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New role of lupeol in reticence of angiogenesis, the cellular parameter of neoplastic progression in tumorigenesis models through altered gene expression

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ABSTRACT

There is a major unmet medical need for effective and well tolerated treatment options for cancer. The search now seeks to identify active biomolecules with multiple targets. Lupeol, an important dietary triterpenoid known as anticarcinogen by inducing apoptosis. But it is still more to reveal the potency of lupeol in the inhibition of neovascularization in cancer context. The study aimed to explore the efficacy of the lupeol in targeting angiogenesis. In this study, the inhibition of neovessel formation was assessed by preliminary antiangiogenesis assays like chorio allontoic membrane (CAM) and rat corneal micro pocket models. Further, validated for the micro vessel density (MVD) in histological sections of peritoneum, solid tumor and xenograft tumor by immunostaining with anti CD31 antibody. Antitumor potency was verified in ascites carcinoma, solid lymphoma and human nueroblastoma xenograft in CAM. Altered angiogenic gene expression by RT-PCR, ELISA and gelatin zymography. Lupeol significantly inhibits the neovessel formation in CAM and in the rat cornea. The similar effect was ascertained in mice and human xenograft tumor models with the regressed growth. Eventually reflecting on the differential transcription of angiogenic genes like MMP-2 & 9, HIF-1α, VEGFa and Flt-1 was noteworthy. It is now evident from our studies that, a new avenue of dietary triterpenoid lupeol by targeting angiogenesis, potentially inferring the multimode action in cancer prevention.

1. Introduction

The new challenge in conventions of anticancer therapy is to recognize the multi compartment nature of the tumor microenvironment which reflexes in the radically different approaches toward the discovery of new treatments. Resistance to apoptosis and progressing angiogenesis are such important parameters which are required for tumor growth, invasion and metastatic dissemination targeted during anticancer therapeutics [1]. The search now seeks to identify active biomolecules which would specifically target these two parameters and there by inhibiting the tumor growth [2]. Therefore, it is necessary to intensify our efforts for better understanding and development of novel treatment and preventive approaches for cancer.

Fruits and other plant derived products have gained considerable attention as they can reduce the risk of several cancer types [3]. Epidemiological and experimental studies provide evidence that some naturally occurring chemical agents in the human diet can reduce cancer risk [4]. Lupeol (Lup-20(29)-en-3b-ol) (Supplementary Fig. 1), a triterpene present in fruits such as mango, olive, strawberry, grapes, figs, etc., vegetables and in several medicinal plants, is used for treatment of number of ailments worldwide [5]. Recent reports showed that lupeol directly induces the apoptosis of tumor cells under *in-vitro* and *in-vivo* situation. These include its beneficial activity against inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity and hepatic toxicity [6–9]. Data emanating from molecular studies with various tumorigenic models suggest that lupeol modulate host systems potentially enabling more robust antitumor responses by aberration of Ras oncoprotein and induction of Fas receptors and its adaptor protein their by inducing apoptosis [10–14]. But

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it is still more to reveal the potency of lupeol in the inhibition of neovessel formation in various cancerous conditions and to unwrap the lupeol as a potent antiangiogenic bullet. The present study explores the efficacy of the lupeol to evaluate the angiopreventive effect.

2. Materials and methods

2.1. Chemicals and others

Lupeol, gelatin type-A porcine skin, TRI reagent, ECM gel, hydron polymer poly-hydroxyethyl-methacrylate (poly HEMA), primers, Anti-VEGF and anti mouse IgG antibodies were obtained from Sigma–Aldrich, USA. Superscript first strand synthesis and PCR supermix for RT PCR from Invitrogen, USA. Anti CD31antibody, Immunostaining kit from Leica Biosystems, Germany. All the other chemicals used were of analytical grade. Firtilized hen's eggs (Giriraja breed) were procured from local market in Shimoga, India. All the photographs were taken using Canon power shot Sx500 IS camera.

2.2. In-vivo and ex-vivo chorioallontoic membrane (CAM) assay

The recombinant $VEGF_{165}$ ($rVEGF_{165}$) induced *in-vivo* and *ex-vivo* CAM angiogenesis models was performed to study antiangiogenis effect of lupeol (10 μM) in fertilized eggs and change in the vascularisation pattern of with or without lupeol treated egg preparations were photographed as described previously [15].

2.3. Animals and ethics

Healthy Swiss Albino male mice weighing 25 ± 2.0 g and male Swiss albino whistler rat weighing 150 ± 5.0 g were grouped (n = 10) and housed in polyacrylic cages and maintained under standard conditions (25 ± 2 °C) with 12 ± 1 h dark/light cycle. All procedures for animal experimentation were approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, Karnataka, India (NCP/IAEC/CL/101/05/2012-13).

2.4. Rat corneal micropocket assay

The $rVEGF_{165}$ (1 μg/pellet) induced neovascularization in rat cornea was examined by treating with or without lupeol (10 μM/pellet), formulated in poly HEMA into rat corneas for treatment [16]. The number of blood vessels and length of the vessels were quantified [17].

