International Journal of Drug Development & Research
| July-Sept 2010 | Vol. 2 | Issue 3 | ISSN 0975-9344 |
Available online http://www.ijddr.in
Covered in Official Product of Elsevier, The Netherlands

EFFECT OF *INDIGOFERA ASPALATHOIDES* LEAF EXTRACT ON HEPATIC XENOBIOTIC ENZYMES ACTIVITY IN HAMSTER WITH DMBA INDUCED ORAL CANCER

Philips Abraham, V Arul [a], Sachu Philip [b], B Padmakeerthiga [a], Nirmal R Madhavan [c] ,S Sethupathy [a]

a-Division of Biochemistry, Rajah Muthiah Medical college, Annamalai University,Chidambaram,Tamil Nadu, India
b- Department of biochemistry, Vinayaka Missions Medical college, Salem,Tamil Nadu,India
c-Department of Oral and Maxiliofacial Pathology, Raja Muthaiah Dental College and Hospital, Annamalai University, Tamil Nadu, India

Abstract
Xenobiotic metabolizing enzymes, which activate procarcinogen in to ultimate carcinogen are known to play a major role in the carcinogenic effect of Poly Aromatic Hydrocarbons .Influence on Hepatic Xenobiotic enzymes and Chemopreventive effect of ethanolic extract of Indigofera aspalathoides(EIA) was tested on DMBA induced hamster buccal pouch carcinoma model. It has been observed that hamster treated with EIA showed a significantly low level of hepatic phase I enzyme and high level of Phase II enzymes, which might be the reason for its chemopreventive effect
Key words : *Indigofera aspalathoides, Xenobiotic metabolizing enzymes ,DMBA*

INTRODUCTION

The use of Medicinal plants as anticancer agents has a long history that began with folk medicine and several drugs currently used in chemotherapy are isolated from plant species or it is derived from plant products(1,2,3). Recently considerable attention has been focused on identifying naturally occurring chemo preventive agents capable of inhibiting, retarding or reversing multistage carcinogenesis(4,5). The liver of tumor bearing animal has evolved as a reliable model for studying malignant transformation and interventions by chemopreventive agents(6).These compounds are known to intercept quantitative changes in hepatic enzymes and metabolites induced by the presence of an extrahepatic tumor(7). A balance between Phase I and Phase II Xenobiotic metabolizing enzyme is essential to reduce the risk of cancer due to carcinogens(8,9). Earlier studies have revealed that *Indigofera aspalathoides* (IA) extract from stem had anti tumour, antiviral and hepato protective effect (10,11,12).So we have designed the present study to explore the influence of ethanolic extract of IA leaves on hepatic xenobiotic enzymes in hamsters with DMBA induced buccal pouch carcinoma.

MATERIALS AND METHODS

Chemicals and reagents:7,12-Dimethyl benz[a]anthracene (DMBA) was purchased from Sigma Chemical Company, St. Louis MD. All the other chemicals used were of analytical grade.

Plant Material: *Indigofera aspalathoides* was collected form Tirunelveli District, Tamil Nadu, in the month of August and September. Leaves were shade dried and powdered. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later it was packed in a soxhlet apparatus and subjected to hot continuous percolation for 8 hrs using 95% Ethanol. The extract was concentrated and dried in a desiccator. (Yield :7.0%w/w)

Animals and treatment

All the experiments were carried out with male Syrian Golden hamsters (*Mesocricetus auratus*) aged 10 -12 weeks weighing between 80 – 100g. They were maintained under standard conditions of temperature and humidity with 12 hrs light- dark cycle and provided standard pellet diet and water ad libitum. They were maintained in accordance with the guidelines of the National Institute of Nutrition, ICMR, Hyderabad, India and experimental design was approved by the Animal Ethical Committee, Annamalai University. (Approval no.474-160/1999/CPCSEA) **Treatment Schedule:**

The animals were randomized into experimental and control group and divided into four group of six animal each. Hamsters in Group I served as untreated control.Right buccal pouch of hamster of Group II and III were painted three times per week with 0.5% solution of DMBA in liquid paraffin with a number 4 brush. Each application

leaves 0.4mg., In addition to DMBA treatment , animals in Group III received an intra gastric administration of 250 mg/kg body weight of ethanol extract from leaves of IA on the days alternate to DMBA application. Group 4 animals were treated with ethanolic leaf extract alone. The experiment was terminated at 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. Before each animal was killed, the right pouch was grossly inspected to evaluate premalignant lesion or tumor development.

Buccal pouch and liver tissues were subdivided and processed for various parameters . Tissue for histopathological examination were immediately fixed in 10% neutral buffered formalin , processed by means of routine histological technique and stained with hematoxylin and eosin. Tissue samples for biochemical analysis were weighed and homogenized according to method described by Ames *et al*(13). All the steps for the preparation of S9 fraction were carried out at 4 °C.

