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Tumour preventive potential of sclareol on 7, 12 dimethylbenz [a] anthracene (DMBA) induced hamster buccal pouch carcinogenesis

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ABSTRACT

Sclareol has demonstrated a broad variety of biological activities that belong to the antioxidant anti-inflammatory and anticancer activities that are used in traditional medicine. In the current study, we intended to explore the anti-tumor possible of sclareol along 7, 12-dimethylbenz (a) anthracene (DMBA) evoked golden Syrian hamster's buccal pouch carcinogenesis. Lipid peroxidation and antioxidants were assessed by the buccal tissue, and plasma of DMBA evoked golden Syrian hamster buccal pouch carcinogenesis of observational animals. We observed 100% of tumor establishment and noted defects in the biochemical restrictions of lipid peroxidation and antioxidants and also histopathological observations of carcinogenesis in the hamster's buccal pouch in DMBA only. Sclareol (20 mg/b.wt) has significantly inhibited the tumor burden, tumor volume, and increased phase II detoxification enzymes, antioxidant level, lipid peroxidation, and also phase I enzymes and has improved the histopathologically changes during carcinogenesis in the buccal pouch of DMBA induced golden Syrian hamster. The outcomes of the present scrutinized study proposed that aiming biochemically and histopathologically that act upon the inheritance of cancer characteristics is an effectual system for chemoprevention. Therefore, our results resolved that sclareol has probably owing to its antioxidant or scavenging properties of free radical and transition act on phase I and II enzymes in regards to the evacuation of metabolites carcinogenesis, which is evoked by DMBA in hamster's buccal pouch.

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INTRODUCTION

Squamous cell carcinoma of the oral cavity has been commonly and globally reported with an occurrence of over 4, 00,000 new reports per year (Saba *et al.*, 2015). It creates the highest rate of mortality and morbidity in India, where this carcinoma form records for 40% of all malignant tumors (Bagan *et al.*, 2010). Any form of tobacco with alcohol intake is known as the major risk factor for the higher incidence of oral cancer in India (Bassiony *et al.*, 2015). DMBA induced an experimental animal model for evaluating the chemoprevention study of oral cancer (Manoharan *et al.*, 2015). DMBA is commonly utilized as a major carcinogen

to develop a tumor in the golden Syrian hamster's buccal pouches (Karthikeyan *et al.*, 2013). It is a sensitive, excellent carcinogen to induce experimental oral carcinogenesis (Tanaka and Ishigamori, 2011). During the metabolism of DMBA, dihydrodiol epoxide by cytochrome p450 initiates the carcinogenic processing by evoking chronic inflammation via overproduces of anti-oxidants like ROS and NOS (Christou *et al.*, 1987). This application is relevant to biologically, physiologically, pharmaceutically, and histopathologically indicate the identical to the mammalian system (Tanaka and Ishigamori, 2011).

The detoxification enzymes are demanded to activate metabolites of the carcinogens such as phase I enzymes (cytochromes P450 and b5) and phase II enzymes (glutathione reductase -GR, glutathione-S-transferase -GST and reduced glutathione –GSH). The status of the above- mentioned detoxification enzymes and reduced glutathione to assess the potent chemoprevention of sclareol in experimental carcinogenesis. The carcinogenic substance of DMBA to converted epoxide due to produce ROS generation to the redox imbalance, which is damaged by biomolecules and deregulation of gene expression, which is due to produce the carcinogenesis including oral carcinogenesis (Silvan and Manoharan, 2013). The enzymatic and non-enzymatic antioxidants are naturally prevention of oxidative damage of ROS production while, disturbances antioxidants in the circulation system, its induced damaged DNA leads to cancer (Ohnishi *et al.*, 2013; Lobo *et al.*, 2010). Measurement of antioxidants also helps in assessing the chemopreventive potential of sclareol.

