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Anti-cell Proliferative, Anti-inflammatory and Anti-angiogenic Potential of Lupeol in 7,12-dimethylbenz(a) Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Authors' contributions

This work was carried out in collaboration between all authors. Author SM designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author SK managed the literature searches. Author AM performed the experimental work and molecular analysis. Author MRN managed the experimental process. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Diverse pharmacological and biochemical effects of lupeol have been reported earlier. The present study utilized the immune expression pattern of proliferating cellular nuclear antigen (PCNA), cyclin D1, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NFkB) to assess the anticancer potential of lupeol in 7,12-dimethylbenz(a) anthracene (DMBA) induced oral carcinogenesis.
Methods: Well differentiated squamous cell carcinoma was appeared in the buccal mucosa of hamsters painted with DMBA thrice a week for 14 weeks. The expression pattern of the molecular

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markers was analysed using immunohistochemistry (PCNA, VEGF), Real Time PCR (NFkB, cyclin D1) and ELISA (COX-2).
Results: We noticed oral tumors in all the hamsters treated with DMBA alone and thus the tumor incidence is 100%. The total number of tumors developed in DMBA alone painted hamsters was 23. Upregulation of cell proliferative (PCNA, cyclin D1), inflammatory (NFkB, COX-2) and angiogenic markers (VEGF) was noticed in oral tumor bearing hamsters. Lupeol administration orally to DMBA painted hamsters completely inhibited the tumor formation (0%) and downregulated the immunoexpression pattern of cell proliferative (PCNA, cyclin D1), inflammatory (NFkB, COX-2) and angiogenic markers (VEGF).
Conclusion: The present results suggest that lupeol exhibited antitumor potential through its anti-cell proliferative, anti-inflammatory and anti-angiogenic potential during DMBA induced oral carcinogenesis.

Keywords: Oral cancer; lupeol; DMBA, angiogenesis; inflammation; cell proliferation.

1. INTRODUCTION

Oral cancer, a life threatening and disfiguring malignant neoplasm of the oral cavity, is the fifth most frequent cancer worldwide. Oral carcinoma constitutes 2-3% of all cancers in Western countries and 40-50% of all cancers in developing countries. Those who are habituated to consumption of tobacco, alcohol, and betel quid are identified as a high risk group for oral cancer development [1,2]. Oral cancer is, however, avoidable and preventable if it is diagnosed and treated earlier [3].

The poor survival outcome of oral cancer patients, due to late diagnosis, warranted to investigate sensitive and specific biomarkers to diagnose oral cancer at an early stage. Though a variety of biochemical markers are available to diagnose oral cancer, they are non-specific and non-sensitive [4]. Oral carcinogenesis arises due to dysregulation in several molecular markers that have a prominent and vital role in cell proliferation (PCNA and Cyclin D1), inflammation (NFkB and COX 2) and angiogenesis (VEGF). The present study thus utilizes these specific biomarkers expression to assess the anticancer potential of the test compound, lupeol.

7,12-dimethylbenz(a)anthracene is widely utilized to induce buccal pouch tumors in golden Syrian hamsters [5]. DMBA mediates oral carcinoma by inducing inflammation and causing oxidative damage to DNA, as well as by inducing DNA mutation through generating excess ROS. Furthermore, DMBA induced biochemical, molecular and histopathological alterations in the buccal mucosa mimics human oral tumors [6]. Researches on chemoprevention of oral cancer utilize DMBA induced hamster buccal pouch carcinogenesis to examine the anticancer potential of bioactive constituents of medicinal plants or synthetic agents [7].

Lupeol, lup-20(29)–en–3β-ol, is a triterpene found in several medicinal plants, vegetables and fruits such as mango, grapes, figures and strawberries [8]. Extensive studies focused the diverse pharmacological effects of lupeol, which include anti-inflammatory, anticancer, hepatoprotective and antioxidant properties [9,10]. *In vitro* and *in vivo* studies documented the anticancer activities of lupeol against several cancer cell lines and experimental models respectively [11,12]. Lupeol explored the chemopreventive and apoptotic efficacy in oral tumor bearing hamsters [12,13]. The aim of the present study is to focus the modulating effect of lupeol on the immunoexpression pattern of PCNA, Cyclin D1, NFkB, COX-2 and VEGF in the buccal pouch tumor bearing golden Syrian hamsters.

