

*Original Research Article***EFFECT OF *PUNICA GRANATUM* (POMEGRANATE) FRUIT EXTRACT ON ANGIOGENESIS**

Ghulam Jilany Khan^{1*}, Muhammad Ovais Omer², Muhammad Ashraf¹, Habib Ur Rehman², Zaheer Ud Din Khan³.

1. Department of Pharmacology & Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
2. Assistant Professor, Department of Pharmacology and Toxicology, College of Pharmacy. Umm Al- Qura University - Al-Abidiyya, Makkah, Kingdom of Saudi Arabia
3. Department of Physiology, University of Veterinary and Animal Sciences, Lahore.
4. Department of Botany, Government College University, Lahore.

ABSTRACT

Angiogenesis, which is the development of blood vessels from previously existing vessels, is a physiological process. It is a common and most important process in the formation and development of blood vessels, so it is supportive in healing of wound and granulation tissue. This study was designed to evaluate the effect of pomegranate whole fruit extract on angiogenesis by using CAM assay and to verify either the extract is angiogenic or anti-angiogenic. To explore the effect on angiogenesis we used CAM model which is an extra-embryonic membrane model in Chicken embryos and SPIP (scanning probe image processor) for quantification of resultant images. For this study fertilized eggs were collected from a local hatchery in Lahore and different aqueous dilutions of whole fruit extract were applied on developing CAM on day 6. A noticeable reduction in surface roughness of the blood vessels was observed. The diameter of tertiary, secondary and primary blood vessels was also reduced as compared to the blood vessels of control group CAM. The maximum effect was seen with 0.5% dilution. The study proved that *Punica granatum* (Pomegranate) fruit extract was anti-angiogenic and can be included in the studies for the development of new drug studies to treat cancer (as an anti angiogenic agent).

keywords: Pomegranate, Angiogenesis, Chorioallantoic membrane assay, Scanning Probe image processing system.

***Corresponding author: Ghulam Jilany Khan**, Pharm.D (PU), MBA (UVAS), M.Phil (UVAS) Department of pharmacology and toxicology, University of Veterinary and Animal Sciences, Out-fall road Lahore. Pakistan. T.: 00923334574904; E.: u4574904@hotmail.com

INTRODUCTION

Angiogenesis is a combination of two Greek words "angeion" and "Genesis" means "vase" and "the birth" respectively. This name is given to the particular growth of new blood capillaries from pre-existing blood vessel plexus. This process is essential to fulfill the nourishment and other demands of tissue. It is a common and most important process in the formation and development of vessels, so it is supportive in healing of wound and granulation tissue [1]. Pathological Angiogenesis is associated with many diseases which are retinopathies, arthritis,

and psoriasis etc. [2]. To maintain natural balance between formulation and inhibitory factors, body controls angiogenesis. When this balance is disturbed, the body results in either too much growth or too much inhibition of vessels. The general concept is that development of tumor is dependent on angiogenesis and needs vascular growth. In the absence of vascular growth the tumor will not be malignant and remain inactive [3]. That's why the anti angiogenic drug development is of great interest for therapeutic purposes.

Various studies were conducted to study angiogenesis. More than 18,000 scientific articles have been published in last 40 years to prove the effects of angiogenesis in different life threatening diseases [4]. And now it is believed that angiogenesis, in which vascular supply from adjacent tissues is derived by cancer cells, is a vital step in growth and metastatic spread [5]. With the help of angiogenesis process, cancerous cells got the sufficient supply of nutrients and oxygen. When a tumor cell has multifaceted system of blood capillaries, the shedding of the cells from the primary tumor begins [6].

Angiogenesis is not often found in healthy condition due to its highly precise nature so we can target the process of angiogenesis with the help of compounds that are safer and have no long term side effects. Since the previous four decades, there have been astonishingly sound revivals of concern in medicinal plants study [7]. In the study of medicinal plants, this new world wide interest has led to the categorization of new molecules and segregation of active chemical compounds from the plants of curative nature [8].