Sclareol is a diterpene compound isolated from Clary sage (Salvia sclarea L.), which is mainly purified from leaves and flowers. This compound commonly used in folk medicine such as essential oil used for the preparation of food, the cosmetic industry, and aromatherapy. Sclareol has potent cytotoxic properties of different types of human cancer cell lines such as gastric carcinoma, colorectal cancer, leukemia and osteosarcoma through the deregulation of c-myc is a protooncogene meddling with the cell progression and promoting apoptosis (Dimas *et al.*, 2001; Hsieh *et al.*, 2017; Mahaira *et al.*, 2011; Mohan *et al.*, 2006). However, the effect of chemoprevention of sclareol on investigational hamster buccal pouch carcinogenesis remains not understandable. Within the current study, we assess the chemoprevention potency of sclareol on DMBA evoked carcinogenesis in the buccal pouch of hamsters by analyzed the biochemical markers of lipid peroxidation and enzymatic antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, and non-enzymatic antioxidants such as reduced glutathione, glutathione-s-transference, vitamin C and vitamin E.

MATERIALS AND METHODS

Biochemicals

DMBA and Sclareol were acquired from Sigma Aldrich in India. Each other, all chemicals are used to applied analytical grade.

Feed and Animals

About 8 to 10 weeks old male hamsters (*Mesocricetus* auratus) and weighing between 90 to 100 g were bought from Biogen laboratory animals, Bangalore, India. The experimental animals were preserved at Central Animal House, Raja Muthiah medical college, Annamalai University, as per guidelines approved by the Institutional Animal Ethics Committee, according to the guidelines of CPCSEA, under a 12 hrs light/ dark cycle with adequate temperature and humidity.

Design of Experiment

An animal ethical committee has approved 36 animals were set up into 6 groups. The group1 animals represented as an untreated control group painted with liquid paraffin alone. Group 2 animals with painted 0.5% of DMBA in liquid paraffin weekly thrice for 112 days, Groups 3, 4, and 5 animals were painted with DMBA as well, orally administrated sclareol at various doses of 10,20 and 40mg/kg b.wt separately, weekly three times for 112 days. Group 6, animals were sclareol treated alone at 40mg/kg b.wt. These animals have sacrificed their lives at the end of the observational periods. Eventually, Biochemical and histopathological studies were executed.

Biochemical estimates

Lipid peroxidation was evaluated by the measurement of TBARS previously described by (Yagi, 1987; Ohkawa *et al.*, 1979). Glutathione peroxidase, catalase, and Superoxide dismutase were followed by (Kakkar *et al.*, 1984; Sinha, 1972; Rotruck *et al.*, 1973). The levels of Vitamin E, vitamin C, and GSH were analysed by (Desai, 1984). Cyt-P450 and Cyt-b5 were assayed by (Omura and Sato, 1964). GR, GST, and GSH were evaluated (Habig *et al.*, 1974; Carlberg and Mannervik, 1985; Beutler and Kelly, 1963).

Pathology of tissue

The Specimen of the sample was fixed with 10% formalin dehydrated within alcohol, diaphanized in

Table 1: Changes of body weight in Initial and final

Treatment schedule	Body weight Initial (g)	Body weight final (g)	Weight gain (g)	Growth rate (g)
Control	120.02±9.13^{a}	184.73±14.06^{a}	64.01±4.87^{a}	0.57±0.04^{a}
DMBA	121.02±9.21^{a}	146.27±11.19^{b}	25.21±1.93^{b}	0.22±0.01^{b}
DMBA + Sclareol (10 mg/kgb.wt)	120.06±9.18^{a}	165.72±12.61^{c}	65.72±12.61^{a}	0.40±0.03^{c}
DMBA + Sclaeol (20 mg/kg b.wt)	123.06±9.42^{a}	174.38±13.34^{a}	51.32±3.92^{c}	0.45±0.03^{d}
DMBA + Sclareol (40 mg/kg b.wt)	124.02±9.44^{a}	180.03±13.70^{ac}	56.01±4.26^{c}	0.50±0.03^{e}
Sclareol alone (40 mg/kg b.wt)	125.02±9.51^{a}	187.23±14.25^{a}	62.21±4.73^{a}	0.55±0.04^{a}

Data are expressed as mean ± SD for n=6 hamsters in each group. (a–d) are used to refer and distinguish the values of the different groups.
Values not sharing a common superscript differ significantly at P<0.05 (DMRT)

