Indian Journal of Experimental Biology
Vol. 47, July 2009, pp. 602-607

Protective potentials of a plant extract (*Lycopodium clavatum*) on mice chronically fed hepato-carcinogens

Surajit Pathak[†], Antara Banerjee[†], Saili Paul & Anisur Rahman Khuda-Bukhsh*
Department of Zoology, Cytogenetics and Molecular Biology Laboratory, University of Kalyani, Kalyani, India, 741 235

Received 3 July 2008; revised 26 March 2009

Chronic feeding of carcinogens p-dimethylamino azobenzene (initiator) and phenobarbital (promoter) for 90 and 120 days elevated activities of acid and alkaline phosphatases, levels of blood glucose and cortisol and decreased the activities of glutathione reductase, succinate dehydrogenase, and blood cholesterol and hemoglobin contents, and levels of serum estradiol and testosterone in mice. Levels of these biomarkers in both liver and spleen tissues were positively altered along with a significant reduction of tumor incidence in liver of carcinogen intoxicated mice treated with spore extract of *Lycopodium clavatum*. The results validate the use of this plant extract in complementary and alternative medicines against hepato-toxicity.

Keywords*:* Acid and alkaline phosphatase, Hepatoprotective, *Lycopodium clavatum*, p-dimethyl aminoazobenzene, Phenobarbital

p-dimethyl amino azobenzene (p-DAB) is an azo dye which is widely used as a coloring agent in polishes, soaps, wax products and also as a food additive. It is included as a Group-2B carcinogen[1]. It produces carcinogenic effect in liver when chronically fed to mice and rats[2]. Teratogenicity and mutagenicity including its dose-response have been reported[3]. Phenobarbital (PB) is an anti-epileptic drug and is also of carcinogenic potential[4] to human, mice and rats when administered orally[5,6]. PB, when fed with known carcinogens, has been reported to promote not only hepatocarcinogenesis in monkeys, but also enhanced thyroid follicular-cell tumors in mice and rats[4]. Phenobarbital, is a strong inducer of p-450 enzymes like CYP2B1 and CYP2B2 which in turn enhance hepatic tumors[7]. The chronic feeding of both p-DAB and PB has been reported to promote tumor growth[8] and induce hepato-toxicity in mice[9]. Chronic feeding of both the carcinogens leads to generation of reactive oxygen species (ROS) over the lapse of time and excessive production of ROS may overrule antioxidant defenses or surpass scavenging ability of the antioxidant defense system resulting in oxidative stress and permanent injury of various tissues other than the primary target organ, the liver.

Simpson[10] and Hana *et al.*[11] concluded from their research that in recent years complementary and alternative medicines have been gaining ground as a supportive medicine in cancer therapy. The spore extract of *Lycopodium clavatum* is used in traditional systems of medicine like Ayurveda, Unani and also in homeopathy as mother tincture (ethanolic crude extract), particularly against symptoms of several liver disorders including cancer. Homeopathic potencies of Lycopodium Clavatum have been generally used in patients with symptoms of liver disorder[11,12] including liver cancer and results indicate that it bears anti-oxidative, anti-proliferative and anti-tumor properties[13,14] However, no systematic study has so far been carried out to examine if it really has any hepato-protective effects. In the present study, the efficacy of ethanolic spore extract of *L. clavatum* in combating hepatotoxicity in carcinogen intoxicated mice has been tested.

Toxicity biomarkers tested include analysis of the activities of acid and alkaline phosphatases (AcP, AlkP), glutathione reductase (GR) and succinate dehydrogenase (SDH) in liver and spleen tissues of carcinogen intoxicated drug (plant extract) fed mice and in controls. Additional studies on blood glucose, hemoglobin, cholesterol, estradiol, testosterone and cortisol, generally used as supportive evidences for predictive risk factor assessment of hepatocarcinogenesis were also considered.

