

Dose response screening of free and encapsulated ellagic acid against 7,12-Dimethylbenz(a)anthracene induced oxidative stress on hamster buccal pouch carcinogenesis

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Abstract

Ellagic acid, a phenolic phytonutrient has become a focus of intense research owing to its role in prevention and treatment of cancer. In the present study, we proposed to screen the dose response effect of free ellagic acid (EA) and ellagic acid encapsulated nanoparticles (EANP) against DMBA induced oxidative stress on hamster buccal pouch model. DMBA (0.5% in mineral oil) was topically applied to the left buccal pouch of male Syrian hamsters 3 times a week for 14 weeks. Treatment groups received EA (20, 40, 80 mg/kg bw) and EANP (10, 20, 40 mg/kg bw) via oral gavage 3 times a week from 10 to 21 weeks. Animals were sacrificed at the end of the experimental period and free radical mediated oxidative damage was estimated using various biochemical markers such as lipid peroxidation and antioxidants (GSH, SOD, CAT & GPx) which are the key indicators for cancer risk at the precancerous stage. DMBA induced positive controls showed altered levels of lipid peroxidation which is associated with diminished cellular antioxidant status. Treatment with EA and EANP significantly augmented the activities of cellular antioxidants and ultimately diminished the levels of lipid peroxidation which point towards suppression of preneoplastic lesions thereby reduces the cancerous risk. Thus from the aforementioned results it is showed that treatment with EA at the dose of 40 mg/kg bw and EANP at the dose of 20 mg/kg bw was found to be the optimal dose which proved antioxidant activity against DMBA induced oxidative stress on hamster buccal pouch carcinogenesis.

Keywords: 7,12-dimethylbenz(a)anthracene, Ellagic acid, Ellagic acid nanoparticles, Lipid peroxidation, antioxidants

Introduction

Oral cancer is the sixth most common malignancy worldwide and one with high mortality ratio among all malignancies. The most

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common intra oral malignancy is squamous cell carcinoma (Gorsky et al. 1994). Some of the major risk factors of OSCC are excessive tobacco chewing with or without betel quid, smoking and alcohol consumption (Gupta and Nandhakumar 1999, Notani 2000). The global incidence of OSCC was estimated to be 4,05,318 about two-thirds of them arising in developing countries. It is found that the five year survival rate in the statistics has not improved for the last few decades (Oral cancer – statistics 2009). Eventhough, a vast number of cancer research has been devoted in the development of antineoplastic drugs, the prognosis of the disease is often challenging due to increased side effects. In recent years, natural dietary agents especially fruits and vegetables have drawn a great deal of attention both from researchers and from the public because of their potential ability to suppress cancers as well as reduce the risk of cancer development (Amin 2009). These dietary substances have a variety of bioactive compounds among which polyphenols have great interest due to their antioxidant activity as well as their possible beneficial implications on human health especially on cancer treatment (Bravo 1998).

Ellagic acid, a well known polyphenolic antioxidant is naturally derived from various fruits and nuts such as raspberries, straw berries, crane berries, black berries, pomegranate, grapes, walnuts, peacans etc. It may occur in its free form or more commonly as a breakdown product of hydrolysable tannins specifically ellagitannins. EA has been reported to be associated with various remedial properties such as anticancer, antidiabetic, atherosclerosis, hepatoprotective and antimicrobial activities (Vattem and Shetty 2005). Numerous scientific reports both on *in vivo* and *in vitro* models validate its use mainly as an anticancer agent. EA has been shown to inhibit the development of cancer in animal models of colon, oesophageal, liver, lung, tongue and skin cancer (Arulmozhi and Mirunalini 2010). Despite its beneficial role against cancer treatment, EA was not up to the mark to meet the clinical trials due to its low bioavailability. In order to overcome this problem, a suitable drug delivery system is needed to render its full potential. Nowadays, researchers are showing considerable interest in developing nanocarriers for effective drug delivery.

