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Homeopathic Remedies, Carcinosin200C and Natrum Sulphuricum200C, Used Intermittently Demonstrate Greater Ameliorative Response in Mice Intoxicated with Liver Carcinogens

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ABSTRACT

Background- In homeopathy, Carcinosin 200C (Car200) and Natrum Sulphuricum 200C (Nat Sulph200), are generally used individually in liver ailments depending on the totality of symptoms. This study was designed to examine if a combined treatment of these two homeopathic remedies can provide better ameliorative effects in mammalian model mice *(Mus musculus)* with reference to the generation of hepatotoxicity, free radicals and liver tumors induced by chronic feeding of two carcinogens, p-dimethylaminoazobenzene (p-DAB) and phenobarbital (PB).

Methods- 42 mice were divided into following groups comprising 6 mice each: normal untreated (control–1), normal+succussed alcohol–fed (control–2; alcohol being "vehicle" of the drugs), p-DAB+PB fed (carcinogen–intoxicated), p-DAB+PB+succussed alcohol fed (carcinogen–intoxicated control–3), p-DAB+PB+Nat Sulph200 fed (intoxicated drug–fed–1), p-DAB+PB+Car200 fed (intoxicated drug–fed–2), and p-DAB+PB+Nat Sulph200+Car200 fed (intoxicated drug–fed-3). Cytogenetical endpoints like chromosome aberrations (CA), micronuclei, mitotic index (MI) and sperm head anomaly (SHA), biomarkers like aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipid peroxidation (LPO), reduced glutathione content (GSH), gamma-glutamyl transferase, lactate dehydrogenase (LDH), glucose–6–phosphate dehydrogenase (G6PD), succinate dehydrogenase (SDH), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were assayed at certain intervals. Additionally, electron microscopical studies (scanning and transmission) and gelatin zymography for matrix metalloproteinases were conducted in the liver at day 90 and 120.

Results- All toxicity parameters were favorably modulated by administration of either of the two homeopathic remedies, but the protection level was greater in mice treated conjointly with both the drugs.

Conclusion- Conjoint use of Car200 and Nat Sulph200 in carcinogen-intoxicated mice ameliorated hepatotoxicity and oxidative stress significantly more than when a single drug was administered and their clinical use in human liver ailments is validated.

Key-words: Carcinosin 200C, Cytogenetical endpoints, Enzymatic biomarkers, Hepatotoxicity, Liver carcinogens, Natrum Sulphuricum 200C

INTRODUCTION

Homeopathy is a holistic method of medical treatment introduced by Dr. Samuel Hahnemann, a German physician, more than two hundred years ago.

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In homeopathy, ultra-highly diluted drugs are often used in micro doses [1] particularly to treat chronic ailments. Natrum sulphuricum 200C (Nat Sulph200) and Carcinosin 200C (Car200) are two such ultra-highly diluted drugs (diluted by a factor of 10^{400}), which are generally used individually against liver disorders based on totality of symptoms, and sometimes their successive use is claimed in homeopathic literature to ameliorate most stubborn cases of liver ailment [1,2]. However, there is no scientific validation that Nat Sulph200 and Car200 have any ameliorative potential against liver toxicity either

when treated alone or in combination in mice intoxicated with liver carcinogens.

The azo dye induced hepatocarcinogenesis in mice has been used quite extensively as a model for studying chronological events leading to severe hepatotoxicity and liver cancer by many earlier workers [3-5]. Mice are chronically fed with p-dimethylaminoazobenzene (p-DAB, initiator) and phenobarbital (PB, promoter) to develop hepatotoxicity and after about 2 months of feeding, tumors are developed in the liver. Tumors spread further aggressively if the feeding of the carcinogen continues and after 90 days, tumors are found to be spread all over the liver, some of which may transform into a malignant state at 120 days of continued feeding. Thus, these intoxicated mice can serve as suitable materials for toxicological studies and give clear evidence if any drug has ameliorative effect on hepatoxicity/ hepatocarcinogenesis by analyzing certain relevant scientific protocols periodically in these experimental mice [6-11], along with observation on certain pathophysiological changes (e.g. tissue damage, necrotic changes etc.) [7]. By adopting such a strategy, the anti-cancer potential of some homeopathic remedies had earlier been evaluated [6-11].

In view of the above, the objectives of this study were: i) to determine the cytogenetical (genotoxic) changes induced by the carcinogens, and to evaluate if and to what extent these changes could be ameliorated by the individual/combined treatment with Nat Sulph200 and Car200; ii) to assay some relevant toxicity-biomarkers like AST, ALT, LPO, GSH, SDH, SOD, CAT, GR, G6PD, GGT and LDH to know the nature of toxicological changes that occur when the carcinogens are chronically administered and to know the extent of favorable modulation, if any, brought forward by the individual/combined treatment of the two homeopathic drugs under study. Further, iii) the changes in MMP, if any, induced by the carcinogens and their possible modulations by the individual/combined treatment of these two homeopathic drugs would be under scrutiny along with iv) the assessment of corresponding ultra-structural/ patho-physiological changes in liver (the major target organ of the carcinogens) studied through scanning and transmission electron microscopes.

MATERIALS AND METHODS

Experimental Protocol- For the development of hepatotoxicity and liver nodules and subsequent hepatocarcinoma, the method of chronic dietary feeding of carcinogens used by several earlier workers [3,5,7-11] was adopted. The major part of the experimental study was carried out in the Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, West Bengal, India from September 2007 to October 2008.

Ethical clearance and acclimatization of animals- 42 randomized healthy inbred adults of Swiss albino mice (*Mus musculus*) of both sexes weighing 25±2 gm were used as materials. All animals were acclimatized seven days prior to the commencement of the treatment and allowed free access to food (50% wheat, 40% gram and 10% powdered milk without any animal protein supplementation) and water, and kept in hygienic condition. Experiments on animals were performed with clearance from the Animal Ethics Committee, University of Kalyani (Vide sanc.No.KU/IAEC/Z-11/07, dt. 18 May 2007) and were conducted under the overall supervision of the Animal Welfare Committee, University of Kalyani.

42 mice were used for each of four fixation intervals namely- 30, 60, 90 and 120 days. For each fixation interval, mice were divided further into 7 sets of 6 mice each. The first set of mice was maintained on a normal diet (Group I, control 1). The second set of mice was fed succussed alcohol ("vehicle" of the drug prepared in the same way as the drug using alcohol from the same stock) in addition to normal diet (Group II, control 2). Another group of mice was kept on a diet mixed with 0.06% p-DAB (Sigma, D-6760) and provided 0.05% aqueous solution of PB instead of water (Group III, carcinogen intoxicated). The fourth group of mice was chronically fed 0.06% p-DAB along with 0.05% aqueous solution of PB plus succussed alcohol (Group IV, carcinogen intoxicated positive control). The fifth set of mice was chronically fed p-DAB + PB + Nat Sulph200 (Group V, intoxicated drug fed 1). The sixth group of mice was fed p-DAB + PB + Car200 (Group VI, intoxicated drug fed 2). A group of mice was fed p-DAB + PB + Nat Sulph200 + Car200 (Group VII, intoxicated drug fed 3). All the experiments were carried out concurrently and in similar environmental setup. After sacrifice, blood was collected from jugular veins, since this proved to yield the adequate amount of blood necessary for all the tests.

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Liver tissues were quickly processed and stored at -20°C pending for further biochemical estimations.

Source and preparation of stock solution of drug- Homeopathic drugs, Nat Sulph200 and Car200, were prepared in 90% ethyl alcohol by dissolving Sodium sulphate and liver cancer tissue, respectively, following the homeopathic potentization/dynamization method of succussion and dilutions at successive steps, as recommended in the Homeopathic Pharmacopoeia of India [12]. These drugs were procured from HAPCO, 165 BB Ganguly Street, Kolkata, India. Car200 was reported to be derived from cancerous tissue of either liver or breast [13] by following the same homeopathic principle of dilutions and succussion.

1ml each of Nat Sulph200 and Car200 were diluted separately with 20 ml of double distilled water to make the stock solution of Nat Sulph200 and Car200, respectively. In the same way, the stock solution of succussed alcohol was also prepared.

Feeding procedure and dose- Each mouse was fed through gavages with the aid of a fine pipette 0.06 ml of stock solution of either Nat Sulph200 or Car200 or placebo, as the case may be that confirmed a single dose. One dose of Nat Sulph200 was fed once a day and Car200 was fed once a week.

Laboratory methodology- Multiple parameters of the study were used to ascertain the possible pathway(s) and mechanism of action.

Cytogenetic assay- The standard cytogenetic protocols like assays of CA, micronuclei (MN), MI from bone marrow cells and SHA from the epididymis of the testis has been adopted in the present study for testing genotoxicity [5,7-11].

Assessment of liver function- Enzymatic assay of AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) was done by following Bhattacharjee *et al*. [9]. GGT (EC 2.32.2) was estimated according to the manufacturer's protocol (Reckon Diagnostics, India).

Biochemical Assays

From whole blood- G6PD (EC 1.1.1.49) was assayed from whole blood by the reagent kit (UV-Kinetic method) according to the manufacturer's protocol (Reckon Diagnostics, India).

From Serum- LDH (EC 1.1.1.27) was assayed from serum according to the manufacturer's protocol (Reckon Diagnostics, India).