Table 2: Tumor formation, tumor size and tumor burden of tumor in observational and control animals

Parameters	Tumor formation	Tumor size	Tumor burdena (mm3)	Hyperplasi	Dysplasia	SCC (%)
Control	-	-	-	-	-	-
DMBA	15/6 (100%)	353.35±26.9	1060.0±80.71	+++	+++	100%
DMBA + Sclareol (10mg/kgb.wt)	5/6 (33%)	83.80±6.41	251.40±19.24	++	++	-
DMBA+ Sclaeol (20mg/kg b.wt)	0/6 (0%)	0	0	0	+	-
DMBA+ Sclareol (40mg/kgb.wt)	0/6 (0%)	0	0	-	+	-
Sclareol alone (40mg/kg b.wt)	-	-	-	-	-	-

+Mild, ++ moderate, +++ severe, - no change.
*Mean tumor burden was calculated by multiplying the mean tumor volume (4/3π) [D1/2][D2/2] [D3/2]

Table 3: The levels of TBARS and antioxidants in plasma

Treatment	TBARS (nmol/10(Plasma)	SOD (Ua/ml Plasma)	CAT (Ub/ml plasma)	GPx (Uc/ml plasma)	GSH (mg/100ml plasma)	Vitamin E (mg/100ml plasma)	Vitamin C (mg/100 ml plasma)
Control	2.25 ± 0.17^{a}	3.00 ± 0.22^{a}	1.13 ± 0.08^{a}	99.99 ± 7.61^{a}	28.30 ± 2.15^{a}	1.67 ± 0.12^{a}	1.79 ± 0.13^{a}
DMBA	5.38 ± 0.40^{b}	1.80 ± 0.13^{b}	0.53 ± 0.04^{b}	75.54 ± 5.78^{b}	17.51 ± 1.34^{b}	0.78 ± 0.05^{b}	0.99 ± 0.07^{b}
DMBA + Sclareol (10 mg/kgb.wt)	3.93 ± 0.29^{c}	1.50 ± 0.11^{c}	0.67 ± 0.04^{cd}	81.41 ± 6.20^{bc}	20.10 ± 1.53^{c}	0.84 ± 0.06^{b}	1.22 ± 0.09^{c}
DMBA + Sclaeol (20 mg/kg b.wt)	3.73 ± 0.28^{c}	1.92 ± 0.14^{b}	0.70 ± 0.05^{d}	83.34 ± 6.37^{bc}	23.21 ± 1.77^{d}	0.99 ± 0.07^{c}	1.29 ± 0.10^{c}
DMBA + Sclareol (40 mg/kg b.wt	$4.40\pm.33^{d}$	2.57 ± 0.19^{d}	0.60 ± 0.04^{bc}	86.71 ± 6.60^{c}	25.20 ± 1.92^{d}	1.32 ± 0.10^{d}	1.53 ± 0.11^{d}
Sclareol alone (40 mg/kg b.wt)	2.29 ± 0.17^{a}	3.05 ± 0.22^{a}	$1.14\pm.08^{b}$	99.96 ± 7.61^{a}	28.20 ± 2.15^{a}	1.66 ± 0.12^{a}	1.75 ± 0.13^{a}

Values are expressed as mean $\pm$ SD (n=6). Values that are not sharing common superscript letter between groups differ significantly at $p<0.05$ (DMRT)

xylene, and embedded into paraffin. Tissue sectors with 5μm thickness were obtained and stained by hematoxylin and eosin. The images were viewed beneath the light microscope (20$\times$), Olympus.

Data mathematical analysis

The results were carried out by mean $\pm$ S.D. The considerable differences among the groups were statistically evaluated by DMRT, followed by using an ANOVA method. The peak value is less than 0.05 was statistically considered.

RESULTS AND DISCUSSION

Body mass and growth rate variation

Body mass variations and the rate of growth of control and observational hamsters in each group were labeled in Table 1. In this study, from 0th week to 14th week, a significantly decreased body weight in the DMBA painted animals (Group 2), as compared with the normal animals. While Sclareol administrated (10, 20, and 40 mg/kg b.wt) orally in tumor animals (Groups 3, 4, and 5), the body mass and rate of growth were increased as compared with DMBA painted animals ($p \leq 0.05$). However, sclareol alone (Group 6) and (control 2) animals were no significant changes. Besides, sclareol with 20mg/kg b.wt has been shown more effective than other doses.

