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VIRTUAL SCREENING AND BIOLOGICAL EVALUATION OF AVIAN ERYTHROBLATOSIS VIRUS E26 ONCOGENE (ETS-1) INHIBITOR

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ABSTRACT

This study was designed to identify and characterize novel inhibitor of Ets-1 by using virtual screening approach combined with biological evaluation of anti-angiogenic ability of compound. Virtual screening of Indian plant anticancer compound database was performed against phosphorylation site (Threonine-38) of Ets-1 by Auto Dock 4.02. A compound with lowest energy score (-4.28kcal/mol) was selected. Biological activity of compound obtained from virtual screening was further evaluated by performing chick chorioallantoic membrane (CAM) assay. Different concentrations (0.5 %, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 6%, and 12%) of aqueous extract of saffron plant containing screened compound (Picrocrocin) were applied to CAMs and scanning probe image processor was used for quantification of membrane vasculature. Results showed that application of 2% and 3% concentration of extract showed reduction in diameter of blood vessels, decrease in abbot curve area and reduction in surface roughness parameters. This study revealed a potential inhibitor of Ets-1 and anti-cancer mechanism of saffron.

Key words: Virtual screening, angiogenesis, cancer, chick chorioallantoic membrane.

INTRODUCTION

Ets-1 is a prototypic member of the E-26 (Ets) family of transcription factors that share a unique DNA-binding domain, the Ets domain (Yordy *et al*., 2000). This family of transcription factors, in cooperation with other families of transcription factors, regulates a diverse variety of genes controlling various cellular functions associated with development, proliferation, differentiation, apoptosis, tissue remodeling, angiogenesis, malignant transformation and metastasis (Dittmer, 2003). Mounting evidence implicates a role for Ets-1 in oncogenesis. Many studies suggest that aberrant expression of Ets-1 is associated with tumorigenesis and tumor progression, such as invasion, metastasis and neo-angiogenesis (Oikawa, 2004). Especially, Ets-1 over-expression has been observed to be associated with increased angiogenesis in a wide variety of human cancers, including those of ovary, prostate, breast and the gastrointestinal tract (Fujimoto *et al*., 2002).

It is well established that Ras-responsive phosphorylation of a conserved residue threonine-38 in the N-terminal region of Ets-1 strongly increases its transcriptional activity (Wasylyk *et al*., 1997). Phosphorylation of threonine-38 has been studied in detail. Phosphorylation of this site by Ras/ Raf/ MEK/ ERK (extracellular-signal-regulated kinase) pathway as a linear transduction pathway dramatically enhances Ets-1 transcriptional activity by preferential hiring of the co-activators CREB (cAMP response element-binding) binding protein (CBP) and p300 which, in turn, enhances the expression of RRE- containing genes that regulate various factors involved in blood vessels formation (Foulds*et al*., 2004). Plainly, Ets-1 transcriptional activity enhances the expression of both vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), as well as regulates the genetic expression of various downstream targets in endothelial cells that promote an angiogenic phenotype such as VEGF receptors, urokinase, and matrix metalloproteinase's (MMPs) (Dittmer, 2003). VEGF and HGF have been implicated as angiogenic growth factors that promote angiogenesis in different types of Ets-1-induced cancers (Oettgen, 2010). Specific inhibition of threonine-38 phosphorylation, therefore, may be useful to prevent angiogenesis, and thus reduce the metastatic potential of various Ets-associated tumors. However, despite all of the recent advances in computational chemistry and/or drug discovery, nothing has been reported till date in the context of Ets-1 small molecule inhibitors.

Inhibition of angiogenesis is an effective approach in cancer treatment research. Ets-1 can be blocked to inhibit angiogenesis. In this study, *in silico* molecular docking-based virtual screening was utilized to explore small molecule inhibitors of threonine-38 phosphorylation. This technique has become a significantly reliable screening method as compared to the high throughput screening for the discovery of ligands (Shoichet *et al*., 2002). To utilize this technique, the 3-D crystal structure of the PNT (Pointed) domain (29-138) of

Ets-1 (RCSB Protein Data Bank code: 2JV3) was examined. During this analysis, a structural pocket adjacent to threonine-38 was identified. It was hypothesized that the small molecules that specifically bind to this pocket would inhibit threonine-38 phosphorylation, and thereby Ets-1 induced angiogenesis. After screening 125 compounds from Indian Plants Anticancer Compounds Database (InPACdb) the top two compounds, as ranked by predicted binding energy scores and the binding pose, were tested for inhibition of angiogenesis by chorioallantoic membrane (CAM) assay.