From liver tissue

Preparation of tissue homogenates- The entire liver tissue was washed twice with ice-cold 0.1 M phosphate buffered saline (1:9), pH 7.4, blotted, dried and weighed. The liver tissue was stored at -20°C for not more than 12 hours before analysis. A 10% tissue homogenate was prepared in PBS by homogenizing the tissue in a glass homogenizer. The homogenate was centrifuged at 2000 g for 15 min at 4°C to remove the cell debris and then the supernatant was centrifuged at 12,000 g for 1 hour at 4°C. The supernatant obtained was used for use in biochemical assays, namely, AST, EC 2.6.1.1, ALT, EC 2.6.1.2, LPO, GSH content, SOD, EC 1.15.1.1, CAT, EC 1.11.1.6, GR, EC1.8.1.7, and SDH, EC 1.3.5.1 activities and estimation of the protein [7-11].

Gelatin-zymography- Liver extracts were thawed and mixed 3:1 with substrate gel sample buffer (10% SDS, 4% sucrose, 0.25M Tris-HCl pH 6.8, 0.1% bromophenol blue). Each sample (20 μg) was loaded under non-reducing conditions onto electrophoretic mini-gels (SDS-PAGE) containing 1 mg/mL of type 1 Gelatin (Sigma, USA). The gels were run at a running buffer at a temperature of 4°C. After SDS-PAGE the gels were washed twice in 2.5% Triton X-100 for 30 mins each, rinsed in water and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 8). The gels were stained with Coomassie brilliant blue R250, and gelatinolytic activity of MMP was detected as clear bands on a blue background [7,10,12,14].

Electron microscopic study- For electron microscopic study of liver on day 90 and 120, the standard gold coating technique using critical point-drier (CPD-Biorad, Microscience Division, Warford, England), and sputter coater (model 198, Agar Sputter Coater, Stansted, United Kingdom) was adopted in case of Scanning electron microscopy (SEM) (LEO, 435 VP, United Kingdom). For Transmission electron microscopy (TEM; CM-10, Philips Electron Optics, Eindhoven, The Netherlands) the ultra-thin sections (60-90 nm, cut by Reichert E Jung,

England) were stained with uranyl acetate and lead citrate. Generally, four serial liver sections obtained from each of four different mice at each fixation interval were analyzed [7-11].

Blinding- The observer was "blinded" about information if the mice were from the treated or control lots. Uniformity in scoring data of the "control" and the "treated" series was all along maintained.

Statistical Analysis- The significance test between different series of the data was conducted by student's t-test. The differences between the drug fed series and p-DAB+PB+Alc fed positive control was mainly considered. Differences among the groups were assessed by two-way ANOVA using the SPSS 16 software package for Windows. The analysis of variance was presented in Table 1 (a-o). A value of $p<0.05$ was considered significant between drug-treated and control groups.

RESULTS

The results of the analysis of variance in respect of various parameters of the study have been summarized in Table 1 (a-o). The levels of significance between the data in comparison have been denoted by asterisk (*) marks; *denoting $P<0.05$ as moderately significant, ** denoting $P<0.01$ as quite strikingly significant and *** denoting $P<0.001$ to highly or strongly significant.

Table 1 (a-o) shows the analysis of variance (All the analyses of two-way ANOVA were done using SPSS 16 software package for Windows).

Table 1a: Chromosome Aberration (CA)

Source	DF	SS	MS	F-value	P-value
Series	3	16.94	5.648	1.28	0.313^{n}
Days	6	1152.34	192.056	43.38	0.000^{***}
Error	18	79.69	4.427		
Total	27	1248.97			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1b: Micronuclei (MN)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.02800	0.009333	2.16	0.128^{n}
Days	6	2.97717	0.496195	114.80	0.000^{***}
Error	18	0.07780	0.004322		
Total	27	3.08297			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1c: Mitotic Index (MI)

Source	DF	SS	MS	F-value	P-Value
Series	3	14.21	4.7362	5.69	0.006^{**}
Days	6	121.52	20.2535	24.35	0.000^{***}
Error	18	14.97	0.8318		
Total	27	150.70			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

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Table 1d: Sperm Head Anomaly (SHA)

Source	DF	SS	MS	F-value	P-Value
Series	3	1.115	0.3716	2.14	0.130^{n}
Days	6	27.431	4.5718	26.38	0.000***
Error	18	3.120	0.1733		
Total	27	31.665			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1e: Aspartate transaminase (AST)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.003010	0.001003	7.93	0.001**
Days	6	0.005999	0.001000	7.90	0.000***
Error	18	0.002277	0.000127		
Total	27	0.011286			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1f: Alanine transaminase (ALT)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.000078	0.000026	4.02	0.024*
Days	6	0.000579	0.000096	14.95	0.000***
Error	18	0.000116	0.000006		
Total	27	0.000773			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1g: Lipid peroxidation (LPO)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.05616	0.018720	16.31	0.000***
Days	6	0.07073	0.011788	10.27	0.000***
Error	18	0.02066	0.001148		
Total	27	0.14755			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1h: Reduced Glutathione content (GSH)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.000006	0.000002	0.70	0.566^{n}
Days	6	0.000517	0.000086	29.48	0.000***
Error	18	0.000053	0.000003		
Total	27	0.000576			

Table 1i: Glucose 6 phosphate dehydrogenase (G6PD)

Source	DF	SS	MS	F-value	P-Value
Series	3	0.0268	0.00894	0.12	0.947^{n}
Days	6	27.7312	4.62187	61.93	0.000^{***}
Error	18	1.3434	0.07463		
Total	27	29.1014			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1j: Gamma glutamyl transferase (GGT)

Source	DF	SS	MS	F-value	P-Value
Series	3	127.7	42.57	0.70	0.562^{n}
Days	6	12595.9	2099.32	34.67	0.000^{***}
Error	18	1089.9	60.55		
Total	27	13813.5			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1k: Lactate dehydrogenase (LDH)

Source	DF	SS	MS	F-value	P-Value
Series	3	5544	1848	0.97	0.429^{n}
Days	6	718345	119724	62.81	0.000^{***}
Error	18	34308	1906		
Total	27	758197			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1l: Succinate dehydrogenase (SDH)

Source	DF	SS	MS	F-value	P-Value
Series	1	3457	3457.1	16.46	0.007^{**}
Days	6	155624	25937.4	123.53	0.000^{***}
Error	6	1260	210.0		
Total	13	160341			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1m: Superoxide dehydrogenase (SOD)

Source	DF	SS	MS	F-value	P-Value
Series	1	0.000026	0.000026	2.04	0.203^{n}
Days	6	0.004675	0.000779	61.74	0.000^{***}
Error	6	0.000076	0.000013		
Total	13	0.004776			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1n: Catalase (CAT)

Source	DF	SS	MS	F-value	P-Value
Series	1	0.0026	0.0026	0.00	0.954^{n}
Days	6	61.8250	10.3042	14.14	0.003^{**}
Error	6	4.3727	0.7288		
Total	13	66.2003			

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Table 1o: Glutathione reductase (GR)

Source	DF	SS	MS	F-value	P-Value
Series	1	4.571	4.571	2.04	0.203^{n}
Days	6	435.429	72.571	32.43	0.000^{***}
Error	6	13.429	2.238		
Total	13	453.429			

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ^{n}= Insignificant

DF= Degrees of freedom, SS= Sum-of-squares, MS= Mean square

Incidence of liver tumors- Tumors were found on autopsy in the carcinogen treated groups as well as in positive controls (Table 2). But the incidence was less in the single drug fed group. In the carcinogen-intoxicated conjoint drug fed 3 groups, values indicating favorable modulations were usually greater than those observed in either Nat Sulph200 or Car200 fed mice.

Table 2: The number of mice with tumor incidence at different fixation intervals and in different series

Series	Number of specimens	Tumor incidence and intensity			
		30 Days	60 Days	90 Days	120 Days
Normal	24	0/6	0/6	0/6	0/6
Normal+Alc	24	0/6	0/6	0/6	0/6
p-DAB+PB	24	0/6	6/6 (3^{+++}, 3^{++})	6/6 (5^{+++}, 1^{++})	6/6 (6^{+++})
p-DAB+PB+Alc	24	0/6	6/6 (4^{+++}, 2^{++})	6/6 (5^{+++}, 1^{++})	6/6 (6^{+++})
p-DAB+PB+Nat Sulph-200	24	0/6	2/6 (1^{++}, 1^{+})	3/6 (2^{++},1^{+})	3/6 (1^{++}, 2^{+})
p-DAB+PB+Car-200	24	0/6	3/6 (2^{++}, 1^{+})	3/6 (3^{++})	3/6 (3^{++}, 1^{+})
p-DAB+PB+Nat Sulph-200+Car-200	24	0/6	2/6 (2^{++})	1/6 (1^{+})	1/6 (1^{+})
Total	168	0/42	19/42	19/42	19/42

(+) = Low intensity tumor; (++) = Moderate intensity tumor; (+++) = High intensity tumor

Six mice were used per set for fixation intervals at 30, 60, 90 and 120 days.