2.5. Tumor models and treatment

Ehrlich ascites carcinoma (EAC) cells and Dalton's lymphoma ascites (DLA) were maintained separately *in-vivo* by intraperitoneal transplantation to develop ascites tumor [18]. Solid Dalton's lymphoma (DL) tumor induction was developed by injecting tumor cells into the thigh of the experimental animals subcutaneously. Lupeol (40 mg/kg body weight) was administered interaperitoneally (i.p) after onset of tumor in both EAC and solid DL tumor bearing mice on 4th and 10th day, respectively on every alternate day and verified for antitumor potency along with survivability analysis as described previously [15].

2.6. Peritoneal angiogenesis and immunohistopathology

Carcinoma induced peritoneal neovascularization was visualized and documented in with or without lupeol treated animals. Corresponding peritoneum tissue sections were processed for Hematoxyline & Eosin (H&E) staining and immunostain with anti CD31 antibodies as per the manufacturers recommendations [19].

2.7. CAM xenograft model

In-vivo CAM xenograft model to assess angiogenesis was performed with slight modification as described previously [16]. In brief, the plastic rings coated with ECM gel were placed on the growing CAM of 5th day of incubation of fertilized eggs under sterilized condition by making window in the egg shell. The rings were then adsorbed with 5×10^6 B16F10 cell suspension along with $rVEGF_{165}$ (10 ng/ml) and treated with or without lupeol (10 ng/ml). On day 12, the eggs were opened and the tumors formed were excised, tumor size were determined and processed for H&E.

2.8. Reverse transcription (RT)-PCR

DL cells treated with or without lupeol *in-vivo* were harvested and total RNA was isolated using TRI reagent. RT was performed using oligo(dT) primers and superscript reverse transcript following the manufacturer's recommendation and amplified using MMP-2 forward primer (fwd): 5′-ACAGTGACACCACGTGACAA-3′ and reverse primer (rev): 5′-GGGATGGCATTCCAGGAGTC-3′, MMP-9 fwd: 5′-CGCTCATGTACCCGCTGTAT-3′ and rev: 5′-TGTCTGCCGGACT CAAAGAC-3′, HIF-1α fwd: 5′-TTCACTGCACGGGCCATATT-3′ and rev: 5′-TCCACCTCTTTTGGCAAGCA-3′, Flt-1 fwd: 5′-TGGGCAGTCAAGT CCGAATC-3′ and rev: 5′-GTGCAAACTCCCACTTGCTG-3′, VEGFa fwd: 5′-GGGGTGTCCCATAGGGGTAT-3′ and rev: 5′-CGCCTTGGCTTGTCA CATTTT-3′. Normalization of angiogenic gene expression was achieved by comparing the expression of GAPDH fwd: 5′-CGCTC ATGTACCCGCTGTAT-3′ and rev: 5′-TGTCTGCCGGACTCAAAGAC-3′ for the matching sample. PCR product were resolved by using 1.5% agarose gel and identified by ethidium bromide staining.

2.9. VEGF – ELISA

The serum (100 μl) from solid DL bearing animals treated with or without lupeol was collected and VEGF levels were quantified by using anti VEGF antibody by ELISA [19].

2.10. Gelatin zymography

Expression of MMP-2 & 9 in whole cell lysate from DL tumor tissue treated with or without lupeol was assessed by gelatin gel zymography as previously described [20]. In brief, the equal concentration of total protein from with or without lupeol treated tumor tissue was resolved in 11% SDS-PAGE gels containing 0.1% (w/v) gelatin type A porcine skin. The gels washed with zymography renaturing buffer (2% Triton X-100), then incubated for 18 h at 37 °C in reaction buffer (50 mM Tris–HCl, 200 mM NaCl, and 5 mM $CaCl_2$). The gels were then stained with Coomossie brilliant blue R 250. Gelatinase activity in the gel slab was quantified by Bio-rad Gel Documentation™ XR+ Imaging System.

3. Results

3.1. Lupeol inhibits the neovascularization in non tumor model systems

To investigate the antiangiogenic activity *in-vivo*, lupeol was tested in different angiogenesis models induced by $rVEGF_{165}$. In CAM model a clear avascular zone around the implanted disc with lupeol was clear evident for the regression of neovessels in the developing embryos in both *in-vivo* and *ex-vivo* CAM (Fig. 1A and