Tumor incidences

We observed a number of tumor formation, tumor size, and burden of tumor of control and observational animals shown in Table 2. We perceived a 100% tumor formation with the mean tumor size (353.35mm^3) and the burden of the tumor (1060.0 mm^3) in hamsters painted with DMBA (Group 2). Conventionally, the various concentration of sclareol in 10, 20, and 40mg/kg b.wt significantly reduced the formation of tumor and tumor size in DMBA painted hamsters (Group 3, 4, and 5). Group I control animals, and Group 6 sclareol alone treated animals showed in Figure 1 there is no tumor formation.

Histopathological evaluations

Figure 2 Shows the microscopic study of the tissue was carried by control and observational hamsters. The microscopic study of tissue in severe hyperplasia, hyperkeratosis, dysplasia, and well separated squamous cell carcinoma of the epithelium was seen

Table 4: The levels of TBARS and antioxidants in buccal tissues

Treatment schedule	TBARS (nmol/100 Protein)	SOD (Ua/mg Protein)	CAT (Ub/mg protein)	GPx (Uc/mg protein)	GSH (mg/100mg protein)	Vitamin E (mg/100mg protein)	Vitamin C (mg/100 mg protein)
Control	67.3±5.12^{a}	6.25±0.47^{a}	47.8±3.63^{a}	7.80±0.59^{a}	6.45±0.49^{a}	1.58±0.12^{a}	1.23±0.09^{a}
DMBA	39.9±3.05^{b}	3.77±0.28^{b}	27.8±2.12^{b}	16.51±1.26^{b}	12.54±0.96^{b}	3.62±0.27^{b}	3.29±0.25^{b}
DMBA+ Sclareol (10 g/kgb.wt)	42.9±3.26^{b}	3.38±0.25^{b}	29.5±2.24^{b}	4.50±1.10^{e}	9.95±0.76^{d}	3.10±0.23^{d}	2.87±0.21^{d}
DMBA+ Sclaeol (20 mg/kg b.wt)	60.2±4.61^{c}	5.60±0.42^{c}	38.5±2.94^{c}	11.23±0.85^{d}	9.32±0.56^{d}	2.41±0.18^{c}	1.32±0.10^{a}
DMBA + Sclareol (40 mg/kg b.wt)	55.2±4.20^{c}	5.45±0.41^{c}	36.5±2.78^{c}	9.47±0.72^{c}	7.32±0.71^{c}	2.89±0.21^{d}	2.3±0.20^{d}
Sclareol alone (40 mg/kg b.wt)	67.3±5.12^{a}	6.37±0.48^{a}	46.8±3.56^{a}	7.75±0.59^{a}	6.44±0.49^{a}	1.56±0.11^{a}	1.30±0.09^{a}

Data are expressed as mean ± S. D values for six hamsters in each group. Units for SOD, CAT and GPx are amount of enzyme. Values not sharing a common superscript letter (a–d) differ significantly at P<0.05 (DMRT)

in hamsters painted with DMBA as compared to control hamsters. Group 3 shows the properties of a neoplasm (hyperkeratosis, hyperplasia, and dysplasia) was seen in DMBA painted with sclareol treated hamsters (10, 20 and 40mg/B.wt). Although, more effect was observed at sclareol 20mg/kg b.wt (Group 4) as compared with other dosages. Only hamsters administered with sclareol showed normal growth patterns and basement membrane. This is an analogy to that of control hamsters (Group 1).

