IJBPAS, March, 2020, 9(3): 540-552

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANTI-ANGIOGENIC ACTIVITY OF COLUMBIN: A DITERPENOID FROM *TINOSPORA CORDIFOLIA*

TAYAL N[1], GHUFRAN M[2], SRIVASTAVA P[1], KANDHWAL S[3], BISWAS S[2] AND SRIVASTAVA N[1*]

1: Amity Institute of Biotechnology, Amity University, Sec-125, Noida, U.P 201313

2: Amity Institute of Molecular Medicine and Stem Cell Research, Amity University, Sec-125, Noida, U.P 201313

3: Government College for women, Madlauda, (Panipat), 132113

Received 9th Oct. 2019; Revised 7th Nov. 2019; Accepted 8th Dec. 2019; Available online 1st March 2020

https://doi.org/10.31032/IJBPAS/2020/9.3.5004

ABSTRACT

The plant *Tinospora cordifolia* is well known in Ayurvedic medicine for its health benefits and clinical applications. Columbin, a natural diterpenoid is the major phytoconstituent isolated from the stem of *Tinospora cordifolia.* Various studies have investigated the anti-inflammatory, anti-microbial, antifeedant and cardio-protective roles of columbin. In this study, our centre of interest was to determine the anti-angiogenic activity of columbin using computational analyses and validating it *in-vitro* and *in-vivo*. Targeting dys-regulated angiogenesis can be a therapeutic intervention against various malignant, ischemic and inflammatory disorders. Molecular docking analyses showed favorable binding affinities of columbin to angiogenic receptors, VEGFR1 and VEGFR2. *In-vitro* experiments displayed columbin cytotoxicity on human umbilical vein endothelial cells (HUVECs) and also decreased endothelial migration and tubular formation. *In-vivo* chick chorioallantoic membrane assay showed decreased vessel formation on columbin treatment. These findings altogether suggest that columbin can suppress angiogenesis *in-vivo* and *in-vivo* and may serve as a potential natural anti-angiogenic therapy.

Keywords: Anti-angiogenic, Columbin, therapeutics, health, Ayurvedic, *Tinospora cordifolia*

1. INTRODUCTION

Phytochemicals are the bioactive components of plants that give them their specific color, flavor and, aroma. Inside the human body, they work with other phytochemicals and nutrients to battle off various diseases. Some phytochemicals are known to have anti-oxidant activity and stimulate the immune system **[4]**. Many other phytochemicals have also been studied to lower the risk of diabetes, high blood pressure and, heart diseases **[1]**. The plant *Tinospora cordifolia* is well known for its medicinal value in the Indian Ayurvedic system. *Tinospora cordifolia* is extensively spreading and climbing shrub with several elongated twinning branches. This plant is very rigid and it can be grown in almost all climates, preferring warmer climatic zones. The stem of this plant is succulent with long filiform fleshy aerial roots emerging from the branches **[2]**. The stem of *Tinospora cordifolia* has been used by traditional practitioners for treating jaundice. In previous reports, *Tinospora cordifolia* has been well researched for its anti-diabetic, anti-spasmodic, anti-inflammatory, anti-oxidant, anti-allergic, anti-stress, hepatoprotective, anti-arthritic and, immunomodulatory activities **[3]**. Herbal medicines are less expensive and safer alternatives to synthetic drugs due to their fewer side effects. Despite its prolonged use in the Ayurvedic system of medicine, *Tinospora cordifolia* has not been explored scientifically for its anti-angiogenic benefits. We examined for the first time the anti-angiogenic property of *Tinospora cordifolia* - its botanical parts and bioactive compounds.

Angiogenesis is the formation of new blood vessels from the existing vasculature. It is a normal path of growth and healing. Physiological angiogenesis takes place during tissue growth and repair, during the female reproductive cycle and fetal development. Pathologic angiogenesis is characterized by either excessive (e.g. cancer) or inadequate (e.g. coronary artery disease) neo vascularization. Neo-vascularization occurs when the balance between pro-angiogenic and anti-angiogenic factors is disturbed **[7]**. For tumors to develop in size they need an extensive network of capillaries to provide nutrients and oxygen and it recruits new blood capillaries by the process of angiogenesis **[6]**. Among angiogenic growth factors, VEGF is the predominant inducer for the angiogenic process. The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play major role in both physiological and

pathological conditions. VEGF-A regulates angiogenesis and vascular permeability by activating two major receptors, VEGFR-1 and VEGF-2 **[7]**. Thus the VEGF-VEGFR system is an important target for anti-angiogenic therapy.

