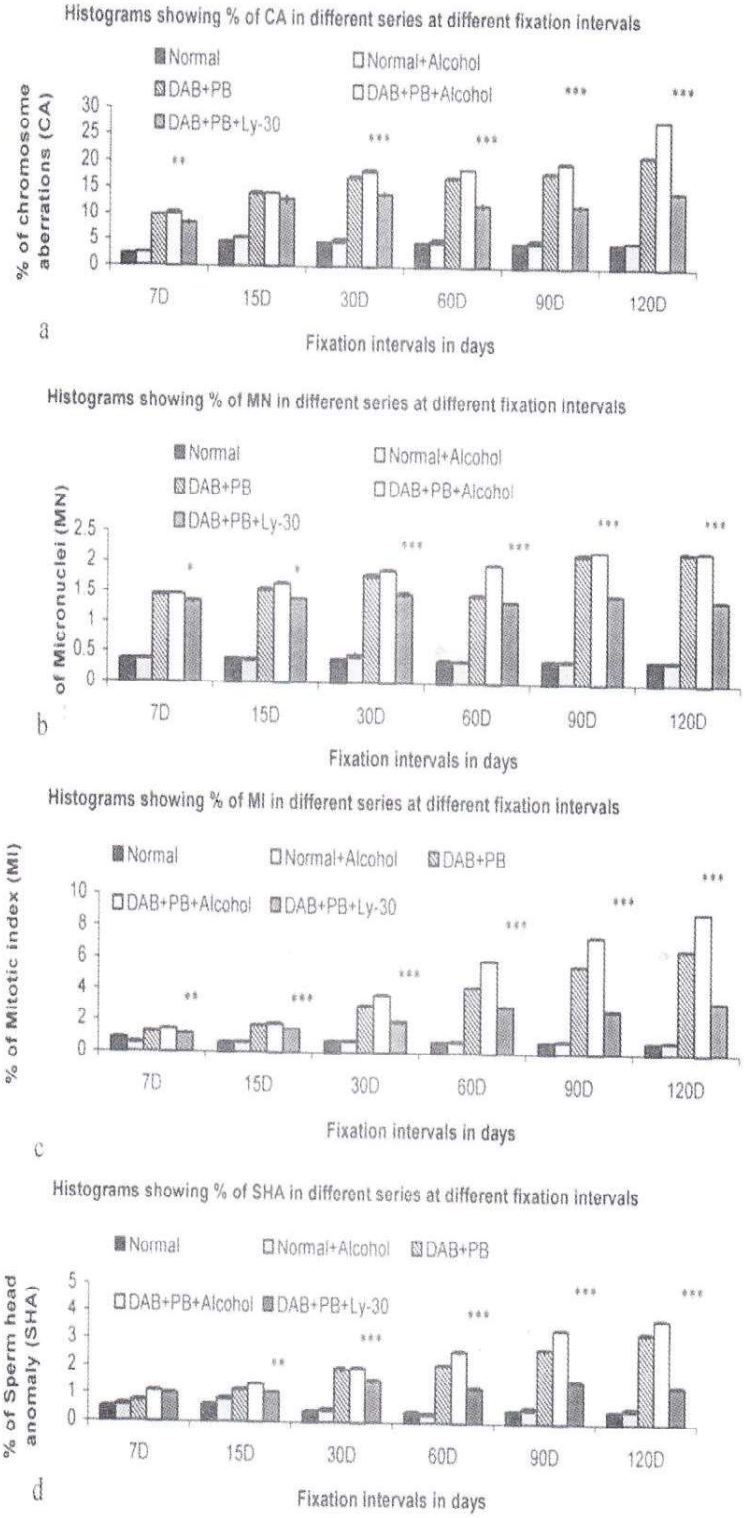

Fig. 2. (a–d) Histograms showing percentage (%) of: chromosomal aberrations (CA) (2a), micronucleated erythrocytes (MN) (2b), mitotic indices (MI) (2c) and abnormal sperm head shapes (SHA) (2d) in different series at different fixation intervals.

