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Effect of *Anisomeles malabarica* (L.) R.Br. Methanolic extract on DMBA - induced HBP Carcinogenesis

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Abstract

In the present investigation, the effect of *Anisomeles malabarica* (L.) R.Br. whole plants extract has been studied on cellular redox status during hamster buccal pouch carcinogenesis. The animals were randomized into experimental and control groups and divided into 8 groups of six animals each. In group 1, the right buccal pouches of hamsters were painted three times per week with a 0.5 percent solution of DMBA in liquid paraffin . Hamsters in groups 2 - 4 painted with DMBA as in group 1, received in addition, intragastric administration of *Anisomeles malabarica* methanolic extract of concentration 125, 250 and 500 mg/kg body weight respectively three times a week on days alternate to DMBA. Animals in groups 5 through 7 were administered *Anisomeles malabarica* metabolic extract alone (125, 250 and 500 mg/kg body weight respectively). Group 8 animals received the same volume of water and served as controls. Administration of AMME to DMBA - painted hamsters reduced the incidence of SCC and mean tumour burden in addition to preneoplastic lesions. In the buccal pouch, AMME reversed the susceptibility to lipid peroxidation while simultaneously increasing GSH-dependent antioxidant enzyme activities, whereas in the liver and erythrocytes, the extent of lipid peroxidation was reduced with elevation of antioxidants. Thus, modified oxidant status together with antioxidant adequacy in the target organ as well as in the liver and erythrocytes induced by AMME may significantly reduce cell proliferation and block tumour development in the HBP. The results of the present study are consistent with the free radical scavenging properties of AMME reported in literature. AMME has been shown to prevent the increase in lipid peroxidation and protect against oxidative DNA damage by improving antioxidant defences. Among the doses used in the present study, the medium dose and higher dose of AMME (250 mg/kg bw and 500 mg/kg bw) were found to be more effective in inhibiting HBP carcinogenesis compared to low dose. The protective effects of AMME against HBP carcinogenesis observed in the present study may be related to the antioxidant and antiproliferative properties of phytochemicals such as flavonoids present in the plant.

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Key words:
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Introduction

Several medicinal plants and their constituents have been reported to prevent multi-stage carcinogenesis [1]. Numerous medicinal plants and their formulations are used for cancer in ethno medical practices as well as traditional system of medicine in India [2].Many plant- based chemopreventive agents are recognized to exert their anticarcinogenic effects

by scavenging ROS and upregulating host antioxidant defense systems [3].

Chemoprevention has evolved as a promising approach to control the incidence of oral squamous cell carcinoma (OSCC). Development of effective chemopreventive agents, however, requires conclusive evidence in a well-established experimental tumor model that closely emulates human OSCC. The hamster buccal pouch (HBP) carcinogenesis model is the most well characterized animal system for the investigation of oral cancer development and intervention by chemopreventive agents. The buccal pouch of the Syrian hamster serves as an excellent target organ for chemointervention because of easy accessibility for examination and follow-up of lesions. Squamous cell carcinoma (SCC) induced by 7, 12-dimethyl benz [a] anthracene (DMBA) in the HBP reiterates many of the features observed in human OSCC. Topical application of DMBA to the HBP induces SCC preceded by hyperplasia, papilloma and dysplasia similar to human OSCC. In addition, it expresses many biochemical and molecular markers that are expressed in humans [4]. Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally occurring constituents of the diet [5].

The current focus of chemoprevention is on intermediate biomarkers capable of detecting early changes that can be correlated with inhibition of carcinogenic progression. Evaluation of intermediate biomarkers reduce the time and cost required to establish the dose-response for effective chemopreventive agents. In particular, the assay of biomarkers in the liver and erythrocytes in addition to the target organ has emerged as a reliable methods for screening putative chemopreventive agents [6].Oxidative stress arising due to overproduction of ROS with depletion in antioxidant defense mechanisms has gained acceptance as a reliable and early index of carcinogen-induced damage and chemoprevention [7].