The stem of *Tinospora cordifolia* contains high crude fibre with low water content **[4]**. Berberine, choline, tinosporide, **columbin**, palmitine and jatrorrhizine are the major phytoconstituents extracted from the stem of this plant **[5]**. The 3D structures of these phytochemicals were retrieved from PubChem database and used as a ligand for targeting receptor proteins VEGFR1 and VEGFR2 (angiogenic receptors for VEGF). Columbin biosynthesized in the chloroplasts is a rare form of diterpene and is structurally related to the labdanediterpenes. It is a clerodanediterpene which belongs to clerodanoids, a large group of bicyclic 20-carbon terpene compounds.

In this study, columbin used was a purified compound (98%) from Toronto Research Chemicals, Canada.

The molecular formula of columbin is represented as $C_{20}H_{22}O_6$with 358.39 g/mol molecular weight. It is white to off-white solid in appearance having melting point/freezing point of >170°C. Columbin is sparingly soluble in chloroform and slightly soluble in DMSO and ethyl acetate. It is a furanolactone whose structure followed 1H NMR and 13C NMR studies.

In the present research, columbin was selected based on its binding affinity with angiogenic growth factor receptor VEGFR1 and VEGFR2 via MMGBSA calculations. As there is no report on anti-angiogenic activity of columbin, *in vivo* chick embryo chorioallantoic membrane assay was performed with varying concentrations of columbin. Further, to substantiate anti-angiogenic behavior of columbin, human umbilical vein endothelial cells were examined for their proliferation, migration and tube formation in the presence of columbin *in-vitro*.

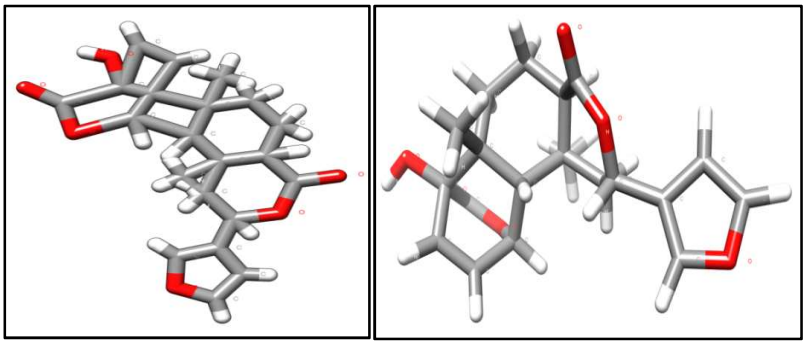

Fig.1.(a) **Fig.1.(b)**

Figure 1: 1.(a) 2-Dimensional Structure of Columbin; 1.(b) 3-Dimensional Conformer of Columbin

2. MATERIAL AND METHODS

2.1. Molecular docking Studies – *In-silico* Analysis

2.1. a. Structure preparation and Receptor Grid Generation

Missing hydrogens were added to stabilize the PDB structure files of vascular endothelial growth factor receptor (both 1 and 2) **[9, 10]**. Missing side chains were added along with hydrogens and structures were minimized after removing water molecules using Protein Preparation Wizard **[11]**. Active sites of each protein were curated from SwissProtKB of UniProtKB Database. The grid dimensions were parameterized to 15*15*15 Angstroms **[12]**. For Columbin 64 possible states were generated at the physiological pH of 7.0 +/- 2.0, which were then minimized using OPLS_2005.

