CHAPTER- 4
Histological and immunohistochemical study of the cell proliferation (cyclin D1) expression in silibinin-loaded nanoparticles in DMBA-induced oral carcinogenesis

4.1. Introduction

Oral cancer is one of the most common cancers and it constitutes a major health problem particularly in developing countries and one-half of all head and neck cancer occur in the oral cavity. In 2011, oral squamous cell carcinomas (OSCC) accounted for nearly 3% of all cancer cases worldwide; its estimated incidence is approximately 275,000 cases per year, with two-thirds of these cases occurring in developing countries (Jemal et al., 2011). The majority of oral cancers (90%) are SCC, etiologically linked independently to lifestyle, with major risk factors being tobacco and alcohol exposure, with reported synergism between the two risk-enhancing behaviors. These exposures create an oral microenvironment that is high at-risk for premalignant initiation and malignant progression (Warner et al., 2014). In addition to smoking, the use of smokeless tobacco has been strongly linked to oral. Since, the oral cavity is more accessible to complete examination; it could be used in early detection of pre-cancerous and cancerous lesions. But, either due to ignorance or inaccessibility of medical care, the disease gets detected in the later stages. Because the conventional therapies, including surgical resection, chemotherapy and radiation are often inadequate in treating this disease, new treatment options are critically needed. Therefore, it is important to establish chemoprevention in an experimental animal tumor model that mimics
specific characteristics of human OSCC before embarking on clinical trials.

A number of complex mechanisms are involved in the genesis and progression of oral cancer. OSSC is a multistep process in which multiple genetic events occur that alter the normal function of oncogenes and tumor suppressor genes. These events can result in the increased production of growth factors. Recent advances in the understanding of the molecular control of these various pathways will allow for more accurate diagnosis and assessment of prognosis and might lead the way for more novel approaches for treatment and prevention (Calixto et al., 2014). The role of biomarkers in cancer detection and progression is a major effort at various laboratories aimed at the development of novel and simple approaches for early detection of human cancer (Linkov et al., 2007). Molecular profiling studies, the major contributors of cancer biomarker discoveries, are based on an association or correlation between a molecular signature and cancer behavior. Chemical agents like poly aromatic hydrocarbon (PAH) appear to be the dominant etiological factor in oral cancer and use of carcinogen induced animal model is required to assess efficiency of new therapeutic approaches (Kleiner et al., 2004; Nagini et al., 2009). Experimental cancer model using hamster buccal pouch (HBP) and topical application of a chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) a member of PAH is an optimal model for the study of oral cancer. The HBP is covered by a thin layer of stratified squamous epithelium that is very similar to the floor of the mouth and the ventral surface of the tongue in humans, which is the most common site of human OSCC. OSCC induced by the application of DMBA to the buccal pouch of the Syrian hamsters are
morphologically and histologically similar to human tumors (Shklar, 1972; Nagini et al., 2009; Siegel et al., 2012). In addition, hamster tumors express many biochemical and molecular markers that are expressed in human oral cancer. It is believed that identification and monitoring of these potentially malignant lesions and conditions allows clinicians to detect and treat early intraepithelial stages of oral carcinogenesis, for example mild, moderate or severe dysplasia and carcinoma in situ, all of which generally precede the development of invasive OSCC. This model has gained wide acceptance as the simplest and most effective system to analyze oral cancer development and investigate approaches to chemoprevention and chemointervention (Shklar, 1998).

Dietary phytochemicals have gained significant recognition in recent years as potential candidates for cancer chemoprevention owing to their ability to arrest or reverse the cellular and molecular processes associated with carcinogenesis (Priyadarsini and Nagini, 2012). Natural products represent one of the most enduring approaches in the development of anticancer targeting drug. Therefore, taking natural compounds for cancer prevention can be a well-justified and effective strategy for people with increased risk for cancer development such as those with premalignant lesions of intraepithelial neoplasia. Among many such natural compounds, silibinin has drawn special attention for its chemoprevention potential because of its safety, multi-targeted anticancer effects and easy accessibility (Amin et al., 2009). Silibinin, the major constituent of the milk thistle extract is consumed widely as a dietary supplement and it displays a remarkable spectrum of pharmacological activities like antioxidant, anti-inflammatory, antiallergic, antimutagenic, antiviral and
antineoplastic, affecting basic cell functions such as proliferation, differentiation and apoptosis (Abascal et al., 2003; Deep and Agarwal, 2010; Sangeetha et al., 2012). However, oral bioavailability of silibinin is quite low, which is mainly attributed to its poor stability and intestinal absorption. Poor bioavailability is the major drawback associated with the failure of many natural chemopreventive agents in clinical settings.