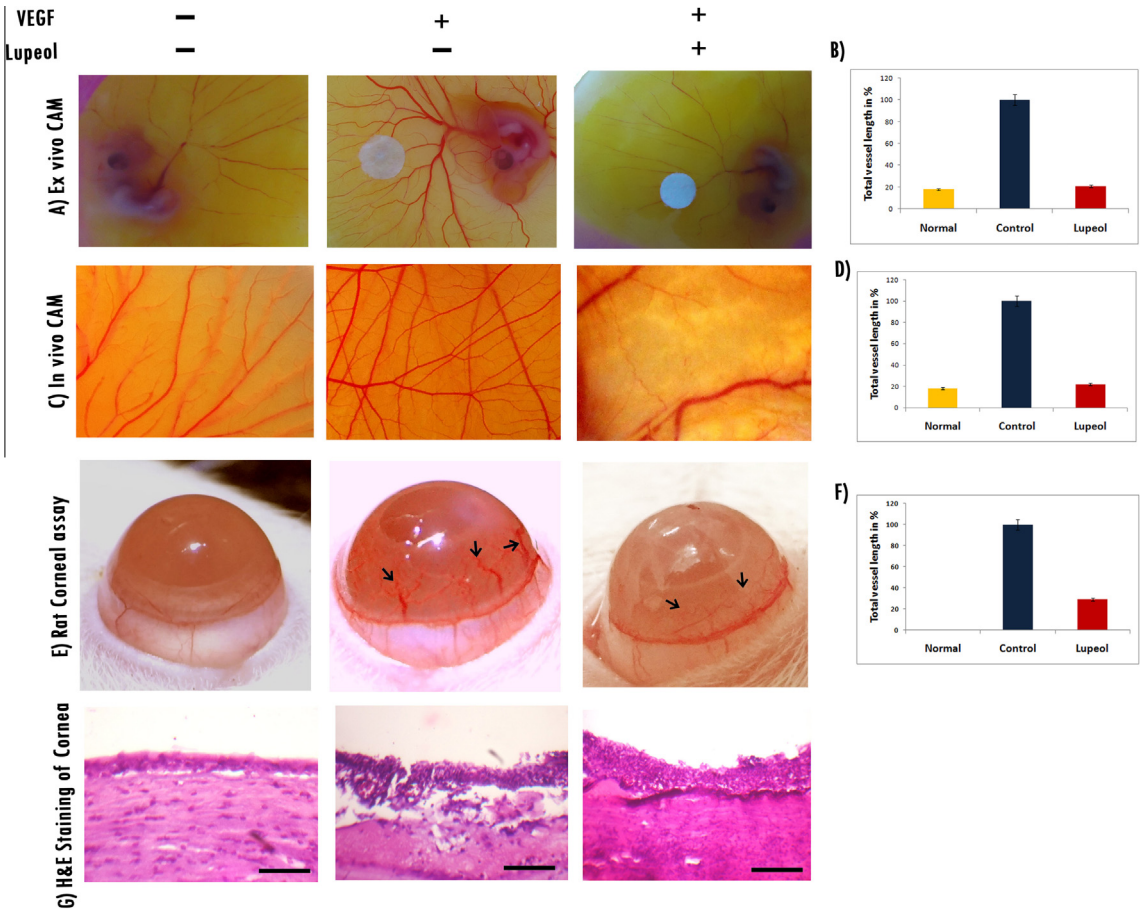

Fig. 1. Lupeol inhibits neovessel formation in rVEGF$_{165}$ induced angiogenesis models: (A)–(D) *in-vivo* and *ex-vivo* CAM assay photographs representing the angio modulatory with depreciation of total vessel length by lupeol treatment. (E) Representative photographs of rat cornea indicating the inhibition of angiogenesis and (F) decrease in total vessel length after lupeol treatment in rat cornea. (G) H&E staining of cornea showing improved corneal gesture with lupeol treatment.

C). The total vessel length of lupeol treated was drastically decreased to ~80% of the untreated in both the assays (Fig. 1B and D). The similar results were mimicked in rat corneal micro pocket assay (Fig. 1E and F). The histopathological analysis of corneal section further revealed the improved corneal gesture compared to untreated (Fig. 1G).

3.2. Lupeol inhibits tumor induced neovascularization in ascites carcinoma and solid lymphoma

Ascites carcinoma induced angiogenesis in mice peritoneum was verified in response to lupeol treatment. Decreased peritoneal angiogenesis was visualized in lupeol treated animals after three doses when compared to extensive angiogenesis in untreated control (Fig. 2A). Histological sections of peritoneum were characterized by a remarkable decrease in MVD and the caliber of detectable vascular channels with 6.4 ± 1.5 MVD/high power field (MVD/HPF) as compared to control 23 ± 1.7 MVD/HPF (Fig. 2B and C). Further, immunostaining observation of same with anti CD31 antibodies, signifies the suppressed and seldom CD 31 positive cells (Fig. 2D). This inhibition of neovessel formation was reflected in the dose dependent decrease in cell proliferation (Fig. 2E), ascites secretion (Fig. 2F) and there by the tumor growth (Fig. 2G) with the expanded life span up to 26 days (Fig. 2H).

