RESEARCH ARTICLE

Anti-cell Proliferative and Anti-angiogenic Potential of Andrographolide During 7,12- Dimethylbenz(a)anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Abstract

Our aim was to explore anti-cell proliferative and anti-angiogenic potential of andrographolide by analyzing the expression pattern of cell proliferative (PCNA, Cyclin D1) and angiogenic (VEGF) markers during 7, 12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. DMBA painting three times a week for 14 weeks in the buccal pouch of golden Syrian hamsters resulted in oral tumors which were histopathologically diagnosed as well differentiated squamous cell carcinoma. Immunohistochemical (PCNA, VEGF) and RT-PCR (Cyclin D1) studies revealed over expression of PCNA, VEGF and Cyclin D1 in the buccal mucosa of hamsters treated with DMBA alone. Oral administration of andrographolide at a dose of 50 mg/kg bw to hamsters treated with DMBA not only suppressed the histological abnormalities but also down regulated the expression of PCNA, VEGF and Cyclin D1. The results of the present study suggest that andrographolide suppressed tumor formation in the buccal mucosa of hamsters treated with DMBA through its anti-cell proliferative and anti-angiogenic potential.

Keywords: Andrographolide - PCNA-VEGF - cyclin D1 - oral cancer -DMBA

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Introduction

Oral cancer, a highly complex multistep process that occurs when squamous epithelium is affected by multiple genetic alterations, is the fifth most common cancer worldwide and 90% of these forms of cancers are squamous cell carcinoma (SCC) (Jemal et al., 2009). While oral carcinoma comprises around 40% of total malignancies in India, in western countries it accounts for only 3-5% of all cancers. High incidence of oral cancer is attributable to the indigenous habit of chewing a mixture of tobacco, areca nut, lime, betel leaf, and spices in several combinations (Chiba, 2001). The status of tumor suppressor genes, oncogenes, cell proliferation markers, angiogenic markers and cell adhesion molecules are utilized as a potential tool to predict the prognosis of patients with SCC (Massano et al., 2006).

Golden Syrian hamsters are commonly utilized as an experimental model of oral carcinogenesis due to their pocket (pouch) like anatomy in the mouth, which retains the carcinogen for a longer time (Manoharan et al., 2012; Singh et al., 2012). DMBA, a polycyclic aromatic hydrocarbon, is widely employed to induce oral carcinoma in experimental animals. DMBA induced experimental oral carcinogenesis in the hamster cheek pouch produces premalignant and malignant changes that resemble premalignancy and malignancy of human oral mucosa (Morris, 1961). Also DMBA induced oral tumors expressed biochemical and molecular characteristics similar to that of human oral tumors (Shklar et al., 2009).

PCNA, a molecular coordinator for DNA replication, is involved in maintaining genome integrity and serve as a molecular platform to recruit proteins involved in DNA synthesis, cell cycle control and DNA damage repair (Prelich et al., 1987). PCNA, a sliding clamp for DNA polymerase delta, encircles DNA and coordinates multiple genetic functions during DNA replication and repair (McAlear et al., 1994). PCNA has been used as a molecular marker for assessing tumor progression and prognosis (Gulbis et al., 1996; Sakurai et al., 2005). Cyclin D1 is involved in promoting cell progression from G1 to S phase (Lu et al., 2009). Cyclin D1 plays crucial role in cell proliferation and differentiation (Jiao et al., 2013). Cyclin D1 overexpression mediates tumor proliferation, lymph node metastasis and prognosis (Kunisaki et al., 2004). Cyclin D1 is over expressed in a large spectrum of human cancers (Bosch et al., 1994).

Angiogenesis, the growth of new blood vessels from pre-existing ones, is a basic requirement for sustained tumor growth (Lingen, 1999). Angiogenesis is essential

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to supply the nutrients and oxygen, which are necessary for tumor growth and metastasis (Pratheeshkumar et al., 2012). Under cancerous conditions, an imbalance occurs between pro-angiogenic and anti-angiogenic factors that favor the promotion of tumor angiogenesis.