2. MATERIALS AND METHODS

2.1 Animals

The source of golden Syrian hamsters is National Institute of Nutrition, Hyderabad, India. The animals were 8 weeks old and weighing 80-120g at the time of purchase. The experimental animals were housed and maintained in the Central Animal House, Annamalai University.

2.2 Experimental Design

Animals were categorized into 4 groups and each group contains six animals. The group I hamsters served as vehicle treated control and received topical application of liquid paraffin on their buccal pouches thrice a week for 14 weeks. Hamsters that were categorized as group II were

painted with DMBA in liquid paraffin thrice a week for 14 weeks on their buccal pouches. Those hamsters received lupeol (50 mg/kg bw) orally thrice a week for 14 weeks on alternate days of DMBA treatment served as group III. Hamsters which received lupeol alone throughout the experimental period are categorized as group IV. Group IV is included in order to access the side effects (toxic) of long term administration of lupeol if any. Cervical dislocation was followed to sacrifice the experimental hamsters.

2.3 Immunohistochemical Staining

The immunoexpression pattern of PCNA and VEGF was studied using immunohistochemical techniques. The buccal mucosa tissue sections incubated with primary antibodies were later incubated with horse raddish peroxidase labeled secondary antibodies. 3,3'-diaminobenzedine was then added to the immune complex. The slides were scored for positively stained cells after the acceptable colour intensity was attained.

2.4 COX-2 activity by Enzyme-linked Immunosorbant Assay [ELISA]

The formation of oxidized N, N, N', N'-tetramethyl-P-phenylenediamine (TMPD) was read at 590nm in a Microtiter plate reader and is utilized as an end point to assess the activity of COX-2 in the buccal mucosa of golden syrian hamsters.

2.5 Expression of NF-kB and cyclin D1 Using Real-time Polymerase Chain Reaction

NFkB and cyclin D1 mRNA expression pattern were quantified using Real-Time PCR according to the amplification procedure recommended by the manufacturer (9700HT RT-PCR, Applied Biosystem, UK), using the primers mentioned in Table 1.

2.6 Statistical Analysis

One way analysis of Variance (ANOVA) followed by Duncan's Multiple Range test (DMRT) was used to compare the statistically significance between the groups. P values less than 0.05 between two groups mediates they are statistically significant.

3. RESULTS

The immunoexpression pattern (Figs. 1 and 2) and scoring of positive staining (Table 2) are analysed in the buccal mucosa of control and experimental hamsters. Higher expression of PCNA and VEGF was noticed in the buccal mucosa of hamsters painted with DMBA alone. Lupeol administration (50 mg/kg bw p.o) to DMBA painted hamsters reduced the PCNA and VEGF expression. This observation was substantiated with the scoring of positively stained cells in the buccal mucosa.

Fig. 3 illustrates the fold variation in the mRNA expression pattern for NFkB and cyclin D1, relative to control (fold variation β-actin normalized). The NFkB and cyclin D1 mRNA expression pattern was significantly increased in hamsters painted with DMBA alone. Lupeol administration to these hamsters reduced the mRNA expression pattern of NFkB and cyclin D1.

Fig. 4 depicts the status of COX-2 activity in the experimental hamsters. Hamsters painted with DMBA alone showed higher COX-2 activity in the buccal mucosa as compared to control hamsters. Lupeol administration to these hamsters orally brought back the COX-2 activity to near normal concentration. The status of COX-2 was found to be statistically insignificant between control hamsters and hamsters treated with lupeol alone.

Table 1. NFkB, cyclin D1 and β-actin primers used in Real-Time PCR

	Primers	Sequences
NFκB	Forward	5'-ATGGACGATCTGTTTCCCCT-3'
	Reverse	5'- CGGTTTACTCGGCAGATCTT-3'
Cyclin D1	Forward	5'-CGGAGGACAACAAACAGATC-3'
	Reverse	5'-GGGTGTGCAAGCCAGGTCCA-3'
β-actin	Forward	5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3’
	Reverse	5'-AGCAGCCGTGGCCATC TCTTGCTCGAAGTC-3'

Table 2. PCNA and VEGF scoring in the buccal mucosa of experimental hamsters (n = 6)

Positive staining	Control	DMBA	DMBA + Lupeol	Lupeol alone
PCNA				
0	0	0	0	1
1+	6	0[a]	5[b]	5[b]
2+	0	4[a]	1	0
3+	0	2	0	0
VEGF				
0	6	0[a]	5[b]	6[b]
1+	0	1	1	0
2+	0	2	0	0
3+	0	3[a]	0[b]	0[b]