Biodegradable polymeric nanocarriers have gained considerable interest in this regard (Peer et al. 2007). These polymeric

nanocarriers by virtue of its size and surface properties can improve the oral bioavailability of hydrophobic drugs due to their specialized uptake mechanism. Chitosan, a polymeric nanocarrier derived from partial deacetylation of chitin which is a polymer of glucosamine and N-Acetyl-D-glucosamine linked together by β (1,4) glycosidic bonds (Gan and Wang 2007). Owing to its beneficial properties such as biocompatible, biodegradable, non-toxic, low-immunogenicity as well as a favourable mucoadhesiveness and the ability to increase membrane permeability it is used in different biomedical and drug delivery applications (Schuetz et al. 2008, Wu and Sailor 2009). Hence chitosan is a promising nanocarrier to release the encapsulated anticancer drug to the acidic environment of tumor tissues.

As we aimed to investigate the anticancer effect of nanoencapsulated EA mainly for its targeted drug delivery against oral carcinogenesis, herein we made an attempt to study the dose response screening of free EA and EANP against DMBA induced oxidative stress on hamster buccal pouch carcinogenesis.

Materials and Methods

Chemicals

Ellagic acid, chitosan, sodium tripolyphosphate, 7,12-Dimethyl benzanthracene (DMBA) were obtained from Sigma-Aldrich chemicals Co. (St. Louis, Mo.USA). All other chemical and reagents were of analytic grade.

Preparation of ellagic acid encapsulated chitosan nanoparticles

Ellagic acid loaded chitosan nanoparticles were synthesized by ionic gelation method using tripolyphosphate as a gelating agent (Li et al. 2010). A known amount of chitosan was dissolved in 1% (V/V) acetic acid and allowed to stir for 1 hr. 3 mg/ml EA was then added

to the freshly prepared chitosan dispersion. The pH of the medium was maintained at 5.0 using 1M NaOH and then further stirred for 1 hr. Finally, 1mg/ml of IPP was added to the chitosan-EA solution under mild magnetic stirring. The resulting mixture was allowed to stir for 1 hr to form a EA encapsulated chitosan nanoparticles. The EA loaded chitosan nanoparticles was isolated by centrifugation and lyophilized. The lyophilized samples were characterized by SEM, FTIR and particles size analyser (Arulmozhi et al. 2011).

Animals and Experimental design

Male Syrian Golden Hamsters weighing (60-80 g), were procured from National Institute of Nutrition, Hyderabad, India. All the animals were housed in well ventilated polypropylene cages and were acclimatized to laboratory conditions under controlled temperature and humidity with an automatic 12h light/dark cycle for 1 week before the commencement of the treatment. The animals were given standard pellet diet and water ad-libitum throughout the experimental period. The experimental protocol was duly approved by the Institutional Animal Ethics Committee (IAEC), Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University and the experiments was performed in accordance with the CPCSEA guidelines.

Figure 1 illustrates the experimental protocol. The chemotherapeutic model of DMBA induced hamster buccal pouch carcinogenesis was modified from Salley et al. 1954. Animals were randomized in to 10 groups of six animals each. Group 1 animals served as control. The left buccal pouches of the hamsters in groups 2 were painted with 0.5% DMBA in mineral oil using No.4 painting brush, thrice a week from 0-14 weeks. Each applications delivers approximately 0.5 mg of DMBA. Group 2 animals served as positive control and received no further treatment. Animals in group 3-8 were painted with DMBA for 0-10 weeks and further the animals were treated with different doses of free EA (20, 40 & 80 mg/kg b.wt) and EANP (10, 20 & 40 mg/kg b.wt) via oral gavage thrice a week from 10-21