In order to achieve maximum response of a chemopreventive agent, novel strategies are required to enhance the bioavailability of potentially useful agents. It is noteworthy that in recent years, nanotechnology is being implemented and assessed in different areas of cancer therapeutics and cancer management. Since most biological processes, including those that are cancer related, occur at nanoscale, nanoparticulate technology is a potential tool to diagnose and treat cancer. In envisioned that nanoparticle-mediated delivery could be useful to limit the toxicity and enhance the bioavailability of the chemopreventive agents. Polymeric nanoparticles have attracted significant attention in the study of drug delivery systems as they offer a means for localized or targeted delivery systems of a drug to specific tissue/organ sites of interest with an optimal release rate (Mu and Seow, 2007). A significant advantage of the biodegradable polymers is their history of safe use, proven biocompatibility and ability to control the time and rate of polymer degradation and the release of the incorporated entity. The encapsulation processes with polymeric nanoparticles are in more advance condition in comparison to other nanoparticle systems.

Prognostic markers are indicators of aggressiveness, invasiveness, metastasis and thus, correlate with survival span
independent of systemic therapy. They will be useful in assessing the risk involved for each patient. Expression pattern of markers of cell proliferation has been utilized as potential tool to assess the molecular pathogenesis of oral carcinogenesis. It has been shown that abnormal expression of cyclin D1 promotes genetic instability in vitro and tumorigenesis in vivo (Izzo et al., 2003). It has been suggested that cyclin D1 may be an important molecular target of oral cancer prevention since its expression has been shown to occur in the early stages of oral carcinogenesis (Mishra and Das, 2009). Moreover, the chemopreventive activity of the silibinin and its nanoparticulate are associated with the rate of cell proliferation which further validates its anti-cell proliferative potential, utilizing cyclin D1 expression as markers of cell proliferation. Hence, the present study was designed to elucidate the protective role of free SIL and SILNPs on DMBA-induced oral cancer by investigating the tumor incidence, tumor volume and tumor burden. Histopathological and immunohistochemical localisation of cell proliferation (cyclin D1) in hamster buccal mucosa tissue was also done to validate the antitumor efficacy of SILNPs in comparision with free SIL against DMBA-induced HBP carcinogenesis.

4.2. Review of Literature

Manikandan et al., (2008) have evaluated chemopreventive potential of Azadirachta indica (neem) leaf fractions based on in vitro antioxidant assays and in vivo inhibitory effects on 7,12-dimethyl benz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. Their results demonstrated that neem leaf fractions exert greater inhibitory effect on HBP carcinogenesis at a lower concentration compared to the crude extract indicating enrichment of phytochemicals with anticarcinogenic properties. The results that the
antioxidant properties of neem leaf fractions may be responsible for modulating key hallmark capabilities of cancer cells such as cell proliferation, angiogenesis and apoptosis in the HBP carcinogenesis.

Yi et al., (2008) have investigated thymoquinone inhibited angiogenesis through suppression of intracellular signalling pathways. The results revealed that inhibited endothelial cell migration, invasion, proliferation and tube formation, effectively inhibited angiogenesis in vitro and in vivo and prevented tumor growth in a xenograft mouse model with a low dosage of thymoquinone by blocking tumor angiogenesis. The results suggest that thymoquinone inhibits tumor angiogenesis and tumor growth and could be used as a potential drug candidate for cancer therapy.

Mattheolabakis et al., (2009) have prepared cisplatin-loaded nanoparticles by modified emulsification and solvent evaporation method. They observed that the cisplatin-loaded nanoparticles have appeared to be effective in delaying tumor growth in HT29 tumor-bearing mice with severe combined immune deficiency. The results further suggest that the group of mice treated with cisplatin-loaded nanoparticles had a higher survival rate compared with the free cisplatin group.

