

## Protective effect of Withaferin-A on tumour formation in 7,12-dimethylbenz[a]anthracene induced oral carcinogenesis in hamsters

S Manoharan\*, K Panjamurthy, Venugopal P Menon, S Balakrishnan & Linsa Mary Alias

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar 608 002, India

Received 2 June 2008; revised 3 November 2008

With an aim to investigate the protective effect of Withaferin-A on 7,12-dimethylbenz[a]anthracene (DMBA) induced oral carcinogenesis in Syrian golden hamsters, tumour incidence, tumour volume and tumour burden and status of detoxication agents, lipid peroxidation and antioxidants in DMBA administered (3 times/week for 14 weeks) hamsters were assessed. Hundred percent tumour formation in DMBA alone administered animal was observed. Oral administration of Withaferin-A (20 mg/kg body weight) to DMBA administered animals for 14 weeks completely prevented the tumour incidence, tumour volume and tumour burden. Also, Withaferin-A showed significant anti-lipid peroxidative and antioxidant properties and maintained the status of phase-I and phase-II detoxication agents during DMBA-induced oral carcinogenesis. The results thus indicate that the protective effect of Withaferin-A is probably due to its anti-lipid peroxidative and antioxidant functions as well as modulating effect on carcinogen detoxication during DMBA-induced oral carcinogenesis.

**Keywords:** Antioxidants, Detoxication agents, Lipid peroxidation, Oral cancer, Withaferin-A

Oral squamous cell carcinoma, a disfiguring disease of human population, is the fifth most frequent cancer worldwide. While oral cancer accounts for 3-4% of all cancers in Western countries, India has recorded highest incidence for oral cancer (40-50% of all cancers) than any other country worldwide<sup>1,2</sup>. Tobacco smoking, betel quid chewing, with or without tobacco, and alcohol consumption are identified as the high risk factors for the development of oral cancer<sup>2</sup>. 7,12-dimethylbenz[a]anthracene (DMBA), a potent organ specific carcinogen, mediates carcinogenic process by inducing chronic inflammation, over production of reactive oxygen species (ROS) and oxidative DNA damage. DMBA is commonly used to induce oral carcinoma in experimental animals since oral carcinoma induced by this carcinogen is morphologically and histologically similar to that of human oral tumours<sup>3,4</sup>.

Overproduction of reactive oxygen species in the human body can induce strand breaks and modify DNA bases, contributing to mutagenesis and carcinogenesis. Mammalian cells however have arrays of non-enzymatic (Vitamin E and reduced glutathione) and enzymatic (superoxide dismutase, catalase and

glutathione peroxidase) antioxidant defense system to scavenge or dispose excessively generated reactive oxygen species<sup>5</sup>. Status of lipid peroxidation and antioxidants in both human and experimental oral carcinogenesis have been reported<sup>6,7</sup>.

Cancer chemoprevention, a novel approach in experimental oncology, deals with the prevention, inhibition or reversal of carcinogenesis by synthetic chemical entities or by naturally occurring plant products. In recent years, several phytochemicals with chemopreventive potential were reported<sup>8,9</sup>. Chemopreventive agents possibly exert their role by inhibiting mutagenesis, cell proliferation or by inducing apoptosis and modulating the activities of detoxication agents<sup>10</sup>.

*Withania somnifera* has been used for centuries as a traditional medicine for various human ailments. Withaferin-A (Fig. 1), a highly oxygenated steroidal lactone, is the principal withanolides in Indian *W. somnifera* and its related Solanaceae species. Diverse pharmacological activities reported for Withaferin-A includes anti-inflammatory, antigenotoxic, antitumour and antioxidant properties<sup>11-13</sup>. To the best of our knowledge, there is no report on protective effect of Withaferin-A on tumour formation in DMBA-induced hamster buccal pouch carcinogenesis. Therefore, the present study has been designed to provide scientific validity for the protective effect of Withaferin-A on

\*Correspondent author

Telephone: +91 - 04144 - 238343

Fax: +91 - 04144 - 238145

E-mail: manshisak@yahoo.com

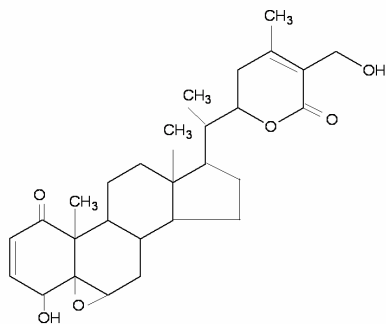


Fig. 1—Molecular structure of Withaferin-A

tumour formation in DMBA-induced oral carcinogenesis in hamsters.

### Materials and Methods

**Chemicals**—The carcinogen, DMBA, was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, purchased from HiMedia Laboratories, Mumbai, India.

**Animals**—Male golden Syrian hamsters 8-10 weeks old weighing 80-120g were purchased from National Institute of Nutrition, Hyderabad, India and were maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed five in a polypropylene cage and provided standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12 hr light/dark cycle. The Institutional animal ethics committee (Register number 160/1999/CPCSFA), Annamalai University, Annamalainagar, India, was approved the experimental design. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use.