Table 2. Mean activities of AST, ALT (nM/100 mg/protein/Min) and AcP (nM/100 mg protein/Min) in liver and spleen of mice treated with *p*-DAB + PB, DAB + PB + Alcohol, DAB + PB + Ly-30, positive and negative controls

	AST				ALT				AcP			
	Liver		Spleen		Liver		Spleen		Liver		Spleen	
Series	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot
7 days fixation interval												
Normal	0.018 ± 0.005		0.017 ± 0.004		0.007 ± 0.001		0.012 ± 0.001		0.046 ± 0.001		0.038 ± 0.002	
Normal + Alcohol	0.024 ± 0.001	−0.006	0.017 ± 0.007	0.000	0.010 ± 0.001	−0.003	0.012 ± 0.002	0.000	0.045 ± 0.001	−0.001	0.042 ± 0.004	−0.004
DAB + PB	0.040 ± 0.004	−0.022	0.032 ± 0.006	−0.015	0.009 ± 0.002	−0.002	0.015 ± 0.003	−0.003	0.081 ± 0.004	−0.035	0.067 ± 0.008	−0.029
DAB + PB + Alcohol	0.043 ± 0.002	−0.003	0.035 ± 0.009	−0.003	0.013 ± 0.001	−0.004	0.021 ± 0.001	−0.006	0.085 ± 0.004	−0.004	0.070 ± 0.008	−0.003
DAB + PB + Ly-30	0.037 ± 0.005	0.006[n]	0.018 ± 0.014	0.017[n]	0.003 ± 0.001	0.010***	0.010 ± 0.003	0.011*	0.074 ± 0.006	0.011[n]	0.048 ± 0.000	0.022*
15 days fixation interval												
Normal	0.011 ± 0.000		0.020 ± 0.000		0.011 ± 0.001		0.010 ± 0.001		0.146 ± 0.010		0.192 ± 0.033	
Normal + Alcohol	0.010 ± 0.001	0.001	0.022 ± 0.001	−0.002	0.011 ± 0.003	0.000	0.017 ± 0.002	−0.007	0.124 ± 0.004	0.020	0.261 ± 0.010	−0.069
DAB + PB	0.042 ± 0.005	−0.031	0.041 ± 0.000	−0.021	0.026 ± 0.002	−0.015	0.025 ± 0.005	−0.015	0.172 ± 0.017	−0.026	0.388 ± 0.000	−0.196
DAB + PB + Alcohol	0.046 ± 0.000	−0.004	0.041 ± 0.000	0.000	0.033 ± 0.001	−0.007	0.028 ± 0.002	−0.003	0.151 ± 0.002	0.021	0.430 ± 0.004	−0.042
DAB + PB + Ly-30	0.025 ± 0.002	0.003[n]	0.016 ± 0.004	0.021***	0.211 ± 0.015	0.135*	0.250 ± 0.086	0.253[n]	0.147 ± 0.000	0.004*	0.008 ± 0.000	0.007
30 days fixation interval												
Normal	0.022 ± 0.001		0.011 ± 0.000		0.006 ± 0.001		0.007 ± 0.000		0.041 ± 0.003		0.055 ± 0.003	
Normal + Alcohol	0.021 ± 0.000	−0.001	0.014 ± 0.001	−0.003	0.008 ± 0.000	−0.002	0.010 ± 0.002	−0.003	0.033 ± 0.006	0.008	0.046 ± 0.001	0.009
DAB + PB	0.048 ± 0.003	−0.026	0.025 ± 0.002	−0.014	0.012 ± 0.002	−0.006	0.023 ± 0.002	−0.016	0.085 ± 0.010	−0.044	0.116 ± 0.006	−0.061
DAB + PB + Alcohol	0.052 ± 0.003	−0.004	0.027 ± 0.001	−0.002	0.019 ± 0.001	−0.007	0.035 ± 0.004	−0.012	0.087 ± 0.004	−0.002	0.122 ± 0.001	−0.006