* Correspondent author
Telephone: 91-33-5828750;extn. 315 (O) 91-33-5828768 (R)
Fax: 91-33-25828282
E-mail: prof_arkb@yahoo.co.in,
khudabukhsh_48@rediffmail.com
Present address: Department of Surgery and Gastroenterology
University of Padova, Padova, Italy

Materials and Methods

Chemical used— Analytical grade chemicals p-DAB (D-6760) (Sigma Chemical, Saint Louis, USA), phenobarbitol (product no- 8294, BDH Chemicals Ltd, England), p- nitrophenol (E-MERCK Japan, Cat. No 6798), GSSG (oxidized glutathione; SRL, India, T830764), FAD (Himedia, RM538) and NADPH (SRL, India, T831301) were used.

Animals— Healthy inbred strain of Swiss albino mice (*Mus musculus*) of either sex were used. They were provided with food and water *ad libitum*. The food was generally made up of wheat, gram and powdered milk without any animal protein supplementation. The experimental protocols were cleared by the Animal Ethical Committee, Kalyani University and done in accordance with the guidelines laid down by the Animal Welfare Committee, University of Kalyani.

Plant extract— The ethanolic extract of spores of *Lycopodium clavatum* was procured from HAPCO, 165, BB Ganguly Street, Kolkata 700014. To prepare the extract, dried plant spores of *Lycopodium clavatum* identified by professional taxonomist are macerated with 90% alcohol (ethanol) and filtered. Filtrate is evaporated to a thick residue at 50ºC. The yield of the extract is about 60%. This extract was re-suspended in 90% alcohol (ethanol) and used for *in vivo* experiments.

Experimental protocol— For the induction of hepatic liver nodules and subsequent hepatocarcinoma, the chronic dietary feeding method used by earlier workers[5,6,15-21] was adopted. The azodye, p-dimethylaminoazobenzene (0.06% p-DAB) was chronically fed followed by chronic feeding of 0.05% phenobarbital (PB).

A group of 35 healthy mice weighing between 25-30 g were used for each of the two fixation intervals (i.e., 90 days and 120 days of treatment). Mice were divided into following 5 different groups of 7 mice each (4 males and 3 females in each group):

i) healthy untreated mice reared under normal laboratory conditions with normal low protein diet (i.e. diet containing no animal protein supplementation) (Group-I, normal control 1); ii) mice fed succussed alcohol (as the homeopathic drugs were also prepared in ethyl alcohol with serial agitations) with normal low protein diet (Group-II, vehicle control 2); iii) mice fed diet mixed with 0.06% p-DAB and provided 0.05% aqueous solution of PB instead of water (Group-III, carcinogen treated); iv) mice chronically fed 0.06% p-DAB along with 0.05% aqueous solution of PB plus succussed alcohol; Group IV (carcinogen treated positive control); v) mice fed p-DAB + PB + 0.06 ml of 0.05 % *L. clavatum* stock solution once a day (i.e. at 08.00 hrs) (carcinogen intoxicated drug fed – Group-V).

Feeding procedure and dose— The stock solution of *Lycopodium* was prepared by diluting 100 µl of ethanolic extract of *L. clavatum* in 20 ml of double distilled water. Similarly, the placebo stock solution was prepared in the same way by diluting 100 µl of 90% ethanol in 20 ml of double distilled water. Each mouse was fed through gavage 0.06 ml of 0.05% stock solution of ethanolic extract of *L. clavatum* or placebo with the aid of a micro pipette.

Mice were sacrificed under chloroform anesthesia at two intervals of fixation, viz. at 90 and 120 days. All the experiments were carried out concurrently and in similar environmental set-up.

Laboratory methodology

Tumor incidence was recorded in carcinogen fed and spore extract fed mice after autopsy.

Assay procedure —Blood (approx 2.5 ml from each mouse) was drawn by ventricular puncture of chloroform anesthetized mice using sterile disposable syringe and needle. Blood was collected in two vials, one containing anti-coagulant (EDTA) and the other without EDTA. Blood was centrifuged at 3000 *g* for 10 min and serum was obtained from without EDTA blood, which was used for serum hormone assays and determination of blood glucose level. Blood with EDTA was used for determination of hemoglobin (Hb) and cholesterol.