Harish Kumar et al., (2010) evaluated the relative chemopreventive potential of the neem limonoids azadirachtin and nimbolide in the hamster buccal pouch carcinogenesis model by analyzing the expression of PCNA, p21, cyclin D1 and glutathione S-transferase pi (GST-P). VF-κB, p53, Fas, Bcl-2, Bax, Bid, APaf-1, cytochrome C, survivin, caspases-3, -6, -8 and -9 and poly (ADP-ribose) polymerase (PARP) by RT-PCR, immunohistochemical and western
blot analysis. The results revealed that both azadirachtin and nimbolide mediate their anti-proliferative effects by downregulating proteins involved in cell cycle progression and transduce apoptosis by both the intrinsic and extrinsic pathways. The results further suggest that nimbolide was found to be a more potent anti-proliferative, apoptosis inducing agent and offers promise as a candidate agent in multitargeted prevention and treatment of oral cancer.

Pugalendhi et al., (2010) have evaluated the effects of genistein and daidzein, in combination, on the expression pattern of various biomolecular markers during DMBA induced mammary carcinogenesis in sprague-dawley rats. Oral administration of genistein+daidzein in combination to DMBA treated rats significantly down regulated the expression of VEGF. The results suggest that the pleiotropic and synergistic effects of genistein and daidzein have reduced cellular proliferation, decreased angiogenesis and induced apoptosis during DMBA induced mammary carcinogenesis in sprague-dawley rats.

Duan et al., (2010) have investigated the antitumor activity of curcumin-loaded chitosan/poly (butyl cyanoacrylate (PBCA) nanoparticles in vitro and in vivo against human hepatocellular carcinoma. The results demonstrated that curcumin nanoparticles and free curcumin suppressed COX-2 and VEGF expression and cell proliferation/survival of hepatocarcinoma carcinoma cells. Further, curcumin nanoparticles inhibited hepatocarcinoma cell growth in murine xenograft models and these outcomes have been accompanied by a potent antiangiogenic response. The results further suggest that curcumin-PBCA nanoparticles provide an opportunity to expand the clinical repertoire of this efficacious agent by enabling ready aqueous dispersion.
El-Rouby, (2011) investigated the chemopreventive role of lycopene in 4-nitroquinoline-1-oxide (4-NQO)-induced tongue squamous cell carcinoma in rats. The histopathological and immunohistochemical findings revealed that lycopene treatment significantly decreased the incidence of tongue carcinogenesis and also decreased the percentage of PCNA positive nucleic. Increased E-cadherin and β-catenin immuneexpression were also further observed in the lycopene treated group. The results suggest that lycopene can exert protective effects against 4-NQO-induced tongue carcinogenesis.

Manoharan et al., (2011) have investigated the protective effect of berberine on expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers during DMBA-induced HBP carcinogenesis. The results revealed that oral administration of berberine brought back the expression of molecular markers to near normal pattern in hamsters treated with DMBA. The results suggest that berberine has potent anti-inflammatory, anti-angiogenic, anti-cell proliferative and apoptosis inducing properties in DMBA induced oral carcinogenesis.

Vinothkumar et al., (2012) have investigated the modulatory effect of geraniol on the expression pattern of cell proliferative (PCNA, cyclin D1, C-fos), inflammatory (Nf-κB, Cox-2), apoptotic (p53, Bax, Bcl-2, Caspase-3 and Caspase-9) and antiogenic (VEGF) markers in DMBA-induced HBP carcinogenesis. Over expression mutant p53, PCNA, Bcl-2 and VEGF accompanied by decreased expression of Bax were noticed in hamsters treated with DMBA alone. Increased expression of C-fos, Cox-2, NF-κB and cyclin D1 and decreased activities of Caspase-3 and
Caspase-9 were also noticed in tumor tissues. Further, oral administration of geranoil not only completely prevented the tumor formation but also prevented the degradation in the expression of above mentioned molecular markers in hamsters treated with DMBA. The results thus suggest that geranoil has potent anti-inflammatory, anti-angiogenic, anti-cellproliferative and apoptosis-inducing properties in DMBA-induced HBP carcinogenesis.