The antioxidant and antiproliferative effect of *Anisomeles malabarica* have created interest in this plants with potential anticancer properties. The results of a study designed to evaluate the effect of *Anisomeles malabarica* methanolic extract at three different dose levels on DMBA- induced HBP carcinogenesis. The extent of lipid peroxidation and the status of GSH and GSH-dependent enzymes in the buccal pouch, liver and erythrocytes were used as biomarkers of chemoprevention.

Materials and Methods

Chemicals

Heparin, bovine serum albumin, 2-thiobarbuturic acid, trichloroacetic acid(TCA), 1,1,3,3-tetramethoxy propane, 2,4- dinitro phenylhydrazine(DNPH),reduced glutathione (GSH),5,5'-dinitrobenzene (CDNB), 7,12-dimethyl benz (a) anthrance (DMBA) were purchased from Sigma chemical company USA.

Collection of plant material and preparation of extract

The whole plant of *Anisomeles malabarica* (Linn.) R.Br. were collected from Aadivaraganallur near Srimushnam in Cuddalore District of Tamil Nadu, India. The plant was botanically authenticated. A voucher specimen was deposited in the Department of Botany, Annamalai University. The whole plant of *Anisomeles malabarica* were dried in shade and powdered. The powdered plant materials were successively extracted with methanol (80°C) by hot continuous percolation method in Soxhlet apparatus [8]for 24 hrs. The solvent from the extracts was recovered under reduced pressure using rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained.

Animals and diets

All the experiments were carried out with male Syrian hamsters,aged 8-10 weeks, weighing 95-

105g,obtained from the central animal house, Rajah Muthiah Medical Collage and Research institute, Annamalai University, India. They were housed six to a polypropelene cage and provided food and water *ad libitum*. The animals were maintained in a controlled environment under standard condition of temperature and humidity with an alternating 12 hours light/dark cycles. All animals were fed standard pellet diet (Mysore snack feed Ltd, Mysore).

Treatment schedule

The animals were randomized into experimental and control groups and divided into 8 groups of six animals each. In group 1, the right buccal pouches of hamsters were painted three times per week with a 0.5 percent solution of DMBA in liquid paraffin [9]. Hamsters in groups 2 - 4 painted with DMBA as in group 1, received in addition, intragastric administration of Anisomeles *malabarica* methanolic extract of concentration 125, 250 and 500 mg/kg body weight respectively three times a week on days alternate to DMBA application [10]. Animals in groups 5 through 7 were administered *Anisomeles malabarica* methanolic extract alone (125, 250 and 500 mg/kg body weight respectively). Group 8 animals received the same volume of water and served as controls.

Since hamsters painted with DMBA induced well defined tumours in 14 weeks, the experiment was terminated 14th week and all animals were killed by cervical dislocation after an overnight fast. The tumour and host tissue were subdivided and variously processed for distribution to each experiment.

Biochemical assays

Fresh homogenized tissues were used for biochemical estimation. Lipid peroxidation was estimated as evidenced substances formation of thiobarbituric acid- reactive substances (TBARS), TBARS were assayed in tissues according to the method described by Ohkawa et al.,[11], GSH was determined by the method of Anderson [12] based on the development of yellow color when 5,5 - dithiobis (2- nitrobenzoic acid) is added to compounds containing sulfhydryl groups. Oxidized glutathione (GSSG) was estimated after oxidation of reduced nicotinamide adenine dinucleotide phosphate by glutathione reductase at 340 nm based on the method of Anderson [12]. Glutathione peroxidase (GPx) activity was assayed by following the utilization of hydrogen peroxide according to the method of Rotruk et al., [13] with minor modifications. Glutathione -S- transferase was assayed by the method of Habig et al, [14]. GGT was assayed by using - glutamyl p – nitroanilide as substrate by the method of Fiala et al., [15]. The protein content was estimated by the method of Lowry et al.,[16].

Statistical analysis

The values are expressed as mean ± SD. Body weight were analysed using Student's *t* test. The data for lipid peroxides and antioxidants were analysed using ANOVA and the group means were compared by LSD test. The results were considered statistically significant at $P<0.05$.