MATERIALS AND METHODS

The 3-D crystal structure of Ets-1 PNT (Pointed) domain 29-138 (Pdb ID: 2jv3) was retrieved from protein data bank (http://www.rcsb.org/pdb). Residues that are assumed to be essential for the phosphorylation of Ets1 (threonine-38 and serine-41) were mapped out in Pymol. The Indian plant anticancer compound database provides 125 compounds with potential anticancer activity and was downloaded free of cost from http:// www.inpacdb.org/ (Umashankar *et al*., 2009).

The program AUTODOCK 4.02 (Morris *et al*., 1998) was used to calculate the potential interactions of the compounds against the targeted residues. AutoDock calculations give several docked conformations that are analyzed by the predicted binding energy. To identify the best solution, the results of docked simulations are clustered and analyzed by conformational similarity, visualizing conformations, visualizing interactions between ligands and proteins, and visualizing the affinity potentials created by AutoGrid (Morris *et al*., 1998). AutoDock Tools were used for creating PDBQT files from traditional PDB files of all the InPACdb compounds and of Ets1. To prepare pdbqt file of rigid protein Ets-1and of ligands: hydrogen atoms were added, kollman charges were added, non-polar hydrogen atoms and charges were merged, aromatic carbons were assigned, and number of rotatable bonds was set for each compound. Grid box was set at targeted residues with 40 points in x, y and z dimensions, 0.375 Aº spacing with x center of -17.803, y center of-5.55 and z center of 4.445. Finally grid parameter file was saved. Protein Ets-1 and ligands were opened one by one. Genetic algorithm (Morris *et al*., 1998) was set by initially giving 10 then 20, 30 and 50 numbers of genetic algorithms (Hussain *et al*., 2012), population size as 150, maximum number of generations as 27000, maximum number of energy evaluations ranges 250000. Other docking parameters were set to default values (Khan *et al*., 2002). By setting all these docking parameters docking parameter file was saved by LGA.

Biological Assay: Biological assay was performed on extract of saffron, in which picrocrocin is present. Saffron stigmas were purchased locally from Government College University, Lahore (Voucher No. "GC.Herb.Bot.1910"). Saffron quality depends on concentration of its constituents which is different in different environmental conditions for example picrocrocin concentration is between 0.79% to 12.94% in Spanish saffron, 1.07-2.16% in Indian saffron and 2.18%to 6.15% in Iranian saffron (Mounira and Charles). For extraction 100 grams of powdered stigmas of *Crocus sativus* were filled in thimble of soxhlet apparatus (CG-1368) (Behr Labor Technik). Extraction was carried out at 100°C-130°C for 72 hours with ethanol. Extract was filtered, dried in rotary evaporator and finally in hot air oven. All 8 concentrations (0.5 %, 01%, 1.5%, 02%, 2.5%, 03%, 3.5%, 4%, 6%, and 12%) of extract were prepared in aqueous solvent and filtered through syringe filters (0.22 µ). All operations were performed in laminar flow hood to maintain aseptic conditions.

For biological assay we adopted Chick Chorioallantoic Assay (CAM). Chick Chorioallantoic is membrane containing complex capillary network, covers chick embryo and serves as gas exchange surface. Because of its vascular nature and easy accessibility it is used to study morphology, physiology of angiogenesis and effects of anti angiogenic substances (Abrar *et al*., 2011)). Total number of 70 laid fertilized broiler chicken eggs of day 5 of incubation were purchased locally, moped with isopropyl alcohol and dried. Eggs were placed in incubator at 70% humidity and 37°C temperature. On broader end of eggs small windows (2 cm) were made aseptically one by one in each egg (Ejaz *et al*., 2010). Eggs with dead embryos and with malformed embryos were excluded. There are 9 groups, each group of 7 eggs. On same day 2-5 ml of albumin from each egg was removed with 21 G needle to detach embryos from shells (Ejaz *et al*., 2010) and 200 µl of 8 concentrations were inoculated in all eggs of respective group as G 0.5%, G 01%, G 1.5%, G 02%, G 03%, G 04%, G 06%, G 12%. A 9th group was treated as control and received just distilled water. Windows were sealed with sterile parafilm tape. All work was performed in laminar flow hood to maintain aseptic environment. All eggs with sealed windows were placed in incubator with windows upright. Statistical analysis was performed by SPSS 13 software (SPSS Inc. Chicago, Illinoise 60606, USA). Analysis of variance (ANOVA) followed by student's t test. $P< 0.05$ was considered statistically significant (Ejaz *et al*., 2010).