The anticancer effect of pomegranate fruit has been associated, at least in part, to a large number of biologically active phytochemicals which are present in the fruit. These effects of pomegranate fruit are considered due to their abilities to contract, decrease, and also repair damage due to oxidative stress and inflammation. Tree, fruit, leaves and bark skin of this fruit have been used for various ailments and some other uses like in tanning industry [9]. The various diseases against which pomegranate extract is useful for prevention are prostate cancer, lymphoma, prostatic hyperplasia, oxidative stress in diabetic hemodialysis, diabetes, rhinovirus infection and common cold, Acquired immune deficiency syndrome (AIDs), cardiovascular protection and atherosclerosis [10].

Pomegranate fruit extract gives about 16% vitamin C of an adult's daily need. It consists of potassium, Vitamin B5 and polyphenols which are tannins and flavonoids [11]. Pomegranate fruit extract is rich in Polyphenones which are hydrolysable tannins called ellagitannins [12]. Other phytochemicals which are present in pomegranate contain gallocatechins, polyphenolic catechins and anthocyanins like delphinidin, prodelfinidins, pelargonidin and cyanidin [13].

To observe the development of blood vessels, Chick chorioallantoic membrane assay model is very helpful to precede research as it is impossible in other systems like mammalian system [14]. Chorioallantoic membrane (CAM) is an extra developing membrane which is generally used in research studies of both processes angiogenesis and anti-angiogenesis [15]. The evaluation of angiogenic or antiangiogenic effect of tested materials can easily be done through CAM in a short time of 24 hours [16], hence this model quickly and accurately explains both angiogenic and anti-angiogenic characteristics of various chemicals and natural extracts.

However, a major drawback is that it is labor intensive due to large number of eggs that are required to obtain consistent results. The assortment of a proper in vivo and in-vitro models, for the evaluation of angiogenic and anti-angiogenic compounds, is very important step [17]. It is possible with the help of a new technique for quantification of angiogenesis [18]. This imaging

of whole process explains the changes in blood vessels completely which gives better understanding of whole process of angiogenesis. Present study was aimed to identify the effect of *Punica granatum* fruit extract on angiogenesis with the help of CAM (Chorioallantoic Membrane) assay by applying different concentrations of its extract.

MATERIALS AND METHODS

An in vivo study was designed to evaluate the effectiveness of Pomegranate fruit extract on angiogenesis by using CAM assay.

Eggs Collection and incubation

Freshly fertilized, forty (40) eggs were collected from a local hatchery. 65 grams was the average weight of all forty eggs. All the eggs, of same weights at day one of incubation were collected from Big Bird (a local hatchery) situated in district Lahore, Pakistan. To diminish contamination from egg surface, spraying on all the eggs were done with 70% ethanol and then all eggs were dried in air. Then all eggs were laid horizontally to make sure the suitable position of embryo. For incubation, all eggs were placed in incubator whose temperature was adjusted at 37.7 °C and humidity 65-70% targeting an average humidity of 68% and rocked through a range of 60° in an hourly cycle for 5 days as described by [19].

Fruit Collection and extraction processing

Fresh fruit of *Punica granatum* was collected from district Lahore of Pakistan. Then these fruits were properly identified and thoroughly checked by the qualified botanist from the department of botany, Government College of Science (GCS), Wahdat Road, Lahore. One fruit from each sample group was deposited to the department for future referencing. Healthy and fresh fruits of good appearance were selected for the study while the inferior quality, partially ripened, devastated and dented fruits were discarded. Then these fruits were washed with distilled water and randomly divided into four groups, each consisted of 2 fruits for extraction. The fruits were then crushed, squeezed, grounded and blended with electronic blender and finally dried in an oven adjusted at 40°C for time of 24 hours, then the fine powder was sieved with the help of 24-mesh with some modifications as described by [20].

The fine powdered sample (10g) was extracted with 100ml of distilled water at 25°C for 24 h in a shaking water bath. The filtration of the extract was done with the help of Millipore filter with a 0.45µm nylon membrane under vacuum at 25°C. This extract was filtered, pasteurized, concentrated and was stored at 4°C till used. Different dilutions with the use of sterilized distilled water were made and again these dilutions were stored at 4°C until use with some modifications [20].

Preparation of Chorioallantoic membranes

On day 5 of incubation, we made windows in eggs aseptically as described by [19] with some modifications. Small window (approximately of a diameter of 2 cm) from the air space side of eggs were made by removing the egg shell and inner egg membrane [21]. On the same day 2 ml albumin was aspirated with a sterile 21 G cannula and the embryo was positioned to uppermost side of the yolk as described by [19]. The eggs were then covered with sterile Para- film tape and placed to the incubator. Incubation temperature was adjusted at 37.7 °C and humidity 65-70% targeting an average humidity of 68% [19].