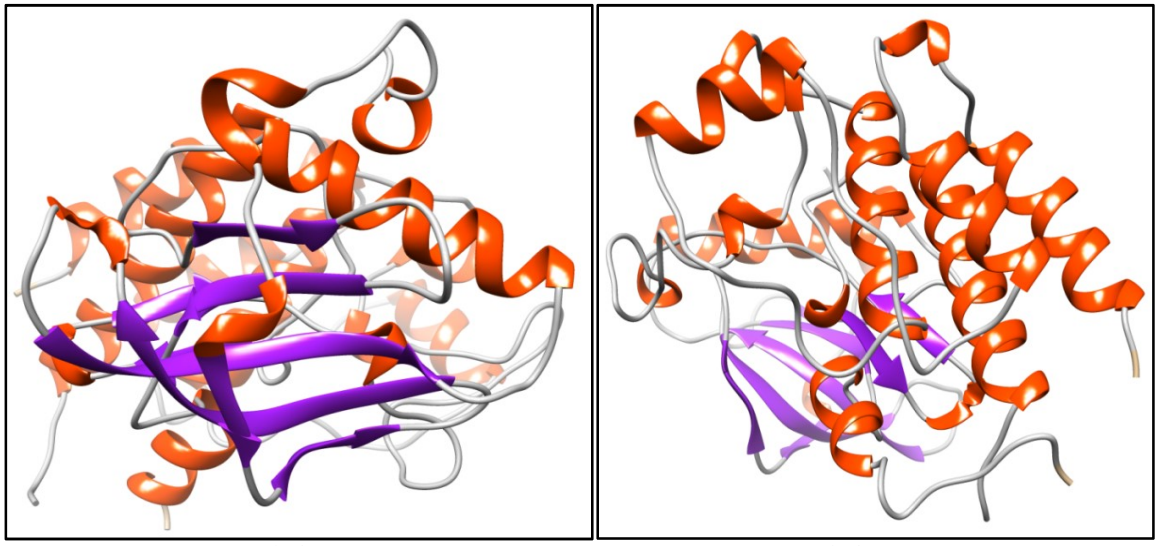

Fig.2.(a) Fig.2.(b)

Figure 2: 2.(a) 3D Structure of computationally minimized VEGFR1; 2.(b) 3D Structure of computationally minimized VEGFR2; Sheets: Purple, Helix: Orange, Loops: Silver

2.1. b. Ligand docking and MMGBSA Calculations

Glide SP pipeline was used to carry out receptor-ligand docking. The scaling factor was set to the default of 0.80 with the partial charge cutoff at 0.15. Ligand structures were sorted based on the Glide score after initial screening and MMGBSA dG was calculated using VSGB for the conformations with best docking scores among all **[14]**.

2.2. CAM (chick chorioallantoic membrane) Assay – *In-vivo* analysis

Fertilized white leghorn chicken eggs were obtained from SPF Eggs' Division, Venky's India Ltd. Pune, India from the day'0' of incubation. Eggs were incubated in an

incubator at 37°C with 60% humidity. Eggs were sub-divided into two sets, one set was labeled untreated and used as control and the other set was treated with columbin. CAM assay was performed on each under aseptic conditions. Briefly, a small window was made in the shell on day 9 of chick embryo development in a laminar hood. The window was resealed with adhesive tape after administering columbin (50µg/ml and 100µg/ml), and eggs were returned to the incubator until day 12 of chick embryo development. Photography was performed on the same day, and eggs were discarded after photography.

2.3. Cell culture studies - *In-vitro* analysis

Human Umbilical Vein Endothelial Cells from pooled donors were purchased from Lonza and cultured in MCDB 131 medium supplemented with 50 µg/ml endothelial cell growth supplement, 20% fetal bovine serum, 2mM L-glutamine, 50µg/ml heparin and antibiotics (100U/ml Penstrep). Cell cultures were maintained at 37ºC in a humidified chamber (humidity-95%) with 5% CO2 in a CO2 incubator. All experiments using HUVECs were conducted between 2 to 5 passages. At the time of experiments, MCDB 131 medium with 2% FBS was used.