**Experimental design**—Hamsters (40) were randomized into 4 groups of 10 each. Group 1 animals which served as control were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups 2 and 3 animals were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group 2 animals received no other treatment. Group 3 animals were orally administered Withaferin-A at a dose of 20 mg/kg body weight, starting one week before the exposure to the

carcinogen and continued on days alternate to DMBA painting, until the sacrifice of the animals. Group 4 animals received oral administration of Withaferin-A alone throughout the experimental period. The experiment was terminated at the end of 15 weeks and all animals were sacrificed by cervical dislocation.

**Extraction and isolation of Withaferin-A from *Withania somnifera* root**—Withaferin-A (WA) was extracted and isolated from commercially available *Withania somnifera* root powder (Kaviraj Pharmaceuticals, Erode, India) as per Subramanian and Sethi<sup>14</sup>. The crude extract was prepared with 95% ethanol and further fractionation was carried out using petroleum ether, ether and chloroform, in that order. The ether and chloroform fractions were subjected to column chromatography (neutral alumina) and thin layer chromatography (silica gel). The final product, Withaferin-A, a steroidal lactone (4 $\beta$ , 27 dihydroxy-1-oxo-5 $\beta$ , 6 $\beta$ , epoxy witha 2-24 dienolide) was obtained as a creamy white crystalline substance, which had  $R_f$  = 0.4 and molecular weight 470. The identity of the isolated Withaferin-A was done by Mass spectral analysis and its identity was confirmed by comparing with the authentic Withaferin-A, purchased from Calbiochem, Darmstadt, Germany. The yield and purity of the isolated Withaferin-A was found to be 0.11% and >90% respectively. For experimental studies, Withaferin-A obtained was first dissolved in a few drops of absolute ethanol followed by dilution with 30% polyethylene glycol-400 (PEG-400) in phosphate-buffered saline (PBS).

**Macroscopic observation**—The number of tumours in the buccal pouches was counted and the diameter of each tumour was measured with a caliper. Tumour volume was measured using the formula  $v = (4/3) \pi [(D_1/2) (D_2/2) (D_3/2)]$  where  $D_1$ ,  $D_2$  and  $D_3$  are the three diameters (mm) of the tumour. Tumour burden was calculated by multiplying tumour volume and the number of tumours/animal.

**Biochemical estimations**—Biochemical studies were conducted on plasma, erythrocytes, and liver of control and experimental animals in each group. Blood samples were collected into heparinized tubes. The plasma was separated by centrifugation at 3,000 rpm for 15 min. After plasma separation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of erythrocytes was lysed with hypotonic buffer at pH 7.4. The hemolysate was separated by centrifugation at 10,000 rpm for 15 min at 20°C.

The erythrocyte membrane was prepared by the method of Dodge *et al.*<sup>15</sup> modified by Quist<sup>16</sup>. The erythrocytes remaining after the removal of plasma were washed three times with 310 mM isotonic Tris-HCl buffer (pH 7.4). Hemolysis was carried out by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes, which contained 20 mM hypotonic Tris-HCl buffer (pH 7.2). The erythrocyte membranes were sediment in a high-speed cooling centrifuge at 20,000 g for 40 min. The supernatant was decanted and the erythrocyte membrane pellet was made up to a known volume using 0.2 M isotonic Tris-HCl buffer (pH 7.4). Aliquots from these preparations were used for the estimation of thiobarbituric acid reactive substances (TBARS) and vitamin E. Liver samples from animals were washed with ice-cold saline and homogenized using appropriate buffer [glutathione-S-transferase (GST) – 0.3 M phosphate buffer, pH 6.5; glutathione reductase (GR) – 0.1 M phosphate buffer, pH 7.4; TBARS – 0.025 M Tris-HCl buffer, pH 7.5; reduced glutathione (GSH) and glutathione peroxidase (GPx) – 0.4 M phosphate buffer, pH 7.0; superoxide dismutase (SOD) – 0.025 M sodium pyrophosphate buffer, pH 8.3; catalase (CAT) – 0.01 M phosphate buffer, pH 7.0] in an all glass homogenizer with teflon pestle and used for biochemical estimations. The status of TBARS and antioxidants were measured in plasma, erythrocytes, and liver homogenate. The activities of detoxication agents were determined in the liver homogenate.

Lipid peroxidation was estimated by the formation of TBARS. TBARS in plasma were assayed by the method of Yagi<sup>17</sup>. Plasma was deproteinised with phosphotungstic acid and the precipitate was treated with thiobarbituric acid at 90°C for 1 hr. The pink colour formed gives a measure of the thiobarbituric acid reactive substances (TBARS), which was read at 530 nm. TBARS in erythrocyte membranes was estimated by the method of Donnan<sup>18</sup>. Absorbance of pink chromogen formed by the reaction of thiobarbituric acid with breakdown products of lipid peroxides was read at 535 nm.