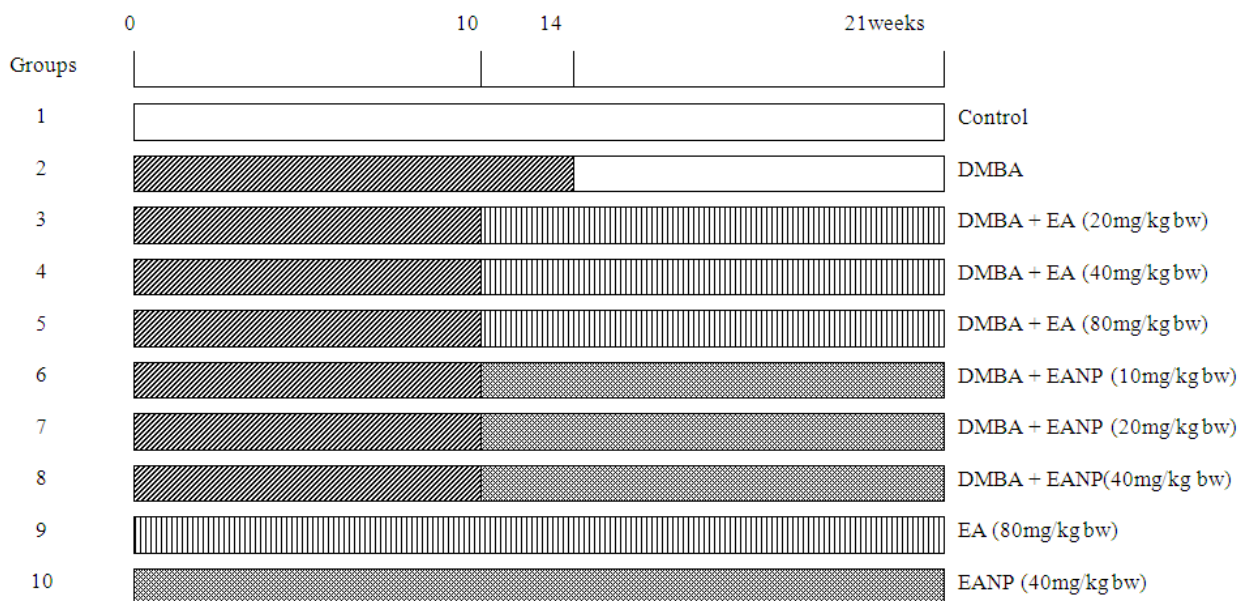


Figure 1: Schematic representation of experimental protocol

weeks. Animals in group 9 & 10 received free EA (80 mg/kg b.wt) and EANP (40 mg/kg b.wt) which served as drug controls. The experimental period terminated by the end of 21 week and all the animals were sacrificed by cervical decapitation after an overnight fast. During sacrifice, blood samples were collected for plasma separation and the left buccal pouches were harvested. The buccal pouches were excised and the tissues were further processed for estimating various biochemical parameters.

Biochemical analysis

The lipid peroxidation was estimated by measuring the levels of TBARS in plasma and buccal tissue. The plasma TBARS was assayed by the method of Yagi 1987 and the buccal tissue was estimated according to the method of Ohkawa et al. 1979. The GSH levels in plasma and buccal was done by the method of Ellman 1959. The activities of enzymatic antioxidants such as superoxide dismutase in plasma and buccal was done by the method described by Kakkar et al. 1984, the catalase activity in plasma and buccal was assayed using the method employed by Sinha 1972 and the activity of GPx in plasma and buccal was determined by Rotruck et al. 1973.

Statistical analysis

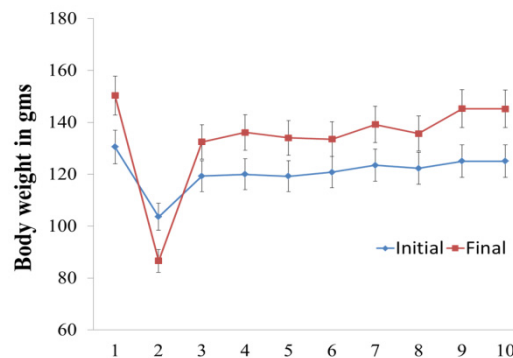
All the datas were expressed as mean ± SD. The comparison between the groups were analysed by one way analysis of variance (ANOVA) followed by Duncans Multiple Range Test (DMRT). The statistical analyses were done using computer software SPSS 11.5. Differences with calculated *P*-values <0.05 were considered as significant.

Results

Figure 2 shows the mean body weight of control and experimental animals. There was a significant decrease in the final body weight of cancer bearing animals (group 2) when compared to group 1 control animals. On the contrary, administration of EA and EANP increased the mean final bodyweight to near normal when compared with group 2 animals. No significant changes were observed in control and control treated groups.

Table 1 shows the levels of TBARS and GSH in plasma and buccal tissue of control and experimental groups. In DMBA induced group 2 hamsters, elevated levels of TBARS were observed in plasma where as in the buccal tissue the levels were found to be reduced when compared to untreated control group. On therapeutic treatment with EA and EANP, it significantly reversed the changes to near normal levels. Among other doses, EA at 40 mg/kg bw and EANP

at 20 mg/kg bw were found to be more effective. Administration of EA and EANP alone showed no significant changes when compared to group 1 control.