In concurrence to these results, the antiproliferative effect of lupeol was also tested in solid DL tumor model. Upon treatment of lupeol from the onset of solid DL tumor, a significant reduction in the neovessel formation around the tumor tissue was evident after five doses. The control group showed 19 ± 1.7 MVD/HPF whereas in treated 5 ± 1.8 MVD/HPF which accounts for ~73% inhibition as observed in the H&E staining (Fig. 2K and L). Impact of decreased MVD resulted in the dose dependent decrease in tumor inhibition (Fig. 2M) and ~61% reduction in the tumor volume after completion of treatment (Fig. 2I and J). Most resolutely, there was approximately 3-fold increase in the life span of treated animals (Fig. 2N). However the morphology and histopathology of the dissected organs like liver and spleen showed reduced infiltration of tumor cells with less histological damage in the respective organs (Supplementary Fig. 2).

3.3. Lupeol exhibits angiopreventive activity in xenograft tumor

Angiopreventive efficacy of lupeol tested in above mentioned models is verified in xenograft human neuroblastoma CAM model. The macroscopic observation of neuroblastoma xenograft tumor treated with lupeol showed a suppressed growth of tumor and regretting allantoic blood vessels surrounding the tumor as compared to rVEGF$_{165}$ alone treatment (Fig. 3A and B). The MVD of control was 11 ± 1.7 MVD/HPF whereas lupeol treated showed 3 ± 1.3 MVD/HPF (Fig. 3C and D) indicating 3-fold decreased count. Altogether these results suggests that the lupeol is capable of inhibiting the neovessel formation in both *in-vivo* and *ex-vivo* angiogenesis assays along with xenograft human tumor.

3.4. Lupeol induces differential expression of angiogenic genes

To further validate the molecular basis of lupeol effect in regulation of angiogenesis, we isolated mRNA from the lupeol treated