DAB + PB + Ly-30	0.026 ± 0.000	0.026	0.016 ± 0.003	0.011*	0.008 ± 0.002	0.011**	0.008 ± 0.001	0.027***	0.082 ± 0.004	0.005*	0.089 ± 0.021	0.033[n]
60 days fixation interval												
Normal	0.026 ± 0.008		0.008 ± 0.007		0.008 ± 0.001		0.010 ± 0.007		0.047 ± 0.007		0.011 ± 0.002	
Normal + Alcohol	0.022 ± 0.003	0.004	0.005 ± 0.001	0.003	0.005 ± 0.001	0.003	0.005 ± 0.001	0.005	0.046 ± 0.003	0.001	0.012 ± 0.001	−0.001
DAB + PB	0.045 ± 0.005	−0.019	0.014 ± 0.008	−0.006	0.009 ± 0.001	−0.001	0.022 ± 0.003	−0.012	0.075 ± 0.011	−0.028	0.047 ± 0.006	−0.036
DAB + PB + Alcohol	0.051 ± 0.002	−0.006	0.018 ± 0.003	−0.004	0.022 ± 0.001	−0.013	0.026 ± 0.008	−0.004	0.081 ± 0.003	−0.006	0.068 ± 0.004	−0.021
DAB + PB + Ly-30	0.028 ± 0.008	0.023*	0.013 ± 0.001	0.005[n]	0.003 ± 0.001	0.019***	0.008 ± 0.001	0.018[n]	0.060 ± 0.004	0.021**	0.056 ± 0.007	0.012[n]
90 days fixation interval												
Normal	0.011 ± 0.001		0.020 ± 0.001		0.005 ± 0.001		0.002 ± 0.001		0.010 ± 0.000		0.065 ± 0.000	
Normal + Alcohol	0.013 ± 0.008	−0.002	0.030 ± 0.007	−0.010	0.006 ± 0.001	−0.001	0.002 ± 0.001	0.000	0.031 ± 0.003	−0.021	0.073 ± 0.008	−0.008
DAB + PB	0.055 ± 0.004	−0.044	0.038 ± 0.002	−0.018	0.014 ± 0.003	−0.009	0.018 ± 0.004	−0.016	0.051 ± 0.008	−0.041	0.095 ± 0.014	−0.030
DAB + PB + Alcohol	0.057 ± 0.001	−0.002	0.044 ± 0.009	−0.004	0.015 ± 0.002	−0.001	0.021 ± 0.001	−0.003	0.053 ± 0.002	−0.002	0.119 ± 0.004	−0.024
DAB + PB + Ly-30	0.049 ± 0.002	0.008*	0.038 ± 0.002	0.013[n]	0.003 ± 0.001	0.012**	0.012 ± 0.001	0.009[n]	0.052 ± 0.001	0.001[n]	0.087 ± 0.004	0.032**
120 days fixation interval												
Normal	0.055 ± 0.002		0.021 ± 0.001		0.002 ± 0.000		0.006 ± 0.003		0.022 ± 0.011		0.105 ± 0.001	
Normal + Alcohol	0.053 ± 0.003	0.002	0.022 ± 0.001	−0.001	0.005 ± 0.001	−0.003	0.006 ± 0.001	0.000	0.109 ± 0.000	−0.087	0.098 ± 0.002	0.007
DAB + PB	0.071 ± 0.010	−0.016	0.033 ± 0.001	−0.012	0.007 ± 0.001	−0.005	0.014 ± 0.001	−0.008	0.126 ± 0.030	−0.104	0.140 ± 0.017	−0.035
DAB + PB + Alcohol	0.109 ± 0.013	−0.038	0.068 ± 0.006	−0.035	0.006 ± 0.002	0.001	0.016 ± 0.002	−0.002	0.129 ± 0.013	−0.003	0.173 ± 0.006	0.033
DAB + PB + Ly-30	0.058 ± 0.009	0.051*	0.022 ± 0.002	0.046***	0.004 ± 0.000	0.002[n]	0.006 ± 0.002	0.010*	0.117 ± 0.022	0.012[n]	0.074 ± 0.010	0.099***