Groups
 1-Control, 2-DMBA, 3-DMBA+EA (20 mg/kg bw), 4-DMBA+EA (40 mg/kg bw), 5-DMBA+EA (80 mg/kg bw), 6-DMBA+EANP (10 mg/kg bw), 7-DMBA+EANP (20 mg/kg bw), 8-DMBA+EANP (40 mg/kg bw), 9-EA alone, 10-EANP alone

Figure 2: Illustration of body weight of control and experimental animals.

In group 2 tumor bearing animals, the GSH levels was significantly decreased in plasma and significantly increased in buccal tissue when compared to group 1 control animals. On supplementation with EA & EANP to experimental animals, the GSH levels was markedly raised in plasma and lowered the levels in buccal tissues when compared with DMBA untreated hamsters. Among other doses in the treatment group, medium dose of EA (40 mg/kg bw) & EANP (20 mg/kg bw) was found to be optimum and their levels were near normal to the control groups. In particular the medium dose of EANP was observed to be non-significant with the controls.

Figure 3 represents the activities of SOD in plasma and buccal tissue of control and experimental animals. In DMBA induced untreated group, the activity of SOD was significantly decreased in plasma and buccal tissue when compared to untreated control. Treatment with EA at the dose of 40 mg/kg bw and EANP at the dose of 20 mg/kg bw significantly increased SOD activity to near normal when compared to other DMBA treated and untreated groups. In addition, no significant changes were observed in control and control treated groups.

Table 1. Effects of EA and EANP on DMBA induced alterations in TBARS and GSH of control and experimental groups

Groups	Treatment	TBARS		GSH	
		Plasma (nm/ml)	Buccal (nm/mg)	Plasma (mg/dl)	Buccal (mg/dl)
1	Control	2.65±0.14	78.75±5.87	31.55±2.21	8.15±0.79
2	DMBA	4.20±0.36 ^{a*}	40.25±2.72 ^{a*}	16.24±1.11 ^{a*}	17.95±1.31 ^{a*}
3	DMBA+EA (20 mg/kg bw)	3.77±0.23 ^{b*}	49.03±3.64 ^{b*}	21.18±1.85 ^{b*}	15.32±1.42 ^{b*}
4	DMBA+EA (40 mg/kg bw)	3.12±0.24 ^{b**}	63.51±4.78 ^{b**}	27.22±2.34 ^{b**}	10.18±0.81 ^{b**}
5	DMBA+EA (80 mg/kg bw)	3.45±0.21 ^{b***}	56.75±3.54 ^{b***}	24.02±2.14 ^{b***}	13.87±0.79 ^{b***}
6	DMBA+EANP (10 mg/kg bw)	3.59±0.25 ^{c*}	54.32±3.12 ^{c*}	22.14±1.86 ^{c*}	14.16±0.94 ^{c*}
7	DMBA+EANP (20 mg/kg bw)	2.98±0.20 ^{c**}	75.28±5.83 ^{NS}	29.84±2.10 ^{NS}	9.02±0.81 ^{NS}
8	DMBA+EANP (40 mg/kg bw)	3.29±0.28 ^{c***}	63.15±4.88 ^{c**}	25.78±2.32 ^{c**}	12.11±1.14 ^{c**}
9	EA (80 mg/kg bw)	2.50±0.24 ^{NS}	80.75±7.66 ^{NS}	33.33±3.11 ^{NS}	8.56±0.77 ^{NS}
10	EANP (40 mg/kg bw)	2.55±0.18 ^{NS}	80.51±4.48 ^{NS}	32.00±2.16 ^{NS}	8.34±0.73 ^{NS}

Values are expressed as mean ± SD. *P*<0.05 NS – non-significant. *a***P*<0.05 of DMBA positive control group compared with control group, *b***b**b***P*<0.05 of EA groups compared with DMBA untreated group, *c*c**c***P*<0.05 of EANP groups compared with DMBA untreated group.