2.3. a. Cell cytotoxicity by MTT

The HUVECs were seeded at a density of 8000 cells per well on a gelatin-coated 96-well plate in their complete media and incubated overnight. The following day, media was removed and cells were treated with increasing concentrations of columbin (0-300µg/ml). The dosing solutions of columbin were prepared from 1mg/ml stock in MCDB131 medium with 2% FBS on the day of the experiment. The concentration of DMSO was <0.1% in all wells including the controls. After 24 hours of treatment, media was removed, cells were washed with phosphate-buffered saline (PBS) and MTT reagent {3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide} was added to each well at a concentration of 0.5mg/ml in MCDB131 medium. After 4 hours of incubation, 100 µl of DMSO was added to dissolve the formazan crystals. The plate was then kept on a shaker for 10-15 minutes and absorbance measurement was taken at 570nm on a plate reader.

2.3. b. Scratch Wound Healing Assay

We used *in-vitro* scratch assay to assess the activity of columbin on the migration of HUVECs.1.5 x 10^5 cells were seeded on 0.25% gelatin-coated 6 well plates in their complete medium. When the cells were 90-95% confluent, cells were serum-starved in MCDB131 medium with 2% FBS. After 16-

24 hours, a scratch was generated by using a 200µl pipette tip similar to a wound. Cells were washed twice with serum-free medium to remove floating cells and debris and were then incubated in MCDB 131 medium with and without columbin. Images were taken in 10X magnification at 0h and 12h. Triple wells were assigned for each condition. The area enclosed by the wound at 0h and after 12 h was quantified using ImageJ software tool.

2.3. c. Endothelial Tube Formation

The effect of columbin on tube formation by HUVECs was examined. Briefly, 120µl of diluted (10mg/ml) growth factor reduced Matrigel was tiled on a pre-cooled 48 well plate and incubated at 37ºC for at least 3 hours for polymerization. 35000 HUVECs/well were seeded on the solidified Matrigel with and without columbin. Each group was taken in triplicate. After 16 hours of culture, tube formation was observed and images were captured in 4X magnification. The tube structures were quantitated by taking an average of 4 randomly selected fields per well. Quantification of the tubes formed was done by using Image J software.

3. RESULTS

3.1. Columbin exhibits favorable binding affinities with VEGF receptors

In the present study by molecular docking, the docking score, interacting residues and binding energies (kcal/mol) were evaluated to estimate the favorable binding of columbin to the angiogenic receptors VEGFR1 and VEGFR2. **Table 1** shows the complete profile of these parameters of columbin with both the angiogenic receptors. Based on the results of docking studies, it can be observed that columbin showed favorable binding with VEGFRs **(Figure 3, 4)**.

3.2. Reduced angiogenesis *in-vivo* after columbin treatment

Chick chorioallantoic membrane (CAM) assay is one of the most reliable angiogenesis assays. The columbin have been administered on CAM and visualized for its effects after 48 h in a concentration and dose dependent manner. The analysis of blood vessels in CAM was done on the basis of thickness of vessel, branching, sprouting of vessel and manual counting. We found that columbin inhibited angiogenesis more at the high concentration of drug (100µg/ml) than 50µg/ml **(Figure 5)**.

3.3. Columbin induced cytotoxicity in HUVECs in a dose-dependent manner *in-vitro*

The cytotoxicity of HUVECs was assessed by the MTT assay. Cells were seeded into 96-well plates at a density of

10,000cells/well. After 24 hours of treatment, the MTT Assay revealed that the cell viability of HUVECs after treating with 50µg/ml of columbin was almost similar to that of untreated cells or control. Therefore, it can be said that no significant cytotoxicity was observed at 50µg/ml. At a concentration of 300µg/ml it was observed that columbin is highly cytotoxic and approximately 70% of the endothelial cells are dead **(Figure 6.d and e)**. A concentration of 100µg/ml was significantly cytotoxic and approximately 50% cell viability was observed at 100 µg/ml of columbin. Therefore it can be concluded that columbin exhibits anti-proliferative effects on HUVECs at 100µg/ml.

3.4. Decreased tube formation by HUVECs after columbin treatment

The formation of tubules is an essential step in angiogenesis. It involves matrix degradation, rearrangement and apoptosis of endothelial cells. Therefore, HUVECs was observed for tube formation in the presence of columbin. Only a few tube like structure were formed in the columbin (100µg/ml) treated group which is the indication of inhibitory effect of columbin on angiogenesis **(Figure 7)**.