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Effect on cytogenetical study- A few representative photomicrographs of normal (Fig. 1a) and abnormal metaphase chromosome spreads are shown in Fig. 1b-d, MN in Fig. 1e-f, and normal (Fig. 1g) and abnormal sperm (Fig. 1h-i). Chronic feeding of p-DAB+PB+Alc produced a considerable increase of CA (Fig. 2), MN (Fig. 3), MI (Fig. 4), and SHA (Fig. 5) (Table 1 a-d for statistical results). The administration of Nat Sulph200 to the carcinogen fed mice reduced CA, MN, MI, and SHA frequencies more noticeably only at day 90 and day 120 as compared to that of carcinogens fed mice. The same was more or less true for the administration of Car200. The CA, MN, MI and SHA frequencies were marginally less in the combined drug-treated series, particularly at the long intervals, as compared to either Nat Sulph200 or Car200 treated mice.

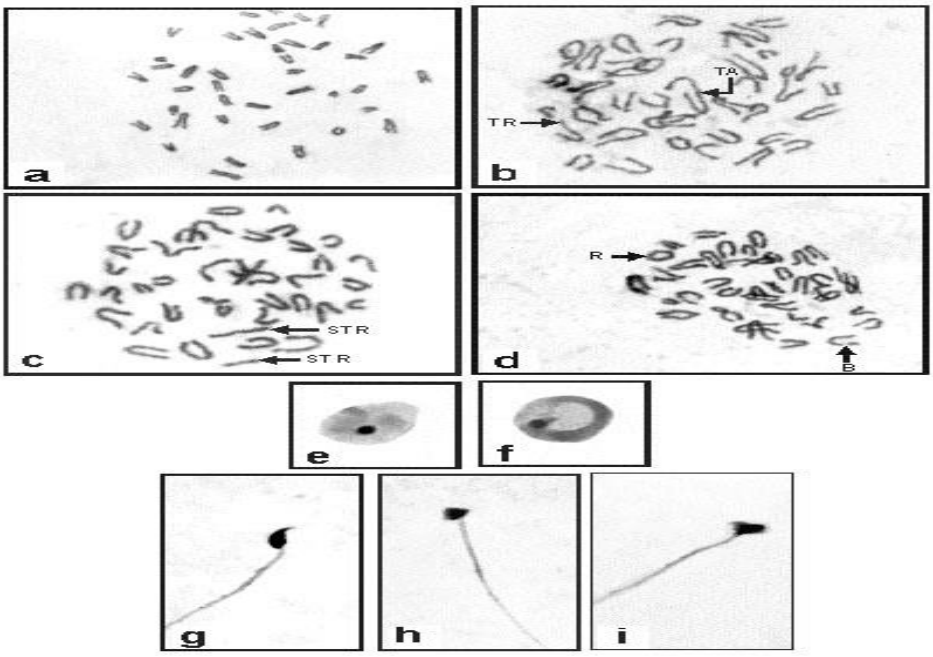

Fig. 1: Representative photomicrographs showing normal metaphase complement **(1a),** Translocation **(1b),** Stretching (STR) **(1c),** Ring (1d) and Break **(1d);** Erythrocyte showing micronucleus **(1e,f);** Sperm with normal head morphology **(1g),** Sperm with abnormal head morphology **(1h,i)**

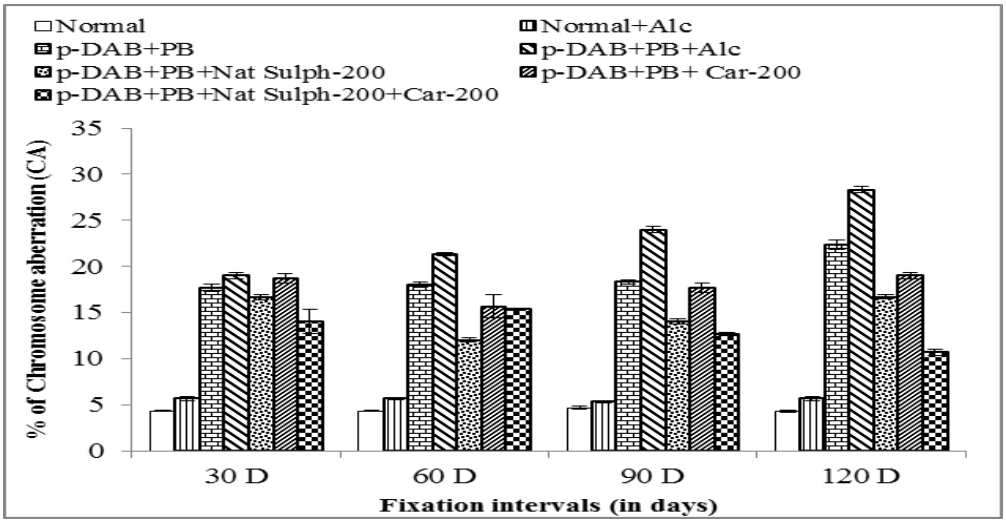

Fig. 2: CA percentage in different series of mice at different fixation intervals
(Data presented as Mean±S.E.)

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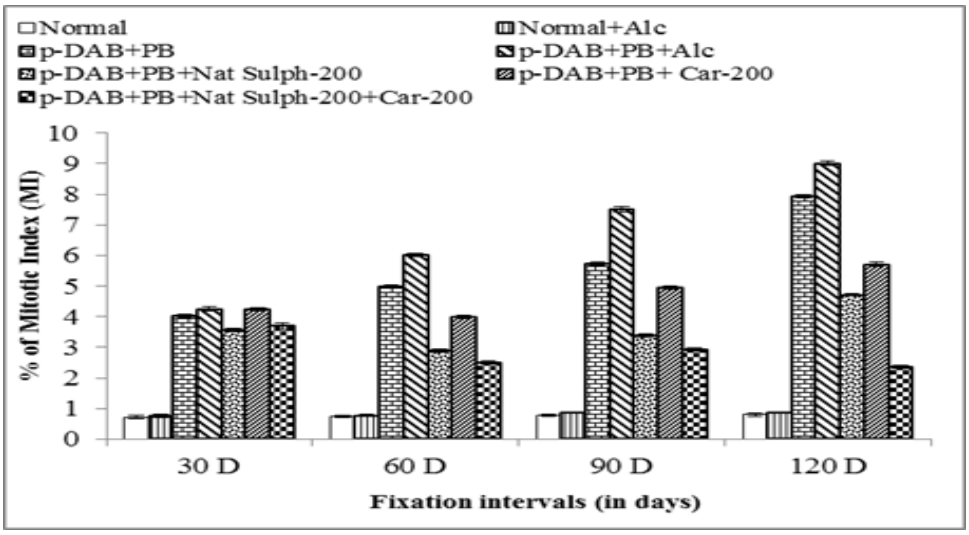

Fig. 3: Micronucleus (MN) percentage in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

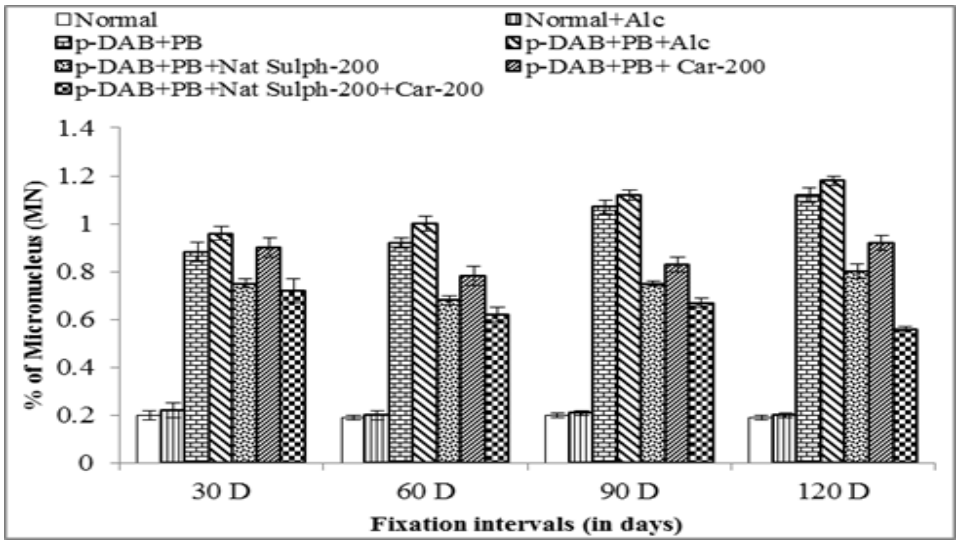

Fig. 4: MI percentage in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

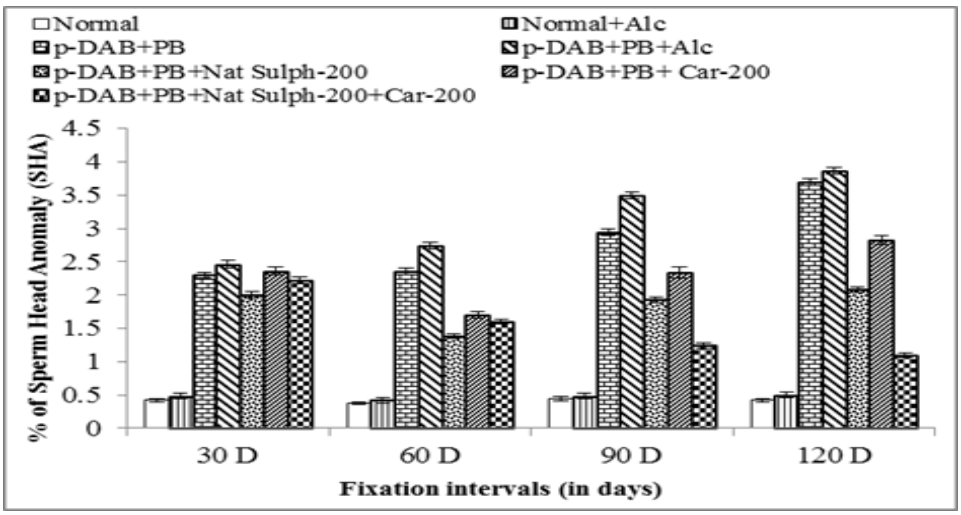

Fig. 5: SHA percentage in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

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Effect on biochemical parameters