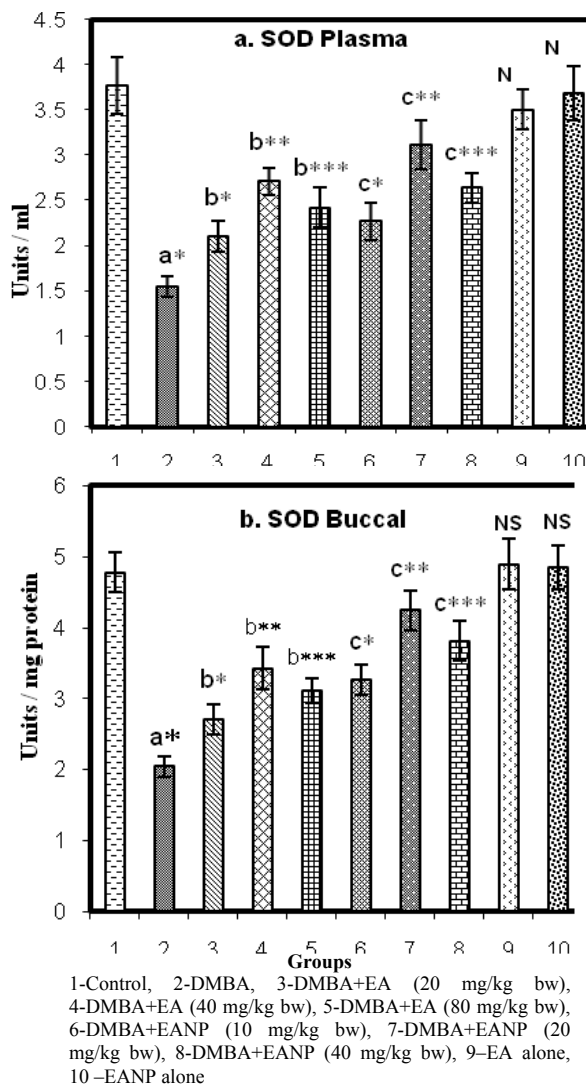


Figure 3: Effect of EA and EANP on the activities of SOD a. plasma & b. buccal of control and experimental animals. Values are expressed as mean \pm SD. Units for SOD was expressed as the amount of enzyme required to inhibit 50% of NBT reduction. $P < 0.05$ NS – non-significant, a* $P < 0.05$ of DMBA positive control group compared with control group, b*b**b**** $P < 0.05$ of EA groups compared with DMBA untreated group, c*c**** $P < 0.05$ of EANP groups compared with DMBA untreated group.

DMBA induced group 2 animals exhibited a significant decrease in catalase activity in both plasma and buccal tissue than that of untreated control group (figure 4). Upon administration with EA and EANP, the medium dose was found to be effective when compared to group 2 and other DMBA treated groups. Control and control treated groups showed no significant changes.

Figure 5 shows the GSH-Px activity of control and experimental animals. In group 2 DMBA induced animals, the GSH-Px activity was significantly lower in plasma while the buccal tissue was found to be higher when compared to group 1 hamsters. However, treatment with EA and EANP reversed these changes to near normal when compared to DMBA induced group. Among other doses of treatment, EA (40 mg/kg bw) and EANP (20 mg/kg bw) showed greater effect and was near normal to the untreated control group. The groups such as control and control treated hamsters showed no significant changes.