Liver and spleen tissues of sacrificed mice were then quickly collected in an ice tray and processed for the estimation of biochemical parameters. Before carrying out the enzymatic estimations the quantitative estimation of total protein was made by the method of Lowry *et al*[22].

For the study of acid and alkaline phosphatases the method of Walter and Schutt[23] was followed. The enzyme phosphatase hydrolyses p-nitrophenol phosphate (substrate). The released p-nitrophenol is yellow in color in alkaline medium. The optimum *p*H for acid and alkaline phosphatases is 4.8 and 10.5 respectively. Hence, as per the normal practice, the activity was expressed in mM of phenol liberated/100 mg of protein at 37°C/min after 30 min of incubation. Therefore, the results were obtained by comparing with the standard solution of the

p-nitrophenol. Glutathione reductase activity was measured according to the procedure of Beutler[24]; one unit of enzyme activity is expressed as µmoles of GSSG oxidized/mg protein/min. For estimation of succinate dehydrogenase activity the method of Slater and Bonner[25] was followed.

For blood glucose determination, standard glucose test kit (enzymatic, GOD-POD method) obtained from Span Diagnostics Limited, India, was used and the level of blood glucose was determined by a Spectrophotometer (Shimadzu, Double beam Spectrophotometer, UV 1700, Japan) at 550 nm. Hemoglobin content was determined by Sahli's method with the help of a hemometer (Marienfield, Germany). The blood cholesterol was measured as per the method of Plummer[26].

Blood serum was tested in both female and male mice for estradiol, testosterone and cortisol assays by using the appropriate diagnostic kit (EQUPAR srl, Saronno, Italy) with the aid of an ELISA Reader (ELDEX 3.8, USA) at 450 nm.

Blinding and statistical analysis of data— The experiment was done single blinded and the levels of significance between different series of data were analyzed by Student′s '***t***'-test and two-way ANOVA (Mini Tab 13.31 Software).

Results

Tumor incidence— In general, in the drug fed mice, not only the number of mice showing the tumor nodules was less than the carcinogen fed mice, but also the tumors (nodules) in the former were less in number and growth as compared to the aggressively grown tumors found in the carcinogen fed mice.

Activities of acid and alkaline phosphatases— The activities of AcP and AlkP of Group-IV mice were significantly increased in liver and spleen tissues at day 90 and 120 as compared to Group-I mice. The AcP an-l AlkP activities in liver and spleen of Group-V mice were significantly decreased at both the fixation intervals (Table 1) as compared to Group-III and Group-IV mice.

Activities of glutathione reductase and succinate dehydrogenase— There was a significant increase in activities of these enzymes in both liver and spleen of the Group-V mice as compared to the reduced activities noted in the Group-III and Group-IV mice (Table 2) at both the 90 days and 120 days intervals.

Blood glucose level— As compared to the higher levels of blood glucose observed in Group-III and Group-IV mice, there was significant decrease in blood glucose level in Group-V mice [Table 3].

Blood hemoglobin (Hb)— There was decrease in Hb contents in both Group-III and Group-IV mice, which were significantly increased in Group-V mice (Table 3).

Blood cholesterol— In Group-V mice, a considerable increase was noted in cholesterol content as compared to both Group-III and Group-IV mice (Table 3).

Serum hormonal assays: estradiol, testosterone and cortisol levels— The estradiol level in female mice and testosterone level in male mice were found to be

Table 1— Mean acid (A) and alkaline (B) phosphatase (mM phenol liberated/100mg protein/min) activities of mice at different fixation intervals

[Values are mean ± SE]

Group		*90 days interval*		*120 days interval*	
		Liver	Spleen	Liver	Spleen
Normal	A	0.010±0.000	0.065±0.000	0.022±0.011	0.105±0.001
	B	0.018±0.000	0.012±0.002	0.006±0.002	0.052±0.008
Normal+Alcohol	A	0.031±0.003	0.073±0.008	0.109±0.000	0.098±0.002
	B	0.024±0.000	0.021±0.006	0.042±0.006	0.058±0.002
p-DAB+PB	A	0.051±0.008	0.095±0.014	0.126±0.030	0.140±0.017
	B	0.043±0.006	0.052±0.008	0.124±0.008	0.094±0.006
p-DAB+PB+Alcohol	A	0.053±0.002	0.119±0.004	0.129±0.013	0.173±0.006
	B	0.045±0.004	0.056±0.006	0.147±0.002	0.169±0.012
p-DAB+PB+ Lycopodium clavatum	A	0.039±0.000***	0.080±0.017n	0.117±0.022n	0.074±0.010***
	B	0.028±0.001**	0.023±0.005**	0.116±0.004***	0.139±0.019 n

=*P* values: <0.01, *<0.001, n=non-significant.