Effect of sclareol on anti-oxidant and lipid peroxidase status in plasma

TBARS's status, enzymatic and non-enzymatic antioxidants in the plasma of the control & observational animals are shown in Table 3. The range of TBARS were improved, in other hands an enzymatic antioxidant's (CAT, SOD, GSH and GPx) activity and non-enzymatic antioxidant's activity (GSH, Vitamins C and E) levels had considerably been reduced ($p \leq 0.05$) in tumor acquired animals (Group 2) as comparing with control animals. Orally administrative sclareol (10, 20 and 40mg/b.wt) to DMBA treated hamsters considerably brought again ($p \leq 0.05$) that the status of TBARS and antioxidant level nearly normal range. However, hamsters treated with sclareol only (Group 6) not showed considerable dissimilarity as comparing control animals (Group 1).

Benefits of sclareol on lipid peroxidation and antioxidant status in the buccal tissue

The levels of TBARS, enzymatic & non-enzymatic antioxidants status in the buccal pouch of control and observational animals have been shown in Table 4. There was a decrease in TBARS levels ($p \leq 0.05$), and interruption in antioxidant's status (vitamin E, GPx and GSH) were raised, CAT and SOD reduced ($p \leq 0.05$) and these were noticed in only DMBA treated animals (Group II) as comparing control animals. Oral administration of sclareol 10, 20 and 40mg/b.wt to DMBA painted animals brought again the previous concentration of TBARS and antioxidant agent nearly normal range. Furthermore, a sclareol dose of 20 mg/kg b.wt efficaciously brought the previous range of TBARS and antioxidants levels again. Only hamsters with sclareol treatment (Group 6) did not show considerable

Table 5: The status of phase I and phase II detoxification enzymes in liver of control and observational animals

Treatment schedule	Cytochrome-p450 (μmoles/mg protein)	Cytochrome-b5 (μmoles/mg protein)	GST (Ua/mg protein)	GR (Ub/mg protein)	GSH (Uc /mg tissue)
Control	0.78 ± 0.05^{a}	1.50 ± 0.11^{a}	29.70 ± 2.26^{a}	19.70 ± 1.50^{a}	2.75 ± 0.08^{a}
DMBA	2.53 ± 0.19^{b}	2.63 ± 0.20^{b}	18.51 ± 1.41^{b}	15.30 ± 1.17^{b}	1.54 ± 0.04^{b}
DMBA + Sclareol (10 g/kg b.wt)	2.30 ± 0.17^{d}	2.47 ± 0.18^{b}	24.90 ± 1.89^{c}	17.40 ± 1.32^{c}	1.82 ± 0.05^{c}
DMBA + Sclaeol (20 mg/kg b.wt)	1.97 ± 0.15^{c}	2.23 ± 0.12^{c}	27.51 ± 2.10^{ac}	18.81 ± 1.43^{a}	2.35 ± 0.07^{d}
DMBA+ Sclareol (40 mg/kg b.wt)	2.10 ± 0.16^{c}	2.10 ± 0.16^{d}	25.30 ± 1.92^{c}	15.70 ± 1.19^{b}	2.15 ± 0.06^{d}
Sclareol alone (40 mg/kg b.wt)	0.77 ± 0.05^{a}	1.49 ± 0.11^{a}	29.75 ± 2.26^{a}	19.67 ± 1.49^{a}	2.69 ± 0.08^{a}

Values are expressed as mean ± S.D (n=6). Values that are not sharing common superscript letter between groups differ significantly at p<0.05 (DMRT)

changes in TBARS and antioxidants status while comparing with untreated control hamsters (Group 1).

Effect of sclareol on Detoxification enzymes

Table 5 shows the status related to phase I (cytochrome P450 and b5) and phase II detoxification agents (GSH, GR, GST) in control and observational animals on every group. Firstly, phase I enzymes was considerably raised, and secondly, phase II enzymes were considerably ($p\leq0.05$) reduced in DMBA treated hamster (Group3). Various concentrations of sclareol at (10,20 and 40mg/kg b.wt) different doses on DMBA painted hamster significantly ($p\leq0.05$) improved phase II and reduced phase I enzymes comparing with control animals. Furthermore, the ability of outcome has been determined in sclareol at 20 mg/kg b.wt comparing with other doses. Sclareol alone handled hamsters (Group 6) have not shown variation in phase I and phase II enzyme activities.