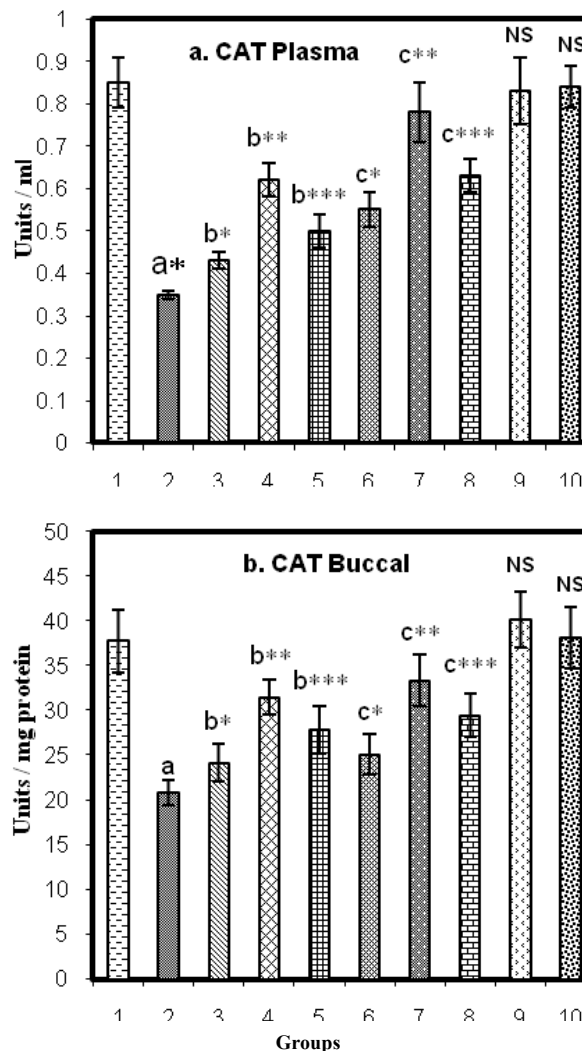
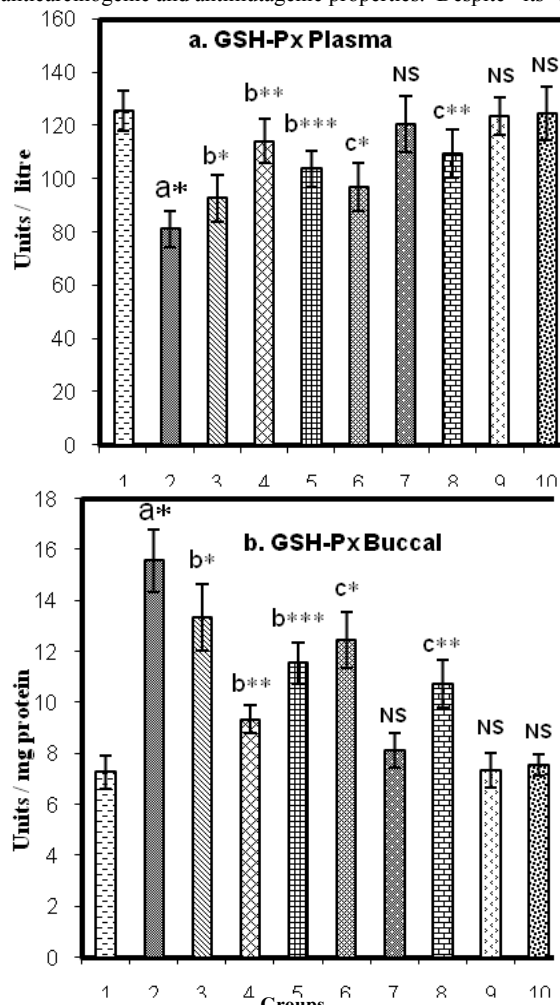


Figure 4: Effect of EA and EANP on the activities of CAT a. plasma & b. buccal of control and experimental animals. Values are expressed as mean \pm SD. Units for CAT was expressed micromoles of H_2O_2 utilized/second. $P < 0.05$ NS – non-significant, a* $P < 0.05$ of DMBA positive control group compared with control group, b*b**b**** $P < 0.05$ of EA groups compared with DMBA untreated group, c*c**** $P < 0.05$ of EANP groups compared with DMBA untreated group.

Discussion

A successful chemotherapy for cancer generally depends upon the effective delivery of the anticancer drug to the target site without causing any intolerable toxicity to the patients (Natalie and Tarun 2007). This can be achieved by nanoparticle drug delivery system which is a far more effective approach in chemotherapy rather than other conventional therapies. To have an effective drug delivery, chitosan a polymeric nanocarrier has been chosen due to its mucoadhesive property which enhances drug transmucosal absorption (Reverchon and Antonacci 2006) and promotes sustained drug release to the target site (Akbuga 1993).