Table 2— Mean activities of glutathione reductase (μmoles of GSSG oxidized/mg protein/min), succinate dehydrogenase (μmol/mg protein/min) at different fixation intervals

[Values are Mean ±SE]

Group	Glutathione reductase (μmol of GSSG oxidized/mg protein/min)				Succinate dehydrogenase (μmol/mg protein/min)			
	90 days interval		120 days interval		90 days interval		120 days interval	
	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen
Normal	12.00±0.63	13.00±0.71	14.00±1.00	16.00±1.30	530.00±1.58	487.00±0.32	550.00±1.26	500.00±0.63
Normal+Alcohol	12.80±0.10	13.60±0.13	13.10±0.16	17.90±0.03	541.00±1.26	448.00±0.63	557.00±0.63	498.00±0.95
p-DAB+PB	4.80± 0.06	5.80±0.95	4.20±0.11	10.10±0.13	280.00±1.90	260.00±1.58	298.00±0.63	240.00±1.26
p-DAB+PB+Alcohol	3.70± 0.09	6.00±0.95	2.90±0.03	8.00±0.63	270.00±0.63	250.00±0.95	300.00±0.95	210.00±1.30
p-DAB+PB+ Lycopodium clavatum	9.00± 1.00***	9.00±1.26^{n}	9.80±0.05***	13.00±0.63***	390.00±0.95***	430.00±0.95***	480.00±1.26***	440.00±1.41***

***=*P*<0.001, n=non-significant.

Table 3— Mean blood glucose (mg/dl), blood hemoglobin (mg/dl), blood cholesterol (mg/dl) levels and estradiol (pg/ml), testosterone (ng/ml) and cortisol (ng/ml) contents at different fixation intervals.

[Values are Mean ± SE]

Group	Blood glucose (mg/dl)		Blood Hemoglobin (mg/dl)		Blood cholesterol (mg/dl)		Estradiol (pg/ml)		Testosterone (ng/ml)		Cortisol (ng/ml)	
	90 days	120 days	90 days	120 days	90 days	120 days	90 days	120 days	90 days	120 days	90 days	120 days
Normal	82.443 ±1.157	100.415 ±0.875	14.60 ±0.20	14.10 ±0.07	53.19 ±0.07	61.70 ±0.09	40 ±0.84	44 ±1.26	3.10 ±0.32	3.54 ±0.06	4.30 ±0.13	4.60 ±0.09
Normal+ Alcohol	84.105 ±0.947	98.746 ±0.478	14.50 ±0.20	14.40 ±0.20	54.05 ±0.081	62.01 ±0.010	38 ±0.63	45 ±0.32	3.40 ±0.63	3.65 ±0.13	4.40 ±0.09	4.50 ±0.13
p-DAB+PB	120.150 ±0.347	141.257 ±0.148	8.60 ±0.40	7.80 ±0.80	25.53 ±0.094	12.77 ±0.08	21 ±0.32	25 ±1.00	0.48 ±0.01	0.32 ±0.01	7.20 ±0.16	8.00 ±1.41
p-DAB+PB+ Alcohol	131.247 ±1.498	155.121 ±0.489	7.90 ±0.10	6.30 ±0.25	21.28 ±0.142	14.89 ±0.10	23 ±0.95	28 ±0.45	0.42 ±0.02	0.30 ±0.06	8.00 ±1.26	10.00 ±1.26
p-DAB+PB+ Lycopodium clavatum	92.108 ±0.474***	96.748 ±0.948***	11.26 ±0.30***	12.90 ±0.10***	46.81 ±0.412***	59.57 ±0.02***	39 ±0.95***	42 ±0.95***	1.08 ±0.01***	1.49 ±0.14***	6.80 ±0.19^{n}	6.20 ±0.02*

*=*P* values: <0.05, *** <0.001, n=non-significant

increased in Group-V mice, as compared to Group-IV mice. However, the cortisol level was found to be significantly decreased in the Group-V mice (Table 3).

