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THE EFFECT OF FRACTIONS OF *INDIGOFERA ASPALATHOIDES* LEAF EXTRACT ON EXTEND OF LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN DMBA INDUCED HAMSTER BUCCAL POUCH CARCINOMA.

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ABSTRACT: Oral cancer is the sixth leading cancer globally and the leading carcinoma in the Indian subcontinent. The relatively high incidence is due to usage of tobacco, and exposure to carcinogens are frontrunners of oxidative stress-induced DNA damage. The practice of chemopreventive agents is considered to be alternative oral carcinoma. A study on novel natural chemo preventive agent-*Indigofera Aspalathoides* was undertaken to evaluate its preventive chemo effect. Male Syrian Golden hamsters aged 10-12 weeks and weighing about 80-100 g were selected. Group 1were control; Group II hamsters were given Intragastric administration of ethanolic fraction of IA; Groups III had Intragastric administration of chloroform fraction of IA, Group IV had Intragastric administration of E IA + DMBA ,Group V had DMBA + Intragastric administration of CIA, and Group VI with DMBA alone. Total antioxidant power and extend of lipid peroxidation was measured by gold standard methods. Our study suggests that both EIA and CIA had a significant anticancer and preventive chemo effect against squamous cell carcinoma. Elevation in the extent of lipid peroxidation and the decrease in the levels of antioxidants were observed in the plasma of DMBA alone treated animals. This study suggests that elevation in the extent of lipid peroxidation and the decrease in the levels of antioxidants were observed in the plasma of DMBA alone treated animals.

INTRODUCTION: Oral cancer refers to a subgroup of head and neck malignancies that develop at the lips, tongue, salivary gland, gingiva, floor of the mouth, oropharynx, buccal surfaces and other intra-oral locations [1]. Oral cancer is estimated by World Health Organisation to be the sixth most cancer worldwide. In India and other Asian countries, oral and oropharyngeal carcinoma comprises up to half of all malignancies. The predictability of oral cancer in all ages has been projected to increase by 2030.

Challenges focus on the most critical initiator and a risk factor for the development of oral cancer alcohol consumption in males and tobacco chewing. Although drinking and smoking are

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independent risk factors, they have a synergistic effect and greatly increase risk together [3]. The prime major carcinogens present in tobacco are polycyclic aromatic hydrocarbons (PAH), aromatic amines and tobacco-specific nitrosamines, and their activity of carcinogen are exerted through DNA adducts [4]. The prime event involved in mutagenesis and carcinogenesis is found to be oxidative stress-induced DNA damage. These DNA damages can result in arrest or induction of transcription, modulation of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis. While major attention has been focused on oxidative stress-induced direct DNA damage, it is also noted that reactive radical species may also cause damage to other cellular components.

Cell membrane phospholipids, which contain poly unsaturated fatty acids, are very sensitive for oxidation and have been found to be frequent target of radical-induced damage and thus it participates in free radical chain reaction [5]. Malondialdehyde (MDA) is one of the major product of lipid per oxidation. Since MDA can react with DNA bases to form DNA adducts, it is highly mutagenic and carcinogenic. It is also used as a marker for extent of lipid peroxidation. Many of the biological effects of antioxidant appear to be related to their ability to scavenge deleterious free radicals and modulate cell signaling pathways [6]. In spite of enormous efforts to search for a cure, cancer still remains a formidable challenge for public health.

There are compromised patients' quality of life after treatment, an increasing trend of chemoresistance, and reoccurrence of secondary tumors. To overcome these, the practice of chemoprevention is considered to be an alternative, more realistic, and fundamental strategy for the management of this dreadful disease [7]. Besides antioxidant vitamins, numerous non-nutritive substances present in the plant-based diet collectively termed phytochemicals have been identified as chemopreventive agents. The antitumor, antiviral and antibacterial property of stem extracts of *Indigofera aspalathoides* (Shivanar Vembu in Tamil) has been used in ayurvedic treatment for many years [8]. Since chemical agents like PAH appear to be the dominant etiological factor in oral cancer, and use of carcinogen-induced animal tumor model is required to assess the efficiency of new therapeutic approaches. An experimental cancer model using hamsters buccal pouch (HBP) and topical application of a chemical carcinogen-7, 12-dimethylbenzanthracene [DMBA], a member of PAH is an optimal model for the study of oral cancer [9]. Hence this study was designed to assess the effect of fractions of *Indigofera aspalathoides* leaf extract on lipid peroxidation and enzymatic antioxidant defense of Superoxide dismutase (SOD), Glutathione peroxidase (GPx), and Catalases in DMBA induced hamster buccal pouch carcinoma.