RESULTS AND DISCUSSION

Amino acid sequence of Ets-1 was retrieved from PDB and is shown below. Alphabets are depicting the single letter code of amino acids. It starts from methionine M (No. 29) and ends in aspartic acid D (No. 138). Threonine-38 and serine-41 are shown in figure 1.

>2JV3:M[29]ECADVPLL**T**[38]PS**S**[41]KEMMSQALKATFSG KGVDFQKFCMSGAALCALGKECFLELAPDFVGD[138]
FTKEQQRLGIPKDPRQWTETHVRDWVMAWNEFSL

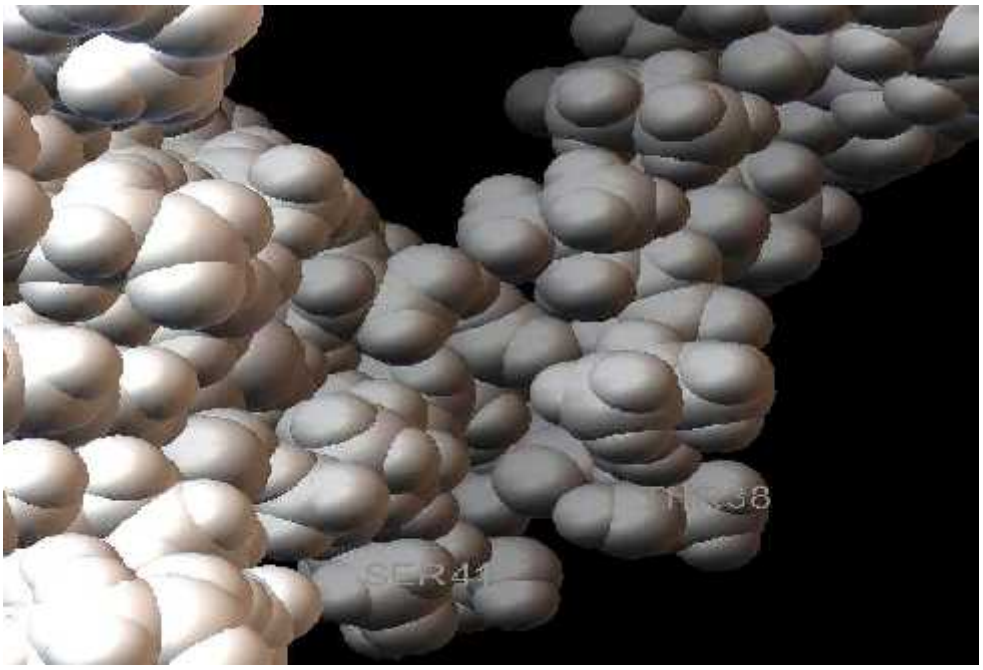


Figure 1: Amino acid sequence of PNT domain of Ets-1.

The established site for phosphorylation on Ets-1 is threonine-38. For phosphorylation at threonine-38 MAPK must dock on pointed domain of Ets-1 where threonine-38 is present (Wasylyk C *et al*., 1997). All 125 compounds of database were docked against phosphorylation site of Ets-1 and 18 compounds with at least one hydrogen bond with either threonine-38 or serine-41 were selected after 10 runs of docking.