Results

Observations:

The body weights, tumour incidence and mean tumour burden in experimental and control animals are shown in table 1. Topical application of DMBA for 14 weeks application of DMBA for 14 weeks significantly decreased the body weight of group 1 animals compared to control ($p< 0.05$). However, no significant weight gain was observed in other groups. The tumour incidence was 100% in group 1 animals. The tumours were exophytic and well defined with a mean tumour burden of 525 mm^3. The animals in groups 2 and 3 showed a tumour incidence of 66.6 and 50% respectively. In group 4 animals significantly reduced the tumour incidence

to 16.6% with a mean tumour burden of 80mm^3. The tumours were smaller than those in group 1 animals. No tumours were observed in animals of groups 5 through 8.

Table 1: Body weight, tumour incidence and mean tumour burden in control and experimental hamsters(mean ± SD; n= 6)

Group	Treatment	Initial weight(g)	Final weight(g)	Tumour incidence(%)	Mean tumour burden[b](mm^3)
1.	DMBA	98 ± 0.35	108 ± 0.48[a]	6/6 (100(%))	525
2.	DMBA+AMME (125mgkg^{-1} bw)	102 ± 0.28	125 ± 0.26	4/6 (66.6%)	350
3.	DMBA+AMME (250mgkg^{-1} bw)	103 ± 0.36	128± 0.65	3/6(50%)	200
4.	DMBA+AMME (500mgkg^{-1} bw)	104 ± 0.43	130 ± 0.58	1/6 (16.6%)	80
5.	AMME (125mgkg^{1}bw)	100 ± 0.51	119 ± 0.34	0	-
6.	AMME (250mgkg^{1}bw)	95 ± 0.37	118 ± 0.39	0	-
7.	AMME (500mgkg^{1}bw)	96 ± 0.42	115± 0.41	0	-
8.	Control	98 ± 0.57	125 ± 0.62	0	-

[a] Signifcantly different from group 8 by Student's *t* test($P<0.05$)
Parentheses = Percentage of lesions; AMME=*Anisomeles malabarica* whole plants MeOH extract; - =Nil.
[b] Mean tumour burden was calculated by multiplying the mean tumour volume ($4/3\pi r^3$;where r = ½ tumour diameter in mm), with the mean number of tumors.

Microscopic Observations

Histopathological observations are presented in table 2. The right buccal pouches of all animals treated with DMBA exhibited pathological changes. Animals in group1 showed hyperplastic squamous epithelium with SCC. Histopathological observation of buccal pouches revealed scattered areas of severe hyperplasia, hyperkeratosis and dysplasia in group 1 and 2 animals respectively. Although the incidence of SCC was low in group 3and group 4 animals. Mild hyperkeratosis and hyperplasia were observed in group 5 through 7, which was treated with *Anisomeles malanarica* methanolic extract (AMME). Since epithelium was normal and continuous in untreated control animals.

Table 2. Histopathological changes in the buccal pouch of hamsters in experimental and control groups (n=6).