0 = negative, less than 5% of cell staining, 1+= weak staining, between 5 and 25% of cells staining, 2+= moderate staining, between 25 and 50% of cells staining, 3+= strong staining, more than 50% of cells staining
[a] Significantly different from control hamsters by χ^2 test ($p<0.05$), [b] Significantly different from DMBA alone treated hamsters by χ^2 test ($p<0.05$)

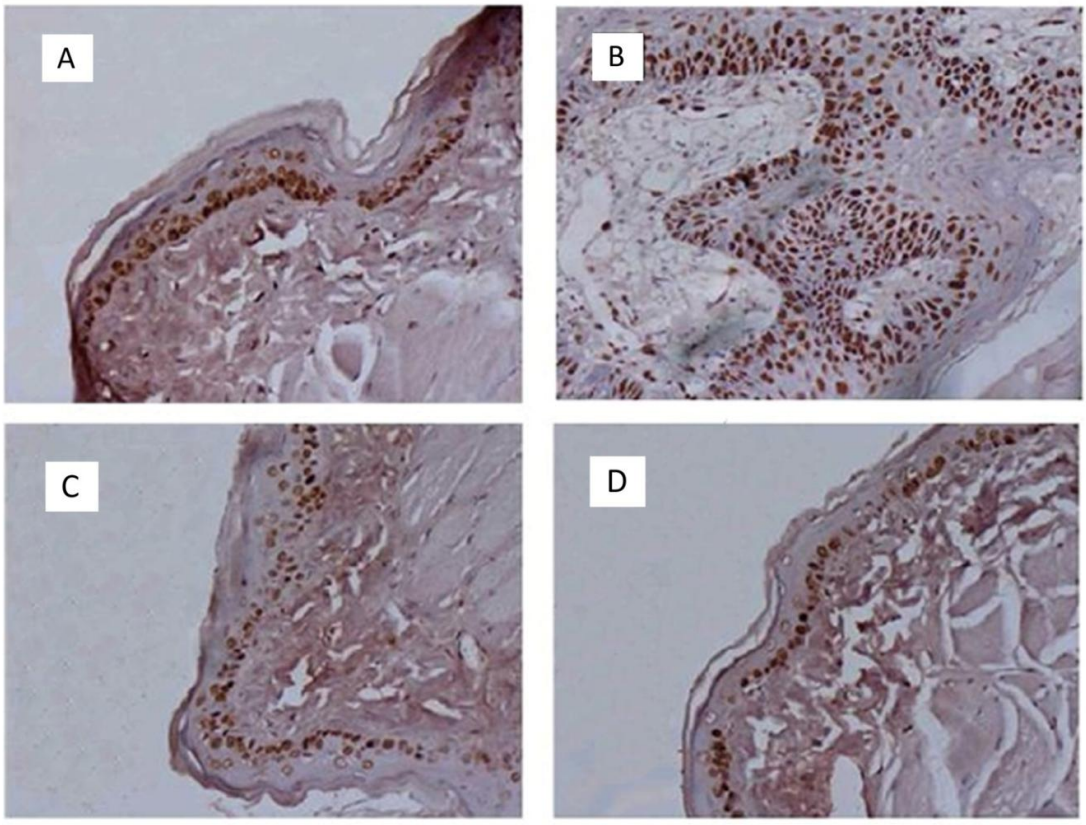

Fig. 1. PCNA expression in the buccal mucosa of experimental hamsters (40x)
A - Control (weak staining), B - DMBA alone (over expression), C - DMBA + Lupeol (down-regulated), D - Lupeol alone (weak staining)

4. DISCUSSION

Oral cancer is associated with abnormal expression of cell-cycle regulatory cyclins. Of three isoforms of D-type cyclins, cyclin D1 is overexpressed in oral cancer tissues [14]. Cyclin D1 has a crucial role in the G1 to S transition in the cell cycle and its over expression result in shortening of G1 phase and thus leads to abnormal cell proliferation [15]. De Falco et al.