Alizadeh et al., (2012) investigated the preventive effects of polymeric nanocarrier-curcumin (PNCC) on colon carcinogenesis in an azoxymethane (AOM)-induced rat tumor. The histopathological and immunohistochemistry examinations revealed that in vivo curcumin nanoparticles inhibited colon cancer growth in animal model. The tumor incidence and tumors were found to be decreased by nanocurcumin in comparison with free curcumin. Further, the nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity, goblet depletion, structural abnormality and the expression of beta-catenin and Bcl-2 proteins were found reduced in nanocurcumin when compared to free curcumin. These results showed that nanoencapsulated curcumin exerts a significant chemopreventive effect on AOM-induced colon cancer through cell proliferation inhibition and apoptosis induction.

Prabhakar et al., (2012) reported the anti-cell proliferation efficacy of ferulic acid by analyzing the expression pattern of cell proliferation markers, PCNA and cyclin D1 in the buccal mucosa of golden Syrian hamster treated with DMBA. They suggested that ferulic acid might have inhibited tumor formation in the buccal mucosa of
hamster treated with DMBA through its anti-cell proliferation potential as evidence by the decreased expression of PCNA and cyclin D1.

Silvan and Manoharan, (2013) have investigated the modulating effect of various molecular markers during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. Oral administration of apigenin results revealed down regulated the expression of VEGF in hamsters treated with DMBA. They concluded that the anti-tumor initiating effect of apigenin could be probably due to its anti-cell proliferative, antiinflammatory, apoptotic and anti-angiogenic potential during DMBA induced hamster buccal pouch carcinogenesis.

She et al., (2013) have prepared heparin doxorubicin (DOX) conjugate based nanoparticle and evaluated the antitumor efficacy in the breast cancer model. The results revealed that nanoparticles have strong antitumor activity, high anti-angiogenesis effects and induced apoptosis on the 4T1 breast tumor model confirmed by mice weight shifts, tumor weights, tumor growth curves, immunohistochemical assessment and histological analysis. The results suggest that dendronized heparin DOX conjugate based nanoparticle could provide useful design and preparation strategies for soft nanoparticles as safe and efficient drug delivery systems.

Arumugam et al., (2014) have studied the effect on administration of an ethanolic fraction of Neem leaf (EFNL) inhibits progression of chemical carcinogen induced mammary tumorigenesis in rat model. Treatment with EFNL inhibited MNU-induced mammary tumor progression. EFNL treatment is found to be highly effective in reducing mammary tumor burden and in suppressing mammary tumor
progression even after the cessation of treatment. The EFNL treatment caused downregulation of angiogenic proteins (angiopoietin and vascular endothelial growth factor A [VEGF-A]). The results suggest that EFNL exert a potent anticancer effect against mammary tumorigenesis by altering key signaling pathways.

Khan et al., (2014) have reported the synthesis, characterization and efficacy assessment of the nanotechnology-based oral formulation of chitosan nanoparticles encapsulating epigallocatechin-3-gallate (Chit-nanoEGCG) for the treatment of prostate cancer (PCa) in a preclinical setting. They studied the antitumor efficacy of Chit-nanoEGCG in subcutaneously implanted 22Rv1 tumor xenografts in athymic nude mice. They observed that the expression of Ki-67, PCNA (cell proliferation markers), CD31 and VEGF (markers of angiogenesis) were decreased in tissues of mice treated with Chit-nanoEGCG than free EGCG. They concluded that nanotechnology based, non-invasive platform has tremendous potential to replace many of the invasive techniques (like radiation therapy or surgery) to treat PCa.

From the above review of literatures, it is confirmed that histopathological and immunohistochemical analysis of tissue section can give valuable information on the histological and immunohistochemical changes during the process of various carcinogenesis. Hence the current chapter was designed to evaluate the histological and immunohistochemical alteration in 7,12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis.
4.3. **Experimental protocol**