Andrographolide, a major active constituent of Andrographis paniculata, exhibited diverse biochemical and pharmacological properties including anti-inflammatory, antihyperglycemic, hepatoprotective, antioxidant and anticancer properties (Trivedi et al., 2007; Dai et al., 2011). Wang et al. (2011) reported that andrographolide suppressed oral tumors through NFkB inactivation. Recently, we demonstrated the antigenotoxic, chemopreventive, pro-apoptotic and anti-inflammatory potential of andrographolide in experimental animal models (Manoharan et al., 2011; 2012; Shanmugam et al., 2012). The present study focuses the anti-cell proliferative and anti-angiogenic potential of andrographolide during DMBA induced hamster buccal pouch carcinogenesis.

Materials and Methods

Golden syrian hamsters

Male golden Syrian hamsters purchased from National Institute of Nutrition, Hyderabad were maintained in the Central Animal House of Annamalai University. The hamsters were housed in polypropylene cages and were maintained under temperature (27±2°C) and humidity (55±5%) with a 12h light/dark cycle. The experimental work was approved by Institutional Animal Ethics Committee (Reg. No 160/1999/CPCSEA).

Experimental design

A total number of 40 hamsters were divided into four categories of 10 hamsters in each. Group 1 hamsters were treated with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Hamsters in groups 2 and 3 were treated with 0.5% DMBA in liquid paraffin three times per week for 14 weeks. Hamsters in group 2 received no other treatment. Group 3 hamsters were orally administered with andrographolide (50 mg/kg bw) three times per week on days alternate to DMBA application, starting 1 week before the exposure to DMBA and continued until one week after the final exposure of the DMBA. Group 4 hamsters were orally administered with andrographolide alone throughout the experimental period. All hamsters were sacrificed by cervical dislocation at the end of experimental period.

Immunoexpression of PCNA and VEGF

The antigen retrieved tissue sections were incubated with respective primary antibodies (PCNA and VEGF) and subsequently treated with secondary antibodies conjugated with horseradish peroxidase. The antigen-antibody complex was then detected using 3,3'-diaminobenzidine. The percentage of positive cells was scored after the slides were counter stained with hematoxylin.

Expression of cyclin D1 using real time PCR

Isolated RNA from the buccal mucosa was reverse transcribed to cDNA with random primers (Table 1) using High cDNA Reverse Transcriptase Kit. Amplification of cDNA was carried out to analyze the expression of cyclin D1, with β-actin as a control. The relative quantification of cyclin D1 was determined using threshold cycle (CT) method.

Statistical analysis

The data for Cyclin D1 is expressed as mean ± standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The statistical evaluation for the score of positively stained cells of PCNA and VEGF was analyzed using Chi-square (χ^2) test. The results were considered statistically significant if the p values were 0.05 or less than 0.05.

Results

Immunohistochemical analysis revealed overexpression of PCNA and VEGF in hamsters treated with DMBA (Figures 1 and 2). Oral administration of andrographolide to hamsters treated with DMBA prevented the abnormal expression of PCNA and VEGF during DMBA induced oral carcinogenesis. The score of positively stained cells for PCNA and VEGF expression in control and experimental hamsters are also given in (Table 2).

The primer melting curve and the fold increase in the Cyclin D1 mRNA expression pattern of control and experimental hamsters in each group is shown in Figures 3 and 4 respectively. Overexpression expression of cyclin D1 mRNA was noticed in hamsters treated with DMBA alone. Oral administration of andrographolide to hamsters treated with DMBA suppressed the expression of Cyclin D1. Similar Cyclin D1 mRNA expression pattern was noticed in control hamsters and hamsters treated with andrographolide alone.

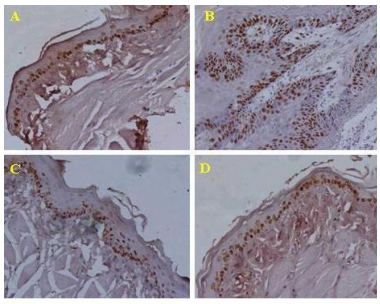

Figure 1. Immunoexpression Pattern of PCNA Protein in the Buccal Mucosa of Control and Experimental Hamsters in Each Group (40×). A and D-Control and Andrographolide alone (expression not detectable); B-DMBA alone (over expressed); C-DMBA+Andrographolide (down regulated