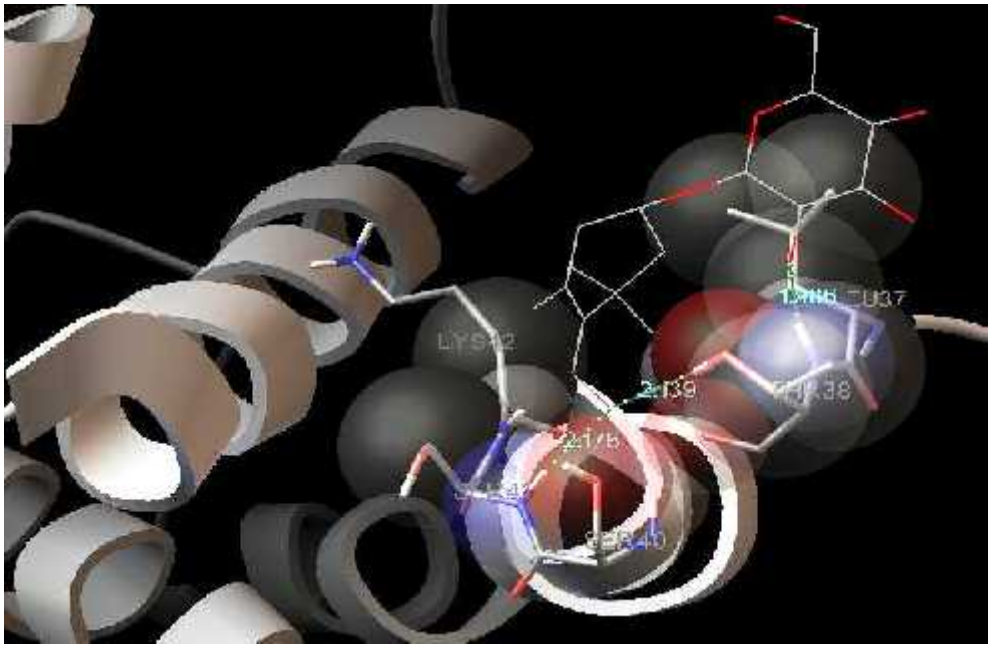


Figure 2: 3 D structure of phosphorylation site residues of Ets-1 After 20 Runs of Docking Energy Barrier -3.5kcal/mol

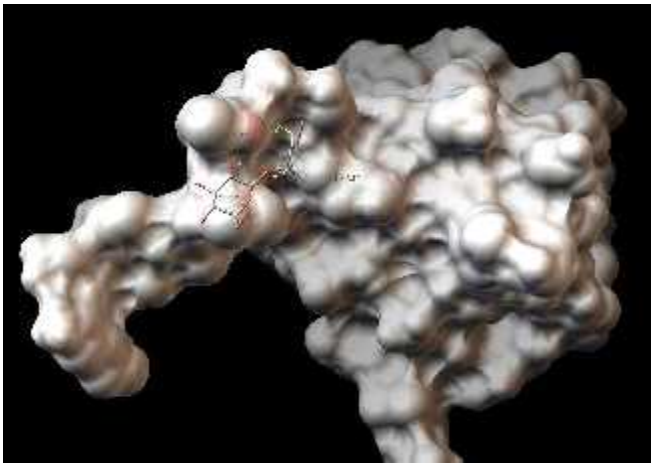
Table 1. Compounds with binding energy less than-3.5 kcal/mol

No. of Compound	Botanical Name	Binding Energy kcal/mol	No. of H-Bonds	Residues involved in H-Binding
01	Picrocrocin	-3.99	03	Ser 41, Thr 38 (2)
02	Lobeline	-3.81	02	Thr 38
03	Cyclophosphamide	-3.77	01	Thr 38
04	Naringenin	-3.63	01	Thr 38
05	Hesperetin	-3.55	02	Thr 38 , Ser 41

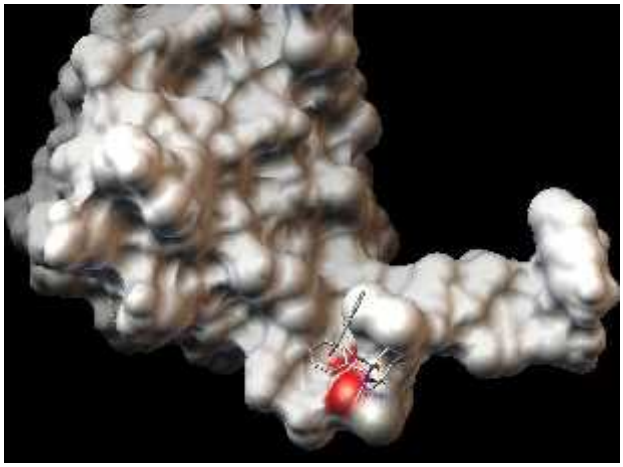
After 50 Runs of Docking

Table 2. Compounds obtained after 50 runs of docking

No. of Comp	Botanical Name	Binding Energy kcal/mol	H- Bonds formed	Inhibitory Constant Ki	Bond Length Aº
01	Picrocrocin	-4.28	Thr38:HG1-O16	170.23 µM	1.988
			NH-H26		2.139
			Ser41: HN-H26		2.176
03	Lobeline	-3.93	Thr38: NH-O16	155.00 µM	2.086