The local institutional animals ethics committee (IAEC), (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, 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 the Indian Council of Medical Research, India. A total number of 36 animals were divided into six groups and each group contained six animals. Group I hamsters served as the control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Group II, III and IV hamsters were treated with 0.5% solution of DMBA in liquid paraffin using a No. 4 sable brush, three times a week for 14 weeks on their left buccal pouches (Shklar, 1999). Group II animals received no other treatment. Groups III and IV hamsters were orally given silibinin at a dose (50 mg/kg body weight/day) and SILNPs (dose equivalent to 50 mg/kg body weight/day of SIL), dissolved in 1 ml of 5% dimethyl sulphoxide (DMSO) and dissolved in distilled water, respectively, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Groups V and VI hamsters received the same dose of SIL (50 mg/kg body weight/day) and SILNPs alone (dose equivalent to 50 mg/kg body weight/day of SIL) as in groups III and IV throughout the experiment period. The dose of silibinin used in this study was chosen based on a dose response study undertaken by us that demonstrated maximum chemopreventive efficacy at this dose which has been reported in previous studies (Kaur et al., 2010; Sangeetha et al., 2012). The experiment was terminated at the end of 16 weeks and hamsters were
sacrificed by cervical dislocation after an overnight fast. The buccal pouches were excised and the tissues were further processed for experiments. The tissue samples were immediately fixed in 10% neutral buffered-formalin and embedded in paraffin. For histopathological study, tissue sections, 3-4 μm in thickness were cut in a rotary microtome and stained with haematoxylin and eosin. The other sections were used for immunohistochemical staining.

4.4. Results

4.4.1. Body weight, tumor incidence, volume and burden observations

The animals were weighed at the beginning and the end of the induction period during the treatment phase which is indicated in Fig. 4.1. In DMBA-alone painted animals, the mean body weight gain was significantly decreased compared to the control group. Administration of SIL and SILNPs to DMBA-painted animals significantly increased the mean final body weight compared to the animals painted with DMBA-alone. No significant differences in the body weights were observed in animals administered SIL and SILNPs alone as well as in the control (Fig. 4.1).

The tumor incidence, tumor volume and tumor burden was observed in the buccal mucosa of hamsters in the control and the experimental animals are shown in Table 4.1. The gross appearance in hamster buccal pouch mucosa of the control and the experimental animals are shown in Fig. 4.2. In the control group, no tumor formation was induced by liquid paraffin treatment. The incidence of 100% tumor formation was observed with mean tumor volume (275 ± 21.0 mm$^3$) and mean tumor burden (852 ± 65.06 mm$^3$) in DMBA-alone painted animals.
Fig. 4.1 Changes in body weight achieved from the control, DMBA, DMBA+SIL and DMBA+SILNPs. Data are presented as mean ± Standard deviation.
The total number of oral tumors in the buccal pouches was counted and the diameter of each tumor was measured with a vernier caliper. Administration of SIL and SILNPs to DMBA-painted animals significantly decreased the tumor incidence (33.3% and 16.6%), mean tumor volume (112 ± 11.77 mm$^3$ and 57 ± 4.85 mm$^3$) and mean tumor burden (268 ± 24.03 mm$^3$ and 79 ± 5.85 mm$^3$) compared to DMBA-painted animals. Conversely, tumors in the SILNPs treatment had a markedly smaller size, suggesting tumor growth was greatly suppressed with much smaller tumor size than that of DMBA+SIL (Table 4.1). Moreover, as seen in Fig. 4.3, a photo of representative tumors taken from animals receiving administration of SILNPs to DMBA painted animals compared to administration of SIL to DMBA painted animals can also illustrate the distinct tumor suppression effects as visual evidence.

4.4.2. Histopathology

The histopathological features observed in the buccal mucosa of hamsters in the control and the experimental animals in each group are shown in Fig. 4.4 and Table 4.2. As seen from Fig 4.4 the tumors observed in DMBA painted animals were 100% well-differentiated squamous cell carcinomas. Two of the six animals treated with DMBA + SIL developed squamous cell carcinoma, while others exhibited moderate to severe dysplasia. One of the six animals treated with DMBA + SILNPs developed squamous cell carcinoma, while others exhibited mild to moderate dysplasia. No tumors were observed in the control animals painted with liquid paraffin alone (group I) as well as SIL and SILNPs alone administered animals (Group V and VI). In groups I, V and VI, the epithelium was normal, intact and continuous. But, administration of SIL to DMBA painted animals decreased tumor
incidence as well as preneoplastic lesions, the inhibitory effect was more pronounced in administration of SILNPs to DMBA painted animals. Histopathological examination of the pouches in groups III and IV animals revealed varying degrees of preneoplastic and neoplastic lesions. As mentioned earlier, in group V (SIL alone) and group VI (SILNPs alone), the epithelium was normal, intact and continuous. Therefore, groups (I-IV) were chosen for further immunohistochemical analysis.