The reduced glutathione level in plasma, erythrocytes, and liver was determined by the method of Beutler and Kelly<sup>19</sup>. The technique involves protein precipitation by meta-phosphoric acid and spectrophotometric assay at 412 nm of the yellow derivative obtained by the reaction of the supernatant with 5-5'-dithiobis-2-nitrobenzoic acid. The level of

plasma Vitamin C was determined by the method of Omaye *et al.*<sup>20</sup>. The dehydro-ascorbic acid formed from the oxidation of Vitamin C by copper, forms a coloured product on treatment with 2,4-dinitrophenylhydrazine whose absorbance was measured at 520 nm. Vitamin E level in plasma and erythrocyte membrane was determined colorimetrically by the method of Desai<sup>21</sup>. Vitamin E presents in the lipid residue forms a pink coloured complex with bathophenanthroline-phosphoric acid reagent, which was measured at 536 nm. Superoxide dismutase activity in plasma and erythrocytes was assayed by the method of Kakkar *et al.*<sup>22</sup>, based on the 50% inhibition of formation of NADH-phenazine methosulphate nitro blue tetrazolium (NBT) formation. The colour developed was read at 520 nm. One unit of enzyme is taken as the amount of enzyme required to give 50% inhibition of nitro blue tetrazolium (NBT) reduction. The activity of catalase in plasma and erythrocytes was assayed by the method of Sinha<sup>23</sup>, based on the utilization of H<sub>2</sub>O<sub>2</sub> by the enzyme. The colour developed was read at 620 nm. One unit of the enzyme is expressed as  $\mu$  moles of H<sub>2</sub>O<sub>2</sub> utilized/min. The activity of glutathione peroxidase (GPx) in plasma and erythrocytes was determined using the method of Rotruck *et al.*<sup>24</sup>, based on the utilization of reduced glutathione by the enzyme. One unit of the enzyme is expressed as  $\mu$  moles of GSH utilized/min.

The activity of glutathione-S-transferase (GST) in liver homogenate was assayed by the method of Habig *et al.*<sup>25</sup>. GST activity was measured by incubating the tissue homogenate with the substrate 1-chloro 2,4 dinitrobenzene (CDNB). The absorbance was followed for 5 min at 540 nm after the reaction was started by the addition of reduced glutathione. Glutathione reductase activity in liver homogenate was assayed by the method of Carlberg and Mannervik<sup>26</sup>. The enzyme activity was assayed by measuring the formation of reduced glutathione when the oxidized glutathione (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH).

The levels of cytochrome P<sub>450</sub> and b<sub>5</sub> in liver were determined according to the method of Omura and Sato<sup>27</sup>. Cytochrome P<sub>450</sub> was measured by the formation of pigment on reaction between reduced cytochrome P<sub>450</sub> and carbon monoxide. The pigment was read with an absorbance maximum at 450 nm. The difference spectrum between reduced and

oxidized cytochrome was used as an index to measure the level of cytochrome b<sub>5</sub>. The activity of DT-diaphorase in liver was estimated according to the method of Ernster<sup>28</sup> based on the measurement of reduction at 550 nm using reduced nicotinamide adenine dinucleotide phosphate as the electron donor and 2,6-dichlorophenol indophenol as the electron acceptor.

For histopathological examination, buccal mucosal tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3 μm sections were cut in a rotary microtome and stained with haematoxylin and eosin.

*Statistical analysis*—The data are expressed as mean ± SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA), followed by Duncan’s Multiple Range Test (DMRT). The results were considered statistically significant if the *P* values were less than 0.05.

**Results**

Table 1 shows the tumour incidence, tumour volume and tumour burden of control and experimental animals in each group. Hundred percent tumour formation with mean tumour volume (428.09 mm<sup>3</sup>) and tumour burden (1669.56 mm<sup>3</sup>) in DMBA alone painted animals (Group 2) was observed. Oral administration of Withaferin-A at a dose of 20 mg/kg

body weight for 14 weeks completely prevented the tumour incidence, tumour volume and tumour burden in DMBA painted hamsters (Group 3). No tumours were observed in control animals painted with liquid paraffin alone (Group 1) as well as Withaferin-A alone administered hamsters (Group 4).

The histopathological features observed in buccal mucosal tissues of control and experimental animals in each group are depicted in Table 2. The buccal pouches from DMBA treated hamsters (Group 2) revealed severe keratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma. A mild to moderate preneoplastic lesions (hyperplasia, keratosis, and dysplasia) were noticed in DMBA painted animals administered with Withaferin-A..

The levels of TBARS, enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (Vitamin E, Vitamin C and reduced glutathione) antioxidants in plasma and erythrocytes of control and experimental animals in each group are shown in Tables 3 and 4 respectively. The level of TBARS was increased, whereas the status of enzymatic and non-enzymatic antioxidants was significantly decreased in DMBA-alone painted animals (Group 2) as compared to control animals (Group 1). Oral administration of Withaferin-A to DMBA painted animals (Group 3) significantly restored the status to near normal concentrations.

