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ANTI-ANGIOGENIC PROPERTY OF BERGENIN IN CHICKEN CHORIOALLANTOIC MEMBRANE.

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ABSTRACT

Angiogenesis is one of the prime factors required for the formation of blood vessels and capillaries from pre-existing blood vessels. It plays a major role during embryonic development but is detrimental during the later stage as uncontrolled angiogenesis will lead to the formation of tumours and other disorders such as neurodegenerative diseases. Hence to combat this irregularity and maintain balance, a major receptor called VEGF needs to be targeted. Therefore many recent studies are based upon experiments using drug molecule from a plant source which has no side effects and effective in blocking the pro-angiogenic factors. In this context, our present study is based on investigating the antiangiogenic effect of Bergenin using the preliminary and widely used in-vivo model called CAM. Bergenin is a natural compound found in rhizomes ofBergenia species.It has various medicinal properties including anti-inflammatory, anti-oxidant,neuroprotective activity andmany more. The CAM Assay in our study is followed by a cell viability assay on macrophages which show Bergenin to be non-toxic. Also, at the highest concentration, Bergenin has shown good anti-angiogenic activity by inhibiting the formation of blood vessels in the chorioallantoic membrane when compared with the standard drug molecule.The above findings indicate that the molecule Bergenin can be further used in high throughput screening of its antiangiogenic property and can be used as a valuable lead molecule in drug discovery and development against cancer and other neuroinflammatory diseases.

Keywords: CAM, Angiogenesis,VEGF, Neuroinflammation.

1. Intoduction:

Angiogenesis is a phenomenon of the formation of new blood vessels from the arterial vascularization that is generated by endothelial cells, necessary for the normal development of organs. It is also a recurrent hallmark not only in cancer but in several neurological constraints such as in Alzheimer's disease (AD), Parkinson's disease (PD), Multiple sclerosis (MS) and brain injury (JiaW 2018;Seabrook TJ 2010) Angiogenesis also has a prominent role in building up the BBB (Blood Brain Barrier) that is obligatory for selective permeability involving a series of changes ceasing in the formation of tight junctions (Rigau V2007;Seabrook TJ 2010)

Even though angiogenesis is decisive during tissue repair and injury, it plays a role in the formation of tumours (Ahmad 2016) Both excessive or insufficient angiogenesis can lead to several disorders (Oktavia 2017) A wide range of diseases are being treated by targeting

leukocyte-vascular interactions (Kim 2015). Blood vessel formation, immune response and oxidative stress can be significantly interconnected because inflammation can stimulate angiogenesis and oxidative stress which is also vice-versa (Joshi 2016) this interplay has been identified in the pathogenesis of several neurodegenerative and metabolic disorders (Vallon 2014). Above all, there is a strong connection between macrophages and angiogenesis. Especially, in tumour formation, macrophage has an active role as it produces VEGF and MMP9 which are pro-angiogenic factors that can readily initiate the tumorprogression (Graney 2020). But during the embryonic stage as in the chicken chorioallantoic membrane, the macrophages are produced by the chick yolk sac as first-generation and these macrophages produce certain factors such as inflammatory factors that promote angiogenesis (Rezzola 2020). Thereafter, macrophages can be a good therapeutic target for the discovery of antiangiogenic drugs.

In the present study, the natural compound Bergenin already claimed by previous studies for having anti-inflammatory and antioxidant activities is used to check its anti-angiogenic property in-vivo using CAM as a model. It is a universally reliable quantitative in-vivo model to check the anti-angiogenic activity that aids in the screening of antiangiogenic efficacy of many drugs and naturalproducts (Oreffo 2020)

Worldwide populations rely on herbal medicines as a major source of health care. Since ancient times herbal medicines are being used to prevent and treat many different ailments (Ngoua-Meye-Misso 2018). However, the mode of action of plant-based drugs varies from that of the commercially available synthetic drugs. Natural products emerging from medicinal plants are vital for pharmaceutical research and drug development by delivering as a source of therapeutic agents (Ahmad 2016, Lu 2016 and Habib-Martin 2017). Presently many medicinal plants and their purified phytochemicals such as Curcumin (Liu, D 2008), Resveratrol (Nih 2018;Tseng 2004) and Quercitin have shown both in-vitro and in-vivo anti-angiogenic activities (Ravishankar 2015)

Bergenin a natural polyphenolic compound sub-classified as an Iso-coumarin mainly isolated from Bergenia species of Saxifragaceae family usually found in eastern and the South Western Ghats of India, Central Asia, Afghanistan and China (Pandey 2017; Singh 2019) It is a prominent polyphenol present in the rhizome of the plant and is considered one of the most important drugs in Ayurveda as it is used in many Ayurveda formulations and traditional medicines since the 7^{th} century (Pandey 2017). It is affirmed to possess various medicinal properties that include antioxidant properties, chemo-protective activity (Gao 2015;Zhang 2013) neuroprotective (Takahashi 2003) and anti-inflammatory activity (Nazir 2007).