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

Table 3. Mean activities of AlkP (nM/100 mg protein/Min), LPO (nM/MDA/mg wet tissue) and GSH (nM/mg wet tissue) in liver and spleen of mice treated (*p*-DAB)+ PB, DAB+PB+Alcohol, DAB+PB+Ly-30, positive and negative controls

Series	AlkP				LPO				GSH			
	Liver		Spleen		Liver		Spleen		Liver		Spleen	
	Activity ± SE	Prot.	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot	Activity ± SE	Prot
7 days fixation interval												
Normal	0.018 ± 0.002		0.026 ± 0.005		0.248 ± 0.011		0.343 ± 0.006		0.004 ± 0.000		0.005 ± 0.000	
Normal+Alcohol	0.025 ± 0.000	−0.007	0.030 ± 0.004	−0.004	0.275 ± 0.037	−0.027	0.345 ± 0.020	−0.002	0.003 ± 0.000	−0.001	0.004 ± 0.001	−0.001
DAB+PB	0.037 ± 0.001	−0.019	0.049 ± 0.002	−0.023	0.329 ± 0.011	−0.081	0.737 ± 0.084	−0.394	0.001 ± 0.000	−0.003	0.003 ± 0.001	−0.002
DAB+PB+Alcohol	0.041 ± 0.002	−0.004	0.064 ± 0.005	−0.015	0.353 ± 0.081	−0.024	0.906 ± 0.050	−0.169	0.001 ± 0.000	0.000	0.001 ± 0.000	−0.002
DAB+PB+Ly-30	0.035 ± 0.003	0.006[n]	0.032 ± 0.010	0.032*	0.337 ± 0.009	0.016[n]	0.489 ± 0.066	0.417**	0.002 ± 0.000	0.001	0.002 ± 0.000	0.001
15 days fixation interval												
Normal	0.012 ± 0.010		0.007±0.002		0.130 ±0.027		0.202±0.019		0.006±0.000		0.006±0.002	
Normal+Alcohol	0.013 ± 0.003	−0.001	0.019±0.001	−0.012	0.174 ± 0.000	−0.044	0.197±0.004	0.005	0.005±0.000	−0.001	0.008±0.000	−0.002
DAB+PB	0.024 ± 0.005	−0.012	0.024±0.005	−0.017	0.309 ± 0.017	−0.179	0.406±0.005	−0.204	0.001±0.000	−0.005	0.001±0.000	−0.005
DAB+PB+Alcohol	0.028 ± 0.000	−0.004	0.047±0.001	−0.023	0.346 ± 0.049	−0.037	0.403±0.004	0.003	0.001±0.000	0.000	0.001±0.000	0.000
DAB+PB+Ly-30	0.025 ± 0.002	0.003[n]	0.016±0.004	0.021***	0.211 ± 0.015	0.135*	0.250±0.086	0.253[n]	0.003±0.000	0.002	0.008±0.000	0.007
30 days fixation interval												
Normal	0.026 ± 0.001		0.034±0.003		0.087 ± 0.002		0.275±0.023		0.005±0.003		0.005±0.002	
Normal+Alcohol	0.057 ± 0.005	−0.031	0.028±0.007	0.006	0.092 ± 0.001	−0.005	0.283±0.015	−0.008	0.003±0.001	−0.002	0.006±0.003	0.001
DAB+PB	0.042 ± 0.003	−0.016	0.070 ± 0.007	−0.036	0.231 ± 0.017	−0.144	0.367±0.050	−0.092	0.001±0.000	−0.004	0.001±0.000	0.004
DAB+PB+Alcohol	0.049 ± 0.006	−0.007	0.112 ± 0.029	−0.042	0.268 ± 0.008	−0.037	0.482±0.062	−0.115	0.001±0.000	0.000	0.001±0.000	0.000
DAB+PB+Ly-30	0.025 ± 0.003	0.024*	0.097 ± 0.012	0.015[n]	0.217 ± 0.041	0.051[n]	0.277±0.093	0.205[n]	0.003±0.000	0.002	0.004±0.000	0.003
60 days fixation interval												
Normal	0.020 ± 0.000		0.047 ± 0.004		0.050 ± 0.003		0.105±0.000		0.005±0.000		0.004±0.000	
Normal+Alcohol	0.022 ± 0.001	−0.002	0.034 ± 0.003	0.013	0.056 ± 0.005	−0.006	0.087±0.057	0.018	0.005±0.001	0.000	0.004±0.000	0.000
DAB+PB	0.031 ± 0.000	−0.017	0.064 ± 0.003	−0.017	0.243 ± 0.063	−0.193	0.355±0.011	−0.250	0.003±0.000	−0.002	0.002±0.000	−0.002
DAB+PB+Alcohol	0.036 ± 0.000	−0.005	0.062 ± 0.004	−0.002	0.252 ± 0.017	−0.009	0.370±0.003	−0.015	0.001±0.000	−0.001	0.002±0.000	0.000
DAB+PB+Ly-30	0.028 ± 0.001	0.008***	0.050 ± 0.003	0.012[n]	0.094 ± 0.006	0.158***	0.141±0.014	0.229***	0.003±0.000	0.002	0.004±0.040	0.002
90 days fixation interval												
Normal	0.018 ± 0.000		0.012 ± 0.002		0.040 ± 0.000		0.105±0.000		0.006±0.000		0.009±0.001	
Normal+Alcohol	0.024 ± 0.000	−0.006	0.021 ± 0.006	−0.009	0.094 ± 0.016	−0.054	0.118±0.007	−0.013	0.006±0.000	0.000	0.008±0.000	−0.001
DAB+PB	0.043 ± 0.006	−0.019	0.052 ± 0.008	−0.040	0.115 ± 0.016	−0.075	0.215±0.004	−0.110	0.005±0.000	−0.001	0.005±0.000	−0.004
DAB+PB+Alcohol	0.045 ± 0.004	−0.002	0.056 ± 0.006	−0.004	0.121 ± 0.003	−0.006	0.469±0.027	−0.254	0.003±0.001	−0.002	0.003±0.001	−0.002
DAB+PB+Ly-30	0.040 ± 0.003	0.005[n]	0.046 ± 0.008	0.010[n]	0.105 ± 0.005	0.016*	0.409±0.030	0.060[n]	0.006±0.001	0.003[n]	0.005±0.000	0.002[n]
120 days fixation interval												
Normal	0.006 ± 0.002		0.052±0.008		0.044±0.000		0.027±0.019		0.006±0.000		0.007±0.000	
Normal+Alcohol	0.042 ± 0.006	0.036	0.058±0.002	−0.006	0.082 ± 0.007	−0.038	0.022±0.006	0.005	0.007±0.000	0.001	0.007±0.000	0.000
DAB+PB	0.124 ± 0.008	−0.118	0.094±0.006	−0.042	0.069 ± 0.012	−0.025	0.048±0.011	−0.021	0.003±0.001	−0.003	0.001±0.000	−0.006
DAB+PB+Alcohol	0.147 ± 0.002	−0.023	0.169±0.031	−0.075	0.148 ± 0.183	−0.079	0.156±0.055	−0.108	0.003±0.001	0.000	0.003±0.001	0.002
DAB+PB+Ly-30	0.116 ± 0.004	0.031***	0.139±0.019	0.030[n]	0.124 ± 0.020	0.024[n]	0.094±0.019	0.062[n]	0.005±0.000	0.002[n]	0.007±0.000	0.004**