Enzymatic markers (From tissue samples)

Aspartate and Alanine aminotransferase (AST and ALT) activities- In mice fed p-DAB+PB and p-DAB+PB+Alc, the activities of AST (Fig. 6) and ALT (Fig. 7) were greater in carcinogen fed series of mice than that of the drug treated ones. The differences were statistically significant for all or most parameters. In Nat Sulph200 fed mice, the positive intervention in activities was observed, particularly at day 90 and day 120 in the liver. Car200 fed mice showed a considerable ameliorative effect in the liver. However, Nat Sulph200 along with Car200 exhibited greater combative effects than single drug fed series in the liver, particularly conspicuous at longer fixation intervals.

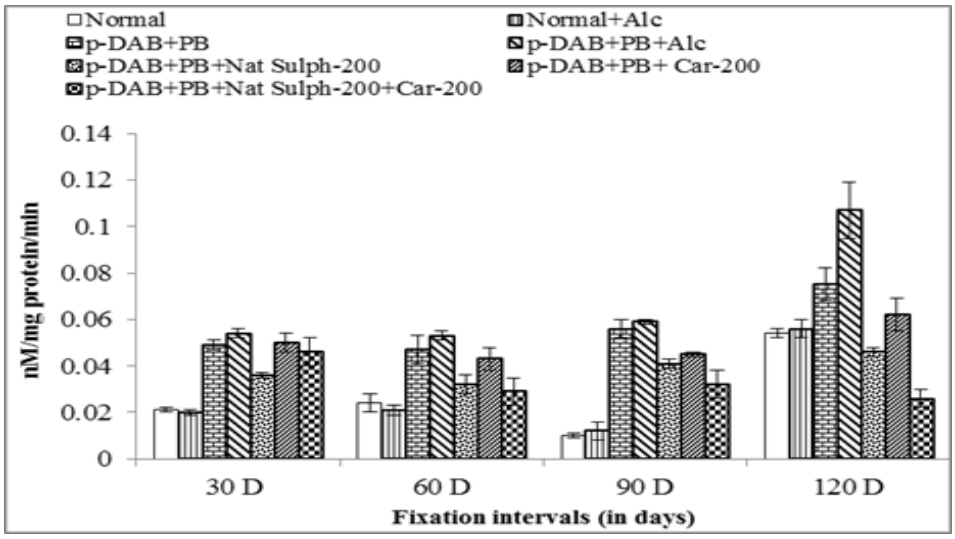

Fig. 6: Activity (nmol/mg protein/min) of AST (Histogram 5) in different series of mice at different fixation intervals (Data presented as mean±S.E.)

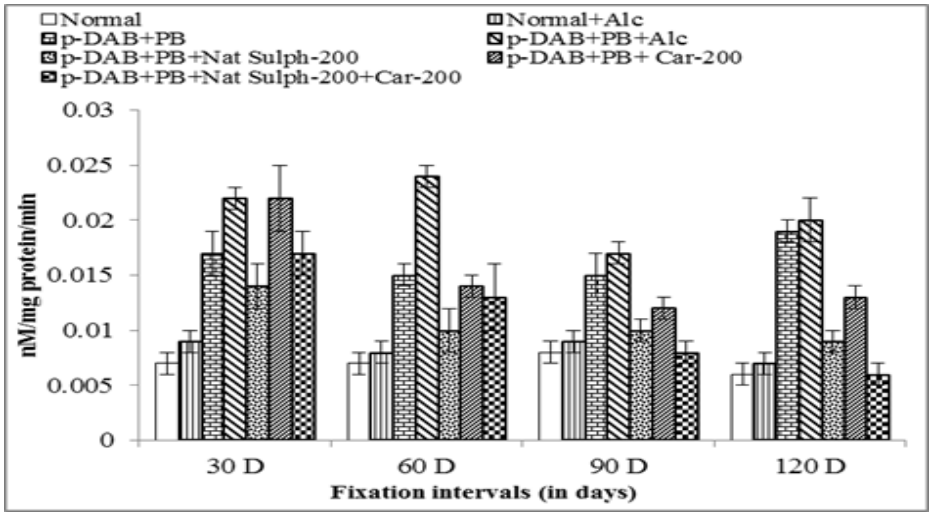

Fig. 7: Activity (nmol/mg protein/min) of ALT (Histogram 6) in different series of mice at different fixation intervals (Data presented as mean±S.E.)

Effect on lipid peroxidation- Lipid peroxidation (Fig. 8) was a significantly decreased in all the drugs-fed mice, when compared with carcinogen fed series at all fixation intervals. Nat Sulph200 or Car200 shows significant decrease in lipid peroxidation at different fixation intervals. Here the combinational therapy showed appreciably better results, particularly at longer fixation intervals.

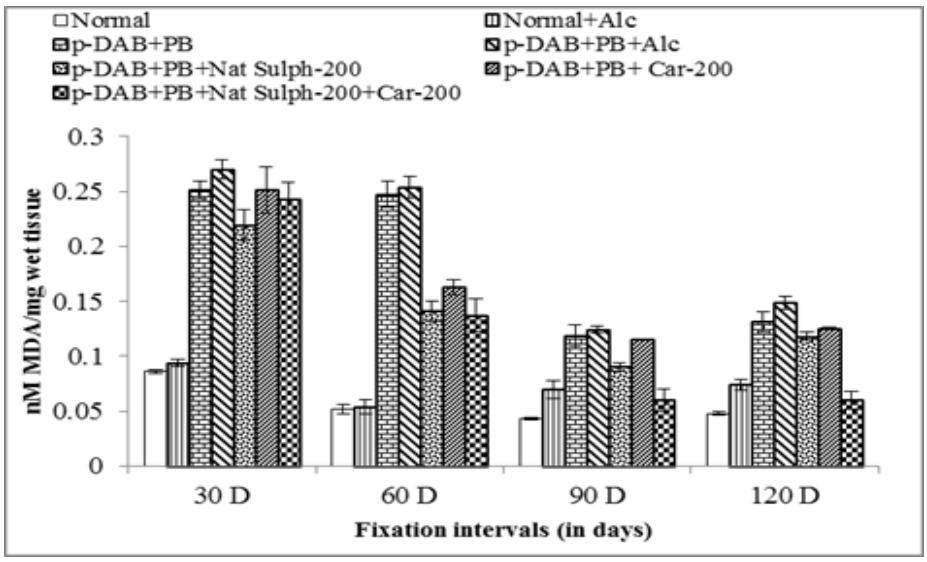

Fig. 8: LPO (nM MDA/mg wet tissue) in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

Effect on GSH content- Chronic feeding of p-DAB+PB and p-DAB+PB+Alc decreased GSH content (Fig. 9) in mice. Administration of Nat Sulph200 to carcinogen fed mice generally showed favorable modulation in GSH content particularly at longer fixation intervals. Administration of Car200 to carcinogen fed mice brought about considerable favourable change in GSH content. Administration of Car200 intermittently with Nat Sulph200 showed values of GSH content under greater control than that of either only Nat Sulph200 or Car200 fed group.

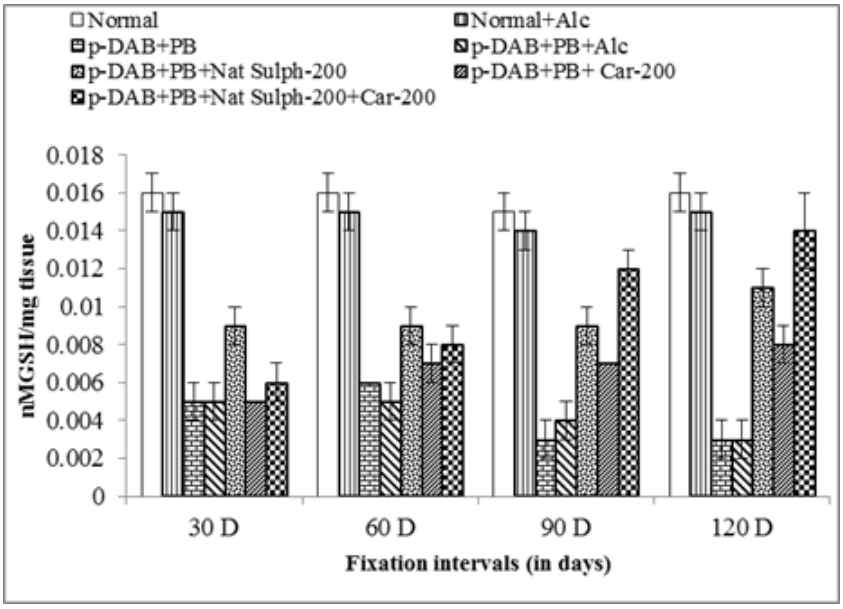

Fig. 9: GSH (nM GSH/mg tissue) content in different series of mice at different fixation intervals (Data presented as Mean±S.E)

Effect on succinate dehydrogenase (SDH) activity- A significant decrease in activities of SDH (Fig. 10) was observed in carcinogen fed series of mice. The Nat Sulph200 administration showed considerable ameliorative effect in SDH activity. A similar trend was observed in Car200 fed series. However, conjoint treatment of Nat Sulph200 and Car200 exhibited greater combative effects on SDH activity as compared to either Nat Sulph200 or Car200 particularly at longer fixation intervals.