Fig. 4.3 Photographs of tumor after excision from (a) DMBA, (b) DMBA+SIL and (c) DMBA+SILNPs treated animals
4.4.3. **Cyclin D1 expression**

Deregulation of cell cycle machinery is an important step in malignant transformation. Cyclin D1 is one of the most important proto-oncogenes and cell cycle regulators. Its protein known as cyclin D1 is expressed in G1 phase of the cell cycle. The cyclin D1 expression in the hamster buccal mucosa of the control and the experimental animals in each group are depicted in Fig. 4.5 and Table 4.3 respectively. In DMBA-alone treated hamsters, the expression of cyclin D1 was significantly higher as compared to the control hamsters. However, the tumor tissues from the DMBA+SIL and DMBA+SILNPs treated hamsters exhibited significantly decreased expression of cyclin D1, compared to those from the DMBA-alone treated hamsters. Moreover, the tumors from the DMBA+SILNPs group showed suppressed expression of cyclin D1, compared to those from any other DMBA-painted animals.

4.5. **Discussion**

Of late, medicinal plants rich in antioxidant phytochemicals have received growing attention as potential chemopreventive agents. Chemoprevention is the use of natural or synthetic substances to halt, delay or reverse malignant progression in tissues at risk for the development of invasive cancer. Several modern anticancer drugs have been developed from traditional medicinal plants and phytochemicals. Silibinin is one of the potent dietary phytochemicals and possesses various pharmacological activities, but the bioavailability of silibinin is quite low owing to degradation by gastric fluid and its poor aqueous solubility. Therefore, to achieve maximum response of an anticancer agent, novel strategies are required to enhance the bioavailability of potentially useful agents. Nanoencapsulation of anticancer drugs
Fig. 4.5 Representative photographs of immunohistochemical staining of cyclin D1 expression of hamster buccal mucosa in the control and the experimental animals (n=6) (x100)
increases drug efficacy, tolerability and therapeutic index of corresponding drugs. Considering the potential of nanoparticles as oral drug delivery system, the present investigation involved development and characterisation of SILNPs with a view to improve its oral bioavailability. Nanoparticles possess the ability to permeabilize the cells more efficiently than which facilitates administration of large quantities of drug to give better efficacy (Nair et al., 2011).

Oral delivery of nanoparticles is important as a mean of continuous exposure of tumor cells to the anticancer drugs of a relatively lower but safer concentration that gives little chance for the tumor blood vessels to grow, resulting in much better efficacy and few side effects than intermittent chemotherapy (Qi et al., 2007). It has been found that the size of the nanoparticles plays a key role in their interactions with the biological cells and the in vivo fate of a particulate drug delivery system (Jin et al., 2009). The smaller size particles seem to have efficient interfacial interaction with the cell membrane compared to larger size particles, thus improving efficacy of the particle based oral drug delivery systems. Therefore, the design of a new drug-delivery vehicle that incorporates the formulation of NPs will prevent drug loss in the delivery route and improve functionality (Asghar and Chandran, 2006). Eudragit® E is a cationic copolymer has been widely used to improve the solubility of poorly water-soluble drugs (Jung et al., 1999). It has a basic site containing tertiary amine groups which are ionized in gastric fluid and therefore it is easy to dissolve in the gastric environment. In the present study SILNPs was synthesized by nanopercipitation method using Eudragit® E (EE) as a drug delivery vehicle and validates the chemopreventive potential of SIL and SILNPs by investigating its protective and antitumor effect of
free SIL and SILNPs in DMBA induced hamster buccal pouch carcinogenesis through histological assessment as well as immunohistochemical expression of cell proliferative marker, cyclin D1.