MATERIALS AND METHODS: All the experiments were carried out with male Syrian Golden hamsters (Mesocricetusauratus) aged 10 – 12 weeks, weighing about 80-100g. They were maintained and fed with a standard pellet diet and water ad libitum, according to the guidelines of the National Institute of Nutrition, ICMR, Hyderabad, India. The study was approved by the Institutional Animal Ethical Committee (IAEC), Annamalai University (Approval No. 474-160/ 1999/ CPCSEA). The study was carried out in 2013 at the Division of Biochemistry, Rajah Muthaiah Medical college.

Preparation of Plant Extracts: *Indigofera aspalathoide* were collected, and the leaves were shade dried and powdered. Removal of chlorophyll and dewaxing from powdered materials was done by treating with Petroleum ether (40-600 °C) by hot continuous percolation method in Soxhlet apparatus 9 for 24 h. Then the ethanol fraction of 5.0% w/w and chlorophyll extract of 2.5% w/w were prepared.

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

Group I	Control
Group II	Intragastric administration of ethanolic fraction of IA (EIA)
Group III	Intragastric administration of chloroform fraction of IA (CIA)
Group IV	DMBA + Intragastric administration of EIA
Group V	DMBA +Intra gastric administration of CIA
Grou VI	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. S9 fraction was prepared according to the method by Ames *et al.* [10].

Total Antioxidant Power was determined by FRAP assay [11], reduced glutathione SOD, Catalase, and Glutathione Peroxidase measured by Anderson *et al.* [12]. The extent of lipid peroxidation was determined by measuring Thiobarbituric acid reactive substance (TBARS) in the buccal pouch homogenates [13].

Statistical Analysis: Data are expressed as mean ± standard deviation. Statistical analysis on the datas for biochemical assays was done with the analysis of variance, and group means were compared by the least significant difference test. Results were considered statistically significant at P <0.05.

RESULT AND DISCUSSION: Table 1. shows the extent of lipid peroxidation and the status of antioxidants in Buccal pouch of different groups of animals. Lipid peroxidation was significantly decreased in tumor tissue compared with buccal mucosa of control animals and treated groups. GSH and FRAP level was found to be significantly high in DMBA alone treated group (Group VI) when compared to normal. Simultaneous administration of EIA and CIA has resulted in significant elevation of GSH level and FRAP value when compared to normal, and DMBA alone treated group.

TABLE 1: THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS), REDUCED GLUTATHIONE (GSH), AND FERRIC REDUCING ANTIOXIDANT POWER (FRAP) IN ORAL POUCH MUCOSA OF CONTROL AND EXPERIMENTAL ANIMALS

Group	Treatment	TBARS	GSH	FRAP
I	Control	85.32 ± 2.92^{a}	0.13 ± 0.023^{a}	263 ± 10.15^{a}
II	EIA	82.6 ± 1.21^{b}	0.19 ± 0.013^{b}	281 ± 10.3^{b}
III	CIA	80.6 ± 0.91^{b}	0.20 ± 0.023^{b}	289 ± 8.87^{b}
IV	DMBA+ EIA	76.33 ± 2.76^{c}	0.39 ± 0.015^{c}	324 ± 11.5^{c}
V	DMBA+CIA	77.9 ± 1.66^{c}	0.38 ± 0.017^{c}	336 ± 9.45^{d}
VI	DMBA	63.47 ± 3.56^{f}	0.35 ± 0.011^{d}	$314 \pm 8.4^{c,e}$

Data are presented as the means ± SD of six animals in each group. Statistical analysis was done using ANOVA followed by DMRT. Values that do not share a common superscript in the same column differ significantly at P<0.05. TBARS expressed as - nM/100 mg protein, GSH as mg/100 g tissue, and FRAP as µM/100 µg of tissue protein

TABLE 2: ACTIVITY OF SUPEROXIDE DISMUTASE (SOD), CATALASE AND GLUTATHIONE PEROXIDASE (GPX) IN BUCCAL POUCH MUCOSA OF CONTROL AND EXPERIMENTAL ANIMALS