Despite the several natural plant products explored for their anti-angiogenic effects and considered as a valuable source of angio-modulators no research on the potential anti-angiogenic activity of the bioactive molecule Bergenin using CAM Assay has been yet addressed to the best of our knowledge. Within this context, the main aim of the present work is to check the biological efficacy of Bergenin in inhibiting blood vessel formation in-vivo using the CAMmodel.

2. Materials andMethods:

2.1 Chemicals andreagents:

Bergenin (98% pure) and retinoic acid CAS NUMBER: 302-79-4 was purchased from (Sigma Aldrich). Ethanol, PBS and DMSO are of analytical grade, Watttman No-1 filter paper, MTT, FBS and DMEM.

2.2 Cell lines:

The RAW 264.7-Mouse Macrophagecell lines were procured from NCCS, Pune.

2.3 Cell culture:

RAW 264.7 cells were maintained in DMEM - High Glucose - (#AL111, Himedia)supplemented with 10% FBS ((#RM10432, Himedia), 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C with a humidified atmosphere of 5% CO2 incubator (Healforce, China).

2.4 Cell Viability Assay:

The toxicity of Bergenin on macrophage cell lines was assessed using the MTT Assay. Briefly, 100 µl of cell suspension having 2 x 10^4cells were seeded in a 96 well plate and allowed to grow for about 24 hours. After 24 hours the cells were exposed to an increased concentration of Bergenin (0-200µM) and incubated for another 24 hours at 37^0 C in a 5% CO2 atmosphere. After incubation,the plates are carefully takenout and the spent media is removed and 0.5mg/ml of the total volume of MTT reagent is added and kept for another 3 hrs in a 5 % CO2 incubator.Then carefully remove the MTT from the cells without disturbing the precipitate and add 100 µl of solubilisation solution (DMSO).It is then placed in the gyratory shaker which will further enhance the solubilisation hence completely dissolving the MTT formazan crystals. The absorbance was read at 570nm using a plate reader.The percentage of cell viability was calculated by comparing it with the control sample treated with DMSO.

$$\% \text{ of Cell viability} = \frac{\text{Absorbance of}_{\text{sample}} - \text{Absorbance of}_{\text{blank}}}{\text{Absorbance of}_{\text{control}} - \text{absorbance of}_{\text{blank}}} \times 100$$

2.4.1 Preparation of sample:

Bergenin was dissolved in 100% DMSO and different aliquots with varying concentration (5µM,10µM, 25 µM, 50 µM,100 µM and 200 µM) was prepared for MTT Assay. Sterile discs of 5 mm diameter were prepared using Whatman No 1 filter paper and the prepared disc was sterilized by autoclaving for 15 minutes. Each disc was loaded with 10 µl of sample and dried so that the final concentration of the discs will be 25 µM, 50 µM,100µM. Retinoic acid serving as positive control is also applied in three different concentrations same as the sample. PBS is used as blank control. All the steps were being carried out under the laminar airflow chamber to maintain the sterile conditions.

2.5 Chorioallantoic Membrane Assay (CAM):

The angiogenic activity was performed according to the method of (Ngoua-Meye-Misso 2018) fertilized brown Leghorn eggs about four days old were collected from the local hatchery and were cleaned with 70% Ethanol under a laminar airflow hood. A small hole of 10 cm2 was made at the pointed end of the egg and 2 to 3 ml of albumin was sucked out from the egg using a 21 gauge needle to bring their developing membrane down from the shell. The eggs were then incubated in an incubator with a temperature of 37^0 C and 80% humidity by placing them horizontally and rotated 5 times a day. After 24hrs of incubation, a window (4x4cm) was made on the surface of the shell with a sharp tool and the shell was carefully pulled out without damaging the inner tissue using the sterileforceps.