3. 5. Slower migration of HUVECs in presence of columbin

Similar to tube formation, endothelial migration is also crucial to angiogenesis. We analyzed the efficacy of columbin on HUVECs migration using scratch assay. As seen in **Figure 8**, cell migration in the presence of columbin was lesser as compared to control or untreated cells after 12hrs on treating with 100µg/ml of columbin. Reduced migration confirms the anti-angiogenic potential of columbin.

Table 1: Ligand-receptor interaction parameters by docking tool

Target	Interacting Residues	Docking Score	MMGBSA(dG) (kcal/mol)
VEGFR1	Arginine 1021	-4.489	-62.13
VEGFR2	Asparagine 921, Arginine 840	-5.673	-64.20

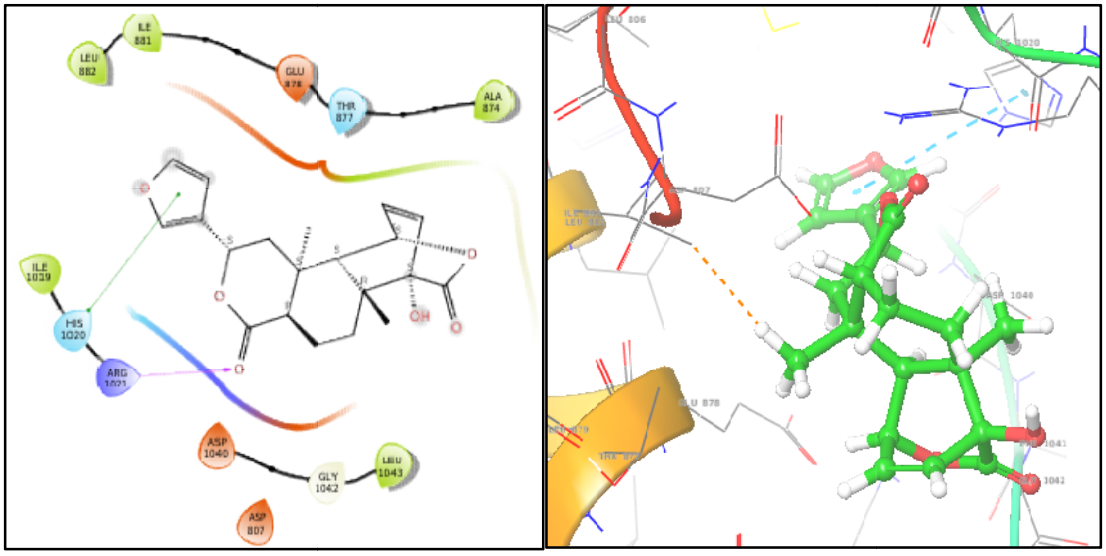

Fig.3.(a) Fig.3.(b)

Figure 3: 3.a) 2Dvisualization of VEGFR1-Columbin interaction diagram; 3.b) 3D visualization of VEGFR1-Columbininteraction diagram