Assay of hepatic Xenobiotic metabolizing enzyme

Cytochrome b5 and cytochrome p450 contents were determined by the method of Omura and Sato(14).Cytochrome b5 reductase was assayed by Strittmater and Verlick(15). UDP Glucuronyl transferase (UGT) and cytosolic Glutothione – S – transferase (GST) activities were determined by using p-nitrophenol(16) and 1-chloro-2,4 dinitrobenzene as substrate(17). DT-Diaphorase (Quinone Reductase) was assayed by the method of Ernster(18).Protein concentration was determined by the method of Lowry et al(19)

Determination of hepatic lipid peroxidation and antioxidant level

Extent of lipid peroxidation was determined by measuring the level of Thiobarbuturic acid reactive substance (TBARS) in the liver homogenates(20). Antioxidant status was analysed by measuring the level of Reduced Glutathione(GSH) by the method of Anderson et al(21).

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis on the datas for biochemical assays were done with analysis of variance, and group means were compared by the least significant difference test. Results were considered statistically significant at $P < 0.0$

RESULTS

Table 1 summarizes the mean body weight tumor incidence and mean tumor burden in control and experimental animals. Topical application of

DMBA for 14 week significantly decreased the mean body weight in Group II. No significant weight decrease was observed in other groups

Table 1: Body weight, Tumor incidence, mean tumor burden

Group	Treatment	Initial weight	Final weight	Tumor incidence	Tumor burden
1	Control	95 ± 4.5	128 ± 4.1	0/6	-
2	DMBA	102 ± 6.1	89 ± 4.7	**6**/6	339.12
3	DMBA + EIA	105 ± 5.6	128 ± 5.9	0/6	-
4	EIA	9.9 ± 6.7	129 ± 3.5	0/6	-

* Values are mean _ standard deviation (*n* _ 6/group).
† Mean tumor burden was calculated by multiplying the mean tumor volume (4/3_r3) by the mean number of tumors (r _ half tumor diameter in millimeters).

The incidence of HBP tumor in Group II was 100%. All tumors were exophytic, with mean tumor burden of 339.12 mm^3, histologically identified as well differentiated squamous cell carcinoma. No tumors were observed in other group. Histopathological examination of pouch of Group III revealed varying degree of dysplasia and hyperplasia, without infiltration.

Table 2: Level of hepatic phase I xenobiotic enzymes

Group	Treatment	Cyt p450	Cyt b_5	Cyt b_5R
1	Control	1.22 ± 0.083	1.6 ± 0.049	0.128 ± 0.008
2	DMBA	10.36 ± 0.52♣	3.6 ± 1.8	0.312 ± 0.0016♣
3	DMBA + EIA	3.12 ± 1.54*	2.1 ± 0.083*	0.234 ± 0.033*
4	EIA	0.94 ± 0.26	1.9 ± 0.035	0.128 ± 0.022

Cytp450=Cytochrome P450 expressed as nmoles of per mg protein (molar extinction coefficient 91/mM/cm of absorbance at 450-490 nm.) Cyt b_5 - Cytochrome b_5 nmoles of cytochrome b_5/mg protein. (molar extinction coefficient of 185 mM/cm between 420-490 nm) . Cyt b_5R= Cytochrome b_5 reductase enzyme activity was expressed as µmoles of NADH utilized /minute /mg protein. Values are expressed as mean± SD for six animals in each group.♣ Significantly different from group 1 ($p < 0.05$). * Significantly different from group 2 ($p < 0.05$)

Table 3: Level of hepatic Phase II xenobiotic enzymes

Group	Treatment	GST	UGT	DT - Diaphorase
1	Control	0.2060 ± 0.00548	0.0640 ± 0.0114	0.112 ± 0.015
2	DMBA	0.1360 ± 0.0089♣	0.0120 ± 0.004♣	0. 11± 0.011
3	DMBA + EIA	0.1540 ± 0.010*	0.0420 ± 0.012*	0.25 ± 0.020*
4	EIA	0.220 ± 0.012	0.0660 ± 0.008	0.16 ± 0.013

GST =Glutathione S-transferase ,expressed as µmoles of CDNB (1-Chloro 2,4 dinitrobenzene); -GSH conjugate formed/min/mg protein),DT-diaphorase as µmoles of 2,6-dichlorophenolindophenol reduced/min/mg protein), UGT= UDP Glucuronyl transferase as µmoles of p nitro phenol disappeared after 20 minutes of incubation at 37^0 c/ mg protein). Values are expressed as mean± SD for six animals in each group.♣ Significantly different from group 1 ($p < 0.05$). * Significantly different from group 2 ($p < 0.05$).