Any dead embryos were disposed of. The sterile disc containing the sample with different concentrations (25 µM, 50 µM and 100 µM) was placed over the blood vessels at the junction on day 5 under sterile conditions. The opening was then covered with sterile surgical tape and

the eggs were incubated for 48 h. The windows were then reopened on day 7 and checked for the inhibition of vessel formation around the area of the disc in terms of number and calibrated by comparing with Retinoic acid used as a positive control. Each experiment was performed in triplicates and the results were assessed visually by counting the vessels with the help of three independent observers.

The images of the blood vessels were capturedusing Nikon D5300 and the results were evaluated and the statuses of the embryos are reported in **Table-1**. The percentage inhibition was calculated by using the formula (Ribatti 2010)

$$\%\text{inhibition} = \frac{\text{Total number of visibleblood vessels}}{\text{Total number of blood vesselssuppressed}} \times 100$$

2.6 Statistical Analysis:

The results obtained were analyzed by calculating their standard mean, among various treatment groups using Graph pad prism 5.0 software. The values are presented as mean ± S.D. One way ANOVA and tukeys post hoc test was performed and p-value <0.005 was considered statistically significant.

3 RESULTS:

3.1 Cell viability Assay:

To determine the toxicity of Bergenin on macrophage cells MTT assay was done. The cells were treated with varying concentration of Bergenin (0-200 µM) for 24hrs. From **figure 4** no toxicity of Bergenin was observed even at the highest concentration when compared to DMSO. Hence the compound Bergenin is proved to be non - toxic on macrophage cells and is safe for further use in in-vivo studies. As there was no significant toxicity observed upto 200µM the subsequent concentrations of 25, 50 and 100 µM were selected for further studies.

3.2 CAM Assay:

The anti-angiogenic activity of Bergenin was interpreted using the CAM Assay with retinoic acid as a positive control. The results were calculated based on the percentage of inhibition of the blood vessels. The changes in the blood vessel density correlate with the percentage of vascularization. Whereas the reduction in the formation of blood vessels is inversely proportional to the degree ofinhibition.

Table 1: In-vivo anti-angiogenic activity of Bergenin in comparison with positive control retinoic acid

Test Sample	Concentration in µM	Embryo status after 48 hrs	% Inhibition of vessel formation
Control (PBS)	-	Living	Nil
Standard	25 µM	Living	32.66 %
	50 µM	Living	49.66 %
	100 µM	Living	80.33 %
Bergenin	25 µM	Living	18.66 %
	50 µM	Living	42.33 %
	100 µM	Living	75.66 %

In the presence of a blank control which is phosphate buffer saline (PBS) 100% of vascularization can be observed corresponding to a normal vascularization of the control having vessels equal to 7. The CAM was treated with three different concentrations as shown in**Table 1**. After 48hrs of observation Bergenin showed 75.66% inhibition of the formation of blood vessels around the disc area with the highest concentration of 100 µM which is comparable to the standard retinoic acid that showed 80.33% at 100 µM concentration. The avascular zone formation around the site of the application represents antiangiogenicactivity as shown in **Figure 6.**

From **Figure 5** The effect of Bergenin on angiogenesis can be seen as a concentration dependent inhibition as it showed the highest inhibition at maximum concentration, whereas at concentrations of 50 µM and 25 µM, the inhibitions were 42.33 % and 18.6 %, respectively, which are lower when compared with that of positive control showing 49.66 % and 32.66 % at 50 µM and 25 µM. Also, the toxicity of the compound was evaluated by recording the status of the embryo as living or dead as shown in **Table-1.**
Among the different doses tested, no dead embryos were found, by which we were able to say that the compound is not toxic and the antiangiogenic activity observed was not due to anytoxicity.

4 Discussion:

The formation and proliferation of capillary tube leading to the growth of vascular endothelial cellsaredescribed as angiogenesis. Some of the widely used angiogenic inhibitors inhibit most of the proteases, blocks the phosphorylation of receptors and disrupt the endothelial tube formation (Yang 2017). Hence, in this context, the present study is carried out to check the anti-angiogenic activity of Bergenin using the CAM method

The chorioallantoic membrane consists of an extra-embryonic membrane and helps in the exchange of gases and nutrients (Irvin 2014) CAM model is extensively used because of its large vascularity and easy availability. It is a widely employed research model that is primarily used to screen the effectiveness of many drugs against angiogenesis, tumour growth and invasiveness (Nowak-Sliwinska 2014).