Group	SOD	GSH	FRAP
I	4.53 ± 0.09^{a}	32.80 ± 1.3^{a}	6.80 ± 0.52^{a}
II	4.77 ± 0.12^{b}	36.23 ± 0.4^{b}	8.70 ± 0.54^{b}
III	4.76 ± 0.076^{b}	36.11 ± 1.2^{b}	9.40 ± 0.61^{c}
IV	4.20 ± 0.09c	28.63 ± 1.77^{c}	13.75 ± 0.73^{d}
V	4.22 ± 0.098^{c}	29.11 ± 1.23^{c}	14.55 ± 0.52^{e}
VI	2.88 ± 0.14^{e}	21.11 ± 2.32^{e}	12.2 ± 0.80^{h}

TABLE 3: TBARS, GSH LEVEL AND TOTAL ANTIOXIDANT POWER (FRAP) IN PLASMA OF CONTROL AND EXPERIMENTAL ANIMALS

Group	Treatment	TBARS	GSH	FRAP
I	Control	2.85 ± 0.21^{a}	36.4 ± 1.1^{a}	203.65 ± 3.82^{a}
II	EIA	2.2 ± 0.1^{b}	41.2 ± 3.1^{b}	213.66 ± 2.71^{b}
III	CIA	44.3 ± 2.1^{b}	1.95 ± 0.11^{b}	217.66 ± 2.66^{b}
IV	DMBA+ EIA	3.3 ± 0.22^{c}	29.1 ± 1.2^{c}	183.83 ± 3.38^{c}
V	DMBA+CIA	3.0 ± 0.1^{c}	33.7 ± 1.8^{c}	190.00 ± 1.21^{c}
VI	DMBA	4.54 ± 0.35^{d}	22.34 ± 3.1^{d}	168.83 ± 3.25^{d}

Data are presented as the means ± SD of six animals in each group. Statistical analysis was done using ANOVA followed by DMRT. Values that do not share a common superscript in the same column differ significantly at P<0.05. SOD expressed as the amount of enzyme required to give 50%

inhibition of NBT reduction/mg protein, Catalase as moles of H_2O_2 utilized/sec/mg protein, and GPx as moles of GSH utilised/min/g protein. **Table 3.** shows the concentrations of TBARS and the status of antioxidants in the plasma of control and treated animals. A marked increase in lipid peroxidation with antioxidant status depletion was observed in tumor-bearing animals. The administration of fractions of IA leaf extract has considerably elevated the antioxidant status. Data are presented as the means ± SD of six animals in each group. Statistical analysis was done using ANOVA followed by DMRT. Values that do not share a common superscript in the same column differ significantly at $P<0.05$.