Table 2 illustrates the effect of administration of EIA on the activity of phase I enzyme. Activity of Cyt p450, Cyt b_5 and Cyt b_5R are significantly elevated in the liver of DMBA painted animals (Group II). Co-administration of EIA significantly decrease the level of these enzymes in Group III animals.

Table 3 explains the effect of administration of EIA on liver Phase II enzymes. Activities of Phase II enzymes were significantly decreased in DMBA treated animals compared with control animals. Administration of EIA increase the Phase II enzyme activity in Group III-.

Table 4 explains the effect of administration of EIA on extend of lipid peroxidation and antioxidant status in liver. TBARS level was significantly increased and glutathione level was decreased in DMBA treated animals compared with control animals. Administration of EIA has significantly improved the antioxidant level and minimized the lipid peroxidation.

Table 4: Level of hepatic lipid peroxidation and antioxidant level

Group	Treatment	TBARS	G-SH
1	Control	23.1 ± 1.5	0.31 ± 0.06
2	DMBA	32.4 ± 2.3♣	0.18 ± 0.03♣
3	DMBA + EIA	28.6 ± 1.5*	0.27 ± 0.016*
4	EIA	22.8 ± 1.1	0.32 ± 0.08

TBARS = thiobarbituric acid reactive substances. Expressed in nmoles/mg protein .GSH = Reduced glutathione; expressed in mg/g tissue.
Values are expressed as mean± SD for six animals in each group.♣ Significantly different from group 1 ($p < 0.05$). * Significantly different from group 2 ($p < 0.05$).

DISCUSSION

In the present study, topical application of DMBA in the hamster buccal pouch has induced well differentiated squamous cell carcinoma. DMBA an indirect procarcinogen is bioactivated by the action of phase I enzymes like cytochrome p450 and produce ultimate electrophilic carcinogen DMBA- 3,4 dihydrodiol 1,2 epoxide. Although phase II enzymes detoxifies DMBA metabolites, some of the diol epoxide derivatives that escape the detoxification bind to adenine residues of DNA causing mutation and carcinogenesis(22). Polycyclic aromatic hydrocarbons such as benzopyrene and DMBA before its bioactivation, upregulate the transcription of Phase I and Phase II enzymes by binding to the xenobiotic

response element (XRE) of the promoter region of the gene(23).

The increase in hepatic Phase I enzyme in DMBA administered animal (Group II) reflects the up regulation of Phase I enzyme by DMBA in liver. Enhanced lipid peroxidation in liver of Group II animals reflects excessive generations of free radicals during carcinogen metabolism. The liver plays a major role in the interorgan homeostasis of GSH, the major cellular non protein thiol(24). GSH in conjugation with GST and other antioxidant enzymes play a crucial role in maintaining the integrity of liver, when challenged by toxic agents(6). Significantly lower level of GSH that is observed in Group II animals when compared to Group I is due to its excessive utilization.

According to Flora *et al* (25), along with Phase I enzymes, concomitant elevation in hepatic phase II enzymes is expected. But in contrast liver of tumor bearing animal showed a significant low level of hepatic phase II enzyme activity. Toxic free radicals that are produced during activation of carcinogen could have caused imbalance in redox status with adverse effect on SH group of functional protein such as Phase II enzyme .

In our study, it has been observed that simultaneous administration of Ethanolic extract of *Indigofera aspalathiodes* (EIA) and DMBA significantly inhibited elevation of hepatic Phase I enzymes in Group III animals. This might be due to 1. Inhibitory action of EIA on Phase I enzymes or blocking actions on bioactivation process. As a result of this , the formation of toxic free radicals was significantly lowered which was reflected by low level of lipid peroxidation and high level of GSH in this group. 2. Phytochemicals present in extract with antioxidant property also might have inhibited the lipid peroxidation by detoxifying the toxic radicals produced during carcinogen metabolism and have strengthened antioxidant defense capacity of the body. 3. Many chemopreventive polyphenolic compounds have been shown to modulate expression of Phase II enzymes through Nuclear factor erythroid 2 related factor (Nrf_2) - Antioxidant response element(ARE) signaling pathway(26).

Conclusion :

It is concluded that EIA could be an effective chemopreventive agent against carcinogenesis based on our observations of its influence on Xenobiotic enzymes in DMBA induced HBP carcinoma.