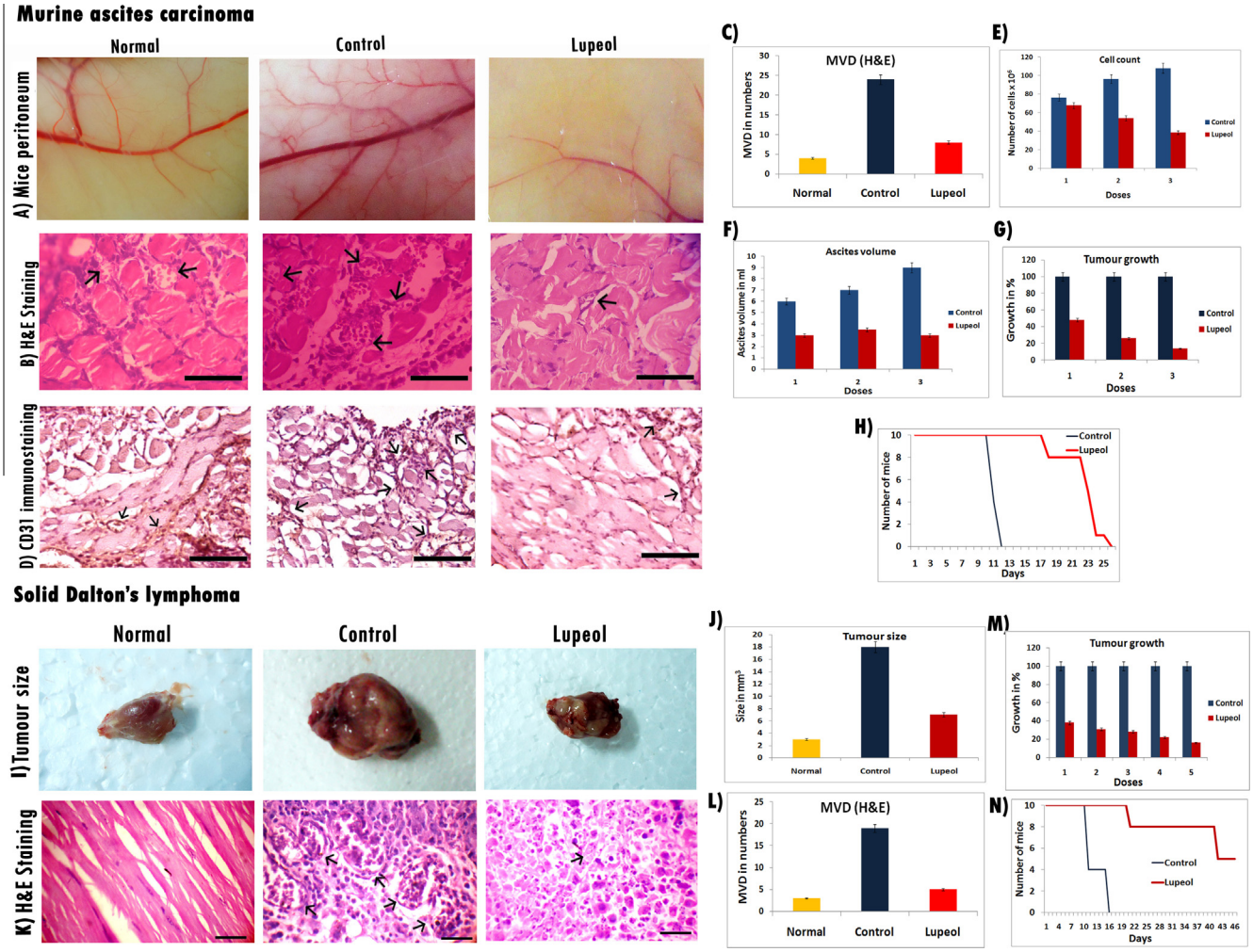

Fig. 2. Angiomodulatory effect of lupeol in ascites carcinoma and solid lymphoma: (A) representative photographs of peritoneum linings of normal and ascites carcinoma bearing mice treated with or without lupeol indicating the reduced neovascularization and (B) and (C) H&E staining of peritoneum and decreased MVD count was clearly evident after treatment with lupeol. (D) Seldom of CD 31 positive cells of peritoneum section after lupeol treatment. (E) Dose dependent decrease of cell count. (F) Ascites volume, and (G) percentage of tumor growth regression after treatment with lupeol. (H) Kaplan–Mayer survivability curve indicating the increased life span of lupeol treated animals. (I) Morphology of normal, control and lupeol treated thigh tissue, showing reduced vasculature around the tumor and (J) measured tumor volume. (K) Representative photographs of H&E staining and (L) with decreased number of MVD/HPF was evident in lupeol treated sections. (M) Dose dependent decrease of tumor growth after treatment with lupeol. (N) Kaplan–Mayer survivability graph showing increase in life span of lupeol treated animals.

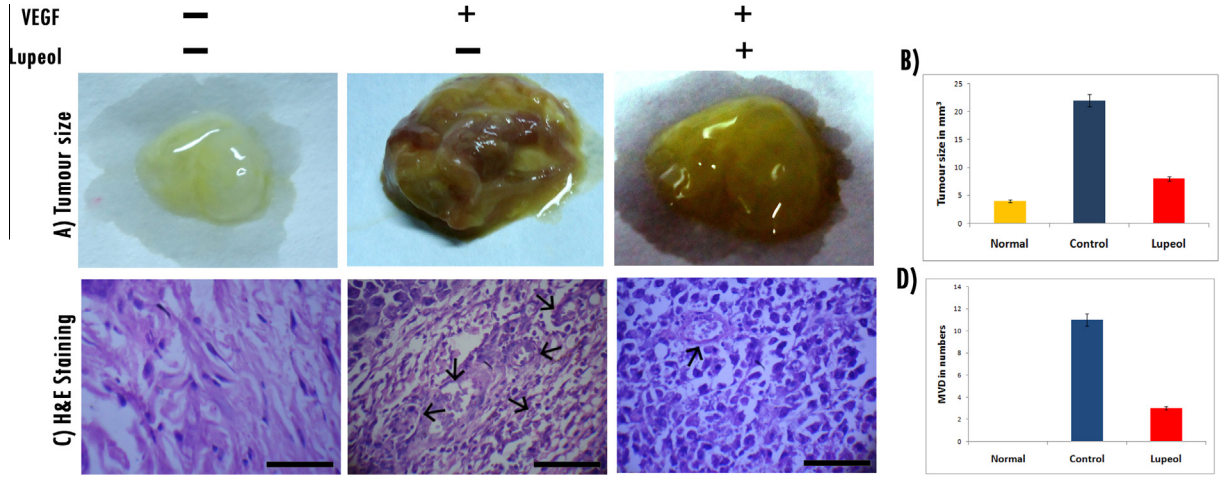

Fig. 3. Lupeol exhibits avasculogenic effect in xenograft tumor (A) $rVEGF_{165}$ induced human neuroblastoma xenograft in CAM showing reduced vasculature and (B) decreased tumor size after treatment with lupeol. (C) and (D) Hematoxylin & Eosin stained tumor mass depicting that extensive vasculatures in control whereas reduced MVD/HPF after treatment with lupeol.

solid tumor and gene expression profile was verified by semi quantitative RT-PCR analysis. The results inferred, there was a complete transcriptional inhibition of MMP-2 & 9, and partial inhibition of HIF-1α, VEGF and flt-1 with 40%, 20% and 37.5%, respectively upon lupeol treatment (Fig. 4A and B).

Eventually the protein expression profile of MMP-2 & -9 resulted no cleavage of substrate gelatin indicating its inhibition by lupeol (Fig. 4C). The ELISA results revealed, lupeol induces dose dependent inhibition in secretion of VEGF in the sera of DL tumor bearing mice (Fig. 4D), inferring 5-fold decrease of VEGF secretion was noteworthy.