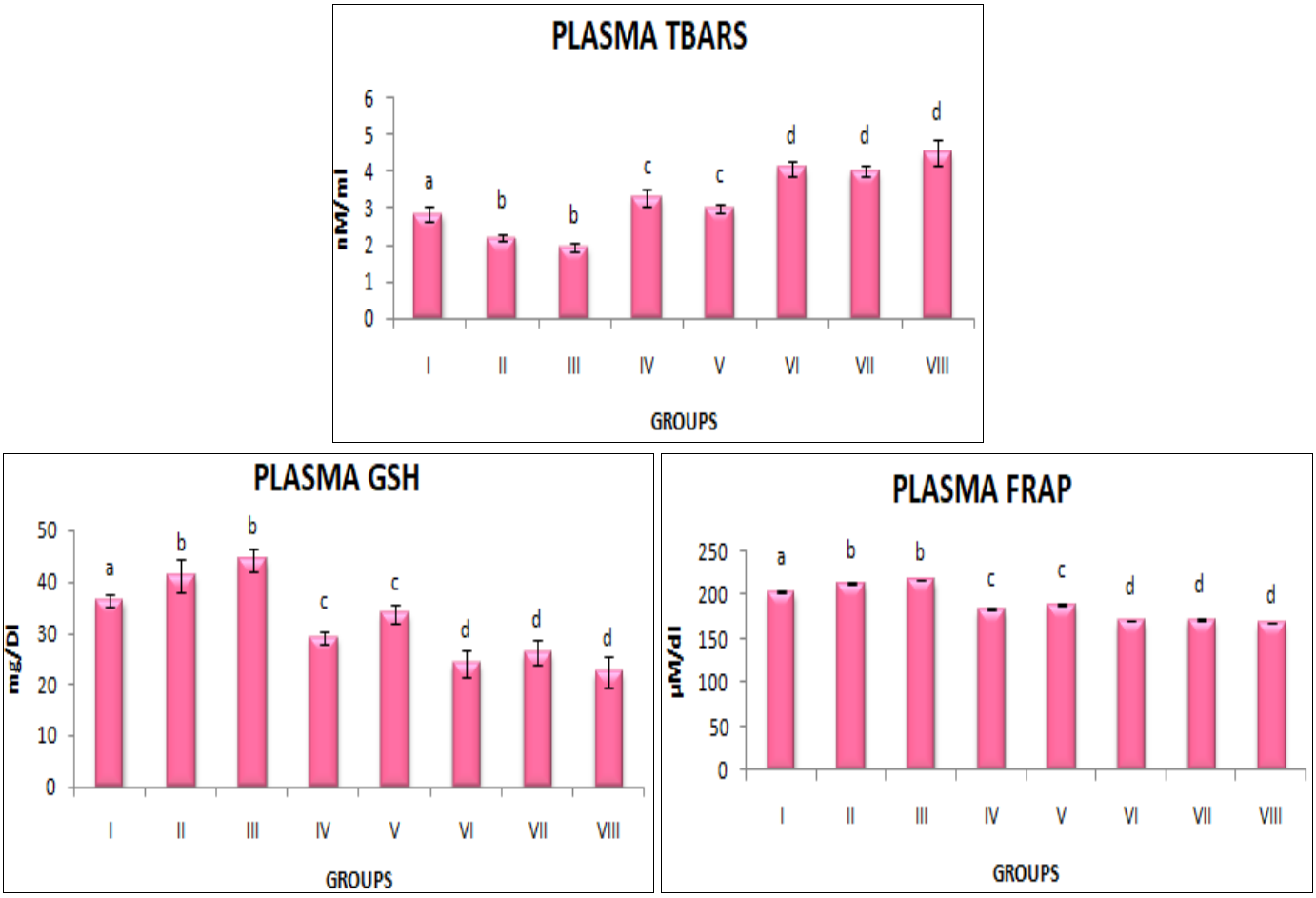

FIG. 1: TBARS, GSH LEVEL, AND TOTAL ANTIOXIDANT POWER (FRAP) IN PLASMA OF CONTROL AND EXPERIMENTAL ANIMALS

DISCUSSION: Oxidative stress arising due to the overproduction of reactive oxygen species (ROS) coupled with a deficiency of antioxidant defense mechanisms has been implicated in the pathogenesis of cancer. Therefore, scavengers of ROS have evolved as effective chemopreventive agents [14]. Many functional foods and diet-derived agents rich in radical scavenging antioxidants are known to prevent the deleterious effects of free radicals; thus, they have assumed importance in cancer chemoprevention in recent years [15]. In tumor-bearing animals (Group VI) high plasma TBARS levels and reduced antioxidant levels were observed. The enhanced lipid peroxidation in circulation reflects excessive free radical generation due to the continuous exposure to carcinogens [16]. The observed decrease in the circulatory total antioxidant power and Glutathione level in DMBA treated animals could be due to increased scavenging of lipid peroxides and sequestration of antioxidants by the tumor cell. In hepatic tissue of tumor-bearing animals, a high TBARS and low antioxidant level were observed [17]. This might be due to the accumulation of toxic radicals produced during the metabolic activation of DMBA by Cytochrome P450 in hepatic microsome [18]. Administration of EIA or CIA along with DMBA (Group IV and Group V) had

improved the total antioxidant power, GSH level and lowered TBARS level in plasma. The free radical scavenging property and antioxidant enhancing capacity of the extract would have played a role in its chemopreventive action, as reported earlier using various phytochemicals [19, 20]. To explore this free radical scavenging property of fractions of IA leaf extract, four different *in-vitro* models were used.

- ✓ DPPH -a lipophilic radical scavenging assay
- ✓ ABTS radical cationdecolorisation assay
- ✓ Hydroxy radical scavenging assay
- ✓ Nitric oxide radical scavenging assay

Analysis using these four models showed that both fractions had an effective free radical scavenging property in a concentration-dependent manner. Phytochemical studies showed the presence of polyphenolic compounds, especially flavonoids, in both CIA and EIA. It is an established fact that all polyphenolic compounds, especially flavonoids, have antioxidant properties [21]. Rice Evan *et al.* had reported that antioxidant property of phenolic compounds depends on major structural features like a number of a hydroxyl group attached to the ring structure, relative position of hydroxyl group, and the presence of unsaturation in the ring, which allows electron delocalization across the molecule for the stabilization of the radical. So phytochemicals present in the extract with antioxidant properties might have been involved in removing toxic radicals produced during carcinogen metabolism [22]. This free radical scavenging property would have inhibited ROS-mediated molecular events in carcinogenesis and have played a role in the chemopreventive effect.