Nowadays, phytonutrients are emerging as a promising agent in prevention and treatment of various cancers. Ellagic acid, a phenolic phytonutrient and a powerful antioxidant is mainly focused on its anticarcinogenic and antimutagenic properties. Despite its well



1-Control, 2-DMBA, 3- DMBA+EA (20 mg/kg bw), 4-DMBA+EA (40 mg/kg bw), 5-DMBA+EA (80 mg/kg bw), 6-DMBA+EANP (10 mg/kg bw), 7-DMBA+EANP (20 mg/kg bw), 8-DMBA+EANP (40 mg/kg bw), 9-EA alone, 10 -EANP alone

Figure 5: Effect of EA and EANP on the activities of GSH-Px a. plasma & b. buccal of control and experimental animals. Values are expressed as mean \pm SD. Units for CAT was expressed as micromoles of glutathione utilized/minute. $P < 0.05$ NS – non-significant, a* $P < 0.05$ of DMBA positive control group compared with control group, b*b***b**** $P < 0.05$ of EA groups compared with DMBA untreated group, c*c** $P < 0.05$ of EANP groups compared with DMBA untreated group.

established scientific records regarding its potential activity against various types of cancers (*in vivo* and *in vitro*), its clinical evidence is however much more limited due to its poor oral bioavailability. In an attempt to explore its therapeutic potential, EA was encapsulated using chitosan nanoparticles and this novel formulation has been applied to evaluate its anticancer activity. In the present study we therefore investigated the anticancer activity of free EA and EANP against DMBA induced oxidative stress on oral carcinogenesis in a dose dependent manner. In this context, the buccal pouch of the Syrian golden hamsters was selected for our study which is a widely used animal cancer model for the carcinogenic induction of buccal

squamous cell carcinoma. DMBA, a chemical carcinogen is associated with oxidative metabolism leading to the formation of reactive metabolites which are capable of generating free radicals thereby eliciting cancerous response (Yuk-Kwan and Li-Min 2010). The investigation of the present study initially focused on the weight of the animals on both control and experimental groups. DMBA induced cancer bearing animals was observed to have a bodyweight loss while the control groups continue to gain weight until the termination of the study. However treatment with EA and EANP to DMBA induced hamsters showed a better bodyweight gain when compared to DMBA induced positive control. Results from the previous workers suggest that ellagic acid inhibits cancer induced by various chemical carcinogens (Hannum 2004, Heur et al. 1992, Mandal et al. 1987) which strongly supports our study.

Oxidative stress, especially LPO is well known to be involved in carcinogenesis (Trush and Kensler 1991). Uncompromised generation of free radicals and lipid peroxidation are together associated during the initial stage of carcinogenesis altering the normal biochemical process (Davis and Kuttan 2001, Rice-Evans and Burdon 1993). Numerous studies have demonstrated that DMBA induced oxidative stress is associated with a wide range of macromolecular damage such as lipids, proteins and nucleic acids thereby producing interrelated derangements of cellular metabolism including peroxidation of lipids. Free radical induced lipid peroxidation is considered as one of the basic mechanism for tissue damage and the extent of tissue damage can be estimated by TBARS in plasma. The increased plasma TBARS were observed in oral cancer patients and also in experimental oral carcinogenesis (Balasenthil et al. 2000, Bhuvaneshwari et al. 2001). Our results also corroborate with the above findings where DMBA induced tumor bearing animals showed increased plasma TBARS while the buccal tissue was found to be decreased which could be due to decreased PUFA content in tumor tissues or abnormal proliferation occurring in oral carcinogenesis (Barrera et al. 1991, Rasheed et al. 2007). Administration of EA & EANP exhibited significant dose dependent alterations in the plasma and buccal TBARS which is presumably due to its ability to inhibit free radical generation (Ahmet et al. 2010). Previous reports also suggest that EA exerts its potent scavenging action on superoxide anion and hydroxyl anion *in vitro* as well as its protective effect against lipid peroxidation (Lino et al. 2001). Hence from the above mentioned doses it is speculated that EA (40 mg/kg bw) & EANP (20 mg/kg bw) effectively scavenged free radicals thereby reduces oxidative damage.

Endogenous antioxidants present in the biological system functions interactively and synergistically to neutralize free radicals and also involved in various actions such as prevention, interception and repair (Devasagayam et al. 2004). GSH, an important non-protein thiol present in mammalian cells which participates in a variety of detoxification reactions (Khanduja and Majid 1993). It is an extremely important cell protectant, directly quenches reactive hydroxyl free radicals, other oxygen-centered free radicals and radical centers on DNA and other biomolecules (Kidd 1997). During cancer conditions, the rate of cancer cell proliferation was accompanied by changes in their intracellular GSH levels and consequently these could be reflected in their antioxidant mechanism (Navarro et al. 1999). In line with the above findings our results showed, depleted GSH levels in plasma while in the buccal tissue the levels were found to be raised in DMBA untreated group. The decreased plasma GSH levels could be due to increased utilization of the antioxidant by the cancer cells in circulation to supply the demand of the growing tumor (Buzby et al. 1980). The increased GSH levels in tumor bearing buccal reflects an increased detoxification capacity, by which tumor cells gain a selective growth advantage over their surrounding normal cells (Mirunalini et