Table 1. List of Primers Used for Real-time PCR Analysis

Genes	Primers	Sequences
Cyclin D1	forward	5'-CGGAGGACAACAAACAGATC-3';
	reverse	5'-GGGTGTGCAAGCCAGGTCCA-3'
β-actin	forward	5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3'
	reverse	5'-AGCAGCCGTGGCCATC TCTTGCTCGAAGTC-3'

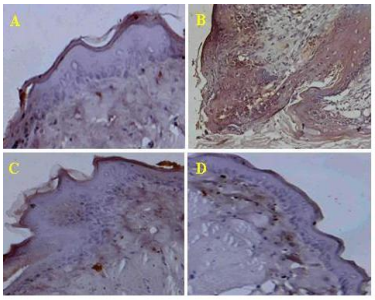

Figure 2. Immunoexpression Pattern of VEGF Protein in the Buccal Mucosa of Control and Experimental Hamsters in Each Group (40×). A) and **D)** Control and Andrographolide alone (expression not detectable); **B)** DMBA alone (over expressed); **C)** DMBA+Andrographolide (down regulated)

Table 2. The Score of Positively Stained Cells of PCNA and VEGF in Control and Experimental Hamsters in Each Group

Groups / Markers	PCNA				VEGF			
	0	1^+	2^+	3^+	0	1^+	2^+	3^+
Control	10	0	0	0	10	0	0	0
DMBA	0	0	1	9	0	1	3	6
DMBA+Andrographolide	0	8	1	1	7	2	1	0
Andrographolide alone	10	0	0	0	10	0	0	0

The percentage positive cells were scored as: 3^+ = strong staining, more than 50% of cells were stained, 2^+=moderate staining, between 20 and 50% of cells were stained 1^+ = week staining, between 1 and 20% of cells were stained, 0 = negative, less than 1% of cell staining

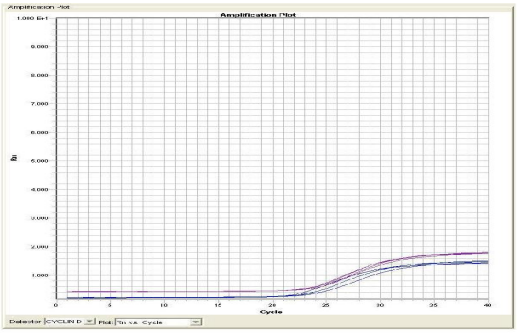

Figure 3. mRNA Expression Pattern of Cyclin D1 in the Buccal Mucosa of Control and Experimental Hamsters in Each Group

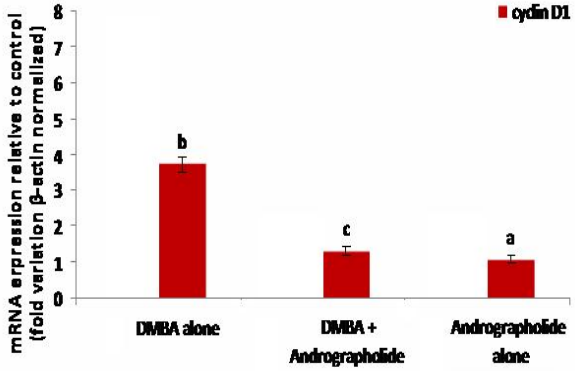

Figure 4. Fold Increase in the mRNA Expression Pattern for Cyclin D1 in Hamsters Treated with DMBA Alone, DMBA+Andrographolide and Andrographolide Alone. Values are expressed as mean±SD for 10 hamsters in each group. Values that do not share a common superscript letter in the same column differ significantly at $p<0.05$ (DMRT)

Discussion

Deregulation of cell proliferation and angiogenesis are two important scenarios occurring in oral carcinogenesis (Johnstone et al., 2006). It has been reported that analysis of PCNA protein expression could play an important role in the prediction of long term survival and prognosis of patients. PCNA has been reported as the ring master of the genome due to active participation in several molecular pathways responsible for the survival and death of mammalian cells (Paunesku et al., 2001). Overexpression of PCNA has been reported in several cancers including oral cancer (Manojprabhakar et al., 2012). Our study supports these finding.