In tumor tissues of the DMBA alone treated animals, a significantly low level of TBARS and a high antioxidant status as reflected by high FRAP value, high glutathione content, increased glutathione peroxidase (GPx) activity was observed when compared to normal. Such observation has also been reported earlier in different tumor tissue such as colon cancer; oral cancer, and breast cancer [23]. It has been reported that an inverse relationship exists between the level of Lipid peroxidation and the rate of cell proliferation, as it prolongs G1 phase of the cell cycle. Aerobic organisms generate free radicals during the production of ATP (Oxidative phosphorylation) in the mitochondria. During Oxidative phosphorylation, due to the leakage of electrons from mitochondria, reactive O_2 species, superoxide anion, and Hydroxyl radical are generated [24]. These ROS can cause lipid peroxidation. Dang and Semenza hypothesized that cell growth modification associated with an increased expression of genes involved in the anaerobic glycolytic pathway is a prerequisite for tumorigenesis [25]. This adaptation is necessary for the survival of solid tumors, as hypoxia exists due to the absence of vascularization.

On the other hand, a low oxidative metabolism can be considered as the physiological status of fast-growing cells. This is clear from the fact that Lymphocytes, enterocytes, and fetal tissues are poorly oxidative, whereas highly oxidative tissues such as the kidney cortex or brain are normally quiescent [26]. Studies have revealed that there exists a positive correlation between the decrease in mitochondrial activity and tumor aggressiveness. Thus the property of low oxidative metabolism exhibited by the cancer tissue might have resulted in the low level of free radical production and lipid peroxidation. Previous studies have shown a significantly low level of total Phospholipid (PL) and Polyunsaturated fatty acid (PUFA) in the cell membrane of in tumor tissues, and it has been reported that in hepatoma, the growth rate of tumor cells was increased with a decrease in the PL content and degree of fatty acid unsaturation [27]. Thus the decline in the lipid peroxidation in the tumor tissue might also be attributed to the low substrate availability.

Tumor cells have been reported to sequester essential antioxidants such as GSH, lipophilic antioxidants from the circulation to meet the demand of growing tumor [28]. This might be the reason behind the observed high level of antioxidant status, GSH, and GPx in the tumor tissue of DMBA alone treated animals (group VI). This increase in antioxidant level might also be attributed to the low level of lipid peroxidation in the tissue. Thus an increased antioxidant level and decreased lipid peroxidation as reported in several malignancies facilitate cell proliferation and confer a selective growth advantage for the cancer cell.

This supports the previous observation that tumor cells are more resistant to the action of free radicals than the normal [25, 29]. Superoxide dismutase (SOD) and Catalase are two important enzymatic antioxidants that act against toxic oxygen-free radicals such as superoxide and hydroxyl radicals in the biological systems. Several reports have cited decreased activities of SOD and Catalase in various carcinogenic conditions [30].

It has been reported that the number of mitochondria was less in tumor than in a normal cell. Since the mitochondrial fraction of SOD forms the major part of total SOD, a decrease in the SOD activity could be attributed to the reduction in the number of mitochondria. This could be the reason for an observed low level of SOD in tumor tissue of DMBA alone treated animals. Hypoxia that occurs due to the insufficiency in vascularization might be another factor that had resulted in the decrease of SOD activity [31].

Sato *et al.* had identified that a decrease in the level of Catalase activity in tumor cells is due to the depression in the Catalase gene expression. Recently it has been demonstrated that this epigenetic silencing found in tumor cells is due to oxidative stress-mediated hypermethylation at the promoter region of the Catalase gene. So the observed decrease in the activity of Catalase could be due to the oxidative insult that occurred as a result of metabolic activation of PAH [31].

CONCLUSION: The present study was carried out to analyze the lipid peroxidation and antioxidant status of *Indigofera aspalathoides* .Based on the evaluation of biochemical and anti-per oxidative properties, both EIA and CIA had a significant anticancer and chemopreventive effect against Oral squamous cell carcinoma. Elevation in the extent of lipid peroxidation and the decrease in the levels of antioxidants were observed in the plasma of DMBA alone treated animals. The above status was significantly reversed in animals co-treated with CIA or EIA, which is suggestive of the antioxidant potential of the extracts. This could be due to the high polyphenol content revealed by our preliminary phytochemical studies.

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