al. 2004). Following treatment with EA and EANP reversed these changes to normalcy. Among other doses of treatment EA at 40 mg/kg bw and EANP at 20mg/kg bw was found to be more effective which might be due to increased free radical scavenging activity thereby alleviates oxidative stress induced carcinogenesis.

Enzymatic antioxidants such as SOD, CAT and GPx are considered as preventive antioxidants which attempt to terminate the formation of reactive oxygen species. SOD a first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide (O_2^-) to H_2O_2 . CAT is a peroxisomal haemprotein that catalyses the removal of H_2O_2 formed during the reaction catalyzed by SOD. SOD and CAT acts mutually supportive antioxidant enzymes, which provide protective defence against ROS (Ashok Kumar and Sudhandiran 2008). During cancerous conditions tumor cells have abnormal activities of antioxidant enzymes (Oberley and Oberley 1986). Several reports have showed decreased SOD and CAT activity in various cancer studies (Floyd 1982, Ramakrishnan et al. 2006). In the current study, we also observed decreased SOD and CAT activities in both plasma and buccal tissue in DMBA untreated group. This decreased activity could be due to inefficient scavenging of free radicals which might be implicated to oxidative inactivation of enzymes (Karthiswaran and Mirunalini 2011).

GSH plays a key role as a cofactor with a variety of enzymes including GSH-Px, which acts as a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging ROS (Micheiels et al. 1994). GSH-Px, neutralizes hydroxyl radicals and singlet oxygen. When present in high concentration in the cells, it protects cells from free radical attack (Arthur 2000). In the present investigation, there was a decline in the plasma GSH-Px activity and rise in the buccal GSH-Px activity in DMBA induced positive control group this may be due to enormous production of free radicals due to DMBA induction. Our results correlate also with the report of Mirunalini et al. 2004.

Numerous reports suggest that phenolic compounds have the ability to scavenge excess free radicals which suggest to hinder the process of carcinogenesis (Arts and Hollman 2005, Chandra Mohan et al. 2005). These studies support well with our findings where supplementation of EA and EANP exhibited remarkable changes in SOD, CAT and GSH-Px in DMBA treated groups. This could be due to the free radical scavenging and antioxidant activity of Ellagic acid. Existing report state that ellagic acid can act as a chain breaking antioxidant whose hydroxyl group can readily react with chain carrying peroxy radicals thus terminating the propagation of free radical mediated reactions (Gil et al. 2000). On the other hand EA besides acting as an antioxidant, enhances the antioxidant defence system thereby inhibits oxidative stress and prevents tissue injury (Ram Sudheer et al. 2007). Previous workers also proved the antioxidant potential of ellagic acid in different cancer models (Khanduja et al. 1999, Majid et al. 1991). Thus our results corroborate well with the above findings.

Taken together, DMBA induced oral cavity cancer increases oxidative stress by diminishing the cellular antioxidant which confirms precancerous stage in hamsters. Administration of EA at the dose of 40 mg/kg bw and EANP at the dose of 20 mg/kg bw was found to be the optimal dose which effectively restored the antioxidant levels to normal. This resulted in decreased oxidative damage which may probably halt or suppress the progression of precancerous cells prior to malignant development. When compared to free ellagic acid, nanoencapsulation of ellagic acid showed greater activity. This may be due to increased bioavailability and sustained drug release.

Conclusion

The results presented in the study highlights the effect of free and nanoencapsulated EA against oxidative stress at the post-initiation stage of oral squamous cell carcinoma using DMBA induced hamster model. Throughout our study we could find that encapsulated EA showed greater activity when compared to free EA which could be due to the improved bioavailability of the drug. Hence this preliminary study of dose fixing will facilitate us to undergo a detailed investigation on ellagic acid encapsulated nanoparticles against oral squamous cell carcinoma which may pave way to establish a novel anticancer agent for an effective treatment against oral cancer.

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