Tumour specific alterations noted in the cyclin D1 gene product indicate the importance of cyclin D1 as a driver of the neoplastic process (Gautschi et al., 2007). Cyclin D1 is also involved in the regulation of apoptosis and it may act as pro-apoptotic or anti-apoptotic factor depending on the proliferative and differentiated state of the cell (Han et al., 1999). Profound studies reported that transcriptional up regulation of endogenous cyclin D1 inhibited apoptotic machinery in human carcinoma cells (Fu et al., 2004; Sun et al., 2012). It has been reported that cyclin D1 amplification increased VEGF production and decreased Fas expression in esophageal tumor cells (Tashiro et al., 2007). Over expression of cyclins is one of the common phenomenons of oral cancer (Miyamoto et al., 2003). Overexpression of cyclin D1 was reported in 30-35% of oral carcinogenesis (Sauter et al., 1999; Silvan et al., 2013).

VEGF is a multifunctional cytokine and is a major angiogenic factor whose biological activity is primarily associated with endothelial cells (Bancroft et al., 2001). VEGF, endothelial specific mitogen, determines the fate of an endothelial cell for the angiogenic process. VEGF regulates the proliferation, migration, differentiation of endothelial cells (Li et al., 2009). Investigation of angiogenic inhibitors could thus serve as a new clinical class of drugs for the treatment of cancers. Although angiogenesis is a common phenomenon in physiological conditions, it is also crucial in pathological conditions such as tumor progression and metastasis. Extensive studies reported overexpression of VEGF in oral carcinogenesis (Silvan et al., 2013). Our results are in line with these findings.

Manoharan et al. (2012) demonstrated the anti-tumor initiating potential of andrographolide in DMBA induced hamster buccal pouch carcinogenesis. This antitumor initiating potential is probably due to its antioxidant potential as well as modulating effect on xenobiotic metabolizing enzymes during DMBA induced oral carcinogenesis. It has been reported that andrographolide has the potential to protect cell surface abnormalities during DMBA induced hamster buccal pouch carcinogenesis this is probably due to its inhibitory effect on enzymes involved in the glycosylation, silalylation and fucosylation process (Singh et al., 2012). Shanmugam et al. (2012) reported the proapoptotic and anti-inflammatory potential of andrographolide during DMBA induced hamster buccal pouch carcinogenesis. They suggested that the anti-tumor

effect of andrographolide could partly be attributed to its apoptotic and anti-inflammatory potential during DMBA induced hamster buccal pouch carcinogenesis. Also, previous studies demonstrated the apoptotic and anti-inflammatory potential of andrographolide in various cancer cells (Cheung et al., 2005; Lee et al., 2011; Pratheeshkumar et al., 2012). It has been demonstrated that andrographolide suppressed VEGF expression in prostate cancer and pulmonary tumors (Zhao et al., 2008). It has been reported that the anti-angiogenic role of VEGF by arresting the cell cycle could play a role in the prevention of pulmonary tumor and its metastasis (Tung et al., 2013). Andrographolide down regulated the expression of cyclin D1 in prostrate cancer (Wang et al., 2011). Also, andrographolide down regulated the expression of PCNA in human skin carcinoma A431 cells (Jing et al., 2013).

In the present study, oral administration of andrographolide at a dose of 50 mg/kg bw down regulated the expression of PCNA, cyclin D1 and VEGF during DMBA induced hamster buccal pouch carcinogenesis. The results of the present study thus revealed the anti-cell proliferative and anti-angiogenic potential of andrographolide during oral carcinogenesis. To conclude, andrographolide could be used as a potent drug to suppress abnormal cell proliferation and angiogenesis occurring in oral carcinogenesis along with the current chemotherapeutic drugs.

Acknowledgements

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References

Bancroft CC, Chen Z, Dong G, et al (2001). Coexpression of proangiogenic factors IL8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK NFkB signal pathways. *Clin Cancer Res*, **7**, 435-42.

Bosch F, Jares P, Campo E, et al (1994). PRAD-1/Cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood*, **84**, 2726-32.

Cheung HY, Cheung SH, Li J, Cheung CS, et al (2005). Andrographolide isolated from Andrographis paniculata induces cell cycle arrest and mitochondrial-mediated apoptosis in human leukemic HL-60 cells. *Planta Med*, **71**, 1106-11.