Nagini et al., (2009) have reported that application of DMBA to the cheek pouch of the golden Syrian hamster produces squamous cell carcinoma that is histologically similar to human oral squamous cell carcinoma. In the present study, 100% tumor formation was observed in hamsters treated with DMBA-alone at the end of the experimental period. The tumors were histopathologically confirmed as well-differentiated squamous cell carcinoma. In addition, administration of SIL and SILNPs to DMBA painted animals significantly reduced and delayed tumor formation. Such an inhibitory effect may be attributed to the repair of the damaged DNA that keep cells alive and healthy. Changes in tumor growth characteristics observed in DMBA+SIL and DMBA+SILNPs treated with hamsters suggest anti-proliferative effects of SILNPs. Lower tumor incidence, smaller tumor size and lesion number may have been attributed to the direct effects of SILNPs which acts as anti-proliferative or its indirect effects via anti-oxidative enzymes. Therefore, the results suggest that SILNPs has significant chemopreventive potential during DMBA-induced oral carcinogenesis compared to DMBA + SIL.

Oral carcinogenesis is a highly complex and a multistep process with multifaceted etiology that arises due to accumulation of heterogeneous genetic changes in the genes involved in the basic cellular functions including cell division, differentiation and cell death. These genetic changes in the affected cells progressively increase the cell proliferation, angiogenesis and inhibition of apoptosis (Vinothkumar et al., 2012). Increased proliferation is well recognized
as one of the fundamental biological changes in carcinogenesis. Markers of cellular proliferation and angiogenesis are often used as biomarkers in cancer studies. Findings of previous studies have shown that cellular proliferation increased in a stepwise fashion from normal mucosa through dysplasia to carcinoma, suggesting that disease progression in the oral mucosa is accompanied by its tumor volume (Macluskey et al., 2000). Tumor number and tumor size are indicators of proliferation and angiogenesis. In recent years, the correlation between cellular proliferation and development of tumor can be seen clearly, an adequate blood flow in tumor stroma was indeed essential for growth of tumor size. Tumor size as a function of time was used to determine the efficacy of therapy and changes in animals body weight. Inhibiting tumor growth and obstructing tumor progression and invasion in this animal model are indicators of anti-proliferative and anti-angiogenic effect of nanoparticles. Cell cycle regulatory genes play a vital role in maintaining normal cell proliferation. It is well known that the metabolic activity of a cellular proliferation may vary with the phase of the cell cycle and the activity of cellular enzymes may fluctuate in a cell cycle-dependent manner (Mishra and Das, 2009).

In the present study, cell proliferation in hamster SCC was measured with cyclin D1 as a proliferative marker and as a key factor related to the cell cycle. The cyclin D1 oncogene is usually amplified or overexpressed in OSCC. Cyclin D1, an important nuclear protein in the G1/S phase of the cell cycle, is involved in the regulation of cell proliferation and differentiation (Mineta et al., 2000; Jirawatnotai et al., 2012;). In the present study, cyclin D1 expression was found to be significantly higher in DMBA induced tumor tissues compared to silibinin and nanoparticulate silibinin treated tissues. Interestingly,
SILNPs treated tissues exhibited remarkably reduced cyclin D1 expression when compared to SIL treated tissues. SILNPs are effective and superior to SIL in inhibiting the tumor formation and suppressing cellular proliferation occurring in DMBA-induced tumor tissues.

Further, enhanced accumulation of SILNPs in tumor tissue might have taken place due to combined effect of enhanced oral bioavailability and enhanced permeation and retention (EPR) effect. EPR effect is the physiology-based principal mechanism of tumor accumulation of large molecules and small particles. It is associated with the unique feature of enhanced vascular permeability and sluggish lymphatic drainage system responsible for spontaneous accumulation and retention of nanoparticles in the tumor cells. This occurs because tumor vasculature is leaky; hence, circulating nanoparticles can accumulate more in the tumor tissue than in normal tissue. Due to their small size and large surface area, nanocarriers are able to penetrate the mucous cell lining and improve bioavailability and it had a more sustained release, a longer circulation time, increased delivery to tissue and an enhanced antitumor effect.

4.6. Conclusion

In conclusion, oral administration of SIL and SILNPs to DMBA painted animals exhibited superior antitumor and antiproliferative effect by tumor suppression and down regulated the expression of cyclin D1. On a comparative basis, SILNPs was found to have more potent antiproliferative potential when compared to free SIL in DMBA-induced HBP carcinogenesis. Thus, the results of the present study further suggest that it confirms not the potential of silibinin in treating oral cancer but also offers an effective way of improving the antitumor efficiency of silibinin by nanodrug delivery systems.