Within the current study, the chemoreceptive outcome of sclareol was noticed in DMBA evoked observational carcinogenesis. 100% tumor development was noticed in DMBA treated animals (Figure 1), which was confirmed by histologically well-distinguished squamous cell carcinoma. Thereby, we pointed out severe hyperplasia, dysplasia, and hyperkeratosis, in hamster painted only with DMBA. Oral treatment of sclareol at 20mg/b.wt to DMBA evoked animals expressively reduced the tumor formation due to preventing the effect of irregular cell proliferation for the duration of oral carcinogenesis. The lipid peroxidation and antioxidant are applied as one of the methods to investigate the chemopreventive capacity of natural products (Waris and Ahsan, 2006).

Lipid peroxidation and protein oxidation are knowingly high in the DMBA group than the control group. The previous researches are reporting that DMBA brings on overcritical oxidative mutilation in the liver invivo (Letchoumy *et al.*, 2006; Manikandan *et al.*, 2007). The oxidative improved DNA addict may also the production of human carcinogenesis (Çakatay *et al.*, 2000). We observed that the effect of sclareol (20mg/b.wt) on plasma and the buccal pouch was considerably decreased in TBARS and lessened activities of superoxide dismutase and catalase in tumor acquiring hamsters would possibly be due to scavenging the extremely generated hydroxyl radicals and superoxide at the location of tumor tissues.

ROS mediated antioxidant stress defended by the crucial role of enzymatic and non-enzymatic antioxidants (Das and Roychoudhury, 2014) and decreased an enzymatical and non-enzymatical antioxidants