Chiba I (2001). Prevention of Betel Quid Chewers oral cancer in the Asian-Pacific area, *Asian Pac J Cancer Prev*, **2**, 263-69.

Dai GF, Zhao J, Jiang ZW, et al (2011). Anti-inflammatory effect of novel andrographolide derivatives through inhibition of NO and PGE2 production. *Int Immunopharmacol*, **11**, 2144-49.

Fu M, Wang C, Li Z, et al (2004). Cyclin D1 normal and abnormal functions. *Endocrinol*, **145**, 5439-47.

Gautschi O, Ratschiller D, Gugger M, et al (2007). Cyclin D1 in non-small cell lung cancer: A key driver of malignant transformation. *Lung Cancer*, **55**, 1-14.

Gulbis JM, Kelman Z, Hurwitz J, et al (1996). Structure of the C-terminal region of p21 (WAF1/CIP1) complexed with human PCNA. *Cell*, **87**, 297-306.

Han EK, Ng SC, Arber N, et al (1999). Roles of cyclin D1and related genes in growth inhibition, senescence and apoptosis. *Apoptosis*, **4**, 213-19.

Jemal A, Siegel R, Ward E, Hao Y, et al (2009). Cancer statistics 2009. *CA Cancer J Clin*, **59**, 225-49.

Jiao J, Huang L, Ye F, et al (2013). Cyclin D1 affects epithelial-mesenchymal transition in epithelial ovarian cancer stem cell-like cells. *Onco Targets Ther*, **6**, 667-77.

Jing S, Yong-gang L (2013). Effect of andrographolide on cell growth, apoptosis and expression of proliferating cell nuclear antigen protein in human skin carcinoma A431 cell line. *J Acta Anatom Sinica*, **44**, 73-8.

Johnstone S, Logan RM (2006). The role of vascular endothelial growth factor (VEGF) in oral dysplasia and oral squamous cell carcinoma. *Oral Oncol*, **42**, 337-42.

Kunisaki C, Shimada H, Akiyama H, et al (2004). Prognostic factors in esophageal cancer. *Hepatogastroenterology*, **51**, 736-40.

Lee KC, Chang HH, Chung YH, Lee TY (2011). Andrographolide acts as an anti-inflammatory agent in LPS-stimulated RAW264.7 macrophages by inhibiting STAT3-mediated suppression of the NF-κB pathway. *J Ethnopharmacol*, **135**, 678-84.

Li D, Zhang C, Song F, et al (2009). VEGF regulates FGF-2 and TGF-β1 expression in injury endothelial cells and mediates smooth muscle cells proliferation and migration. *Microvasc Res*, **77**, 134-42.

Lingen MW (1999). Angiogenesis in the development of head and neck cancer and its inhibition by chemopreventive agents. *Crit Rev Oral Biol Med*, **10**, 153-64.

Lu C, Dong J, Ma H, et al (2009). CCND1 G870A Polymorphism contributes to breast cancer susceptibility: a meta-analysis. *Breast Cancer Res Treat*, **116**, 571-75.

Manoharan S, Singh AK, Suresh K, et al (2011). Protective efficacy of andrographolide on 7, 12-dimethylbenz(a) anthracene induced genotoxicity in bone marrow cells of golden Syrian hamsters. *J Cell Tissue Res*, **11**, 2751-58.

Manoharan S, Singh AK, Suresh K, et al (2012). Anti-tumor Initiating Potential of Andrographolide in 7, 12-dimethylbenz[a]anthracene Induced Hamster Buccal Pouch Carcinogenesis. *Asian Pac J Cancer Prev*, **13**, 5701-08.

Manojprabhakar M, Vasudevan K, Karthikeyan S, et al (2012). Anti-Cell Proliferative Efficacy of Ferulic Acid Against 7, 12-dimethylbenz(a) Anthracene Induced Hamster Buccal Pouch Carcinogenesis. *Asian Pac J Cancer Prev*, **13**, 5207-11.

Massano J, Regateiro FS, Januario G, et al (2006). Oral squamous cell carcinoma: Review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **102**, 67-76.