Statistical analysis of data— The levels of significance between different series of data were analyzed by student′s t-test and two-way ANOVA (Mini Tab 13.31 Software). Analyzing the data by two-way ANOVA, significances at 5 % level were revealed in case of AcP activity in liver due to days and AlkP activity in spleen due to series and also at 1 % level in spleen for AcP and AlkP activity due to days. The activity of GR, SDH were also significant at 5 % level due to series in both liver and spleen tissues. Statistical analysis of blood glucose, hemoglobin, estradiol, testosterone and cortisol was found to be significant at 5 % level both due to days and due to series and that of cholesterol at 1 % level due to series.

Discussion

The chronic feeding of the carcinogens induced hepato-toxicity as revealed from the increased levels of AcP and AlkP. The cytosolic enzymes, acid and alkaline phosphatases (AcP and AlkP), are enzymes which catalyze the splitting off of phosphoric acid from certain monophosphoric esters, a reaction of considerable importance in several body processes, including neoplastic growth. Acid and alkaline

phosphatases have been particularly implicated to cellular damage and toxicity[27-29], 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 organ of the carcinogens, the gradual increase of acid and alkaline phosphatases could be implicated to progression of tumor growth. The administration of the spore extract substantially brought down the levels of the former biomarkers in mice chronically intoxicated with the carcinogens. This was also reflected in the reduced occurrence of liver tumor nodules in carcinogen fed mice which also received the plant extract.

L. clavatum manifested its effects on some mitochondrial enzymes, like glutathione reductase and succinate dehydrogenase. These are all recognized bio-markers of oxidative stress and indicators of faulty internal "respiratory" metabolism. Glutathione reductase is a ubiquitous enzyme known to be associated with the hexose monophosphate shunt of glucose metabolism[30,31].

SDH catalyses the oxidation of succinate to fumarate and transfers the resultant reducing equivalents, directly to the respiratory chain[32]. Thus, modulation in SDH by the extract speaks for its anti-stress and anti-oxidant effects during the process of carcinogenesis.

Interestingly enough, there was noticeable alteration in some gonadal hormones like testosterone in male and estradiol in female, two important hormones playing a great role in reproductive activities and yet again in the *L. clavatum* fed mice there was evidence of positive alteration in their hormonal levels.

While in the p-DAB+PB fed mice both Hb and cholesterol level decreased significantly there was an increase in serum estradiol and cortisol levels. However, there was a decrease in the level of serum testosterone presumably indicating a direct relationship with the increase in toxicity as a result of chronic feeding of p-DAB+PB. There may be a positive correlation between increased sperm head anomaly and alteration in testosterone level. In the *L. clavatum* fed mice, positive changes in the levels of these hormones were encountered. Interestingly in an analogous situation a decrease in testosterone level was observed in human male patient[33,36]. Therefore alteration in levels of sex hormones could also reflect toxicity states and could be used in predictive risk factor assessment as proposed earlier[33-35]. Further, the alteration in sex steroid levels may not always be suggestive of an actual damage of the sex organs but may actually owe their origin to the damage in liver[33]. Hormonal imbalance in testis, ovary and other reproductive organs due to carcinogen feeding had also been reported[36-38], but positive amelioration by *L. clavatum* appears to be the first report in mice model.

Blood cholesterol is known to be decreased in severe liver damage. In the present investigation the cholesterol content also decreased considerably in the carcinogen fed mice and there was considerable improvement in blood cholesterol level in the drug fed mice.