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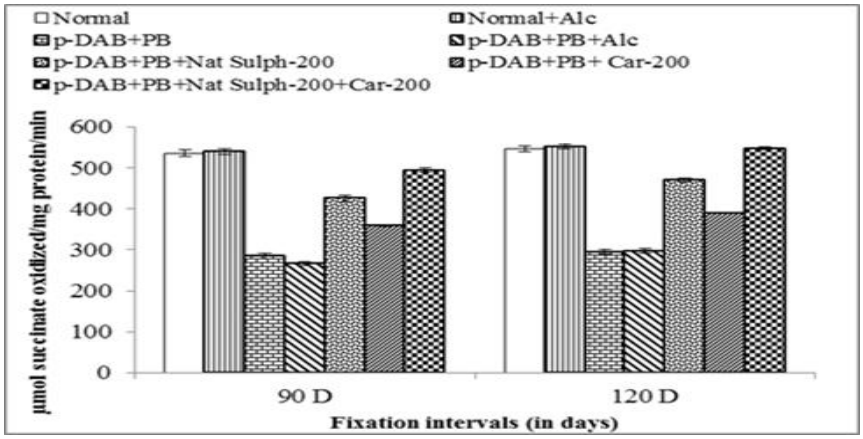

Fig. 10: Activities of SDH (µmol succinate oxidized/ mg protein/min) in different series of mice at 90 and 120 days fixation interval. (Data presented as Mean±S.E.)

Effect on SOD, CAT and GR activities Chronic feeding of p-DAB+PB and p-DAB+PB+Alc decreased activities of SOD (Fig. 11), CAT (Fig. 12), and GR (Fig. 13). Administration of homeopathic remedies appeared to show these data to be significantly close towards normal. Administration of p-DAB+PB+Nat Sulph200 reduced the toxic changes in the carcinogen intoxicated mice more noticeable at longer fixation intervals. Car200 exhibited more or less similar positive modulating effect but the combined series of Nat Sulph200 plus Car200 manifested apparently better able to restrict the toxicity level.

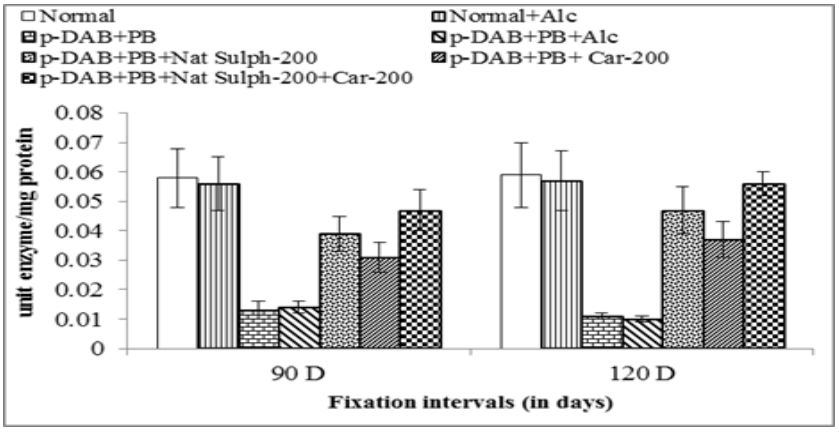

Fig. 11: Activities of SOD (units/mg protein) in different series of mice at 90 and 120 days fixation interval. (Data presented as Mean±S.E.)

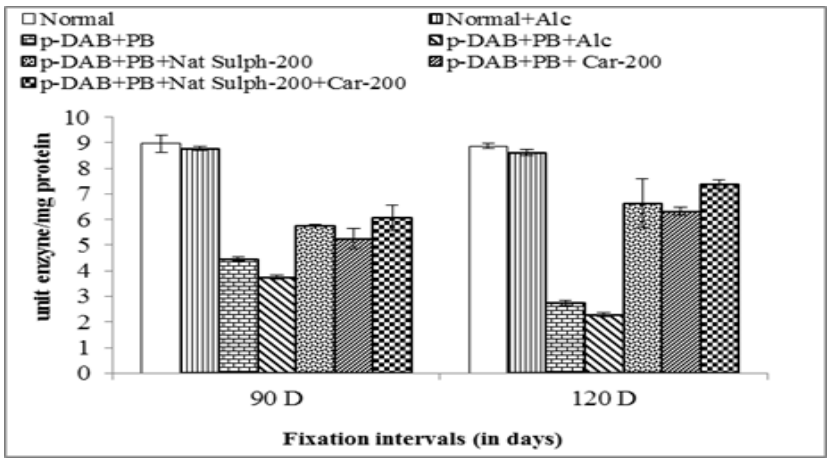

Fig. 12: Activities of CAT in different series of mice at 90 and 120 days fixation interval. (Data presented as Mean±S.E.)

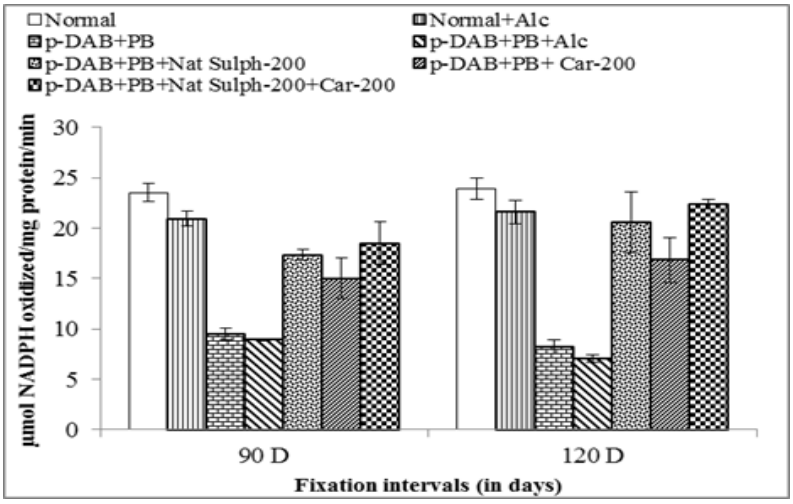

Fig. 13: Activities of GR (µmol NADPH/mg protein/min) in different series of mice at 90 and 120 days fixation interval. (Data presented as Mean±S.E.)

From whole blood sample

Glucose 6-phosphate dehydrogenase (G6PD) activity There was generally a decline in the activity of G6PD (Fig. 14) in carcinogen fed series. The levels of G6PD activity in all the drugs-fed series (single or combined) were close to the normal controls. In p-DAB+PB+Nat Sulph200 fed mice, a significant ameliorative effect was also noted as compared to controls. A more or less similar effect was observed in the p-DAB+PB+Car200 fed mice at longer fixation intervals. Further, in p-DAB+PB+Nat Sulph200+Car200 fed mice, the positive modulating effect was more discernible than in either Nat Sulph200 or Car200 fed mice.

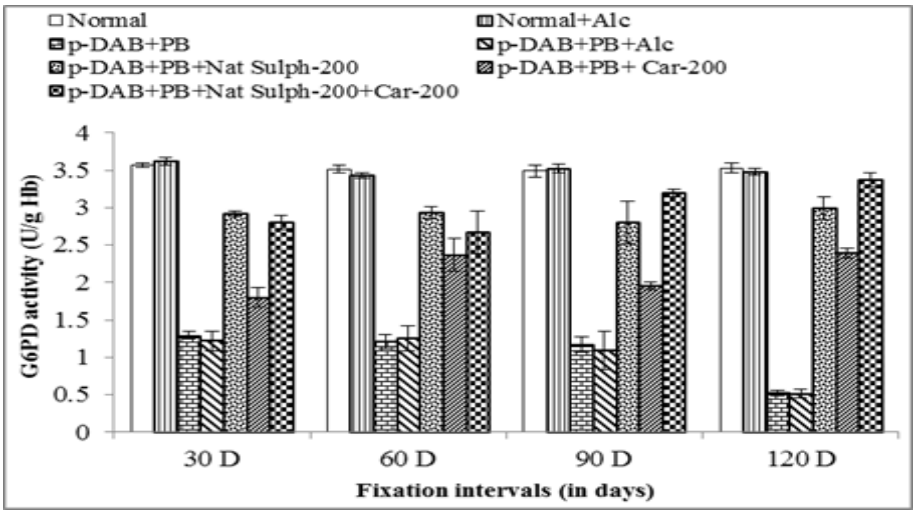

Fig. 14: Activities of G6PD (U/g Hb) in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

Enzymatic markers from serum sample

Gamma glutamyl transferase (GGT) and Lactate dehydrogenase (LDH) activities The GGT (Fig. 15) and LDH (Fig. 16) activities were significantly increased in mice chronically fed p-DAB+PB and p-DAB+PB+Alc. Administration of Nat Sulph200 or Car200 separately along with p-DAB+PB brought about considerable positive intervention in the levels of serum GGT and serum LDH. But p-DAB+PB+Nat Sulph200+Car200 produced palpably better intervention in comparison to either Nat Sulph200 or Car200 fed mice (Table 1e-o for all statistical results).