[16] has shown the transition of cyclin D1 between nuclear and cytoplasmic compartments via nuclear pores. Cyclin D1 is abnormally expressed in the nucleus of oral cancer tissues. Real-Time PCR analysis revealed over expression of cyclin D1 - mRNA in oral carcinogenesis [6]. Dysregulation of cyclin D1 mRNA due to cyclin D1 3' UTR polymorphism has been reported. It has also been reported that cyclin D1 gene mutation may enhance the stability of cyclin D1 mRNA/protein [15]. It has been well documented that downregulation of cyclin D1 implies better prognosis as well as improves survival outcome of patients [17]. Manoharan et al. [18] demonstrated that cyclin D1 was significantly increased in the buccal mucosa of hamsters treated with DMBA alone as compared to control hamsters. The present study also demonstrated upregulation of cyclin D1 mRNA in hamsters treated with DMBA alone painted hamsters.

PCNA, an evolutionarily well-conserved eukaryotic protein, has a major role in the cell-cycle control, DNA synthesis and DNA repair. Extensive studies reported that immunohistochemical expression of PCNA could be used to assess cell proliferation [19]. Immunohistochemical expression also revealed overexpression of PCNA in 70-80% of the oral cancers. Profound studies focused PCNA and cyclin D1 as a prognostic indicator of oral carcinogenesis [20]. Abnormal expression of PCNA is documented in several malignancies, including oral cancer [21]. Silvan et al. [22] pointed out that PCNA was overexpressed in the buccal mucosa of DMBA treated hamsters as compared to control animals. PCNA overexpression was confirmed in DMBA alone painted hamsters as evidenced by the presence of brown staining in the nuclear and cytoplasmic region [17].

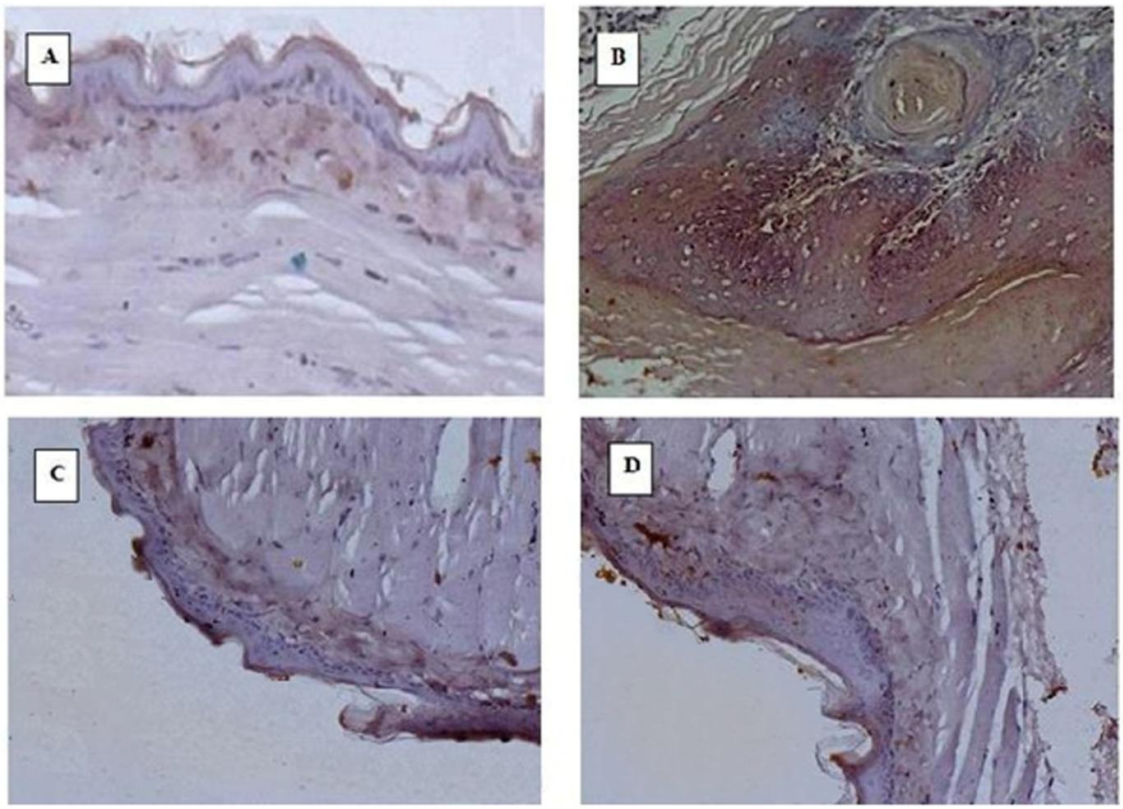

Fig. 2. VEGF expression in the buccal mucosa of experimental hamsters (40x).
A - Control (negative staining), B - DMBA alone (over expression), C - DMBA + Lupeol (down-regulated), D - Lupeol alone (negative staining)