Table 1—Incidence of oral neoplasms in control and experimental animals in each group (n = 10)

Parameters	Group 1 Control	Group 2 DMBA	Group 3 DMBA + Withaferin-A	Group 4 Withaferin-A alone
Tumour incidence (Squamous cell carcinoma)	—	100%	—	—
Total number of tumours	—	39	—	—
Tumour volume (mm <sup>3</sup> )	—	428.09	—	—
Tumour burden (mm <sup>3</sup> )	—	1669.56	—	—

Table 2—Histopathological changes in the buccal pouch of hamsters in control and experimental animals

Parameters	Group 1 Control	Group 2 DMBA	Group 3 DMBA + Withaferin-A	Group 4 Withaferin-A alone
Hyperkeratosis	0	++	+	0
Hyperplasia	0	++	+	0
Dysplasia	0	++	+	0
Well-differentiated squamous cell carcinoma	0	++	0	0

0=Absent; ++=Severe; +=mild

Table 3—Status of plasma TBARS and antioxidants in control and experimental animals  
[Values are mean  $\pm$ SD from 10 animals in each group]

Parameters	Group 1 Control	Group 2 DMBA	Group 3 DMBA + Withaferin-A	Group 4 Withaferin-A alone
TBARS (nmoles/ml)	2.54 $\pm$ 0.34 <sup>a</sup>	4.32 $\pm$ 0.38 <sup>b</sup>	2.99 $\pm$ 0.44 <sup>c</sup>	2.50 $\pm$ 0.12 <sup>a</sup>
GSH (mg/dl)	24.5 $\pm$ 2.2 <sup>a</sup>	17.42 $\pm$ 1.1 <sup>b</sup>	22.40 $\pm$ 1.9 <sup>c</sup>	24.40 $\pm$ 2.1 <sup>a</sup>
Vitamin C (mg/dl)	1.37 $\pm$ 0.13 <sup>a</sup>	0.78 $\pm$ 0.08 <sup>b</sup>	1.27 $\pm$ 0.06 <sup>c</sup>	1.38 $\pm$ 0.14 <sup>a</sup>
Vitamin E (mg/dl)	1.21 $\pm$ 0.09 <sup>a</sup>	0.81 $\pm$ 0.06 <sup>b</sup>	1.12 $\pm$ 0.12 <sup>c</sup>	1.23 $\pm$ 0.08 <sup>a</sup>
SOD (U*/ml)	2.49 $\pm$ 0.24 <sup>a</sup>	1.92 $\pm$ 0.14 <sup>b</sup>	2.75 $\pm$ 0.27 <sup>c</sup>	2.50 $\pm$ 0.25 <sup>a</sup>
CAT (U**/ml)	0.49 $\pm$ 0.04 <sup>a</sup>	0.30 $\pm$ 0.02 <sup>b</sup>	0.40 $\pm$ 0.08 <sup>c</sup>	0.52 $\pm$ 0.14 <sup>a</sup>
GPx (U***/l)	110.9 $\pm$ 9.4 <sup>a</sup>	87.5 $\pm$ 8.6 <sup>b</sup>	102.98 $\pm$ 6.2 <sup>a</sup>	112.4 $\pm$ 11.8 <sup>a</sup>

Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT)

\*Amount of enzyme required to inhibit 50% NBT reduction

\*\* $\mu$ mole of H<sub>2</sub>O<sub>2</sub> utilized /sec.

\*\*\* $\mu$ mole of glutathione utilized/min

Table 4—TBARS and antioxidant status in erythrocytes of control and experimental animals  
[Values are mean  $\pm$ SD from 10 animals in each group]

Parameters	Group 1 Control	Group 2 DMBA	Group 3 DMBA + Withaferin-A	Group 4 Withaferin-A alone
Erythrocyte TBARS (pmoles/mg Hb)	1.98 $\pm$ 0.14 <sup>a</sup>	2.59 $\pm$ 0.22 <sup>b</sup>	2.17 $\pm$ 0.22 <sup>c</sup>	1.94 $\pm$ 0.25 <sup>a</sup>
Erythrocyte membrane TBARS (nmoles/mg protein)	0.38 $\pm$ 0.09 <sup>a</sup>	0.97 $\pm$ 0.12 <sup>b</sup>	0.47 $\pm$ 0.10 <sup>c</sup>	0.37 $\pm$ 0.10 <sup>a</sup>
Erythrocyte membrane Vitamin E ( $\mu$ g/mg protein)	2.29 $\pm$ 0.15 <sup>a</sup>	1.57 $\pm$ 0.13 <sup>b</sup>	2.12 $\pm$ 0.19 <sup>c</sup>	2.32 $\pm$ 0.17 <sup>a</sup>
Erythrocytes GSH (mg/dl)	47.31 $\pm$ 3.23 <sup>a</sup>	30.5 $\pm$ 2.3 <sup>b</sup>	44.1 $\pm$ 3.21 <sup>c</sup>	48.21 $\pm$ 3.46 <sup>a</sup>
Erythrocyte lysate SOD (U*/mg Hb)	2.01 $\pm$ 0.20 <sup>a</sup>	1.47 $\pm$ 0.12 <sup>b</sup>	1.81 $\pm$ 0.16 <sup>c</sup>	2.03 $\pm$ 0.26 <sup>a</sup>
CAT (U**/mg Hb)	1.17 $\pm$ 0.11 <sup>a</sup>	0.85 $\pm$ 0.08 <sup>b</sup>	1.08 $\pm$ 0.08 <sup>a</sup>	1.21 $\pm$ 0.14 <sup>a</sup>
GPx (U***/g Hb)	13.52 $\pm$ 1.28 <sup>a</sup>	8.01 $\pm$ 0.62 <sup>b</sup>	12.25 $\pm$ 1.22 <sup>c</sup>	13.76 $\pm$ 1.27 <sup>a</sup>

Values not sharing a common superscript letter differ significantly at  $P < 0.05$  (DMRT)

\*Amount of enzyme required to inhibit 50% NBT reduction.