In the current study, the anti-angiogenic activity of Bergenin was evaluated by using three different concentrations of the compound and the concentrations are standardized from the viability assay performed. According to the MTT Assay performed, Bergenin was proved to be non-toxic at concentrations ranging from 0-200µM which is consistent with previous studies (Yang 2017). The vascularization was accounted for by comparing with the PBS containing disc (blank) and the inhibition of blood vessels were compared with retinoic acid serving as a positive control. The extent of angio- suppressive activity was validated by observing the changes in the formation of blood vessels around the disc.

Retinoic acid is proven to act as a good antiangiogenic agent according to many studies. In a study (Suzuk 2004) retinoic acid has shown the antiangiogenic potential by down targeting Tie2 signaling along with a decreased number of endothelial cells, leading to the reduction of vascular network formation thus inhibiting the process of angiogenesis. RA was also proved to inhibit tumour growth in the thyroid along with decreased VEGF expression with reduced angiogenesis (Hoffmann 2007)

Hence retinoic acid was chosen as a positive standard to compare the antiangiogenic activity of the test compound. Among three different concentrations of Bergenin tested the highest concentration showed the highest percentage of inhibition comparable to that of retinoic acid. The inhibition may be due to the presence of galloyl moiety and lactone group present in the natural compound Bergenin. Earlier studies have noted the structural based relationship of molecules containing the lactone and galloyl group and their effectiveness in inhibiting angiogenesis (Liu, Y.R 2018) (Hernández 2014).

According to previous In-silico investigations, Bergenin has been computationally proven as a potent compound to target the NWGR motif of CRD of galectin-3 which is the most sought after target in designing anticancer drugs. These findings can further rationalize the anticancer activity of Bergenin. Also, Bergenin has druggable properties as well as a good safety profile according to the modern toxicity studies (Jayakody 2018). However, further expression studies and strong in-vivo studies on the antiangiogenic activity of Bergenin is needed to understand the exact mechanism of action and drug potency after which the compound may be used in combination with a variety of other anti-angiogenic and anticancer agents to potentiate the therapeutic efficiency.

5 Conclusion:

The active compound Bergenin has exhibited a potent dose-dependent anti-angiogenic activity using CAM assay as an in-vivo model owing to the presence of the lactone and galloyl groups. It has also proven to be non-toxic to macrophage cells according to our studies, hence allowing its wide usage in different in-vivo and in-vitro experiments leading towards the inhibition of angiogenesis. This investigation has also made a way for researchers in considering Bergenin as a lead compound for inhibiting angiogenesis. As the use of synthetic angio-suppressive agents pose a variety of harmful side effects researchers and investigators are trying to identify and bring about active bio-molecules from plant sources to combat many ailments. Thus this study also supports the pharmacological and traditional use of Bergenin as a neuroprotective and chemoprotective agent via suppressing the formation of angiogenesis.

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Authors' contributions: All authors contributed equally to the research article.

Figure 1: Graphical abstract depicting the entire study.

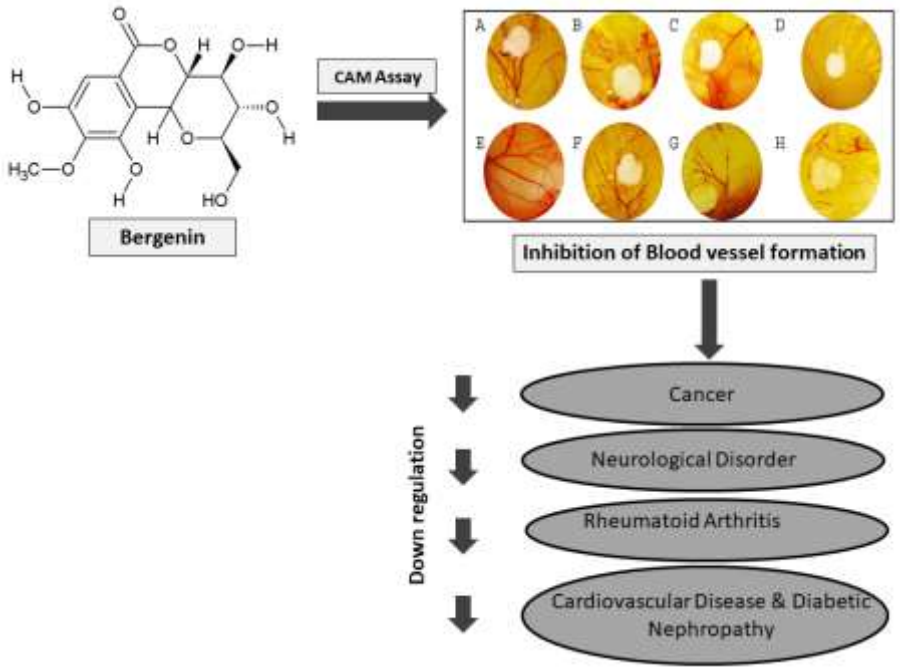


Figure 2: Schematic representation of the interconnections between Angiogenesis, Oxidative stress and Inflammation