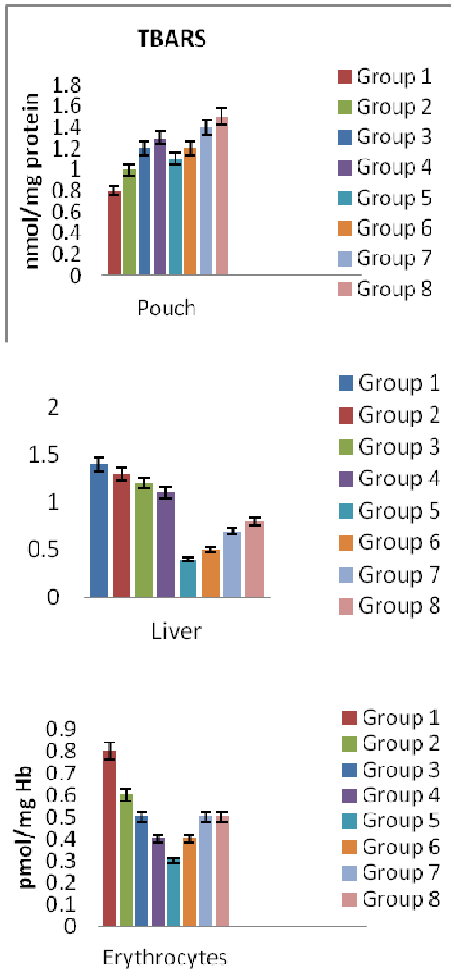
Group	Treatment	Keratosis	Hyperplasia	Dysplasia
1.	DMBA	+ + + (6/6)	+ + + (6/6)	+ + + (6/6)
2.	DMBA+ AMME (125mgkg $^{-1}$ bw)	+ + + (6/6)	+ + + (6/6)	+ + + (5/6)
3.	DMBA+ AMME (250mgkg $^{-1}$bw)	+ + (3/6)	+ + (3/6)	+ + (3/6)
4.	DMBA+ AMME (500mgkg $^{-1}$ bw)	+ (2/6)	+ (2/6)	+ (2/6)
5.	AMME (125mgkg $^{-1}$ bw)	+ (1/6)	+ (1/6)	-
6.	AMME (250mgkg $^{-1}$ bw)	+ (1/6)	+ (1/6)	-
7.	AMME (500mgkg $^{-1}$ bw)	+ (1/6)	+ (1/6)	-
8.	Control	-	-	-

+ = mild; ++ = moderate; +++ =severe; - =no change; AMME= *Anisomeles malabarica* methanolic extract

Biochemical findings:

Figure - 1 illustrates the extent of lipid peroxidation in the buccal pouch, liver and erythrocytes of control and experimental animals. Topical application of DMBA for 14 weeks, significantly lowered lipid peroxidation in the buccal pouch of group 1 animals compared with untreated controls (group 8) animals($p< 0.001$). In contrast to the buccal pouch, the liver and erythrocytes of group 1 animals exhibited significantly elevated levels of TBARS compared to control ($p < 0.001$). Administration of AMME at doses of 250,500 mg/kg were significantly increased ($p < 0.05$ and $p < 0.001$ respectively) the lipid peroxides in groups 2 and 3 compared with group 1 animals. In contrast to the buccal pouch, the extent of lipid peroxidation in the liver and erythrocytes of group 1 animals was significantly elevated ($p< 0.001$) compared with group 8 animals. Administration of AMME alone effectively reduced the extent of lipid peroxidation ($p < 0.05$ and $p < 0.001$, respectively) in the buccal pouch, liver and erythrocytes of hamsters in groups 5 and 6 compared with controls (group 8).

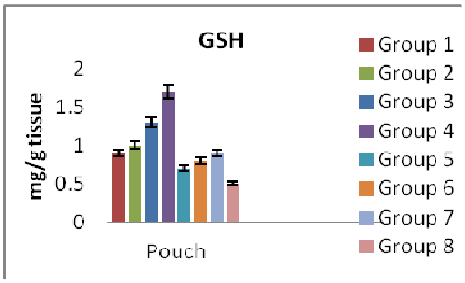
The levels of GSH and the activities of GSH-dependent enzymes like glutathione peroxides (GPx), glutathione S- transferase (GST) and gamma glutamyl transpeptidase (GGT) in the buccal pouch, liver and erythrocytes of experimental and control animals are illustrated in Figures 2 and 3. In hamsters painted with DMBA (group 1), GSH and GSH-dependent enzymes were significantly increased in the buccal pouch and lowered in the liver and erythrocytes ($p < 0.001$) compared with untreated controls (group 8). Administration of AMME + DMBA painted hamsters markedly elevated the enzymatic antioxidant levels in groups 2 – 4 in all the three tissues. AMME alone painted hamsters the enhancing GSH and GSH-dependent enzymes activities was optimum in group 6 animals.