\*\* $\mu$ mole of H<sub>2</sub>O<sub>2</sub> utilized /sec

\*\*\* $\mu$ mole of glutathione utilized/min

Hamsters treated with Withaferin-A alone (Group 4) showed no significant difference in TBARS and antioxidants status as compared to control animals (Group 1).

The status of phase-I detoxication agents (cytochromes P<sub>450</sub> and b<sub>5</sub>) was significantly increased whereas phase-II detoxication agents (GSH, GST, GR and DT-diaphorase) were decreased in the liver of DMBA-painted animals (Group 2) as compared to control animals (Group 1) (Table 5). Oral administration of Withaferin-A to DMBA-painted animals reverted the status of phase-I and phase-II detoxication agents to near normal range in the liver. Oral administration of Withaferin-A alone (Group 4) showed no significant difference as compared to control animals.

## Discussion

The protective effect of Withaferin-A (20mg/kg body weight) was assessed on tumour formation in DMBA-induced hamster buccal pouch carcinogenesis by monitoring the percentage of tumour bearing animals, tumour volume and burden as well as by analyzing the status of phase I and II detoxication agents, lipid peroxidation and antioxidants in DMBA painted animals. Different doses of Withaferin-A (10, 15 and 20 mg) were assessed to find out the effective chemopreventive dose in DMBA induced oral carcinogenesis. A dose of 20 mg/kg body weight Withaferin-A has shown potent chemopreventive potential in DMBA treated hamsters as compared to rest of the doses. Due to these reasons, the dose of 20 mg/kg body weight was chosen for the present study. Hundred per cent tumour formation in DMBA alone

Table 5—Status of Phase-I and Phase II detoxification agents in the liver of control and experimental animals  
[Values are mean  $\pm$ SD from 10 animals in each group]

Parameters	Group 1 Control	Group 2 DMBA	Group 3 DMBA + Withaferin-A	Group 4 Withaferin-A alone
Phase-I detoxification agents				
Cytochromes P <sub>450</sub> (U <sup>X</sup> / mg protein)	0.62 $\pm$ 0.04 <sup>a</sup>	1.68 $\pm$ 0.12 <sup>b</sup>	0.70 $\pm$ 0.09 <sup>c</sup>	0.63 $\pm$ 0.04 <sup>a</sup>
Cytochrome b <sub>5</sub> (U <sup>Y</sup> / mg protein)	1.02 $\pm$ 0.10 <sup>a</sup>	2.20 $\pm$ 0.26 <sup>b</sup>	1.25 $\pm$ 0.24 <sup>c</sup>	1.07 $\pm$ 0.06 <sup>a</sup>
Phase-II detoxification agents				
GSH (mg / g tissue)	2.55 $\pm$ 0.22 <sup>a</sup>	1.30 $\pm$ 0.12 <sup>b</sup>	2.05 $\pm$ 0.18 <sup>c</sup>	2.59 $\pm$ 0.30 <sup>a</sup>
GST (U <sup>A</sup> /mg protein)	23.68 $\pm$ 1.84 <sup>a</sup>	12.4 $\pm$ 1.07 <sup>b</sup>	18.76 $\pm$ 1.53 <sup>c</sup>	22.65 $\pm$ 1.43 <sup>a</sup>
GR (U <sup>B</sup> /mg protein)	18.57 $\pm$ 1.11 <sup>a</sup>	8.80 $\pm$ 0.89 <sup>b</sup>	15.41 $\pm$ 1.10 <sup>c</sup>	17.93 $\pm$ 1.08 <sup>a</sup>
DT-diaphorase (U <sup>C</sup> /mg protein)	0.50 $\pm$ 0.03 <sup>a</sup>	0.44 $\pm$ 0.01 <sup>b</sup>	0.47 $\pm$ 0.03 <sup>c</sup>	0.51 $\pm$ 0.04 <sup>a</sup>

Values not sharing a common superscript significantly differ at  $P < 0.05$  (DMRT)

<sup>X</sup> $\mu$ mole of cytochrome/g tissue; <sup>Y</sup> $\mu$ mole of cytochrome/g tissue; <sup>A</sup> $\mu$ mole of 1-chloro-2,4-dinitrobenzene conjugated with reduced glutathione/ min; <sup>B</sup> $\mu$ mole of NADPH oxidized/hr; <sup>C</sup> $\mu$ mole of 2,6-dichlorolindophenol reduced/min

painted animals was observed. Oral tumour was histopathologically confirmed as well-differentiated squamous cell carcinoma. Severe hyperplasia, dysplasia and keratosis were also observed in DMBA painted animals. The tumour cells exhibited epithelial and keratin pearl formation. Although oral administration of Withaferin-A to DMBA painted animals completely prevented the tumour formation, moderate hyperplasia, dysplasia and keratosis were observed. Control animals as well as those treated with Withaferin-A alone showed well-defined oral epithelial tissues. The present study thus suggests that Withaferin-A inhibited the abnormal cell proliferation during DMBA-induced oral carcinogenesis.