4. Discussion

Drug based strategies for chemoprevention may predominantly rely upon targeted therapies with tolerable but defined toxicities for treatment. Multi targeting agents capable of intervening with a number of critical pathways responsible for tumorigenesis will have a merit over other single targeting agents [5]. One such plant based chemopreventive molecule the triterpene lupeol known for its biological activities [6] has a widespread distribution in diverse plant families, these compounds are also easier to obtain through diet than most treatment currently available [21–23]. Reports have shown that lupeol exhibits anti cancer properties and much focus has been placed on lupeol induced apoptosis [10–13]. The broad spectrum targets of lupeol in cancer treatment are still remained to be elucidated. Recently You et al. shown that lupeol exhibits antiangiogenic property in an *in-vitro* tube formation model of human umbilical venous endothelial cells [24]. No further research progressed in relevance to antiangiogenic potential of lupeol neither *in-vitro* nor *in-vivo*, which grasped our attention to study the plausible effect of lupeol in the modulation of neovessel formation.

In the current study we have chosen *in-vivo* non tumerogenic and tumerogenic models to exhibit lupeol's angioprevention potency. VEGF is predominantly required for the stimulation of neovessels [25]. Hence, the VEGF induced angiogenesis in CAM models and rat corneal micro pocket models were tested for the efficacy of lupeol. These model systems' being the gold standard methods of angiogenesis has valued our results. Our findings deduced that, the VEGF induced angiogenesis was inhibited by lupeol in both *ex-vivo* and *in-vivo* CAM model and in rat cornea (Fig. 1).

In-vivo experiments have demonstrated that establishment of tumor and haematogenous tumor remobilization is angiogenesis dependent and angioprevention will affect the growth and metastatic potentials [26]. Murine ascites and solid tumor are suitable model system for preliminary screening and place a critical role in drug development [27]. The tumor implantation in both mice model system induces a set of local inflammation and there by stimulates the increasing permeability of the surrounding blood vessels and hence results in massive angiogenesis supporting the tumor growth. Our data showed that, the lupeol was able to inhibit the proliferation of neovessls and this is reflected in the reduction of tumor growth in both ascites and solid tumors of different origins. The *in-vivo* effect of lupeol on proliferation of endothelial cells is especially reduced visibility when observing blood vessel formation in the peritoneum of EAC bearing mice and dissected DL solid tumor. Staining for CD 31, an endothelial cell surface marker, clearly suggest that lupeol primarily acts on endothelial cells, and hence inhibition of angiogenesis and directing to tumor regression (Fig. 2). The above said result of angioprevention by lupeol was reproduced in human neuroblastoma xenograft CAM model (Fig. 3). The important concern in drug development process is target specific in action minimizing the toxicological side effects. So for lupeol has been reported to exhibit no toxicities in animal

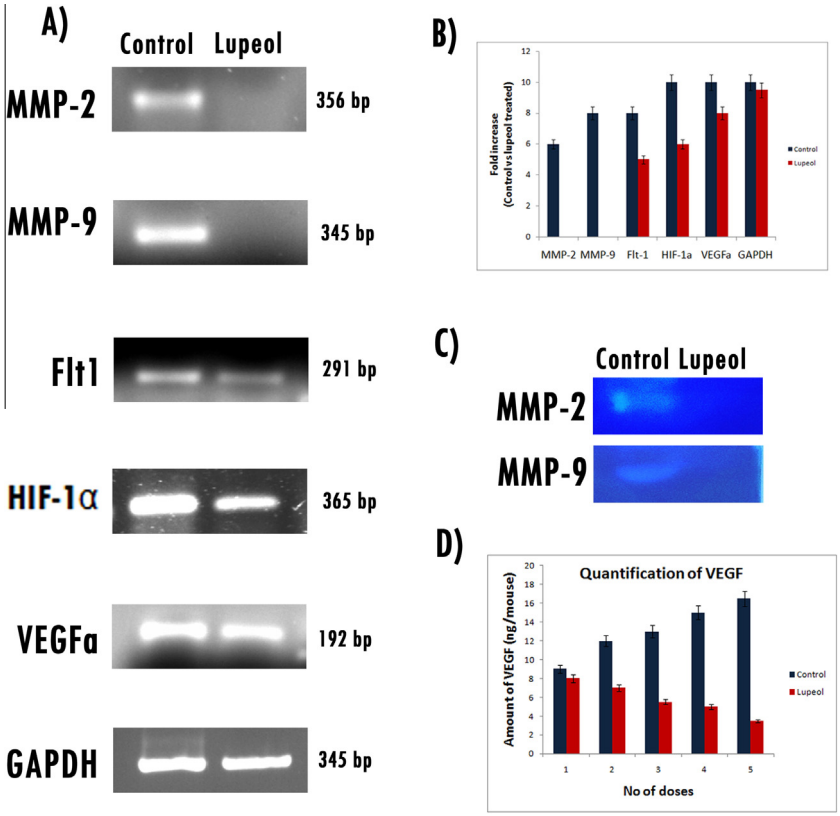

Fig. 4. Lupeol induces differential gene expression. (A) Lupeol induces complete transcriptional inhibition of MMP-2 & -9 and partial inhibition of Flt1, HIF-1α and VEGFa. (B) Comparative evaluation of fold increase in gene expression. (C) Translational inhibition of MMP-2 & -9 as observed in gelatin zymography after treatment with lupeol. (D) Dose dependent reduction of VEGF in sera of lupeol treated tumor bearing mice.

studies and no adverse effect in rats and mice even after the treatment ranging from 40 to 200 mg/kg body weight [28,29]. In our study the 40 mg/kg body weight of treatment has no toxicological significance (Supplementary Fig. 2).