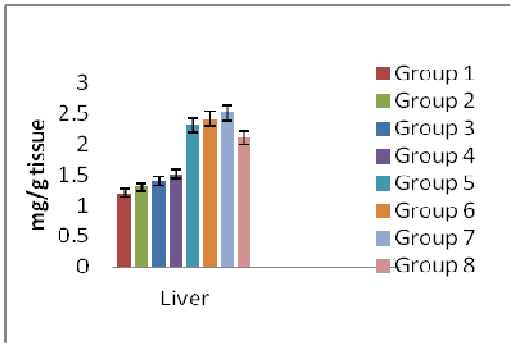


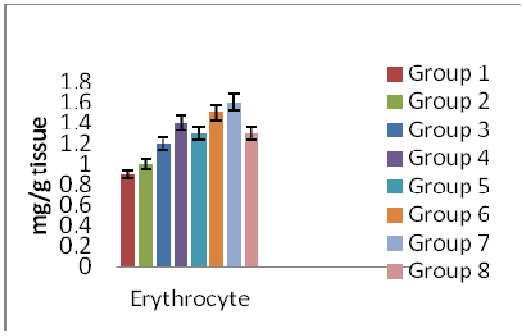
1) DMBA
2) DMBA + AMME (125mg/kg) bw
3) DMBA + AMME (250mg/kg) bw
4) DMBA + AMME (500mg/kg) bw
5) AMME (125mg/kg)
6) AMME (2500mg/kg)
7) AMME (500mg/kg)
8) Control

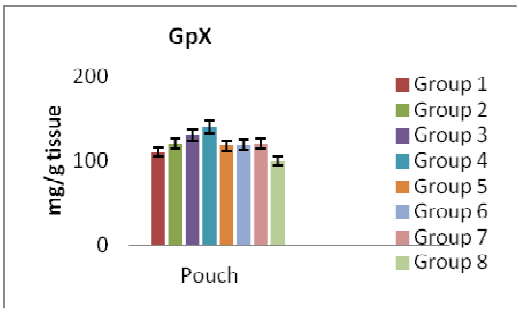
Figure 1. The levels of TBARS in the buccal pouch, liver abd erythrocytes of control and experimental hamsters

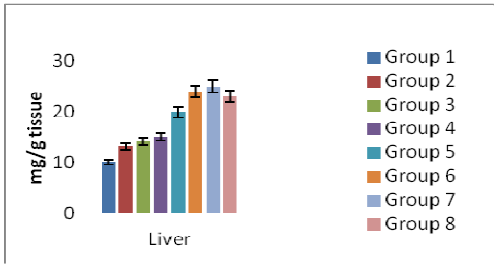
* Significantly different from group 8 ($P< 0.05$)
** Significantly different from group 8 ($P< 0.001$)
♠ Significantly different from group 1 ($P< 0.05$)
♠♠ Significantly different from group 1 ($P< 0.001$)