3(a): Picrocrocin with Ets-1



3(b): Lobelin with Ets-1

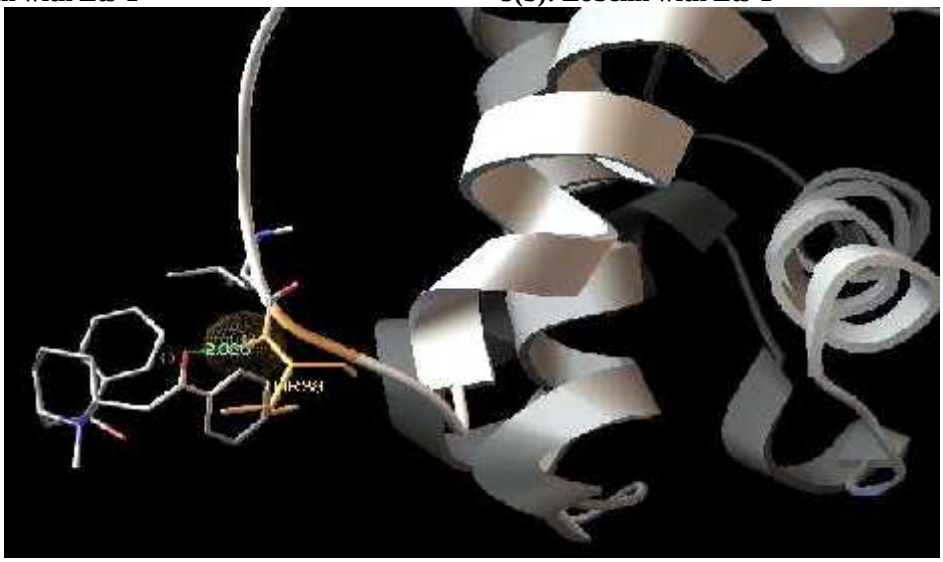
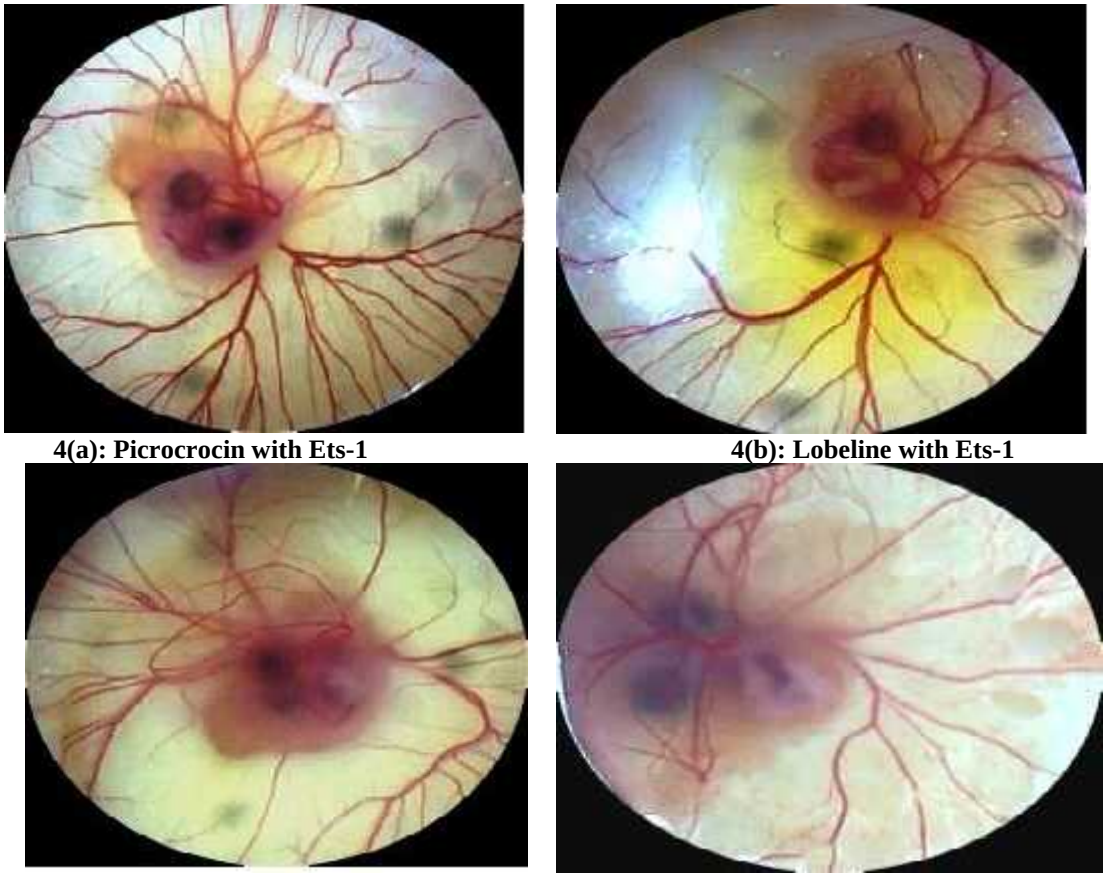