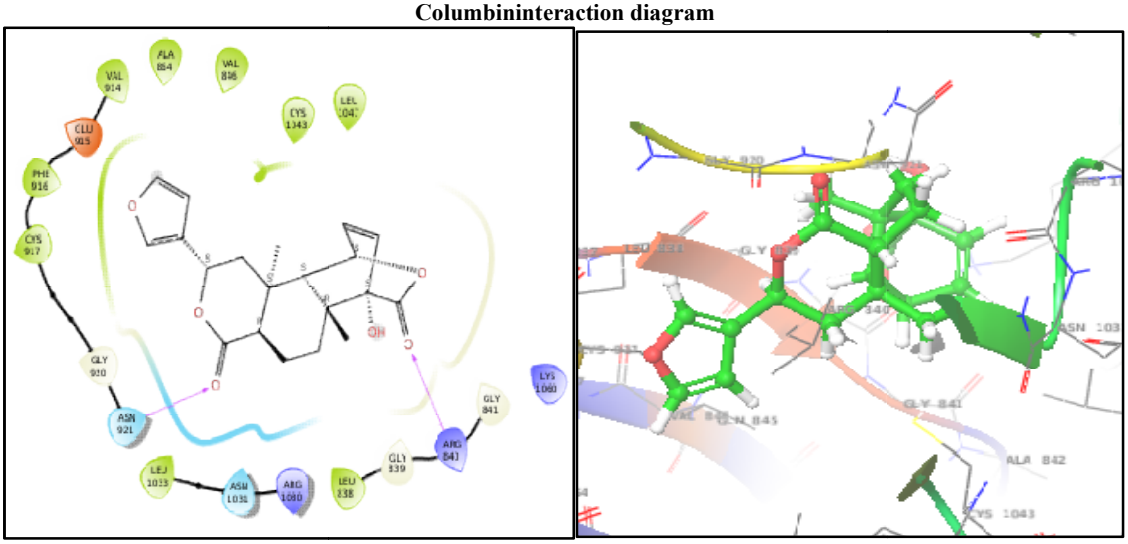

Fig.4.(a) Fig.4.(b)

Figure 4: (a) 2D visualization of VEGFR2-Columbin interaction diagram; (b) 3D visualization of VEGFR1-Columbin interaction diagram

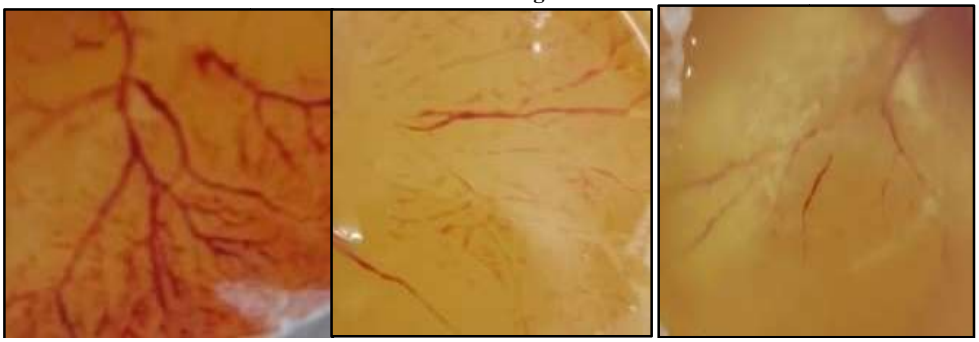

Fig.5.(a) Fig.5.(b) Fig.5.(c)

Figure 5: The effect of columbin on angiogenesis of CAM. Vessel formation in 8-day-old chick embryos in absence and presence of columbin after 48 hours. (a) Untreated CAM; (b) 50µg/ml columbin treatment; (c) 100µg/ml columbin treatment

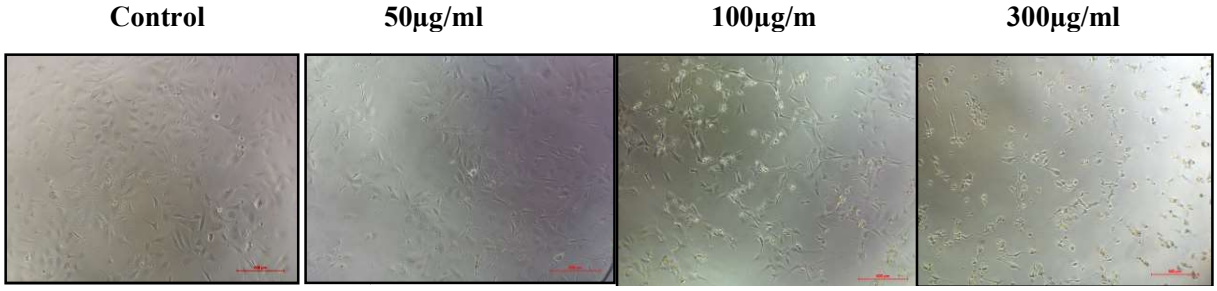

Fig.6.(a)

Fig.6.(b)

Figure 6: Colimbin cytotoxicity of columbin in HUVECs(a) Morphology of HUVECs after 24 hours in control and columbin treated groups(e) Graphical representation of cell viability at different concentrations of columbin (p**= p<0.0001). The images were captured under a phase-contrast microscope at a magnification of 10X**