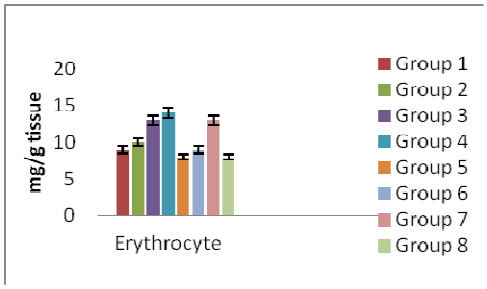








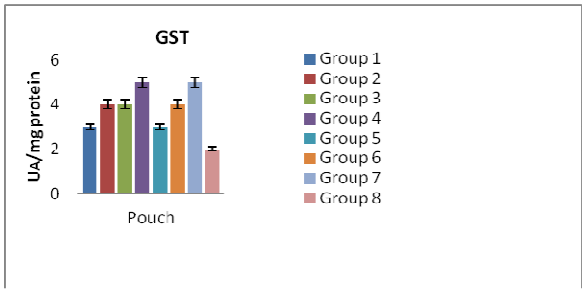


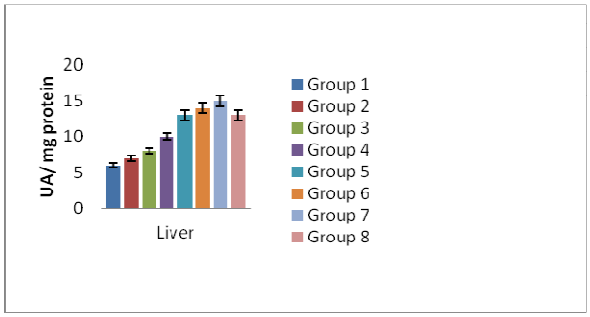


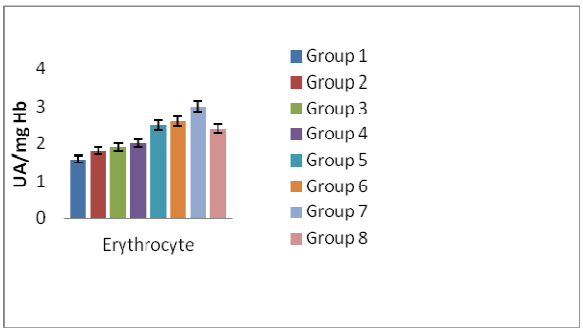
1) DMBA
2) DMBA + AMME (125mg/kg) bw
3) DMBA + AMME (250mg/kg) bw
4) DMBA + AMME (500mg/kg) bw
5) AMME (125mg/kg)
6) AMME (2500mg/kg)
7) AMME (500mg/kg)
8) Control

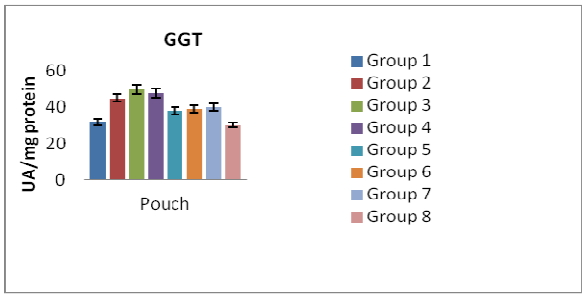
Figure 2: Effect of AMME on levels of GSH and the activity of GPx in the buccal pouch, liver and erythrocytes of control and experimental hamsters

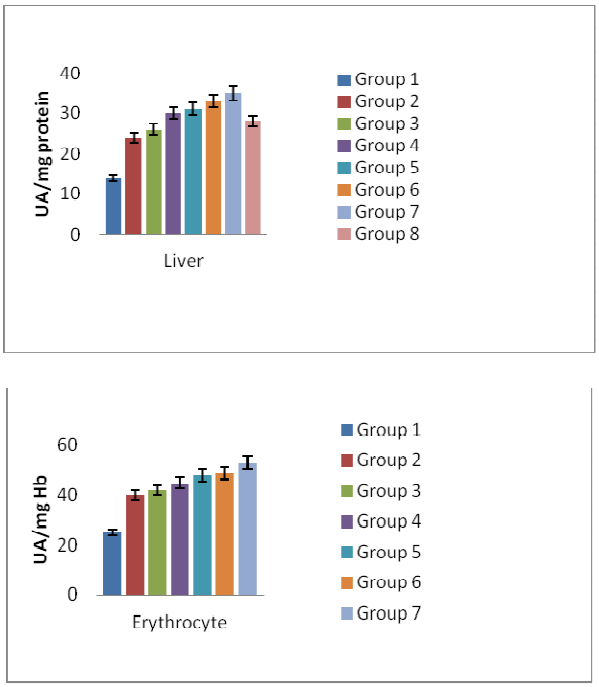
* Significantly different from group 8 (P< 0.05)
** Significantly different from group 8 (P< 0.001)
♠ Significantly different from group 1 (P< 0.05)
♠♠ Significantly different from group 1 (P< 0.001)
A = µmoles of GSH utilized/min











1) DMBA
2) DMBA + AMME (125mg/kg) bw
3) DMBA + AMME (250mg/kg) bw
4) DMBA + AMME (500mg/kg) bw
5) AMME (125mg/kg)
6) AMME (2500mg/kg)
7) AMME (500mg/kg)
8) Control

Figure 3: The activities of GST and GGT in the buccal pouch, liver and erythrocytes of control and experimental hamsters

* Significantly different from group 8 (P< 0.05)
** Significantly different from group 8 (P< 0.001)
♠ Significantly different from group 1 (P< 0.05)
♠♠ Significantly different from group 1 (P< 0.001)
A = µmoles of GSH – CDNB conjugate formed/min
B = µmoles of p- nitroaniline formed/h.