Figure 3: Interactions of Picrocrocin and Lobelin with Ets-1



4(a): Picrocrocin with Ets-1 **4(b): Lobeline with Ets-1**

Figure 4: Binding poses of Picrocrocin and Lobelin with Ets-1

CAM Assay: Treated and control groups were opened after 24 hours of incubation. Images of each control and treated CAMs were taken and their optical resolution was enhanced by adobe photoshop 7.0 and quantified by novel scanning probe image processor (SPIP). Respective x, y and z dimensions of each image were set.

By applying SPIP vessels length, diameter, area and 3D roughness of control and treated CAMs were quantified (Ejaz *et al*., 2004). Best anti-angiogenic results (Reduction in diameter of blood vessels, surface roughness and abbot curves) were obtained in groups treated with 2% and 3% solutions of extract.

Diameters of primary, secondary and tertiary blood vessels of control were 12 µm, 08 µm and 06 µm, 2% treated were 02 µm, 01 µm, 0.3 µm,3% treated CAM were 03 µm, 02 µm and 01 µm, respectively (Table 4). Surface roughness represents the neovascularization. All parameters of surface roughness of treated were significantly less ($P \leq 0.05$) than values of control group (Table 5). Abbot curves (Graphical representation of heights of blood vessels) were also measured. Areas of abbot curves of control group, 2% treated and 3% treated were 0.0545 mm², 0.0538 mm² and 0.0541 mm².

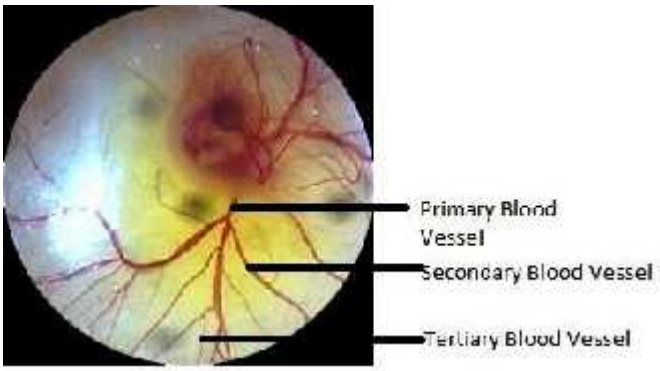
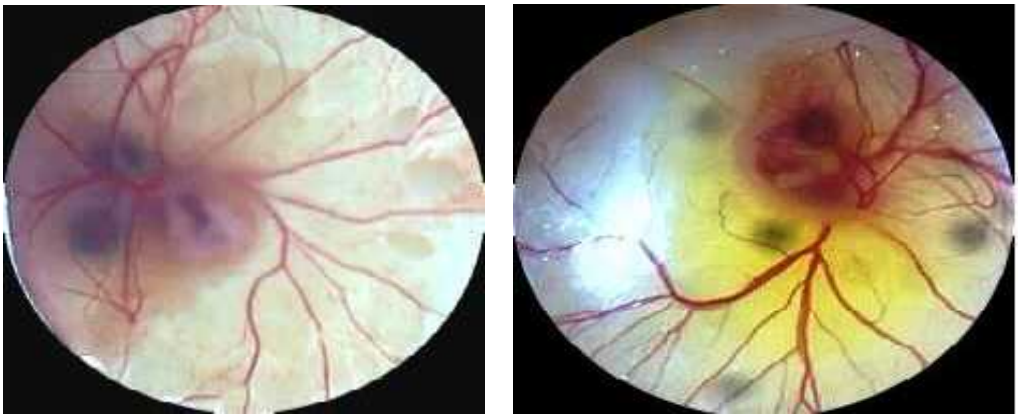
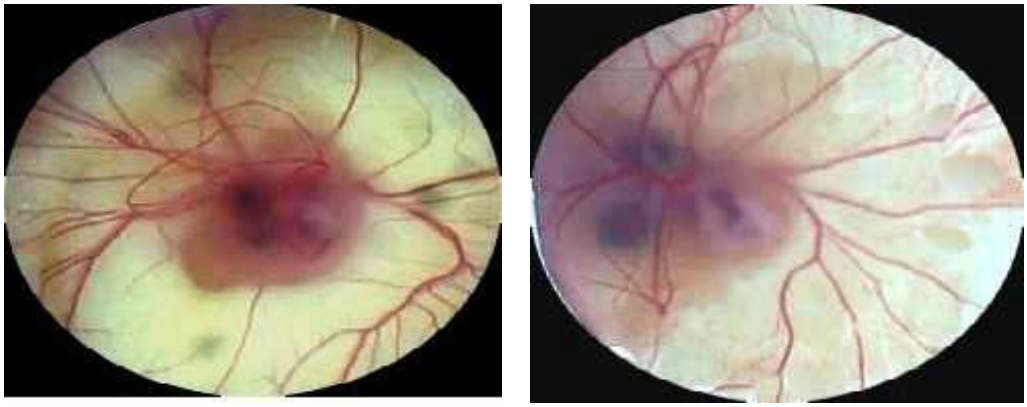