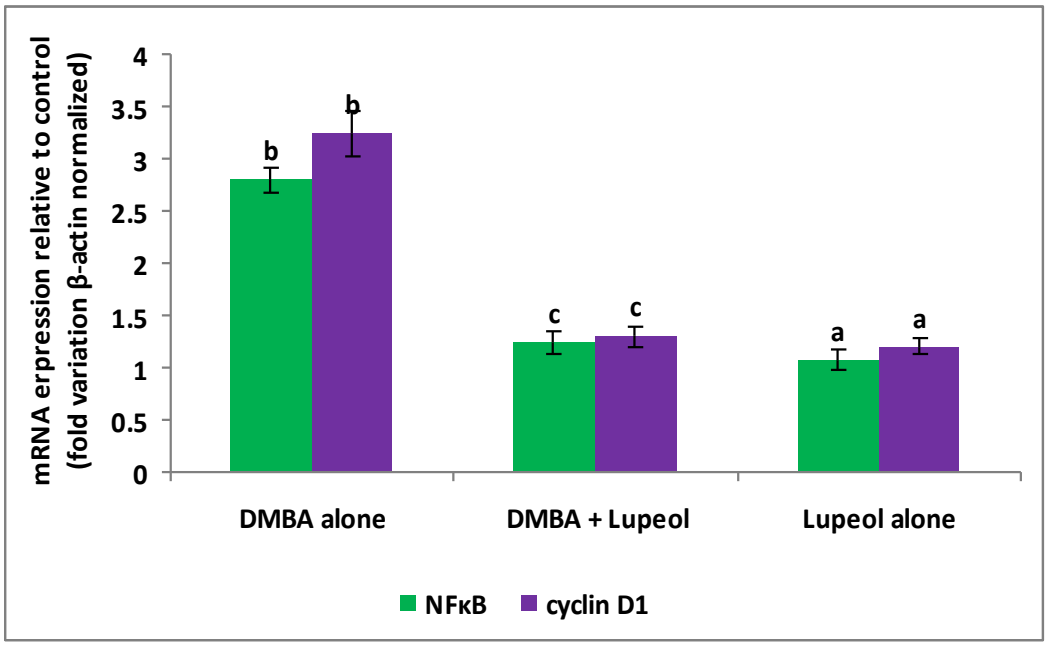

Fig. 3. mRNA expression relative to control hamsters (n = 6)
b and c - Statistically significant (p < 0.05), b and a - Statistically significant (p < 0.05)
c and a - Statistically significant (p < 0.05)