Discussion

Based on histological observations, repeated application of DMBA in liquid paraffin produces hyperplastic changes after 4 weeks, papillomatous outgrowth at about 7-9 weeks, early invasive carcinomas at about 10-12 weeks and finally well-differentiated squamous cell carcinoma at around 14-16 weeks in right buccal mucosa; these observations concur very well with earlier. Oral carcinogenesis is a multifocal disease preceded by distinct premalignant lesions. DMBA induced precancerous and cancerous lesions in hamsters resemble human oral precancerous and cancerous lesions.

Cell proliferation, a hallmark of neoplastic transformation has been negatively correlated with lipid peroxidation. Lipid peroxidation are recognized to prolong the G1 phase of the cell cycle and decrease the rate of cell proliferation [17]. Cell that proliferate rapidly such as spermatozoa have low concentrations of lipid peroxidation, whereas neurons that seldom divide show high rates of lipid peroxidation [18]. Thus, the decreased susceptibility of HBP tumours to lipid peroxidation appears to be one of the mechanisms through which tumour cells maintain a rapid rate of cell proliferation.

In DMBA - induced HBP tumours, decreased the lipid peroxidation was associated with enhanced of GSH – redox cycle enzymes. Overexpression of GSH and GSH – dependent enzymes has also been documented in a wide variety of malignancies including OSCC [19,20]. The results of the present study as well as those reported by Diplock et al.,[21] provide strong evidence for increased antioxidant activity in tumours. This may serve to maintain a reduced environment protecting tumour tissues against oxidative stress and ensure cell survival.

In contrast to HBP tumours, the liver and erythrocytes of tumour bearing hamsters showed enhanced lipid peroxidation and decreased activities of GSH redox cycle enzymes. This can lead to accumulation of toxic radical species including $O_2^{\cdot-}$, H_2O_2, and –OH with deleterious effects on cellular macromolecules including oxidation of critical sulfhydryl groups and conformational changes in functional proteins as well as DNA strand breaks with consequent shift in the redox status towards oxidative stress [22,23,24]. Thus, the tumour and host tissues appear to comprise two separate metabolic compartments with respect to the redox status.

Administration of AMME to DMBA - painted hamsters reduced the incidence of SCC and mean tumour burden in addition to preneoplastic lesions. In the buccal pouch, AMME reversed the

susceptibility to lipid peroxidation while simultaneously increasing GSH-dependent antioxidant enzyme activities, whereas in the liver and erythrocytes, the extent of lipid peroxidation was reduced with elevation of antioxidants. Thus, modified oxidant status together with antioxidant adequacy in the target organ as well as in the liver and erythrocytes induced by AMME may significantly reduce cell proliferation and block tumour development in the HBP.

The results of the present study are consistent with the free radical scavenging properties of AMME reported in literature. AMME has been shown to prevent the increase in lipid peroxidation and protect against oxidative DNA damage by improving antioxidant defenses.

Among the doses used in the present study, the medium dose and higher dose of AMME (250 mg/kg bw and 500 mg/kg bw) were found to be more effective in inhibiting HBP carcinogenesis compared to low dose. The protective effects of AMME against HBP carcinogenesis observed in the present study may be related to the antioxidant and antiproliferative properties of phytochemicals such as flavonoids present in the plant.

Conclusion

The results of the present study suggest that AMME exerts its inhibitory effects on HBP carcinogenesis by modulation of lipid peroxidation and enhancement of GSH redox cycle enzymes. There is also a distinct possibility that synergistic interactions between AMME constituents such as polyphenolic compounds and flavonoids may contribute to the anticancer properties of *Anisomeles malabarica*.

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