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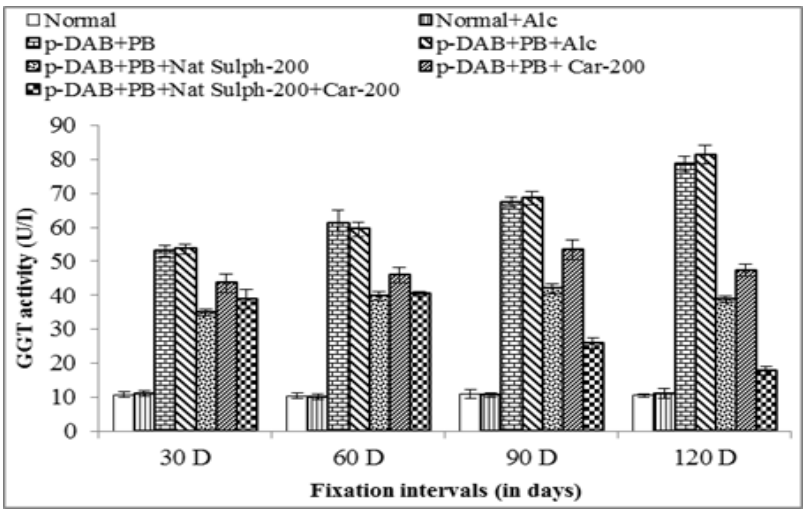

Fig. 15: Activities of serum GGT (IU/L) in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

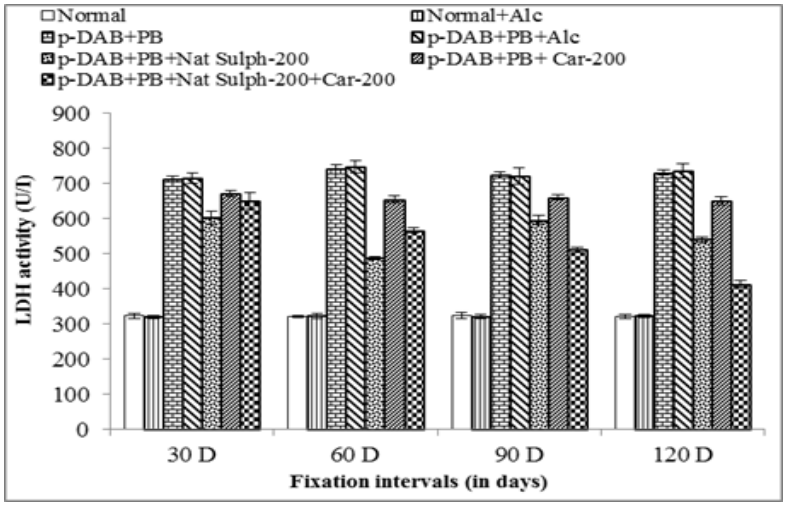

Fig. 16: Activities of serum LDH (IU/L) in different series of mice at different fixation intervals (Data presented as Mean±S.E.)

Ultra structural studies of liver tissue

Scanning electron microscopic (SEM) studies- In normal control series of mice (Fig. 17a), cells were small in size as compared to carcinogen treated series. Hepatic cell boundaries were recognizable. In p-DAB+PB+Alc treated series (Fig. 17b) there was an increase in the number of hepatocytes. In p-DAB+PB+Nat Sulph-200 fed series (Fig. 17c) tissue necrosis was not evident. Hepatic cells appeared to be healthy. In p-DAB+PB+Nat Sulph200+Car200 fed series (Fig. 17d) positive alterations were observed at the ultra-structural level. In the carcinogen treated combined drug fed series less number of damaged hepatocytes was found as compared to single drug fed mice. Tissue necrosis was less persistent in combined series.

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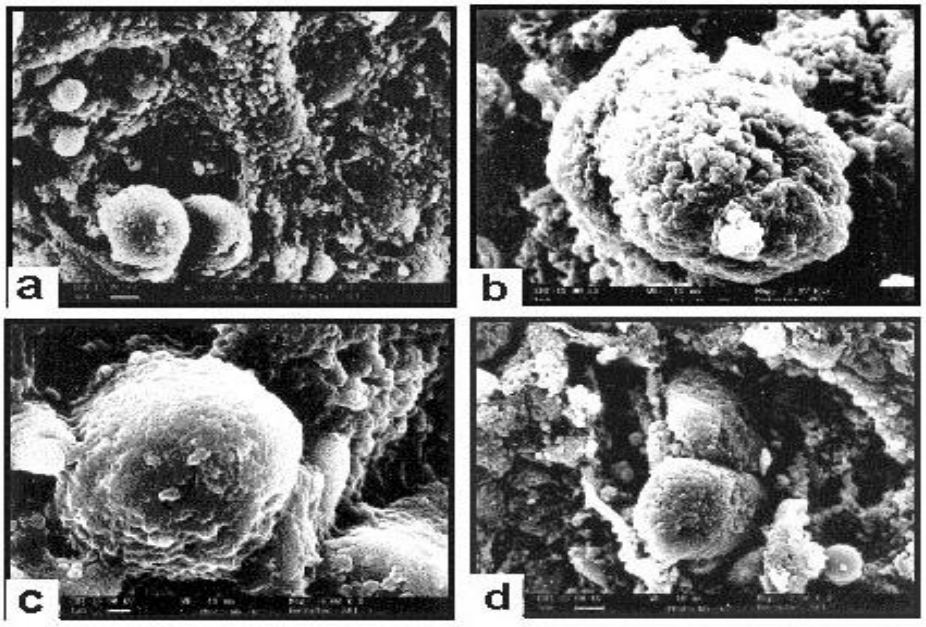

Fig. 17: Representative photomicrographs of liver sections under SEM showing features of normal (17a), p-DAB+PB+Alc (17b) and p-DAB+PB+Nat Sulph200 (17c) and p-DAB+PB+Nat Sulph200+Car200 (17d)

Transmission electron microscopic (TEM) studies- Distinct membrane bound intracellular organelles were observed in the normal group of mice (Fig. 18a) along with normal euchromatinized nuclei. Mitochondria appeared to be normal without any swelling and prominent cristae. Endoplasmic reticulum was continuous with ribosomes attached to its surface. Lipid droplets were absent. In p-DAB+PB+Alc treated series (Fig. 18b) nuclear membrane appeared to be broken at places along with dispersed nucleoplasm and heterochromatinisation. Mitochondria were small and numerous with obliterated cristae. Lipid droplets were numerous in number. In p-DAB+PB+Nat Sulph-200 fed series (Fig. 18c) nucleus was fairly round with evenly distributed nucleoplasm. Mitochondria were round and fewer in number. Few lipid droplets were present. In p-DAB+PB+Nat Sulph-200+Car-200 fed series of mice (Fig. 18d), nuclear membrane appeared to be continuous and mitochondria were few in number but some had an orientation of cristae which was more or less like the normal and some of the endoplasmic reticulum was continuous with few bound ribosomes.

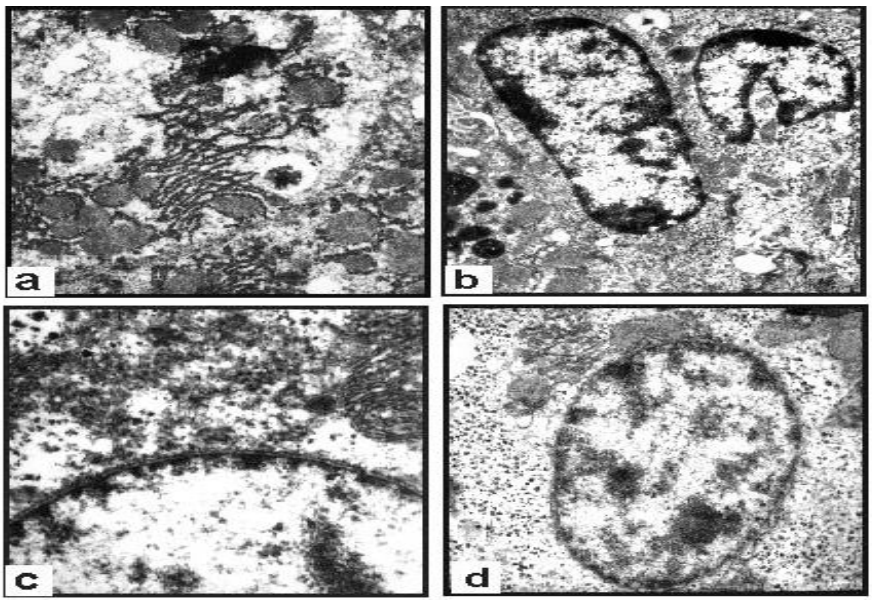

Fig. 18: Representative photomicrographs of liver sections under TEM showing features of normal (18a), p-DAB+PB+Alc (18b), p-DAB+PB+Nat Sulph200 fed (18c) and p-DAB+PB+Nat Sulph200+Car200 (18d)

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Structural changes at pathological level

Development of tumor in liver- Out of 24 normal mice kept as control group, 6 each was sacrificed at day, 30, 60, 90 and 120. At autopsy, liver tumors could not be observed in any of them. The same was true for all 24 mice fed only alcohol. However, in p-DAB+PB treated mice tumors were observed in all 6 mice at day 60 onwards. The same was true for mice fed alcohol along with the carcinogens (Table 2). In mice fed p-DAB+PB+Nat Sulph200, 2 mice had tumors at day 60 and 3 each had tumors at day 90 and day 120. In mice fed p-DAB+PB+Car200, 3 mice each had tumors at day 60, day 90 and day 120. In mice fed Car200 along with p-DAB+PB+Nat Sulph200 tumors were found in 2 mice at day 60 and 1 each at day 90 and day 120. The incidence and growth of tumors found in combined drug fed series were less, both numerically and qualitatively.