Chemopreventive potential of medicinal plants or their active constituents can be assessed by analyzing the status of phase I and phase II detoxication agents such as cytochrome P<sub>450</sub>, cytochrome b<sub>5</sub>, DT-diaphorase glutathione-S-transferase, glutathione reductase and reduced glutathione<sup>29</sup>. Chemopreventive agents convert DNA damaging entities (2,3-epoxide, 7,8-diol-9,10-epoxide, methyl carbonium ion etc.) into excretable metabolites through the induction of detoxication agents<sup>30</sup>. Glutathione-S-transferase (GST) catalyzes the conjugation of reduced glutathione to a large number of electrophilic compounds produced by cytochrome P<sub>450</sub> enzymes. Since electrophiles can form DNA adducts and mutation by binding with DNA, GST plays an important role in protecting cells against the cytotoxic and mutagenic effects of these reactive compounds<sup>31</sup>. Profound evidence suggests that the activity of DT-diaphorase (DTD) is induced in co-ordination with the activity of GST. DTD activity was

elevated in several tumour types including non-small cell lung carcinoma, colorectal carcinoma, liver cancers and breast carcinomas as compared to the surrounding normal tissues<sup>32,33</sup>.

Liver plays an important role in the modulation of the process of carcinogenesis, as it is the primary site for biotransformation of xenobiotics including carcinogens and anticancer drugs<sup>34</sup>. Liver also plays a crucial role in the detoxification process and thus measurement of the activities of detoxification agents such as glutathione-S-transferase, glutathione reductase and reduced glutathione help to assess the chemopreventive potential of the test compound<sup>7,35</sup>. Decreased activities of the xenobiotic biotransformation enzymes, cytochrome P<sub>450</sub>, cytochrome b<sub>5</sub> and glutathione-S-transferase, were reported in the hepatic tissue of tumour bearing mice<sup>36</sup>. Elevated levels of cytochrome P<sub>450</sub> and b<sub>5</sub> in liver suggest the metabolic activation of DMBA in the hepatic tissues. Lowered activities of phase-II detoxication agents observed in the liver of DMBA painted animals indicate that the activities of glutathione-S-transferase, glutathione reductase and reduced glutathione are impaired due to the accumulation of toxic metabolites during oral carcinogenesis. Oral administration of Withaferin-A to DMBA painted animals reverted the status of detoxication agents in the liver, which suggest that Withaferin-A may have induced the activities of detoxication agents to excrete dihydrodiol-epoxide, the active metabolite of DMBA.

Oxidative stress has been implicated in multistage carcinogenesis and in the pathogenesis of several diseases<sup>5</sup>. An increase in TBARS and decline in

enzymatic and non-enzymatic antioxidants status was noticed in DMBA alone painted hamsters as compared to control animals. Metabolic activation of DMBA generates highly toxic and diffusible reactive oxygen species that cause extensive cell damage or adducts on the biomolecules, contributing to malignant transformation<sup>37</sup>. Assay of lipid peroxidation by-products (TBARS) in plasma or serum helps to assess the severity of tissue damage<sup>38</sup>. Erythrocytes membranes are particularly susceptible to oxidative stress due to its high content of poly unsaturated fatty acids, which are more vulnerable to oxidative damage<sup>39,40</sup>. The erythrocytes are particularly susceptible to peroxide stress due to high content of iron, a potent catalyst for the production of ROS and due to continuous challenge with high oxygen tension<sup>41</sup>. Susceptibility of erythrocytes to peroxide stress has been demonstrated in various diseases including oral cancer<sup>6,7,42</sup>. Increase in plasma TBARS in tumour bearing animals is probably due to the consequence of overproduction and diffusion from erythrocyte membrane and damaged host tissues with subsequent leakage into plasma.

Enzymatic and non-enzymatic antioxidants play a crucial role in scavenging or disposing lipid peroxidation by-products when they are excessively generated in the body. Tumour tissues sequester nutrients and antioxidants from circulation to combat the deleterious effects of reactive oxygen species and for their abnormal growth<sup>43</sup>. Vitamin E, the major lipophilic antioxidant, protects cell membranes against lipid peroxidation mediated oxidative stress both by free radical scavenging and by membrane stabilizing mechanism<sup>39,40</sup>. Vitamin C, the most important antioxidant in the plasma, scavenges a variety of oxidants<sup>40</sup>. Reduced glutathione is the most powerful intracellular antioxidant and the molar ratio of reduced glutathione to oxidised glutathione serve as an important marker of the antioxidative capacity of the cell<sup>35,40</sup>. Decreased levels of Vitamin E, Vitamin C and GSH were reported in both human and experimental oral carcinogenesis<sup>6,7</sup>. Lowered levels of Vitamin E, Vitamin C and reduced glutathione in plasma and erythrocytes were probably due to their utilization by oral malignant tumours.