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

Micronucleated erythrocytes

Data on occurrence of micronuclei in normochromatic (NCE) erythrocytes and polychromatic erythrocytes (PCE) have been provided in Fig. 2, Histogram 2b and also representative photomicrographs have been shown in Fig. 1g-i. The percentages of MN were highest in the p-DB+PB plus alcohol fed mice at all the fixation intervals. The least number of MN was noticed in mice fed with Lycopodium-30, at all fixations of interval except on day 15.

Mitotic index

In Lyco-30 fed mice, the MI was much less than in the p-DAB plus PB fed mice and the extent of suppression was statistically significant ($p < 0.05$ through $p < 0.001$. The mitotic index in the p-DAB plus PB fed mice was also appreciably higher than in the normal control series ($p < 0.001$) (Fig. 2, Histogram-2c).

Sperm head anomaly

Quite a high incidence of sperm showing some form of abnormal head morphology (Fig. 1j and k) has been recorded in the different treatment series (Fig. 2, Histogram-2d). In Lyco-30 fed mice, the percentages of sperm with abnormal head morphology was considerably reduced and the differences with regard to controls were statistically significant at day 7, 15, 30, 60, 90 and 120 ($p < 0.05$ through $p < 0.001$).

Biochemical assay

AST and ALT activities

The AST activities in mice fed p-DAB+PB+alcohol were appreciably enhanced ($p < 0.05$ to $p < 0.001$) in liver and spleen at all fixation intervals as compared to normal controls. However, the AST activities in the group of mice fed Lyco-30 along with the carcinogens were appreciably decreased at all the fixation intervals ($p < 0.05$ to $p < 0.001$). Similar results were also obtained in the spleen (Table 2).

In a similar trend, the ALT activities in liver and spleen tissues of p-DAB+PB+alcohol fed mice were increased appreciably at all fixation intervals ($p < 0.05$ to $p < 0.001$) as compared to normal controls, and the activity was reduced considerably at all the fixation intervals in the group of mice fed Lyco-30 along with the carcinogens ($p < 0.05$ to $p < 0.001$) (Table 2).

