ABSTRACT

Indigofera Aspalathoides has been an important ingredient to prepare oils used for the skin diseases and studies with the stem extract demonstrates that it has antitumor, antiviral and antibacterial effect. In our study chemopreventive effect of ethanolic and chloroform extract of Indigofera aspalathoides(EIA) and (CIA) was tested on DMBA induced buccal pouch carcinoma in hamster. The biochemical analysis of Phase 1 xenobiotic enzymes and histopathological examination of oral tissues were done using gold standard methods. The activity of Phase 1 enzymes were significantly elevated in...
the buccal pouch of DMBA painted animals when compared to control animals. The expression of CYP1B1 was significantly elevated in the buccal pouch of DMBA painted animals when compared to control animals. Also the expression of AHR, ARNT and CYP1B1 in buccal pouch showed a significant elevation in the level of these protein in the DMBA alone treated group when compared to control animals.

This study shows that there is a lack induction of cytochrome P4501A (CYP1A) and other aryl hydrocarbon receptor (AHR)-dependent responses after exposure to AHR agonists, suggesting an overall down-regulation of the AHR signaling pathway.

Keywords: Oral cancer, Indigofera aspalathoides, chemopreventive, DMBA.

INTRODUCTION

Oral cancer, a malignant neoplasm of the mouth is major public health problem in the Indian subcontinent. In the world, annually more than 3,00,000 new cases are being diagnosed with Oral squamous cell carcinoma. Oral cancer is estimated by World Health Organisation to be the fifth most frequent cancer in occurrence and sixth most cancer worldwide. India has one of the highest incidences of Oral cancer in the world and constitutes 12% of all cancers in men and 8% of all cancers among women. It accounts for 30-40 percent of all malignancies in India. Every year about 85,000 new cases of oral cancer are reported to be diagnosed. The high incidence in India has been linked with the habit of betel quid chewing incorporating tobacco and tobacco smoking.

Despite therapeutic advances in the treatment of squamous cell carcinoma of Head and Neck (SCCHN), the overall survival from disease has remained unchanged over the last 20 years. For most countries age adjusted death rate from oral cancer have been estimated at 3-4 per 1,00,000 men and 1.5 – 2.0 per 1,00,000 for women. In India and other Asian countries, oral and oropharyngeal carcinoma comprises up to half of all malignancies.

Tobacco carcinogenicity is more evident and about one fourth of oral cancer cases are attributed to cigarette smoking. More than 60 carcinogens are present in the cigarette smoke and the most important are polycyclic aromatic hydrocarbons (PAH), aromatic amines and tobacco specific nitrosamines. The activity of carcinogen is generally exerted through DNA adducts. PAH are regarded as potentially genotoxic and carcinogenic to human. Research at molecular level has identified toxicity of PAH is mediated through transcription factors namely AHR and ARNT. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. Upon binding to a ligand, AhR translocates into the nucleus and dimerizes with the AhR nuclear translocator. This complex binds to the specific DNA region and thereby activates CYP1 gene family which now known to consist of 3 individual forms CYP1A1, CYP1A2 and CYP1B1. Studies have revealed that PAH as such are inert, procarcinogens but are oxidized to ultimate carcinogen (3,4 diol-1,2 epoxide form) by a set of phase I enzymes like CYP1B1 and microsomal Epoxide hydrolase (mEH). If these reactive molecules are not further metabolized by phase II conjugation, they may binds covalently to cellular macromolecules, including DNA causing errors in DNA replication that leads to mutation, damage to RNA and proteins.
As proposed by Wattenberg any agents or compounds that can inhibit the formation of carcinogen from its precursor or that can enhance the detoxification of carcinogens by modulating cellular metabolism can effectively inhibit the initiation stage of cancer and can be considered as effective candidate that can prevent cancer. Site of action of these agents can be considered as good chemopreventive target. *Indigofera Aspalathoides* commonly known as *Shivanar Vembu* in Tamil (Subfamily-Faboideae) is a low under shrub widely distributed in South India and Sri Lanka. It has been used as an important ingredient to prepare oils used for the skin diseases and syphilis. Studies with stem extract indicates that it has antitumor, antiviral and antibacterial effect.