McAlear MA, Howell EA, Espenshade KK, et al (1994). Proliferating cell nuclear antigen (pol 30) mutations suppress cdc44 mutations and identify potential regions of interaction between the two encoded proteins. *Mol Cell Biol*, **14**, 4390-97.

Miyamoto R, Uzawa N, Nagaoka S, et al (2003). Prognostic significance of cyclin D1 amplification and overexpression in oral squamous cell carcinomas. *Oral Oncol*, **39**, 610-18.

Morris AL, (1961). Factors influencing experimental carcinogenesis in the hamster cheek pouch. *J Dent Res*, **40**, 3-15.

Paunesku T, Mittal S, Protic M, et al (2001). Proliferating cell nuclear antigen (PCNA) ringmaster of the genome. *Int J Radiat Biol*, **77**, 1007-21.

Pratheeshkumar P, Sheeja K, Kuttan G (2012). Andrographolide

induces apoptosis in B16F-10 melanoma cells by inhibiting NF-κB-mediated bcl-2 activation and modulating p53-induced caspase-3 gene expression. *Immunopharmacol Immunotoxicol*, **34**, 143-51.

Pratheeshkumar P, Budhraja A, Son Yo, et al (2012). Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR-2 regulated AKT/MTOR/P7OS6K signaling pathways. *PLoS One*, **7**, 47516.

Prelich G, Tan CK, Kostura M, et al (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature*, **326**, 517-20.

Sakurai S, Kitano K, Yamaguchi H, et al (2005). Structural basis for recruitment of human flap endonuclease1 to proliferating cell nuclear antigen. *EMBOJ*, **24**, 683-93.

Sauter ER, Nesbit M, Litwin S, et al (1999). Antisense Cyclin D1 Induces Apoptosis and Tumor Shrinkage in Human Squamous Carcinomas. *Cancer Res*, **59**, 4876-81.

Shanmugam M, Singh AK, Nagarethinam B, et al (2012). Pro-apoptotic and anti inflammatory potential of andrographolide during 7, 12-dimethylbenz[a]anthracene induced hamster buccal pouch carcinogenesis. *J Exp Integr Med*, **2**, 313-19.

Shklar G (1999). Development of experimental oral carcinogenesis and its impact on current oral cancer research. *J Dent Res*, **78**, 1768-72.

Silvan S, Manoharan S (2013). Apigenin prevents deregulation in the expression pattern of cell-proliferative, apoptotic, inflammatory and angiogenic markers during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. *Arch Oral Biol*, **58**, 94-01.

Singh AK, Manoharan S, Suresh K, et al (2012). Modulating effect of andrographolide on cell surface glycoconjugates status during 7, 12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. *Int J Res Pharm Sci*, **3**, 200-5.

Sun Y, Luo D, Liao DJ, et al (2012). Cyclin D1 protein plays different roles in modulating chemo responses in MCF7 and MDA MB231 cells. *J Carcinog*, **11**.

Tashiro E, Tsuchiya A, Imoto M (2007). Functions of Cyclin D1 as an Oncogene and regulation of cyclin D1 expression. *Cancer Sci*, **98**, 629-35.

Trivedi NP, Rawal UM, Patel BP (2007). Hepatoprotective effect of andrographolide against hexachlorocyclohexane-induced oxidative injury. *Integr Cancer Ther*, **6**, 271-80.

Tung YT, Chen HL, Tsai HC, et al (2013). Therapeutic Potential of andrographolide isolated from the leaves of Andrographis paniculata nees for treating lung adenocarcinomas. *Evid Based Complement Alternat Med*, **305898**.

Wang LJ, Zhou X, Wang W, et al (2011). Andrographolide inhibits oral squamous cell carcinogenesis through NF-κB inactivation. *J Dent Res*, **90**, 1246-52.

Wang SC, Nakajima Y, Yu YL, et al (2006). Tyrosine phosphorylation controls PCNA function through protein stability. *Nat Cell Biol*, **8**, 1359-68.

Zhao F, He EQ, Wang L (2008). Anti-tumor activities of andrographolide a diterpene from Andrographis paniculata by inducing apoptosis and inhibiting VEGF level. *J Asian Nat Prod Res*, **10**, 467-73.