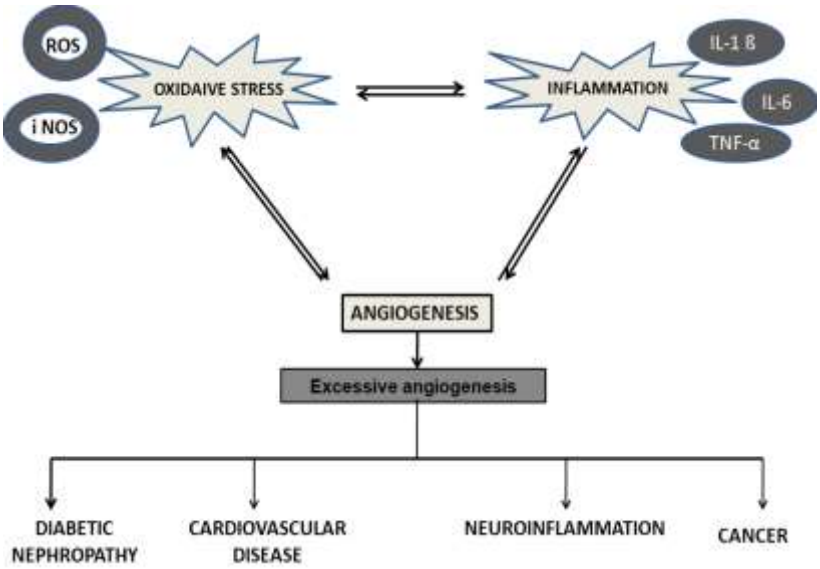


Figure 3: Structure of Bergenin

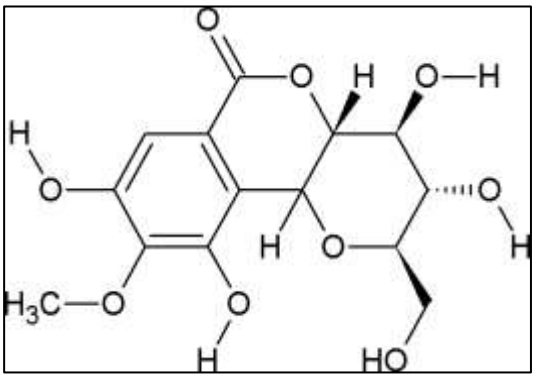


Figure 4: Graph showing the percentage of viable cells (RAW 264.7 Macrophage cells) after treatment with different concentrations of Bergenin (5-200 µM) for 24 hours (*** p< 0.001).

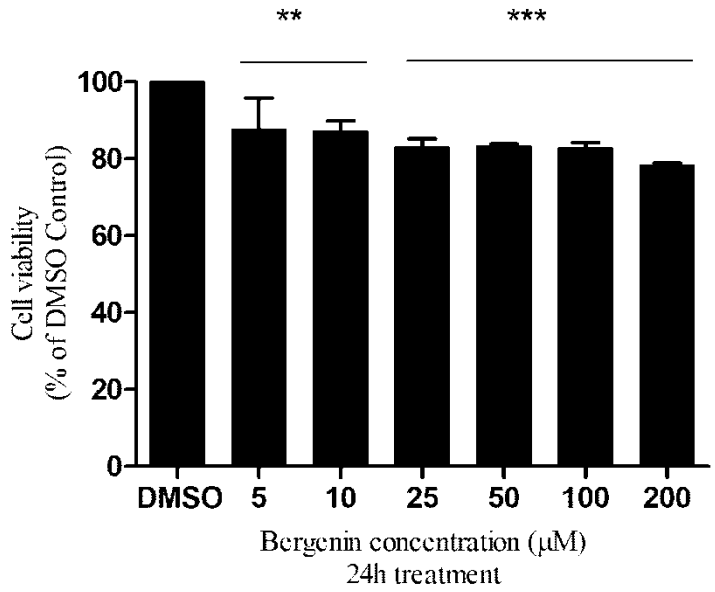


Figure 5: Percentage of inhibition of Angiogenesis with different concentrations of Bergenin compared with standard (retinoic acid) assessed for 48 hours (***** p< 0.05, ******* p< 0.001, ns= non-significant).