However, although there was a general trend of reduction in the activity of these toxicity marker enzymes (e.g. AST and ALT) at all the fixation intervals, the reduction in their activities was not to the same extent, sometimes showing a minor wave like pattern of increase and decrease in consecutive fixation intervals.

Acid phosphatase activity

The acid phosphatase activity in the p-DAB+PB+alcohol fed series was significantly increased in liver and spleen tissues at all the fixation intervals as compared to normal controls (Table 2). The AcP activity in liver of p-DAB+PB+Lyco-30 fed mice was significantly decreased at day 15, 30 and 60 ($p < 0.05$ to $p < 0.01$) but the decrease was statistically not significant at day 7, 90, 120. Interestingly, the AcP activity in the liver tissue was slightly higher than in the spleen at all fixation intervals except at day 90.

Alkaline phosphatase activity

The AlkP activity in the p-DAB+PB+alcohol fed series was notably increased ($p < 0.05$ to $p < 0.001$) in liver and spleen tissues at all fixation intervals as compared to normal controls (Table 3). But the AlkP activity in liver of the drug fed mice was significantly decreased at all the fixation intervals ($p < 0.05$ to $p < 0.001$) (Table 3). However, at day 30 day, the AlkP activity was found to be drastically

increased in spleen tissue but it was decreased at day 90, and again increased at day 120, the reason for which was not understood. Interestingly enough, the activity of AlkP was also found to be increased at day 120 in the drug fed group of mice.

Lipid peroxidation activity

LPO also significantly decreased in drug fed series as compared to carcinogen fed series in all fixation intervals in both the tissues ($p < 0.05$ to $p < 0.001$) (Table 3). Apparently, LPO activity was more pronounced in spleen than in the liver tissue.

Reduced glutathione activity

An increased level of reduced glutathione was observed in the Lyco-30 fed mice at all fixation intervals, as compared to p-DAB+PB+alcohol-30 fed series (Table 3). This protective effect of the remedy was seen both in spleen and liver at all fixation intervals, except at day 15 for spleen tissue.

Discussion

The present study revealed that chronic feeding of p-DAB and PB for varying periods of time exhibited both genotoxic and cytotoxic effects in mice, as revealed from the various types of chromosomal, nuclear and sperm head abnormalities, and an elevated toxicity level of the biomarkers. It has earlier been well established that in majority of malignant tumors, the neoplastic cells undergo chromosomal alterations, often of highly complex nature, usually exhibiting both structural and numerical aberrations [20] as also observed in the present study. Therefore, quite logically, a palpable reduction in chromosomal and nuclear damage by the administration of the potentized homeopathic drug would clearly point to its ability to combat carcinogenesis at the chromosomal and genomic level. Dave et al. [20] suggested that random breakage lesions on chromosomes were linked to tumorigenesis in the target issue. The treatment of carcinogens alone or in combination with alcohol-30 also elevated frequency of micronuclei and caused an increase in the MI of the bone marrow cells. There was also a clear decrease in MN and MI in the drug fed mice, which would also support their anti-carcinogenic role. p-DAB is known to be metabolized to monoaminoazo-zobenezene (MAB) by N-demethylation, and subsequently to aminoazobenezene (AAB) or to N-hydroxy- N-methyl-4-aminoazobenzene (N-OH-AAB). Covalent binding of these metabolites with DNA is considered as a major carcinogenic factor [21, 22] which also causes various chromosomal aberrations by their interaction with the chromosomal DNA. Therefore, the molecular mechanism of protection could lie in the ability of the potentized drug either to interfere with the binding of the metabolites to DNA by some unknown pathway, or else, could interfere with the catabolic pathway of p-DAB itself.

All micronuclei are derived from either broken or intact chromosomes that failed to incorporate into daughter nuclei following mitosis, and thus provide an indirect measurement of the induction of structural CA's. Occurrence of MN in the carcinogen treated mice in both shorter and longer fixation intervals could be due to their chronic effect on the chromosomes in different stages of cell cycle or due to their clastogenic and spindle poisoning effect. The increase in the number of abnormal sperm in the carcinogen treated mice would suggest that they also had spermatotoxic effects, and the repair/protection rendered by the administration of the homoeopathic remedy would speak for their role in protecting the gonadal tissue as well. Aminoazodyes produce reactive oxygen species(ROS). ROS, in turn, react with cellular components which generate hepatotoxicity. The process of lipid peroxidation is initiated by the attack of a free radical on unsaturated lipids and the resulting chain reaction is terminated by the production of lipid breakdown products, lipid, alcohol, aldehyde and malonaldehyde. Thus there is a cascade of peroxidative reaction which ultimately leads to the destruction of lipid and thus the liberation and quantification of malonaldehyde reflects the state of toxicity which may effect membrane structure [8-10]. In the present study, there was a gradual rise of LPO activity from day 7 to day 120 in the carcinogen fed series and a corresponding decrease in the drug fed mice, which will again lend support to the antitoxic effect of the drug. Apparently, the drug also showed its protective ability in spleen.