Gelatin zymography

Matrix metalloproteinase activity- At 90 days fixation interval, in p-DAB+PB and p-DAB+PB+Alc treated series, there were two bands of which the one near about 92 kDa (Fig. 19a) of mice and the bands belonged to the MMP family (from analysis of substrate specificity and proximity of molecular weight 92 kDa, it appeared to be MMP-9). In carcinogen treated drug fed series of mice, expression of a single band was observed near about 92 kDa and the expression of MMP appeared to be somewhat less than that of carcinogen-treated series of mice. Car200 intermittently fed with p-DAB+PB+Nat Sulph200 yielded better efficacy to reduce the expression of MMP as compared to either Nat Sulph200 or Car200 treated series. Similar results were obtained for 120 days also (Fig. 19b).

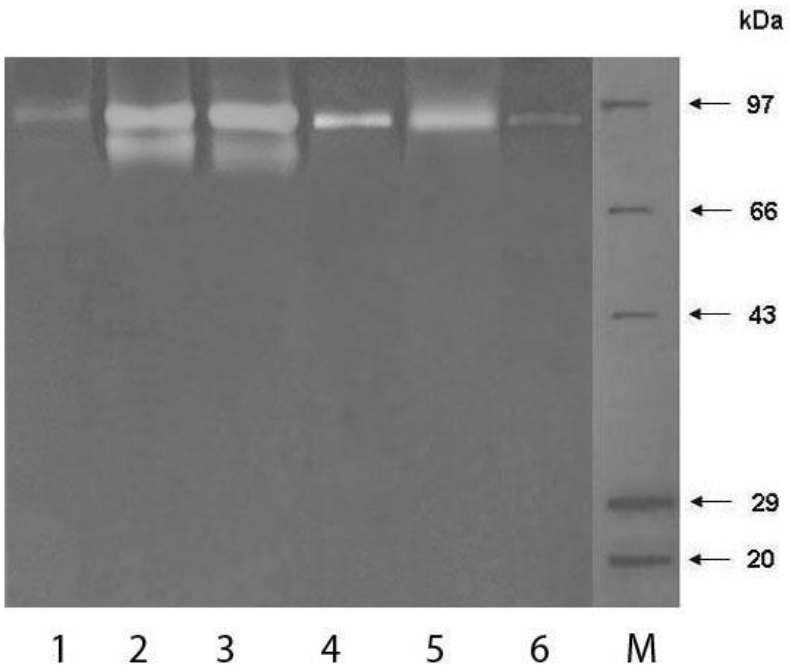

Fig. 19a: Gelatin zymogram of liver samples showing the expression of MMP in experimental mice sacrificed at day 90; Lane 1= Normal, Lane 2= p-DAB+PB, Lane 3= p-DAB+PB+Alc, Lane 4= p-DAB+PB+Nat Sulph200, Lane 5= p-DAB+PB+Car200, Lane 6= p-DAB+PB+Nat Sulph200+ Car200, and M= Molecular weight marker

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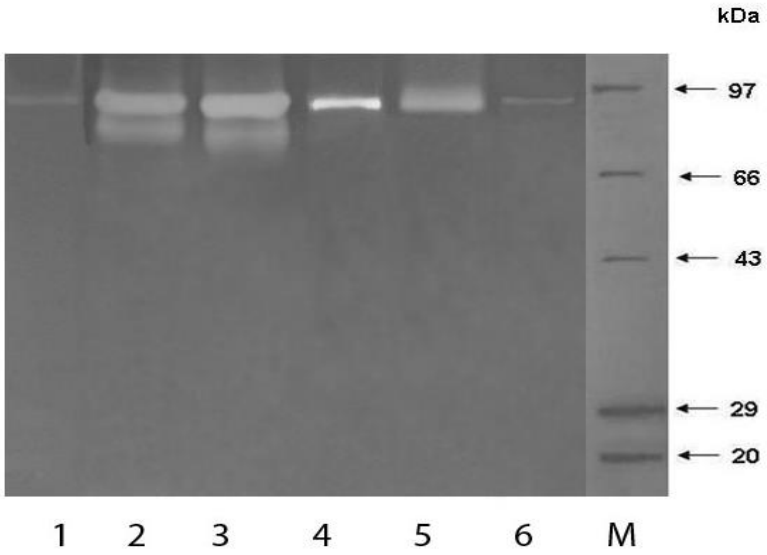

Fig. 19b: Gelatin zymogram of liver samples showing the expression of MMP in experimental mice sacrificed at day 120; Lane 1= Normal, Lane 2= p-DAB+PB, Lane 3= p-DAB+PB+Alc, Lane 4= p-DAB+PB+Nat Sulph200, Lane 5= p-DAB+PB+Car200, Lane 6= p-DAB+PB+Nat Sulph200+ Car200, and M= Molecular weight marker

DISCUSSION

Our results confirmed that chronic feeding of the carcinogens had considerable clastogenic and genotoxic effects in mice as evidenced from the increased frequencies of different chromosomal aberrations, micronuclei and sperm with head anomalies in the carcinogen treated mice. Both the homeopathic remedies claimed to have profound beneficial effects in liver, reduced the deleterious effects of the carcinogens and the conjoint treatment of the two remedies was generally more effective than either of the two drugs administered alone. With the progress of carcinogenesis, the MI of mice was also elevated. Interestingly the homeopathic remedies were successful in decreasing the MI considerably. That means that the drugs have favorable influence on DNA replication and also on protection/repair of DNA, as claimed in some earlier studies by us as well [15-19]. Further, the ultra-highly diluted homeopathic drugs could also protect the sperm head morphology damaged by the carcinogens. Thus the homeopathic remedies behaved not only as anti-genotoxic and anti-tumorigenic agents, but also as anti-spermatotoxic ones.

A more critical analysis of the significance of the changes brought about by the homeopathic remedies could also point out their favorable role in combating the carcinogenetic process which involves transformation of proto-oncogenes into oncogenes, presumably intervening in the process of carcinogenesis at the molecular level. In this study, the drugs modulated the activities of AST and ALT enzymes, which are gene controlled. Further, recently these biomarkers have been implicated to hepato-cellular injury or necrosis of some striated muscles [20,21] and have also been directly implicated to hepatotoxicity generated either as a result of cellular injury or disorder or malfunctioning of hepatocytes because raised levels of ALT had been detected in various hepatic disorders. Therefore, the ability of successful reduction of the AST and ALT could be viewed as supportive evidence of the homeopathic drugs' ability to induce intrinsic regulatory measures in the expression of relevant genes to bring about the functioning of them back to proper and normal level [18,22].

Similarly, SDH, an important mitochondrial enzyme reported to control superoxide scavenging activity of respiratory chain [23], is a membrane bound dehydrogenase linked to the respiratory chain. SOD is a vital defense enzyme, which is capable of scavenging superoxide ($O_2^{\bullet -}$) anion from H_2O_2 also reduces the harmful effects of free radicals derived from secondary reaction [24]. Dismutation of superoxide anion ($O_2^{\bullet -}$) to oxygen (O_2) is catalyzed by SOD [25].

We found SDH and SOD activities to be reduced in p-DAB+PB and p-DAB+PB+Alc treated mice while in the carcinogen treated drug fed mice this activity was replenished. Therefore, this would lend further support

to the contention that the homeopathic remedies favorably acted in a regulatory manner to bring about the changes that would lead the cells to the recovery process.

The enzymatic antioxidants had more protective effects against active and massive oxidative attack due to the ability to decompose ROS. SOD and CAT are well recognized antioxidant enzymes *in vivo* condition. The production of H_2O_2 in cell causes not only cellular damage but it can also cause mutagenic effect. SOD and CAT have the ability to remove ROS. The reaction of superoxide anion radicals ($O_2^{\bullet-}$) and dismutation to hydrogen peroxide (H_2O_2) is catalyzed by SOD and degradation of H_2O_2 is mediated by CAT [26,27]. Therefore, the ability of homeopathic remedies to replenish CAT activity should be considered as a favorable effect in rendering internal environment of the cell relatively free of toxic elements.