Lowered activities of plasma and erythrocytes enzymatic antioxidants were reported in several types of cancers<sup>35,39,43</sup>. Lowered activities of enzymatic antioxidants are probably due to exhaustion of these enzymes to scavenge excessively generated reactive

oxygen species in the system. Oral administration of Withaferin-A at a dose of 20 mg/kg body weight to DMBA painted animals brought back the status of enzymatic antioxidants and non-enzymatic antioxidants to near normal range. The present results thus suggest that Withaferin-A has potent free radical scavenging activity and antioxidant property during DMBA-induced oral carcinogenesis.

The present study thus demonstrated the protective effect of Withaferin-A on tumour formation in DMBA-induced hamster buccal pouch carcinogenesis. Although the mechanism by which Withaferin-A exerts its protective efficacy is unclear at present, its anti-lipid peroxidative, and antioxidant properties may play a possible role. Induction of detoxication cascade by Withaferin-A during DMBA-induced oral carcinogenesis may also contribute to its protective efficacy.

#### Acknowledgement

Financial support from Indian Council of Medical Research (ICMR), New Delhi, to Mr. K. Panjamurthy, in the form of ICMR-SRF is gratefully acknowledged. Thanks are due to Dr C R Ramachandran, Faculty of Dentistry, Rajah Muthaiyah Medical College and Hospital, Annamalai University, Annamalainagar for pathological examinations.

#### References

- 1 Moore S R, Johnson N W, Pierce A M & Wilson D F, The epidemiology of mouth cancer: A review of global incidence, *Oral Dis*, 6 (2000) 65.
- 2 Muwonge R, Ramadas K, Sankila R, Thara S, Thomas G, Vinoda J & Sankaranarayanan R, Role of tobacco smoking, chewing and alcohol drinking in the risk of oral cancer in Trivandrum, India: a nested case-control design using incident cancer cases, *Oral Oncol*, 44 (2008) 446.
- 3 Miyata M, Furukawa M, Takahasi K, Gonzalez F J & Yamazoe Y, Mechanism of 7,12-dimethylbenz[a]anthracene induced immunotoxicity: Role of Metabolic activation at the target organ, *Jpn J pharmacol*, 86 (2001) 302.
- 4 Shklar G, Development of experimental oral carcinogenesis and its impact on current oral cancer research, *J Dent Res*, 78 (1999) 1768.
- 5 Ray G & Husain S A, Oxidant, antioxidants and carcinogenesis, *Indian J Exp Biol*, 40 (2002) 1213.
- 6 Manoharan S, Kolanjiappan K, Suresh K & Panjamurthy K, Lipid peroxidation and antioxidant status in patients with oral squamous cell carcinoma, *Indian J Med Res*, 122 (2005) 529.
- 7 Manoharan S, Kavitha K, Senthil N & Renju G L, Evaluation of anticarcinogenic effects of *Clerodendron inerme* on 7,12-dimethylbenz[a]anthracene induced hamster buccal pouch carcinogenesis, *Singapore Med J*, 471 (2006) 1038.