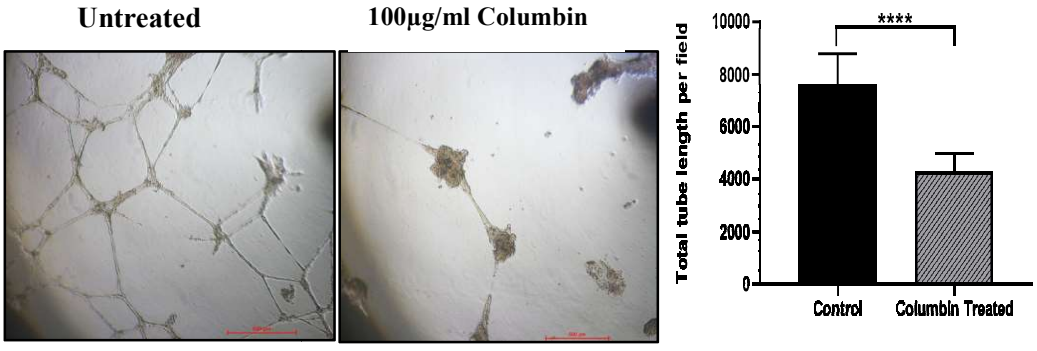

Fig.7(a) **Fig.7(b)**

Figure 7: The effect of columbin on HUVEC tube formation. Tube formation bu HUVECs on Matrigel after 16hrs at a) Untreated HUVECs b) HUVECs treated with 100µg/ml columbin. c) Graphical representation of total tube length per field in both untreated and treated groupsp**= p<0.0001. The images were captured under a phase-contrast microscope at a magnification of 4X**