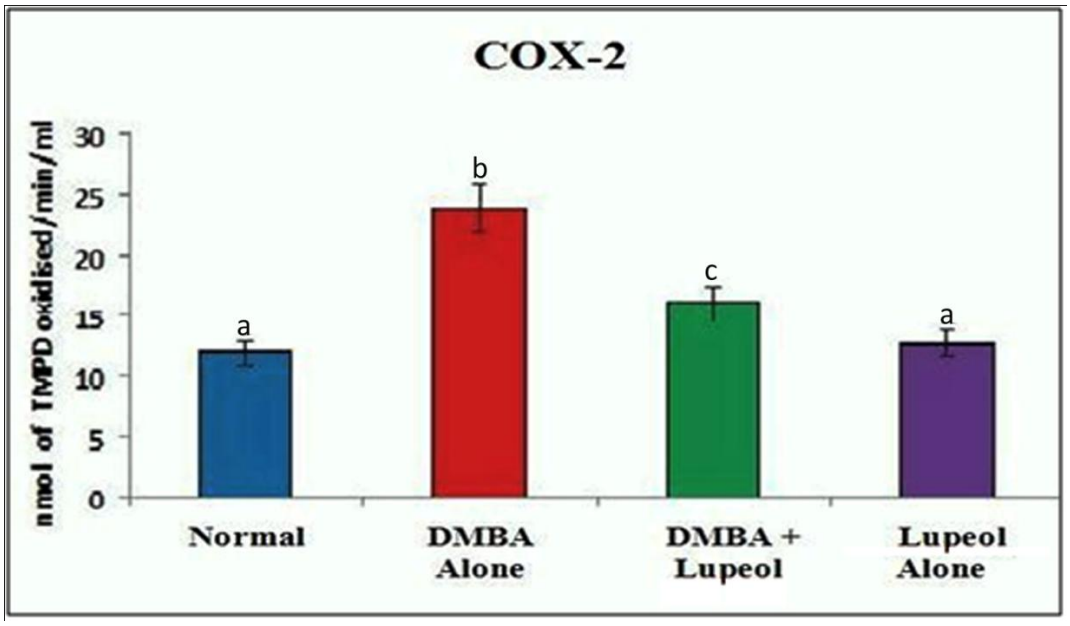

Fig. 4. Status of COX-2 in the buccal mucosa of experimental hamsters (n = 6)
b and c - Statistically significant (p < 0.05), b and a - Statistically significant (p < 0.05), c and a - Statistically significant (p < 0.05), a and a - Not significant

Nuclear factor-kB, a transcription factor, plays pivotal role in several key biological processes including tumor initiation and progression [23]. NFkB mediates carcinogenesis and its progression by upregulating factors involved in angiogenesis (VEGF) and cell survival (anti-

apoptotic proteins) and by decreasing the immune response [24]. Agents that could inhibit the activation and translocation of NFkB could be considered as a potent anti-inflammatory and anti-neoplastic agent [25]. Over expression of NFkB was reported in prostate, breast, pancreas and oral cancers [26]. It has been suggested that COX-2 contributes to tumor development and progression by stimulating mitogenesis and metabolic activation of procarcinogens [27]. Pu et al. [28] reported that cyclooxygenase-2 played a vital role in the development of oral carcinoma via catalysing the synthesis of inflammation including prostaglandins. COX-2 activity was upregulated not only in inflammatory tissues but also in the precancerous and cancerous tissues [29]. COX-2 overexpression could result in the suppression of apoptotic proteins and in the stimulation of proteins, involved in the cell proliferation. It has been suggested that while COX-2 is involved in the early stages of oral carcinogenesis, NFkB is involved in the late stages. Overexpression of COX-2 and NFkB has been reported in patients with oral dysplasia and cancers [26]. Baskaran et al. [30] showed increased COX-2 activity and higher expression in the buccal mucosa of DMBA treated golden Syrian hamsters as compared to control animals. Our results also lead credence to the previous observations.

Angiogenesis plays critical role in inflammation, tumor promotion and metastasis [31]. VEGF, a key regulator of angiogenesis increases microvasculature and vascular permeability [32]. VEGF was overexpressed in various cancers, including oral cancer [33]. Manoj Prabhakar et al. [34] reported that the angiogenic factor VEGF was overexpressed in hamsters painted with DMBA. Our results correlate these findings.

In the present study, lupeol administration to DMBA treated hamsters significantly downregulated cell proliferation markers (PCNA, cyclin D1), inflammatory markers (COX-2, NFkB) and angiogenic marker (VEGF) in tumor bearing hamsters. Nita et al. [35] demonstrated that lupeol induced cell cycle arrest in rats with skin cancer by decreasing the expression of PCNA. Chen et al. [36] reported that lupeol acetate exhibited potent anti-inflammatory activity by effectively inhibiting COX-2 expression in experimental animal model. Vijay Avin et al. [37] suggested that lupeol significantly inhibited the new blood vessel formation by downregulating VEGF expression.

The present study thus explored the antitumor initiation efficacy of lupeol (pre-initiation phase) with the help of various molecular targets that have a pivotal role in cell proliferation, inflammation and angiogenesis. Further studies are, however, needed to confirm its efficacy in the post-initiation phase of DMBA induced hamster buccal pouch carcinogenesis (i.e. administration of lupeol in the tumor bearing hamsters).

5. CONCLUSION

Based on the present findings, we conclude that lupeol explored its anticancer potential by modulating the expression of PCNA, cyclin D1, NFkB, COX-2 and VEGF in favour of inhibiting tumor formation in the buccal mucosa of DMBA treated hamsters.

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CONSENT

Not applicable.

ETHICAL APPROVAL

The present experimental design was approved by Annamalai University animal ethics committee (Reg.no.:160/1999/CPCSEA).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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