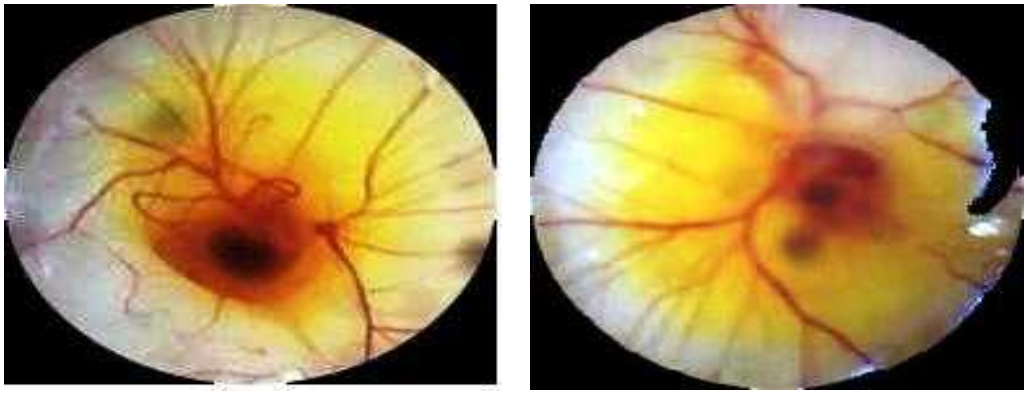
Figure 5. Image of CAM showing primary, secondary and tertiary blood vessels



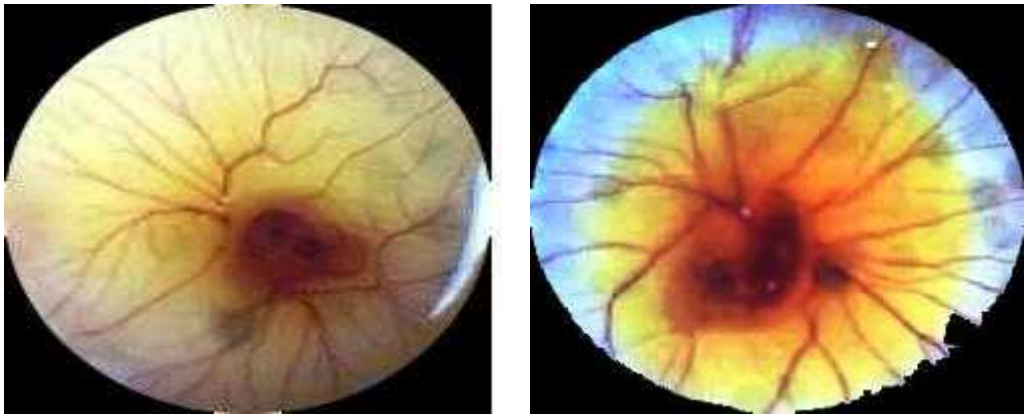
Control 0.5%



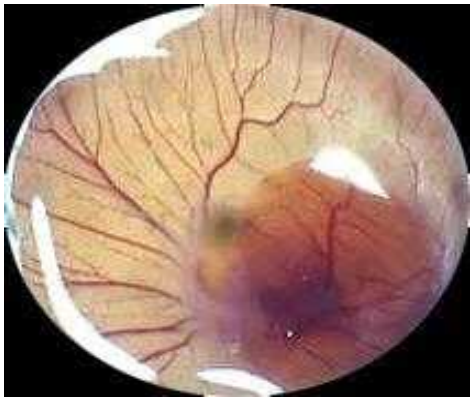
01% **1.5%**



02% **03%**



04% **06%**



12%

Figure 6: Images of treated and control CAMs

Table 3. Diameters of blood vessels of all groups

Groups	Primary Blood Vessel (µm)	Secondary Blood Vessel (µm)	Tertiary Blood Vessel (µm)
Control	12	08	06
0.5%	12	09	07
01%	10	09	06
1.5%	07	06	05
02%	02	01	0.3
03%	03	02	01
04%	05	03	02
06%	06	06	04
12%	08	05	04