This study has been designed to examine the inhibitory effect of plant extract on initiation step of carcinogenesis and also for investigating the efficacy of chemo preventive agents and its influence on AHR–ARNT signalling pathways specifically on Cytochrome p450 enzymes.

**Materials and methods**

**Experimental Animals**

All the experiments were carried out with male Syrian Golden hamsters (*Mesocricetus auratus*) aged 10 – 12 weeks weighing about 80–100g. They have morphological, histological and biochemical similarities to human oral squamous cell carcinoma and have easy accessibility for application of drug and follow up studies on lesion. Hamsters were maintained under standard conditions of temperature and humidity with 12 hours light- dark cycle and were provided standard pellet diet and water ad libitum, in accordance with the guidelines of the National Institute of Nutrition, ICMR, Hyderabad, India and experimental design was approved by the Institutional Animal Ethical Committee (IAEC), Annamalai University (Approval No. 474-160/ 1999/ CPCSEA).

**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.

**Preparation of Plant Extracts**

Leaves of *Indigofera Aspalathoides* were shade dried and powdered removal of chlorophyll and dewatering from powdered materials was done by treating with Petroleum ether (40-60°C) by hot continuous percolation method in Soxhlet apparatus for 24 hrs. It was suspended in 2% tween 20 for further studies. Then the marc was successively subjected to Chloroform (76-78°C) and ethanol extraction for 24 hrs each separately to obtain the chloroform fraction - 2.5% w/w and ethanol fraction – 5.0 % w/w fraction respectively. The extracts were concentrated and dried in desiccators.

**Experimental design.**

The animals were randomized into experimental and control groups and divided into six groups of six animals each.

Group I  Served as untreated control.
Group II  DMBA + Intra gastric administration of ethanolic fraction of IA (EIA)
Group III  DMBA + Intra gastric administration of chloroform fraction of IA (CIA)
Group IV  Intra gastric administration of ethanolic fraction of IA (EIA)
Group V  Intra gastric administration of chloroform fraction of IA (CIA)
Group VI 0.5% solution of DMBA alone.

The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. Buccal pouch, and Blood samples were collected for analysis. The buccal pouch tissues obtained were subdivided into four parts. The first portion of the tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction, the second part was processed using lysis buffer for Western blot analysis. Third portion was used for the preparation of S9 fraction which was used for biochemical analysis. The remaining tissues were fixed in 10% formalin, which were used for histopathological and immuno histochemical analysis.

S9 fraction was prepared according to Ames et al. Tissue samples were weighed, washed with 0.15 M KCl and homogenized in an all glass homogenizer with Teflon pestle using 50 mM Tris HCl buffer of pH 7.4 containing 0.15 M KCl and 2 mM EDTA. Homogenate was centrifuged for 10 minutes at 9000g. All the steps were done at 4°C. Supernatant collected as S9 fraction was used for the enzymatic studies. Cytochrome p450 contents and Cytochrome b5 were determined by the method of Omura and Sato et al. Cytochrome b5 reductase was assayed according to methods of Strittmater and Verlick et al.

**Reverse transcriptase-Polymerase Chain Reaction.**

With the isolated RNA, Reverse transcriptase polymerase chain reaction was performed using a commercially available Kit (Genei, Bangalore) to study the expression of AHR, ARNT, CYP1B1, Bax and Bcl-2. Beta Actin was used as the internal standard. Primer sequence and band length of the amplicon are given in the table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<th>Size</th>
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<tr>
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<td></td>
<td>R</td>
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<td>CYP1B1</td>
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<tr>
<td>AHR</td>
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<tr>
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<tr>
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<td>R</td>
<td>5'-CAGCAGTGACGAGGATGAA-3'</td>
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</table>

**Table I:** Primer sequence and band length of the amplicon

**STATISTICAL ANALYSIS**

The data are expressed as mean ± SD. Statistical analysis on the data for biochemical assays were done with analysis of variance, and group means were compared by the least significant difference test. Protein expressions determined by immunohistochemistry was analyzed by Chi square ($\chi^2$) test. Results were considered statistically significant at p < 0.001.
Results and Discussion.