- 8 Mohan K V, Letchoumy P V, Hara Y & Nagini S, Combination chemoprevention of hamster buccal pouch carcinogenesis by bovine milk lactoferrin and black tea polyphenols, *Cancer Invest*, 26 (2008) 193.
- 9 Surh Y J & Chun K S, Cancer chemopreventive effects of curcumin, *Adv Exp Med Biol*, 595 (2007) 149.
- 10 Sharma S, Stuzman I D, Kelloff G J & Steele V E, Screening of potential chemopreventive agents using biochemical markers of carcinogenesis, *Cancer Res*, 54 (1994) 5448.
- 11 Sharada A C, Solomon F E, Devi P U, Udupa N & Srinivasan K K, Antitumour and radiosensitizing effects of Withaferin-A on mouse Ehrlich ascites carcinoma *in vivo*, *Acta Oncol*, 35 (1996) 95.
- 12 Bhattacharya S K, Satyan K S & Ghosal S, Antioxidant activity of glycowithanolides from *Withania somnifera*, *Indian J Exp Biol*, 35 (1997) 236.
- 13 Panjamurthy K, Manoharan S, Menon V P, Nirmal M R & Senthil N, Protective role of Withaferin-A on 7,12-dimethylben[a]anthracene induced genotoxicity in bone marrow of Syrian golden hamsters, *J Biochem Mol Toxicol*, 22 (2008) (in press).
- 14 Subramanian S S & Sethi P D, Withaferin-A from *Withania somnifera* coagulants roots, *Curr Sci*, 38 (1969) 267.
- 15 Dodge J F, Mitchell G & Hanahan D J, The preparation and chemical characterization of haemoglobin free ghosts of human red blood cells, *Arch Biochem Biophys*, 110 (1968) 119.
- 16 Quist E E, Regulation of erythrocyte membrane shape by  $Ca^{2+}$ , *Biochem Biophys Res Commun*, 92 (1980) 631.
- 17 Yagi K, Lipidperoxides and human disease, *Chem Physiol Lip*, 45 (1987) 337.
- 18 Donnan S K, The thiobarbituric acid test applied to tissues from rats treated in various ways, *J Biol Chem*, 182 (1950) 415.
- 19 Beutler E & Kelly B M, The effect of sodium nitrate on RBC glutathione, *Experientia*, 19 (1963) 96.
- 20 Omaye S T, Turnbull T D & Sauberlich H E, Selected method for the determination of ascorbic acid in animal cells, tissues and fluids, in *Methods of Enzymology* (Academic Press, New York), 1979, 3.
- 21 Desai F D, Vitamin E analysis methods for animals tissue, edited by S Feischer and L Packer, *Methods of Enzymology* (Academic Press, New York), 105, 1984, 138.
- 22 Kakkar P, Das B & Viswanathan P, A modified spectrophotometric assay of superoxide dismutase, *Indian J Biochem Biophys*, 21 (1984) 130.
- 23 Sinha K A, Colorimetric assay of catalase, *Anal Biochem*, 47 (1972) 389.
- 24 Rotruck J T, Pope A L, Ganther H E & Swanson A B, Selenium: biochemical roles as a component of glutathione peroxidase, *Science*, 179 (1984) 588.
- 25 Habig W H, Pabst M J & Jakoby W B, Glutathione-S-transferase the first enzymatic step in mercapturic acid formation, *J Biol Chem*, 249 (1994) 7130.
- 26 Carlberg I & Mannervik B, Glutathione reductase, in *Methods of Enzymology* (Academic Press, New York), 113, 1985, 484.
- 27 Omura T & Sato R, The carbon monoxide binding, pigment of liver, *J Biol Chem*, 239 (1964) 2370.
- 28 Ernster L, DT-diaphorase, in *Methods of Enzymology*, (Academic Press, New York) 1967, 309.
- 29 Wilkinson J & Clapper M, Detoxication enzymes and chemoprevention, *Proc Soci Exp Biol Med*, 216 (1997) 192.
- 30 Chandra Mohan K V P & Nagini S, Dose response effects of tomato lycopene on lipid peroxidation and enzymatic antioxidants in the hamster buccal pouch carcinogenesis model, *Nutr Res*, 23 (2003) 1403.
- 31 Coles B F & Kadlubar F F, Detoxication of electrophilic compounds by glutathione-s-transferase catalysis: determinants of individual response to chemical carcinogens and chemotherapeutic drugs? *Biofactors*, 17 (2003) 115.
- 32 Danson S, Ward T H, Beutler J & Ranson M, DT-diaphorase: a target for new anticancer drugs. *Cancer Treat Rev*, 30 (2004) 437.
- 33 Karezewski J M, Peters J G & Noordhoek J, Quinine toxicity in DT-diaphorase-efficient and deficient colon carcinoma cell lines, *Biochem Biopharmacol*, 57 (1999) 27.
- 34 Koul A, Ghara A R & Ganger S C, Chemomodulatory effects of Azadirachta India on the hepatic status of skin tumour bearing mice, *Phytother Res*, 20 (2006) 167.
- 35 Kavitha K & Manoharan S, Anticarcinogenic and antilipidperoxidative effects of *Tephrosia purpurea* (Linn.) Pers. in 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinoma, *Indian J Pharmacol*, 47 (2006) 1038.
- 36 Subapriya R, Velmurugan B & Nagini S, Modulation of xenobiotic-metabolizing enzymes by ethanolic neem leaf extract during hamster buccal pouch carcinogenesis, *J Exp Clin Cancer Res*, 24 (2005) 223.
- 37 Freig D T, Reid T M & Loeb C A, Reactive oxygen species in tumourigenesis, *Cancer Res*, 54 (1994) 1890.
- 38 Gutteridge J M C, Lipid peroxidation and antioxidants as biomarkers of tissue damage, *Clin Chem*, 14 (1995) 1819.
- 39 Manoharan S, Kolanjiappan K, Kayalvizhi M & Sethupathy S, Lipid peroxidation and antioxidant status in cervical cancer patients, *J Biochem Mol Biol Biophys*, 6 (2002) 225.
- 40 Vijayamalini M & Manoharan S, Lipid peroxidation, vitamins C, E and reduced glutathione levels in patients with pulmonary tuberculosis, *Cell Biochem Funct*, 22 (2004) 19.
- 41 Clemens M R and Waller H D, Lipid peroxidation in erythrocytes, *Chem Phys Lipids*, 45 (1987) 251.
- 42 Eritsland J, Safety considerations of polyunsaturated fatty acids, *Am J Clin Nutr*, 71 (2000) 197S.
- 43 Kolanjiappan K, Ramachandran C R & Manoharan S, Biochemical changes in tumor tissues of oral cancer patients, *Clin Biochem*, 36 (2003) 61.