G6PD acts as an antioxidant enzyme by providing nicotinamide adenine dinucleotide phosphate (NADPH) to reduce oxidative stress [28,29]. GR catalyzes the reduction of oxidized glutathione (GSSG) to GSH [30]. Protective potential against free radical damage had been shown by glutathione (GSH) and GSH-related enzymes [31]. The modulations of the activity of these antioxidant enzymes are in conformity with the favorable changes observed in the other toxicity biomarkers. These enzyme activities were reduced in the carcinogen treated mice and increased in the carcinogen intoxicated mice after the homeopathic drug treatment.

Polyunsaturated aliphatic acids and oxidative degradations are involved in the process of LPO [32]. This process leads to the formation of diversified products including many reactive electrophiles. A product of LPO, malondialdehyde is able to bind to macromolecules like DNA and it shows mutagenic and carcinogenic potentials [33].

In carcinogen fed mice LPO level is enhanced as a result of oxidative stress. Oxidative stress results when the balance between the productions of ROS overrides the antioxidant capacity of the target cell [34] and this may in turn lead to development of cancer [35]. Antioxidants function by stopping free radicals from attacking other healthy molecules and causing a chain reaction. Hence antioxidants are necessary for free radical induced cellular damage in the tissues and organs [36].

In carcinogen fed mice activities of antioxidant enzymes may probably be attributed to the enhanced level of LPO, which would indicate that they were more prone to oxidative stress as compared to carcinogen treated drug fed series of mice. Thus, the ability of homeopathic remedies to bring down LPO level was a strong indication that these remedies could in some way block the ROS or had the ability to induce antioxidant activity, since increased LPO was known to impair membrane function by decreasing membrane fluidity and altering the activity of membrane bound enzymes and receptors [37].

GSH, a prominent cellular reductant also protects the membrane polyunsaturated fatty acids from peroxidation and had an antioxidant function [38]. Therefore, while decrease in GSH indicates higher toxicity the replenishment would indicate the lack of toxicity or toxicity level lowered. This was in agreement with our observations because while a considerable decrease was noted in GSH content of mice fed carcinogens there was a clear indication of the drugs being successful at replenishing GSH level to a considerable extent.

GGT is a membrane bound enzyme that catalyzes the degradation of glutathione and other γ-glutamyl compounds by hydrolysis of the γ-glutamyl moiety or by its transfer to a suitable acceptor. Enhanced activity of GGT may occur due to oxidative stress, which has the capability to increase the transport of glutathione precursors into cells [39,40]. LDH, a cytoplasmic enzyme that catalyzes the reversible conversion of pyruvate to lactate [41,42].

The azo-dye, p-DAB is known to be metabolized to mono-amino azobenzene (MAB) by N-dimethylation and subsequently produced amino azobenzene (AAB). Reactive electrophiles are produced by azo-dye [43,44]. The azo-dye also produces free radicals, which generate the formation of reactive oxygen species (ROS) [45]. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function [46]. An inescapable side product of oxidative metabolism is ROS, which mediate mutagenesis and alter signalling pathways in chemically induced carcinogenesis.

Oxidative stress, a condition in which antioxidant level is lower than normal, results when the balance between the productions of ROS overrides the antioxidant capacity of the target cell. Oxidative stress caused by ROS accumulated in different organs will persistently

destroy the cells, which may lead to diseases. Therefore, ROS is a key parameter that controls tumor progression and angiogenesis by regulating the expression of various oncogenic molecules [27].

ROS are atoms or small molecules that have unpaired valence shell electrons. They readily accept another electron or transfer their unpaired electron to another molecule [47]. Excessive production of ROS may overrule antioxidant defences or surpass scavenging ability of the antioxidant defence system resulting in oxidative stress and permanent tissue injury [48]. Cells are furnished with enzymatic antioxidative mechanisms which take part in the process of elimination of free radicals.

Therefore, positive alterations as a consequence of administration of the homeopathic remedies could be considered significant in terms of their role in combating carcinogens or protecting the liver and other organs from carcinogen inflicted damage. Administration of the homeopathic remedies brought about considerable modulation in the activities of the antioxidant enzymes and reduced oxidative stress to a considerable extent. This would further support the contention that homeopathic remedies favorably acted in a regulatory manner to bring about those changes which would protect the cells from oxidative damage and lead to the subsequent recovery process.

A critical analysis of the results would reveal that chronic feeding of p-DAB and PB induces the development of hepatic tumors, which are prominent as solid white or reddish nodules in varying numbers depending upon the intensity of its growth. A careful analysis would suggest that the administration of Nat Sulph200+Car200 was able to combat the development of liver tumors better than that of Nat sulph-200 or Car200 administered alone. Thus, the intermittent administration of Car200 along with Nat Sulph200 apparently showed an additive action in either protecting the liver from carcinogenic action of p-DAB and PB or by simulating regression of the tumors.

An analysis of studies made through SEM and TEM in mice liver fixed at day 90 and day 120 would support the contention that the homeopathic remedies were able to combat the necrotic damage and other hepatocellular injuries inflicted by the carcinogens. Thus the protection at morphological tumor incidence was also supported by positive ultra-structural changes in the liver.

In TEM study, many mitochondria, distorted nuclei and large black lipid droplets were observed in liver tissues of carcinogen-fed mice. Similarly, in SEM studies, damaged hepatocytes and hepatic chords were observed in the carcinogen treated mice. On the other hand, in the carcinogen-intoxicated mice, the administration of the homeopathic drugs reduced the occurrence of these damages considerably, which would suggest their ability to protect the liver at the ultra-structural level.

MMPs are zinc-dependent endopeptidases that degrade constituents of extra cellular matrix (ECM). Tumor angiogenesis, tumor growth, local invasion and subsequent distant metastasis are important events during various stages of tumor progression and these processes take place as MMPs degrade the constituents of ECM and basement membrane [49]. As compared to adjacent normal tissues, high expression and increased activity of MMP-9 have been noticed in malignant tissues [50].

In the present study, the MMPs were overexpressed in the carcinogen treated mice while there was a reduction in their expression level in the drug fed mice. Therefore, the expression of MMPs or rather the lack of it in drug-fed group renders strong evidence in favor of their anti-tumorigenic effects at the gene expression level. In the present study over expression of MMP (presumably 9, substrate specific molecular weight) was evident (depicted from the number and intensity of the bands) at the longer fixation intervals in mice fed p-DAB+PB or p-DAB+PB+Alc. In the drug-fed carcinogen-intoxicated mice, no over expression (only a single band) of these metalloproteinases was noticed. The present findings appear to add further support to the contention that the potentized homeopathic drugs could be strong candidates for effective use in alleviating hepatocarcinogenesis.

In the present investigation, there were convincing evidences of the efficiency of the potentized homeopathic remedies, Nat Sulph200 and Car200 and the ultra low doses of the ultra highly diluted remedy could bring about multiple changes in both cytogenetical as well as in so many other biomarkers.

Khuda-Bukhsh [15-19] advocated a working hypothesis, which suggested that the homeopathic remedy has the ability to trigger relevant gene(s) into action by acting as a “molecular switch” and thereby initiating a cascade of chain reactions in downstream genes that can regulate the expression of right kind of proteins necessary for recovery from the diseased or disordered state of gene

functioning. Saha *et al.* [51] showed that evidence from the global microarray analysis that the potentized homeopathic drugs: *Hydrastis canadensis* and *Marsdenia condurango* could induce epigenetic modifications and alter gene expression profiles of numerous cancer-related genes in HeLa cells *in vitro* condition.

CONCLUSIONS

Thus, in recent years complementary and alternative medicines (CAM), particularly the efficacy of ultra-highly diluted homeopathic medicines have been gaining ground with scientific explorations that would support their beneficial use, particularly in cancer therapy [52-59] and other difficult-to-cure diseases. Analysis of the result of the present study would validate the use of ultra-highly diluted remedies successfully in clinical practices, at least as supportive medicines.

In the context of the results of the present study, further in-depth studies on the efficacy and exploration of mechanism of action of ultra-highly diluted homeopathic drugs *in vivo* condition with different animal models and in vitro with suitable cell free systems should be highly encouraged and recommended for future research, particularly to combat diseases like cancer, neurological and psychological disorders, because of relatively less or negligible toxic side-effects of these remedies. Evidence based results in homeopathy can be of great help in understanding its impact on the entire medical system, to get introduced and integrated as an authentic medical science into mainstream medical practices.

CONTRIBUTION OF AUTHORS

Research Concept- Khuda-Bukhsh AR.
Research Design- Khuda-Bukhsh AR.
Supervision- Khuda-Bukhsh AR.
Materials- Bhattacharjee N.
Data Collection- Bhattacharjee N.
Data analysis and interpretation- Khuda-Bukhsh AR, Bhattacharjee N
Literature search- Khuda-Bukhsh AR, Bhattacharjee N
Writing article- Khuda-Bukhsh AR, Bhattacharjee N
Critical Review- Khuda-Bukhsh AR, Bhattacharjee N
Article editing- Khuda-Bukhsh AR
Final approval- Khuda-Bukhsh AR
Research support- Khuda-Bukhsh AR, Bhattacharjee N

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