Table I illustrates the effect of administration of fractions of *Indigofera aspalathiodes* (IA) on the activity of cyt p 450, b5 and b5R. The activity of Phase 1 enzymes were significantly elevated in the buccal pouch of DMBA painted animals (Group VI) when compared to control animals (Group I). Co administration of EIA and CIA significantly decreased the level of these enzymes in the animals of Group II and III.

Figure I shows the histopathological changes observed in the buccal mucosa of control and experimental animals in each group are shown in Figure 2. The Control Group, hamsters buccal mucosa showed normal epithelium where as well defined tumour mass was present in buccal pouch painted with DMBA alone. Also, histopathological examination also showed squamous epithelium with dysplastic changes and infiltration into deeper tissue and well differentiated squamous cell carcinoma.

Figure II shows the effect of administration of fractions of *Indigofera aspalathiodes* (IA) on the expression of CYP1B1. It got significantly elevated in the buccal pouch of DMBA painted animals (Group VI) when compared to control animals (Group I). Co administration of EIA and CIA inhibited the elevation in expression of CYP1B1.

Figure III, IV and V shows the expression of AHR, ARNT and CYP1B1 in buccal pouch of control and experimental animals. Percentage of expression of the proteins were calculated by keeping the expression in control group as 100%. A significant elevation in the level of these proteins was observed in the DMBA alone treated group, when compared to control animals. Administration of EIA or CIA along with DMBA had significantly inhibited the expression of these proteins when compared to group VI animals.

DISCUSSION

High incidence of oral cancer in India is attributed to the habit of consumption of tobacco in the form of cigarette smoking and betel quid chewing. One of the major carcinogens present in the tobacco is polycyclic aromatic hydrocarbon (PAH). 7,12-Dimethyl benzanthracene (DMBA) is a type of PAH produced during the incomplete combustion of carbon-containing compounds and is predominantly found in tobacco smoke and motor vehicle exhaust emissions. As it is a potent site specific carcinogen, it is extensively used as a prototype agent in mutation research and cancer research.

Experimental evidences reveal that highly reactive intermediates like dihydrodiol epoxides, radical cations and O-Quinone derivatives are formed in the mammalian tissues during the metabolic activation of DMBA. These electrophilic compounds form stable depurinating DNA adducts and also cause the excess generation of ROS. The formation of these DNA adducts and ROS that cause oxidative damage has been suggested as the ultimate step in the carcinogenic mechanism of DMBA.

In the present study, topical application of DMBA alone to the Hamster buccal pouch (HBP) of Group IV resulted in well differentiated SCC with very high tumor burden. Tumor incidence was found to be 100% in this group. Simultaneous administration of EIA or CIA with DMBA had totally inhibited the occurrence of SCC in Group II and III animals respectively. This reveals that both fractions have remarkable chemopreventive activity against DMBA induced carcinogenesis.
In the study, a marked elevation in the level of buccal pouch phase I detoxification enzymes was observed in DMBA alone treated (Group VI) animals when compared to control. It is well documented that Cytochrome p450 enzymes are substrate inducible and induction occurs at the level of transcription. On cellular entry, PAH activates the cytosolic protein Aryl Hydrocarbon Receptor (AHR). Activated AHR then translocates to the nucleus and binds to Aryl hydrocarbon receptor nuclear translocator (ARNT). This AHR-ARNT complex in turn binds to Xenobiotic response element (XRE) and upregulates a battery of AHR regulated genes which encode both phase I and Phase II xenobiotic metabolizing enzymes. This might have accounted for the elevation of buccal pouch phase I xenobiotic enzyme levels in the Group VI animals. But the administration of EIA or CIA along with DMBA (Group II and III animals) had restricted this elevation and activity of these enzymes were found to be significantly lower when compared to Group VI animals. This might be either due to inhibitory action of phytochemicals on Phase I enzymes or its blocking action on the bioactivation process.