The endothelial cell proliferation during tumor condition is chain of events associated with the activation of many pro and anti angiogenic factors. During tumor angiogenesis the hypoxic tumor micro environment induces the HIF-1α a transcription factor which regulates VEGF secretion. The secreted VEGF binds to its receptor such as flt-1/flk-1 on endothelial cells resulting in the activation of matrix metallo proteases which helps in the degradation of basement membrane there by resulting in the sprouting of neovessels [30]. In pathophysiological condition, targeting of these events is highly prerequisite. In current approach we have used the solid tumor model to analyze the molecular basis of lupeol effect in relevance to angiogenic genes expression both at transcription and translational level. The results indicated the differential gene expression by altering the levels of MMP-2 & -9, VEGFa, HIF-1α, and flt-1. The inhibition of MMP-2 & -9 in transcriptional levels clearly correlated with the data obtained in the protein expression level as observed by gelatin zymography. As in normal angiogenesis tumor angiogenesis appears to rely heavily on VEGF. Increased VEGF expression is closely associated with an increase in MVD [25,30]. In accordance with this view the neutralization of VEGF may halt the tumor formation. Our study revealed that the quantification of VEGF by ELISA indicated a dose dependent decrease amount of VEGF in serum of DL solid bearing lupeol treated animals. Since there is a inhibition of angiogenesis by lupeol it supports the view that lupeol suppress the expression of VEGF, MMP-2 & -9, there by inhibiting the tumor formation (Fig. 4).

In conclusion, the lupeol is capable to suppress the neovessl formation and downregulation of angiogenic genes such as of MMP-2 & -9, VEGFa, flt-1 and HIF – 1α which are associated in tumorigenic condition. Our present investigation demonstrated the new role of dietary triterpenoid lupeol as a potent antiangiogenic drug. Further investigation is needed to understand the signaling mechanism in pathologic angiogenesis and potentially attenuate its activity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.090.