In the present study, the results of several widely accepted protocols would unequivocally suggest that there were positive modulations in all the parameters of study in the *L. clavatum* fed mice, which were not observed in the control group. Therefore, this study puts forward evidence that *L. clavatum* can combat and reduce damages produced by the chronic feeding of carcinogens in mice to a considerable extent, which also puts scientific credentials to validate its potentials for use against hepato-toxicity.

Acknowledgement

This work was financially supported by an Extra-Mural Research Grant of AYUSH, Ministry of Health and Family Welfare, Government of India, New Delhi.

References

1 International Agency for Research on Cancer (IARC), On the evaluation of carcinogenic risk of chemicals to man, Group 2B, supplement 7, (1987) 440.

2 Nesnow S, Argus M, Bergman H, Chu K, Frith C, Helmes T, Mcgaughy R, Ray V, Slaga T J, Tennant R & Weisburger E, Chemical carcinogenesis: A review and analysis of the literature of selected chemicals and the establishment of the gene-tox carcinogen database: A report of the US. Environmental Protection Agency gen-tox program, *Mutat Res,* 185 (1987)1.

3 Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K & Sasaki YF, DNA damage induced by red food dyes orally administered to pregnant and male mice, *Toxicol Sci,* 61 (2001) 92.

4 International Agency for Research on Cancer (IARC), Summaries and Evaluations, Phenobarbital and its sodium salt, Group 2B, 79 (2001)161.

5 Kitagawa T, Pitot H C, Miller E C & Miller J A, Promotion by dietary phenobarbital of hepatocarcinogenesis by 2-methyl-N, N-dimethyl-4-aminoazobenzene in the rat, *Cancer Res,* 39 (1979) 112.

6 Kitagawa T & Sugano H, Enhancement of azodye hepatocarcinogenesis with dietary phenobarbital in rats, *Gann,* 68 (1977) 255.

7 Murray G I, The role of cytochrome P450 in tumour development and progression and its potential in therapy, *J Pathol,* 192(4) (2000) 419.

8 Biswas S J, Pathak S & Khuda-Bukhsh A R, Assessment of the genotoxic and cytotoxic potential of an anti-epileptic drug, phenobarbital, in mice: A time course study, *Mutat Res,* 563 (2004) 1.

9 Pathak S & Khuda-Bukhsh A R, Assessment of hepatocellular damage and hematological alterations in mice chronically fed p-dimethyl aminoazobenzene and phenobarbital, *Exp Mol Pathol,* 83 (2007) 104.

10 Simpson C A, Complementary medicine in chronic pain treatment. *Phys Med Rehabil Clin N Am,* 17(2) (2006) 451.

11 Hana G, Bar-Sela G, Zhana D, Mashiach T & Robinson E, The use of complementary and alternative therapies by cancer patients in northern Israel, *Isr Med Assoc J,* 7 (2005) 243.

12 Boericke W, *Pocket manual of Homeopathic Materia Medica*. Indian 4th edition, (Sett Dey and Co, Calcutta, India) 1992, 409.

13 Gebhardt R, Antioxidative, antiproliferative and biochemical effects in HepG2 cells of a homeopathic remedy and its constituent plant tinctures tested separately or in combination, *Arzneimittelforschung,* 53(12) (2003) 823.

14 Es S, Kuttan G, Kc P & Kuttan R, Effect of homeopathic medicines on transplanted tumors in mice, *Asian Pac J Cancer Prev,* 8(3) (2007) 390.

15 Daoust R, The mitotic activity in rat liver during DAB carcinogenesis, *Cancer Res*, 22 (1962) 743.

16 Daoust R & Molnar F, Cellular population and mitotic activity in pre neoplastic liver parenchyma, *Proc Am Assoc Cancer Res,* 4 (1963) 13.

17 Daoust R & Cantero A, The numerical proportions of cell types in rat liver during carcinogenesis by 4-dimethylaminoazobenzene, *Cancer Res,* 19 (1959) 757.

18 Palekar S D & Sirsat S M, Studies on hepatocyte in azo dye carcinogenesis, *Indian J Exp Biol,* 4 (1966) 73.

19 Biswas S J & Khuda-Bukhsh A R, Cytotoxic and genotoxic effects of the azo-dye *p*-dimethylaminoazobenzene in mice: A time-course study, *Mutat Res,* 587 (2005) 1.