It has been reported that Curcumin an anticancer agent, reduce the rate of metabolic bioactivation of PAH by specifically inhibiting Cytochrome p450 monooxygenases. On the other hand Flavanoids like Resveratrol reduce the level of Phase I enzymes by acting as an AHR antagonist. It was observed that CYP1B1 level in DMBA alone treated animals were significantly higher than in the control group, which was consistent with earlier reports. It has also been reported that level of CYP1B1 is positively correlated to the expression level of AHR and ARNT, which in turn depends on the dosage and duration of Ligand Exposure. Earlier studies have identified a higher level of expression of AHR and ARNT in tumor tissue and also in tissues that are exposed to ligands. Our study also showed a significantly higher level of AHR and ARNT expression in group VI animals, when compared to control. Thus, these transcription factors by binding to XRE might have caused the induction in the expression of CYP1B1 in the buccal pouch of group VI animals. Expression of CYP1B1 in buccal pouch of group II and III animals in which fractions of IA leaf extract was administered along with DMBA was not elevated to the extent observed in Group VI and was almost nearer to that of control values. This could be due to inhibitory action of the extract at the level of transcription or translation of enzyme.

The Reverse Transcriptase PCR analysis had shown that AHR and ARNT levels were not elevated in these groups (II and III) of animals. Reinhard Wanner et al. had demonstrated that administration of retinoic acid reduced the expression of both AHR and ARNT in proliferative keratinocytes. Several in vivo studies reported that the treatment with AHR ligand in mice and rat led to the elevation of AHR level which suggest that its expression is regulated by its own ligand, at least at the pretranslational level.

Recently it has been documented that sulforaphane inhibited the inducing action of ligand and prevented the elevation in expression of AHR. It was noted in the study that the level of Phase I enzymes and CYP1B1 are not significantly decreased in EIA or CIA alone treated groups which shows that they had no influence on activity and expression of enzymes and receptors in the normal condition. The present study demonstrates that the phytochemicals present in the extract might have influenced the ligand mediated action on
expression of AHR and ARNT and this might have affected the transcription of Cytochrome P450 monoxygenases.

**CONCLUSION**
Oral cancer can be preventable by the intervention of risk factors. Its early detection can minimize its detrimental effects and can improve the quality of life of the patient. The present study gives an important insight into the fact that there is a lack induction of cytochrome P4501A (CYP1A) and other aryl hydrocarbon receptor (AHR)-dependent responses after exposure to AHR agonists, suggesting an overall down-regulation of the AHR signaling pathway.

**REFERENCES**


<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Cyp450</th>
<th>Cypb5</th>
<th>Cypb5R</th>
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<td>I</td>
<td>Control</td>
<td>0.12 ± 0.016a</td>
<td>0.31 ± 0.033a</td>
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<td>II</td>
<td>EIA</td>
<td>0.115 ± 0.0107a</td>
<td>0.27 ± 0.022a</td>
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<tr>
<td>III</td>
<td>CIA</td>
<td>0.117 ± 0.010a</td>
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<td>IV</td>
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<td>0.17 ± 0.010b</td>
<td>0.59 ± 0.04 b</td>
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<tr>
<td>V</td>
<td>DMBA + CIA</td>
<td>0.208 ± 0.008c</td>
<td>0.62 ± 0.056b</td>
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<td>VI</td>
<td>DMBA</td>
<td>0.27 ± 0.02d</td>
<td>1.08 ± 0.099c</td>
<td>2.3 ± 0.21c</td>
</tr>
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</table>

Cytochrome P450 and Cytochrome b5 expressed as (μ)micromoles of per mg protein. Cytochrome b5 reductase enzyme activity was expressed as μmoles of NADH utilized /minute /mg protein.

Values are expressed as means ± S.D. of each group. *Value differ significantly from group I (at P < 0.05)

Figure I
Figure II. Expression of CYP1B1

Figure III. Analysis on Expression of AHR by IHC

Figure IV. Expression of ARNT
Figure V. Expression of CYP1B1