References

[1] D. Hanahan, A.W. Robert, Hallmarks of cancer: the next generation, Cell 144 (5) (2011) 646–674.
[2] M. Cesca, F. Bizzaro, Z.E.G. Massimo, Tumor delivery of chemotherapy combined with inhibitors of angiogenesis and vascular targeting agents, Front. Oncol. 259 (3) (2013) 1–7.
[3] N.T. Burkert, J. Muckenhuber, F. Grobschadl, E. Rasky, W. Freidl, Nutrition and health – the association between eating behavior and various health parameters: a matched sample study, PLoS One 9 (2) (2014) e88278.
[4] P. Alessia, P. Gaetano, T. Ugo, Triterpenoids as new promising anticancer drugs, Anti Cancer Drugs 20 (2009) 880–892.
[5] K. Pranav, Chaturvedi, B. Kulpreet, S. Yogeshwer, Lupeol: connotations for chemoprevention, Cancer Lett. 263 (2008) 1–13.
[6] B.C.G. Margareth, J.S. Mieanda, Biological activites of lupeol, Int. J. Biomed. Pharm. Sci. 3 (1) (2009) 46–66.
[7] M.A. Fernandez, B. Heras, M.D. Garcia, M.T. Sáenz, A. Villar, New insights into the mechanism of action of the anti-inflammatory triterpene lupeol, J. Pharm. Pharmacol. 53 (2001) 1533–1539.
[8] J.F. Vasconcelos, M.M. Teixeira, J.M. Barbosa-Filho, A.S. Lucio, J.R. Almeida, L.P. Queiroz, The triterpenoid lupeol attenuates allergic airway inflammation in a murine model, Int. Immunopharmacol. 8 (2008) 1216–1221.
[9] V. Sudhakar, S.A. Kumar, P.T. Sudharsan, P. Varalakshmi, Protective effect of lupeol and its ester on cardiac abnormalities in experimental hypercholesterolemia, Vasc. Pharmacol. 46 (2007) 412–418.
[10] M. Saleem, K. Satwinderjeet, K. Mee-Hyang, M.A. Vaqar, A. Farrukh, M. Hasan, Lupeol, a fruit and vegetable based triterpene, induces apoptotic death of human pancreatic adenocarcinoma cells via inhibition of Ras signaling pathway, Carcinogenesis 26 (11) (2005) 1956–1964.
[11] M. Imtiyaz, M. Sleem, M.A. Vaqar, B.H. Bilal, M. Hasan, Suppression of cFLIP by lupeol, a dietary triterpene, is sufficient to overcome resistance to TRAIL-mediated apoptosis in chemoresistant human pancreatic cancer cells, Cancer Res. 69 (3) (2009) 1156–1165.
[12] P. Sahdeo, K. Neetu, S. Yogeshwer, Induction of apoptosis by lupeol and mango extract in mouse prostate and LNCaP cells, Nutr. Cancer 60 (1) (2007) 120–130.
[13] P. Sahdeo, M. Esha, N. Nidhi, R. Preeti, G. Jasmine, S. Yogeshwer, Induction of apoptosis by lupeol in human epidermoid carcinoma A431 cells through regulation of mitochondrial, Akt/PKB and NFκB signaling pathways, Cancer Biol. Ther. 8 (17) (2009) 1632–1639.
[14] S.C. Kang, Y.L. Sue, J.S. Yoon, Lupeol is one of active components in the extract of chrysanthemum indicum linne that inhibits LMP1-induced NF-κB activation, PLoS One 8 (11) (2013) 1–11.
[15] B.R. Vijay Avin, T. Prabhu, V. Lakshmi Ranganatha, F. Aiyesha, B.T. Prabhakar, A.K. Shaukath, Synthesis and tumor inhibitory activity of novel coumarin analogs targeting angiogenesis and apoptosis, Eur. J. Med. Chem. 75 (2014) 211–221.
[16] B.N. Nishanth, B.P. Salimath, Crosstalk between VEGF and novel angiogenic protein regulates tumor angiogenesis and contributes to aggressiveness of breast carcinoma, Cell Signal. 25 (1) (2013) 277–294.
[17] S. Shivakumar, B.T. Prabhakar, K. Jayashree, M.G.R. Rajan, B.P. Salimath, Evaluation of serum vascular endothelial growth factor (VEGF) and microvessel density (MVD) as prognostic indicators in carcinoma breast, J. Cancer Res. Clin. Oncol. 135 (4) (2009) 627–636.
[18] M. Belakavadi, B.T. Prabhakar, B.P. Salimath, Butyrate-induced proapoptotic and antiangiogenic pathways in EAT cells require activation of CAD and downregulation of VEGF, Biochem. Biophys. Res.Commun. 335 (4) (2005) 993–1001.
[19] B.T. Prabhakar, S.A. Khanum, S. Shashikanth, B.P. Salimath, Antiangiogenic effect of 2-benzoyl-phenoxy acetamide in EAT cell is mediated by HIF-1α and down regulation of VEGF of in-vivo, Investig. New Drugs 24 (2006) 471–478.
[20] C.L. Chen, S.K. Huang, J.L. Lin, Upregulation of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases in rapid atrial pacing-induced atrial fibrillation, J. Mol. Cell. Cardiol. 45 (2008) 742–753.
[21] M. Saleem, Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene, Cancer Lett. 285 (2) (2009) 109–115.
[22] S. Imam, I. Azhar, M.M. Hasan, M.S. Ali, S.W. Ahmed, Two triterpenes lupanone and lupeol isolated and identified from *Tamarindus indica* linn, Pak. J. Pharm. Sci. 20 (2007) 125–127.
[23] E.L. Nguemfo, T. Dimo, A.B. Dongmo, A.G. Azebaze, K. Alaoui, A.E. Asongalem, Anti-oxidative and anti-inflammatory activities of some isolated constituents from the stem bark of Allanblackia monticola Staner L.C (Guttiferae), Inflammopharmacology 17 (2009) 37–41.
[24] Y.J. You, H.N. Nguyen, K. Yong, B. Ki-Hwan, Z.A. Byung, Antiangiogenic activity of lupeol from Bombax ceiba, Phytother. Res. 17 (2003) 341–344.
[25] H.L. Goel, A.M. Mercurio, VEGF targets the tumor cell, Nat. Rev. Cancer. 13 (12) (2013) 871–882.
[26] J. Folkman, Angiogenesis: an organizing principle for drug discovery, Nat. Rev. Drug Discov. 6 (2007) 273–286.
[27] J.E. Talmadge, R.K. Singh, I.J. Fidler, A. Raz, Murine models to evaluate novel and conventional therapeutic strategies for cancer, Am. J. Pathol. 170 (3) (2007) 793–804.
[28] A. Al-Rehaily, K.E.H. El-Tahir, J.S. Mossa, S. Rafatullah, Pharamacological studies of various extracts and the major constituent lupeol obtained from hexane extract of *Teclea nobilis* in rodents, Nat. Prod. Sci. 7 (2001) 76–82.
[29] S.P. Preetha, M. Kanniappan, E. Selvakumar, M. Nagaraj, P. Varalakshmi, Lupeol ameliorates aflatoxin B1-induced peroxidative hepatic damage in rats, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143 (2006) 333–339.
[30] X. Liang, F. Xu, X. Li, C. Ma, Y. Zhang, W. Xu, VEGF signal system: the application of antiangiogenesis, Curr. Med. Chem. 21 (7) (2014) 894–910.