20 Pathak S, Bhattacharjee N, Das J K, Choudhury S C, Karmakar S R, Banerjee P, Paul S, Banerjee A & Khuda-Bukhsh A R, Supportive evidences for anti-cancerous potential of an alternative medicine in hepatocarcinogenesis of mice, *Forsch Komplementarmed,* 14 (2007)148.

21 Pathak S, Das J K, Biswas S J & Khuda-Bukhsh A R, Protective potentials of a potentized homeopathic drug, Lycopodium-30, in ameliorating azo dye induced hepatocarcinogenesis in mice, *Mol Cell Biochem,* 285 (2006) 121.

22 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with Folin-Phenol reagent, *J Biol Chem,* 193 (1951) 265.

23 Walter K & Schutt C, Acid and alkaline phosphatase in serum (Two point method) in *Methods in enzymatic analysis,* edited by H U Bergmeyer, (Academic Press, New York, USA) 1974, 856.

24 Beutler E, *Red cell metabolism – Manual of biochemical methods*. (Grune and Stratton, New York) 1975, 69.

25 Slater E C & Bonner W D, The effect of fluoride on succinic oxidase system, *Biochemical J,* 52 (1952) 185.

26 Plummer D T, *An introduction to practical biochemistry*. 3rd edition, (Tata McGraw-Hill Publication Company Ltd, New Delhi) 1988, 197.

27 Plaa G L, Amdun A M, Doull J & Klasser C D, *Toxic responses of the liver*. 4th edition, (Pergamon Press, Oxford) 1991, 55.

28 Timbrell J A, *Principles of biochemical toxicology*. (Taylor and Francis, London). 1991.

29 Vinitha R, Thangaraju M & Sachdanandam P, Effect of administering cyclophosphamide and vitamin E on the levels of tumor-marker enzymes in rats with experimentally induced fibrosarcoma, *Japan J Med Sci Biol*, 48 (1995)145.

30 Beutler E & Yeh M K Y, Erythrocyte glutathione reductase, *Blood,* 5 (1963) 573.

31 Warsy A S & El-Hazmi M A F, Glutathione reductase deficiency in Saudi Arabia, *Eastern Mediterranean Health J,* 6 (1999) 1208.

32 Hederstedt L & Rutberg L, Succinate dehydrogenase — A comparative review, *Microbiol Rev,* 45 (1981) 542.

33 Kuper H, Mantzoros C, Lagiou P, Tzonou A, Tamimi A, Mucci L, Benetou L, Spanos E, Stuver S & Trichopoulos D, Estrogens, Testosterone and Sex Hormone Binding Globulin in relation to liver cancer in men, *Oncology,* 60 (2001) 355.

34 Zhang K & Chow P K H, The effect of Megestrol Acetate on growth of HepG2 cells *in vitro* and *in vivo, Clin Cancer Res,* 10 (2004) 5226.

35 Cauley J A, Lucas F L, Kuller L H, Stone K, Browner W & Cummings S R, Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer: Study of Osteoporotic Fractures Research Group, *Ann Intern Med,* 130 (1999) 270.

36 Yamamoto R, Iishi H, Tatsuta M, Tsuji M & Terada N, Roles of ovaries and testes in hepatocellular tumorigenesis induced in mice by 3'-methyl-4-dimethylaminoazobenzene, *Int J Cancer,* 49(1) (1991) 83.

37 Yamamoto R, Iishi H, Tatsuta M, Tsuji M & Terada N, Suppressive effect of estrogen on hepatocellular tumorigenesis induced in mice by 3'-methyl-4-dimethylaminoazobenzene, *Exp Toxicol Pathol,* 45 (1993) 325.

38 Tsutsui S, Yamamoto R, Iishi H, Tatsuta M, Tsuji M & Terada N, Promoting effect of ovariectomy on hepatocellular tumorigenesis induced in mice by 3'-methyl-4-dimethylaminoazobenzene, Virchows *Arch B cell Pathol Incl Mol Pathol,* 62 (1992) 371.