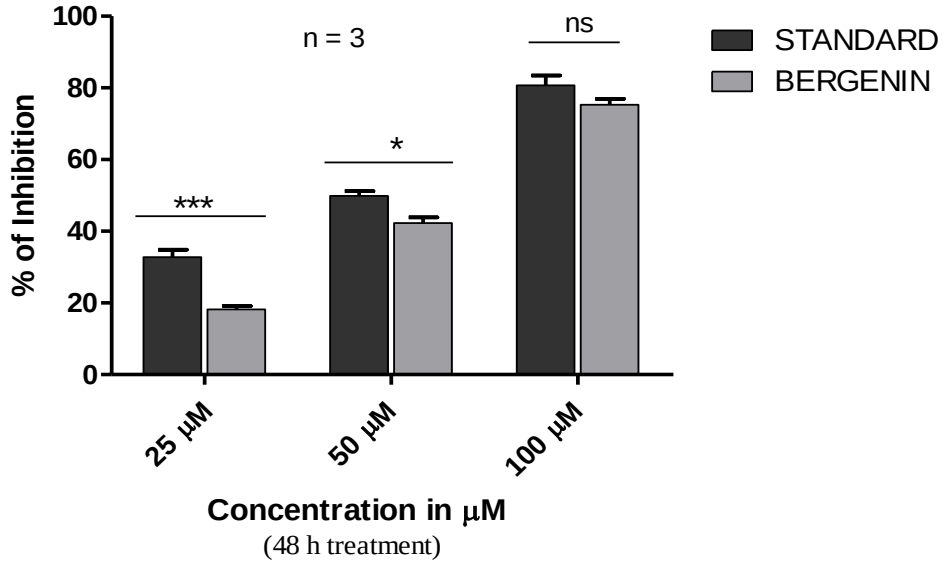
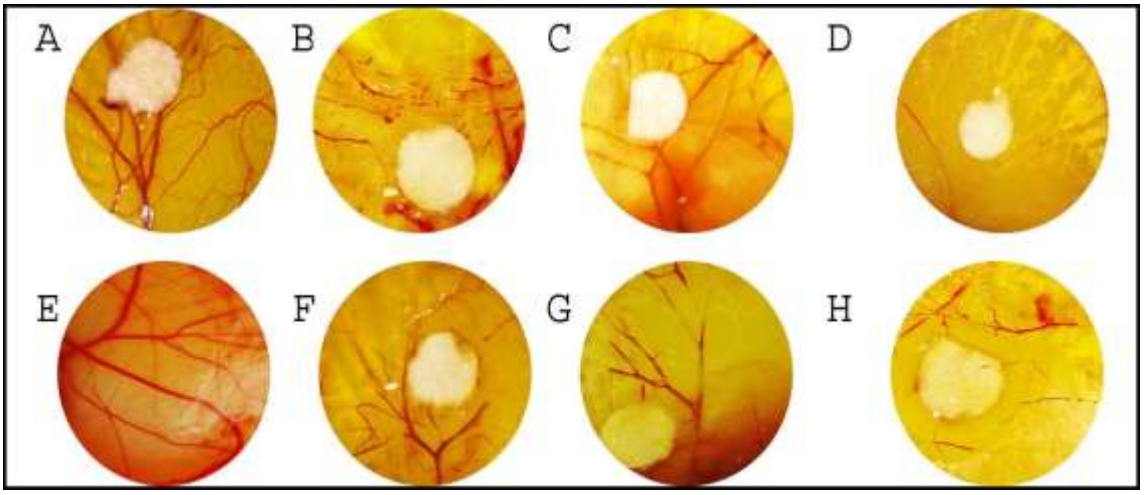


Figure 6: Effect of Bergenin on the suppression of vasculature of CAM in a dose dependent manner **(A)** Control (10 µl PBS); **(B,C,D)** Standard (25 µM, 50 µM, 100 µM); **(E)** Untreated; **(F,G,H)** Bergenin (25 µM, 50 µM, 100 µM);



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