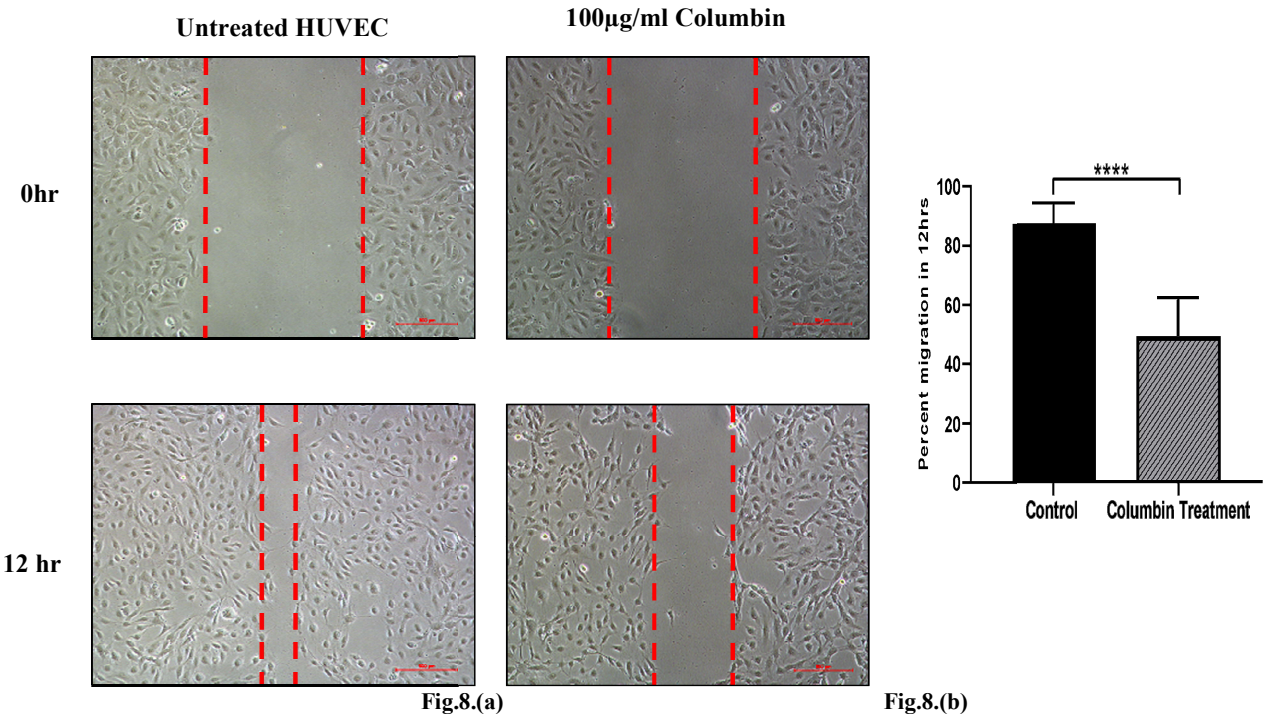

Figure 8: Effects of columbin on migration of HUVECs*in-vitro*. a) Relative migration of HUVECs observed after 12 hours in untreated and columbin treated group; b) Graphical representation of per cent migration in both groups after 12hrs of incubation p**= p<0.0001. Images were captured using a phase-contrast microscope at a magnification of 10X**

4. DISCUSSION

Targeted therapies are widely researched and developed for their efficacy against numerous disorders. However, targeted therapies using synthetic compounds are well known for their various side-effects and high cost. Traditional medicines have shown an advantage over their synthetic substitutes in terms of their low cost and fewer side effects. Amongst the bioactive compounds obtainable from *Tinospora cordifoilia*, columbin has been examined in some studies for its antifeedant activity[15]. To date, no study has discussed the angiogenic behavior of this compound. We studied the anti-angiogenic potential of columbin both *in-vitro* and *in-vivo* so that it may be explored further for its use against angiogenic ailments.

Pathological angiogenesis can be intervened by either targeting VEGF or its cognate receptors, VEGFR1 and VEGFR2. We predicted columbin to have an anti-angiogenic function from our observation of its docking with the two major VEGF receptors (VEGFR1 and VEGFR2). Computationally we were able to predict the binding topology of columbin with the crystal structure of VEGFR1 and VEGFR2. The MMGBSA dG also confirmed the

efficient H-bonding of the Arginine 1021 of VEGFR1 and Asparagine 921 of VEGFR2. Since columbin forms a hydrogen bond with the Asparagine's hexose ring's oxygen, which is the active site of 2-anilino-5-aryl-oxazolea (VEGFR2 inhibitor), therefore we can confidently account for the inhibitory nature of columbin along with its binding topology in 3D space. *In vivo* study using the CAM model, revealed that the anti-angiogenic potential of columbin was better at 100µg/ml as compared to 50µg/ml when observed after 48 hours of incubation. There was less number of vessels and network branching around the implanted disc in developing embryo at this concentration. Since angiogenesis is a cumulative effect of endothelial cell proliferation, migration and tube formation, we assessed the *in-vitro* anti-angiogenic efficacy of columbin by MTT assay, scratch wound healing assay and matrigel tube formation assay. *In-vitro* experiments with HUVECs did not show a significant cytotoxic effect of columbin at 50µg/ml. However, at a concentration of 100µg/ml, 50% decrease in cell viability was observed. Therefore we used this concentration for further experimentation with HUVECs. In concordance with our previous results, the percent cell migration was reduced to almost half in the columbin treated cells as compared to the untreated control. HUVECs have the ability to differentiate and make tubule like structures when seeded on a basement membrane protein. We observed that the addition of columbin reduced tube formation by HUVECs when compared to untreated HUVECs which formed a network of tubes. These observations confirm the anti-angiogenic potential of columbin which may be attributed to the competitive binding of columbin with VEGF receptors. Further investigations using animal models will validate the anti-angiogenic role of columbin observed in our study.

5. CONCLUSION

On the basis of above studies it can be concluded that columbin treatment could significantly suppress the process of angiogenesis in HUVECs grown *in vitro*, angiogenesis of CAM *in vivo*. Also, molecular docking of columbin with angiogenic receptors justified its role in the inhibition of angiogenesis. Therefore this bioactive compound may be explored as an important drug candidate in cancer and other angiogenic ailments where excess angiogenesis leads to dreadful conditions. Further experimental studies are required to clarify the anti-angiogenic molecular mechanism of columbin treatment.

6. Conflict of Interest

We have no conflict of interest to declare.

7. Acknowledgments

The author is thankful to Indian Council of Medical Research (ICMR), Ansari Nagar, New Delhi 110029 for providing support for this study.

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