Reference:

1. *Wang, H.K. Plant-derived anticancer agents currently in clinical use or clinical trials. The Investigation Drugs Journal. 1998; 1: 92–102*

2. *Cragg GM, Boyd MR, Cardellina JH, Newman DJ, Snader KM, McCloud TG. Ethnobotany and drug discovery experienceof the US National Cancer Institute. In: Chadwick, D.J., Marsh, J.(Eds.), Ciba Foundation "Ethnobotany and the search for new drugs"Symposium, 185. Wiley and Sons, Chichester,1994, UK, pp. 178–196.*

3. *Cragg GM, Newman DJ.. Antineoplastic agents from natural sources: achievements and future directions. Expert Opinion on Investigational Drugs . 2000; 9: 1–15*

4. *Morse MA, Stoner GD . Cancer chemoprevention: principles and prospects. Carcinogenesis 1993;14: 1737–1746.*

5. *Kelloff GJ, Hawk ET, Crowell JA, Boone CW, Nayfield SG, Perloff M, Steele VE, Lubet , Sigman. Strategies for identification and clinical evaluation of promising chemopreventive agents. Oncology .1996;10: 1471– 1480.*

6. *Balasenthil S, Nagini. Garlic exerts hepatoprotective effects during 4-nitroquinoline 1-oxide induced oral carcinogenesis in rats.Asia pacific J Clin Nutr.2000;9(2):136-138*

7. *Johnson IT. Plant anticarcinogens. Eur J Cancer Prev1997;6:515-525.*

8. *Parke DV, The cytochromes P450 and mechanisms of chemical carcinogenesis, Environ. Health Perspect. 1994;102: 852–853.*

9. *Sreelekha TT, Ramadas k, PandeyM, Thomas ., Nalinakumari, Pillai. Genetic polymorphism of CYP1A1,GSTM1 and GSTT1 genes in Indian oral cancer, Oral Oncol. 2001;37: 593–598.*

10. *Rajkapoor B, Anandan R, Jayakar B Anti-inflammatoryactivity of Indigofera aspalathoides in rats. Biomedicine .2003;23:118–121*

11. *Rajkapoor B, Jayakar B, Murugesh . Antitumour activity of Indigofera aspalathoides on Ehrlich ascites carcinoma in mice. Indian J Pharmacol. 2004 ; 36:38–40*

12. *Rajkapoor B, Murugesh N, Chodon D, Sakthisekaran D. Chemoprevention of N- nitroso diethylamine induced phenobarbitol promoted liver tumors in rat by extract of Indigofera aspalathoides. Biol Pharm Bull. 2005;28:364–366*

13. *Ames B N, William E, Durston, Edith Yamasaki, Frank D. Lee .Carcinogens Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection.Proc Natl Acad Sci U S A. 1973 ; 70(8): 2281–2285*

14. *Omura T, Sato R. The carbon monoxide binding pigment of liver. J Biol Chem 1964;239:2370–8.*

15. *Strittmatter P, Rerlick SF. A microsomal cytochrome reductase specific for diphosphopyridine nucleotide. . J Biol Chem .1956;* ***221:****277– 86*

16. *Isselbacher K J, Chrabas MF, Quinn RC. The solubilization and partial purification of glucuronyl transferase from rabbit liver microsomes. J Biol Chem1962;237:3033 – 3036*

17. *Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-transferases, the first enzymatic step in mercapturic acid formation. J Biol Chem .1974;249:130 –9.*

18. *Ernster L. DT-diaphorase. Methods Enzymol .1967;10:309 –17*

19. *Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.*

20. *Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–358.*

21. *Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods in enzymol. 1985 : 113; 548– 51*

22. *Schimada T.,Xenobiotic metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbon. Drug. Metab. Pharmacokinet. 2006;21:257-276*

23. *Ciolino HP, MacDonald CJ, Yeh GC. Inhibition of carcinogen activating enzymes by 16-fluoro-5-androsten-17-one. Cancer Res 2002;62:3685–90.*

24. *Fernadez- checa JC, Takikawa H, Horie T,Ookhtens M, Kaplowitz N. Canalicular transport of reduced glutathione in normal and mutant Eisai hyperbilirubinemic rats .J Biol Chem. 1992;267:1667-70*

25. *Flora SD, Ramel C. Mechanisms of inhibitors of mutagenesis and carcinogenesis. Classification and overview. Mutat Res. 1988;202: 285– 306*

26. *Xiang- lin tan, Simon D spivack. Dietary chemoprevention strategies for induction of Phase II Xenobiotic metabolizing enzymes in lung carcinogenesis: A review. Lung cancer.2009:65:129 – 137*

Author Information: Philips Abraham is working Division of Biochemistry, Rajah Muthiah Medical college, Annamalai University,Chidambaram,Tamil Nadu, India

Article History:----------

Date of Submission: 23-03-2010

Date of Acceptance: 04-06-2010

Conflict of Interest: None

Source of Support: Nil