Reduced glutathione (GSH), a tripeptide containing cysteine, is an important thiol compound present in cells. It plays an important role in regulation of cellular proliferation and cellular defense. It has

a nucleophilic thiol group and it can modify substances in one of the three ways, (i) by chemical reaction with a reactive metabolite to form a conjugate, (ii) by donation of a proton or hydrogen atom to reactive metabolites or free radicals or (iii) by conjugation catalysed by a glutathione transferase or else other metabolites may chemically oxidize to GSGG from GSH [24]. Therefore exposure of cells to the carcinogens leads to GSH depletion. Positive alteration in endogenous GSH is encountered in the present investigation after the administration of the homeopathic remedy, which is in conformity with the activities of the other biomarkers. Further, GSH being an antioxidant is able to inhibit lipid peroxidation, and is also involved in hepatic detoxification, thus it can also help in the cytoprotective process.

Phosphatases are enzymes which catalyze the splitting off of phosphoric acids from certain monophosphoric esters, a reaction of considerable importance in several body processes including neoplastic growth. Acid and alkaline phosphatases have been directly implicated to the extent of cellular damage and toxicity [25-27], particularly of liver and cardiac tissue. The level increases mainly as a result of liver, bile duct or gall bladder dysfunction. Since liver is the primary target of the carcionogens, the gradual increase of alkaline phosphatase levels becomes significant in effectively denoting the alteration of toxicity levels during tumor growth. In the present investigation, the homeopathic remedy brought forth appreciable reduction in the levels of phosphatase activities in the drug fed mice, which would again convincingly denote its ability to combat cancer growth.

Similarly, the activities of enzymes like AST and ALT have also been linked to hepatocellular injury or necrosis of striated muscle [28, 29]. Determination of ALT activity is a relatively sensitive indicator of hepatic damage and release of ALT from the cytosol can occur secondary to cellular necrosis or as a result of cellular injury with membrane damage and bleb formation [30]. Thus, the changes in activity and concentration of tumor marker enzymes like AST, ALT, AcP and AlkP in tissues like liver, kidney and spleen could reflect the state of toxicity particularly in relation to tumor growth [27]. Incidentally, from the findings of the present study a fairly strong correlation can be substantiated between modulations in the cytogenetic endpoints and the enzyme biomarkers, which were consistent in their expected pattern of expression, both in the carcinogen treated series as well as in the drug fed series, depicting an overall positive influence of the remedy in ameliorating the toxic effects of the carcinogens in mice.

How the ultra low doses of the remedy, which are not theoretically expected to possess even a single molecule of the original drug substance, could bring about multiple changes in both cytogenetic and enzyme biomarkers, is rather unclear at the present state of our knowledge. Incidentally, Khuda-Bukhsh [31,32] proposed a hypothesis that one mechanism through which the potentized homeopathic drugs act could be through regulation of expression of certain relevant genes, since all the cytogenetical and biochemical markers tested are generally under the strict control of a specific genetic regulatory mechanism.

Finally, the liver's unique metabolism system and its relationship to the gastrointestinal tract make it an important target of many drugs and various chemicals to generate toxicity here. Detoxification of liver is therefore a significant event. Hepatic drug metabolism and chemical carcinogen, often with an imbalance between the generation of toxic metabolites and detoxification processes, can influence the degree of hepatotoxicity. Any drug, which can antagonize this hepatotoxic effect can also become a strong candidate for being considered for use as a supportive therapy, particularly in many forms of liver ailment, including cancer. Further in-depth studies in both animals and in human subjects are, however, necessary before Lyco-30 can be recommended as one probable drug for future use in CAM for supplementing cancer therapy. Therefore, we would encourage other workers to replicate such works and to confirm or refute these findings.

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