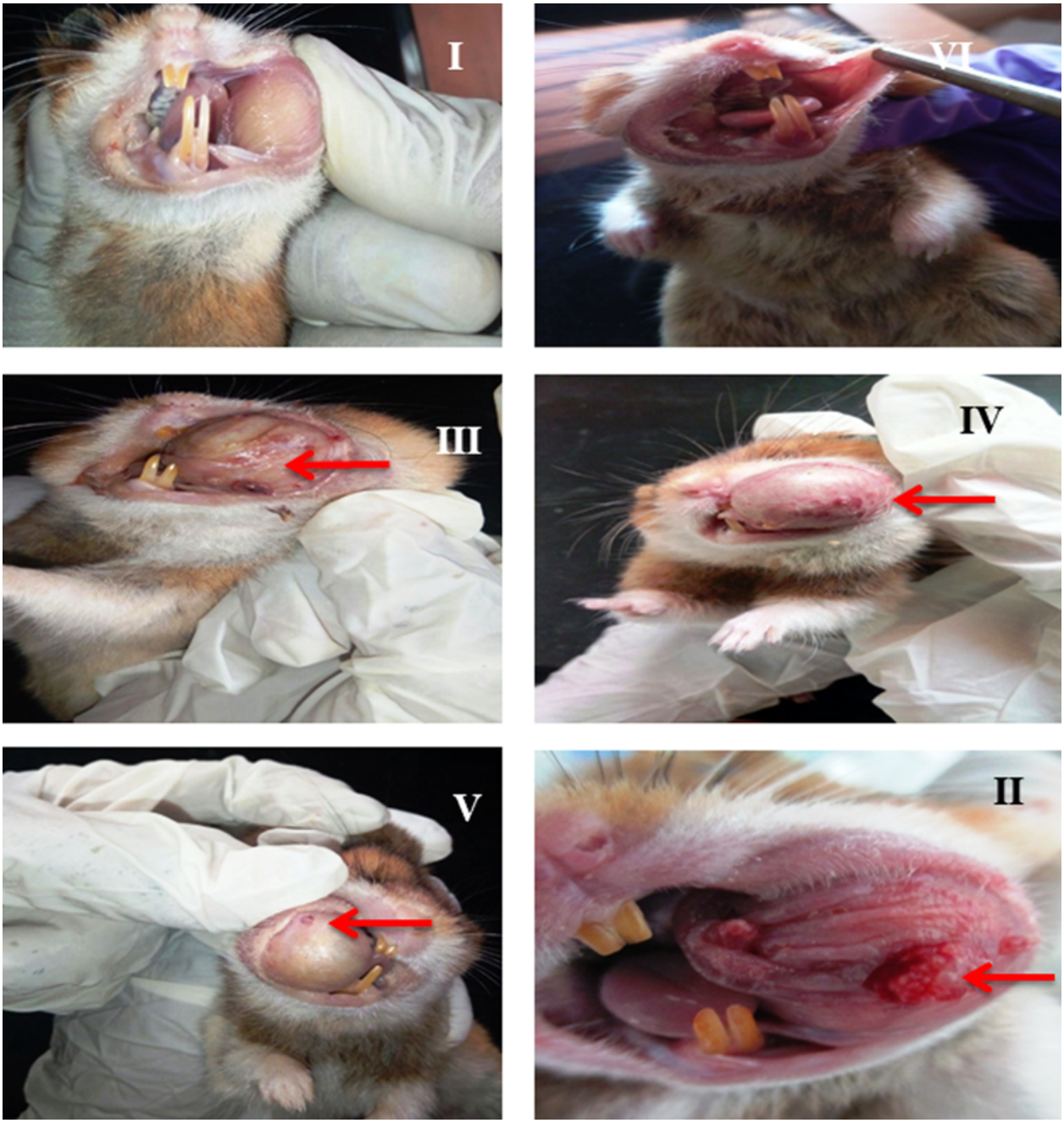

Figure 1: Gross appearance of buccal pouch mucosa of control and experimental animals Group I and Group VI: Normal buccal pouch Group II: well differentiated squamous cell carcinoma. Group III and IV: Precancerous oral epithelial layers. GroupV: Oral Administration of 20mg/b.wt sclareol was decreased tumour mass

level in the plasma of tumor acquiring an animal supports that meet their nutrient demands and supporting the raised level of lipid peroxidation byproducts in the circulation. Our observations of the results are recently finding a clear demonstration that sclareol has anticancer activity in human colon cancer and possesses the chemotherapeutical for the treatment of human cancer (Hatziantoniou *et al.*, 2006). Oral treatment of sclareol significantly enhanced the level of enzymatical and non-enzymatical antioxidants, which suggests that on the antioxidant role and free-radically scavenging property during the oral carcinogenesis.

The reaction of phase I and phase II detoxification enzymes had been reduced by the chemopreventive agents in favor of the excrement of carcinogenic metabolism (Silvan *et al.*, 2011). The Major function of glutathione-reductase creates re-formation and conservation of the level of rock-bottom glutathione in the circulation (Mohan *et al.*, 2006). The