Table 4: Average values of roughness parameters of 2% treated group, 3% treated group and control group

Parameters	2 % Treated Group	3 % Treated Group	Control Group
Sa (nm)	30816.02	33322.78	47378.76
Sq (nm)	37357.46	41686.64	57189.6
Ssk	-1.2805646	-0.756942	-0.466840
Sku	2.907134	5.337434	3.218864
Sy (nm)	215851	278553	296705
Sz (nm)	215851	278553	296705
Ssc 1/nm	0.22505824	0.23529868	0.00896885
Sdq	8.69887	8.93286	12.02602
Sdr (%)	2474.04	2428.95	4864.86
Sci	1.12826	0.93152	2.04654

Sa: average roughness, **Sq:** root mean square deviation, **Ssk:** sekewness of surface,
Sku: kurtosis of surface, **Sdr:** developed surface area, **Sci:** core fluid retention,
Sy:lowest valley, **Sz:** maximum height of surface, **Ssc:** arithmetic mean summit,
Sdq:root mean square slo

Out of main roughness parameters Sa (average roughness) is most commonly used. Average values of surface roughness for 2% and 3% treated and control CAMs were30816.02 nm, 33322.78 nm and 47378.76 nm respectively indicating more roughness of surfaces of blood vessels of control CAM. Sq (root mean square deviation); root mean square of surface, it's a dispersion parameter. Sq average values of 2% and 3% treated solutions were 37357.46 nm, 41686.64 nm respectively and of control was 57189.6 nm. This shows reduction in anti-angiogenic surfaces. Sz (maximum height of surface): is indicates the heights of five highest peaks and depths of five deepest pits. Average Sz values of 2% and 3% treated CAMs were215851 nm, 278553 nm and of control CAM was 296705 nm. Reduction in values of treated samples indicates reduction in blood vessel development area as there was less heights and peaks in treated CAMs than of control CAM. Ssk (sekewness of surface): indicates asymmetry of surface deviations about mean plane. For symmetrical distribution of surface

height value of skewness is zero and for asymmetrical surface height distribution skewness may be negative or positive. Average values of 2% treated and 3% treated CAMs were -1.2805646, -0.756942 respectively and for control its value was -0.466840. This result shows that surface height of control was more symmetrical than the blood vessels of treated CAMs. Sku (kurtosis of surface): is measure of peak endness or sharpness of surface height distribution. It indicates spread of surface height distribution. A symmetrical surface has kurtosis value of 3. Value less than 3 indicates well spread distribution of surface. In present study average values of Sku of blood vessels of CAMs treated with 2% and 3% solutions were 2.907134 and 5.337434 respectively, and of control was 3.218864, indicating distribution of treated CAMs was more close to center whereas blood vessels of CAM of control group were well spread all over the CAM. Sdr (developed surface area ratio): is ratio of increment of interfacial area of surface of sampling area. This parameter describes the spacing or amplitude of blood vessel. Average values of this parameter for 2% and 3% treated CAMs were 2474.04 % and 2428.95 % respectively, and for control average value was 4864.86 %. This result indicates greater amplitude of blood vessels of control than of treated CAMs. Sci (core fluid retentio): is ratio of void volume of unit sampling area at core zone over the root mean square deviation. These values indicate fluid retention in blood vessels. For symmetrical surface value of this parameter is 1.56. Average values of Sci for 2% and 3% treated CAMs were1.12826 and 0.93152 respectively, and for control it was 2.04654. This result indicates greater fluid retention in blood vessels of control CAMs as compared to blood vessels of treated CAMs (Ejaz *et al*., 2004).

Conclusion: In this study, we have demonstrated that threonine-38 phosphorylation can be targeted to inhibit Ets-1-induced angiogenesis, and this information awaits further research particularly in cancer models. Stated simply, this study may give insight to develop novel anticancer drugs.

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