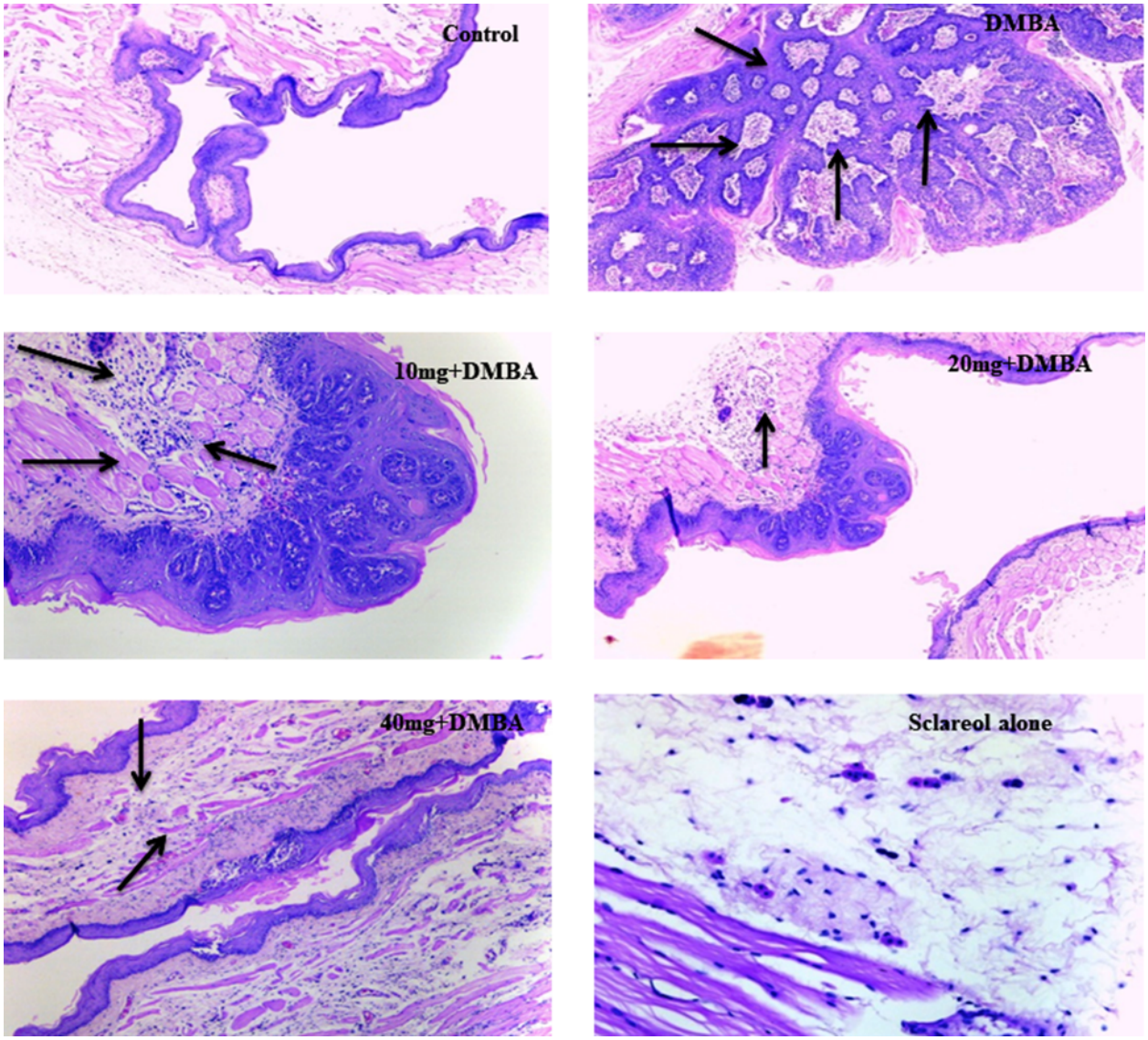

Figure 2: Histopathological features observed in the buccal mucosa of control and experimental animals. Well defined tumor mass present in hamster buccal pouch painted with DMBA. Tumour mass was decreased in DMBA induced cancer animals treated with sclareol. No significant abnormalities were noted in control and sclareol alone animals. H&E images were observed in (100X) Olympus microscope. Buccal pouch epithelium from DMBA group exhibits well-differentiated OSCC. Buccal pouch epithelium of DMBA and sclareol administrated exhibiting dysplasia, Control and sclareol alone animals exhibiting normal buccal pouch

raised action of phase I and diminished action of phase II detoxification enzymes in the tumor acquiring an animal's liver had been reported by former studies (Manoharan *et al.*, 2010). Defective behavior of detoxification agents in the buccal mucosa pointed out towards the opposite results of toxic DMBA metabolites, dihydrodiol epoxides (Manoharan *et al.*, 2010).

We observed the behavior of phase I and the behavior of phase II enzymes, and reduced glutathione levels were drastically modified in the hamster liver (treated by only DMBA), which suggests that their liver is highly exposed to carcinogenesis. Oral administration of sclareol contributed to the behavior of phase I and behavior phase II detoxification agents to almost standard level in their liver detoxification potential. The existing study thus proposes that sclareol regulated the behavior of phase I and behavior of phase II detoxification agents to stimulate the evacuation of the carcinogenic metabolite of DMBA and considerably upgrade the condition of antioxidants and lipid peroxidation in DMBA evoked hamster's buccal pouch carcinogenesis.

CONCLUSIONS

We concluded that the dietary phytochemical of sclareol has contributed to the prevention of cancer progression by lipid peroxidation and antioxidant grade in DMBA-evoked golden Syrian hamster's buccal pouch carcinogenesis. Moreover, the anticancer activity of sclareol has used in anticancer drugs for chemotherapeutics.

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