Chorioallantoic membrane assay

Four groups of eggs were made, each having ten eggs. Group A was kept as control which received distilled water and groups, B, C and D received 0.1%, 0.3% and 0.5% dilution (made with the use of distilled water) of pomegranate fruit extract respectively. In order to reduce the risk of contamination, prepared dilutions (whose pH was adjusted in the range of 6.5 -7.5) were filtered through 0.2 μ m syringe filter. At day six of incubation the windows on eggs were opened and 200 μ l fluid of each dilution was introduced on CAM which is in developing stage. Windows were covered again with parafilm tape and eggs were placed in incubator for next 24 hours in the same conditions as described above.

Image acquisition and image probing system

To evaluate the surface roughness, a combination of technical image processing system and image probing system of software was used [19]. In order to obtain the quality images of the resultant CAM from different dimensions, Nikon D 7000 Japan with pixel dimensions of 1200X1800, resolution of 300 dpi, bit depth of 24 and an ISO speed of 400 was used. Webster camera was used for detailed 3D images..

After taking the images, the contrast between blood vessels and other tissues was adjusted with the use of adobe Photoshop 6.0 (adobe system software, Ireland) and quantification of all images was done with the help of software Scanning Probe image processing system (SPIP), its principle is based on particular system of roughness quantification [22]. For the determination of different roughness parameters, particular x, y and z dimensions of each image were quantified and evaluated with the detailed effects of each sample. With the help of calibration and measurement, the diameters of different blood vessels were determined. Surface roughness is a major parameter in 3D image analysis [23], several other parameters including Abbott curve of vascular area, and angular spectrum of neo-vasculature on CAMs were also measured. Thus blood vessels were evaluated at micrometer (10^{-6}), nanometer (10^{-9}) and pecometer (10^{-12}) scale to determine the detailed effects of pomegranate fruit extract on angiogenesis.

Statistical Analysis

All parameters were measured with the help of multiple range tests LSD by using ANOVA as mean of standard deviation. Analysis of variance (ANOVA) was performed to assess various parameters of quantification which were given above between control and treated samples; at $P < 0.05$, significant statistical results were measured as described by [24].

RESULTS

In the present study we have observed the effect of pomegranate whole fruit extract on angiogenesis using chicken Chorioallantoic membrane (CAM) assay. After the application of various dilutions, a significant decrease in formation of blood vessels was observed as compared to control group. The following results were obtained in control samples as well as with the treatment of different dilutions on other groups; in control group we observed branching pattern of blood vessels which was most likely a tree and it spreads to the largest part of CAM areas. The vascular structural design of the CAM seemed as it was originating from the main branch of the blood vessel termed as “Y” branch, which further made primary, secondary and tertiary branches (Figure 1).

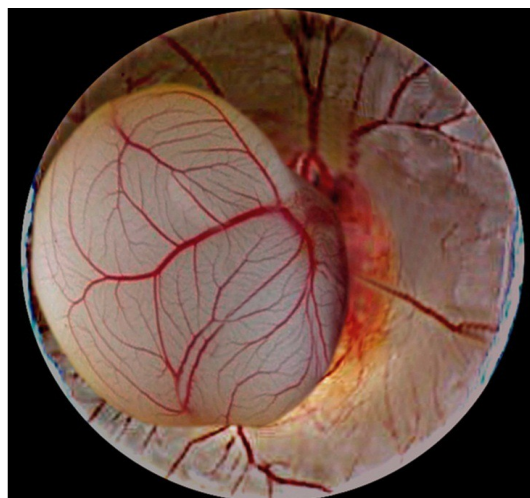
**Control CAM****CAM Vascular structure of Control group****CAM Treated with 0.1%****CAM Vascular structure of 0.1% Treatment**

Figure 1. The vascular structural design of the CAM seemed; branch of the blood vessel termed as “Y”; primary, secondary and tertiary branches.

When we applied different concentrations of Pomegranate fruit extract on CAM, these dilutions caused clear changes in blood vasculature of the CAM. Antiangiogenic activities (reduction in blood vessels) were seen after the introduction of various concentrations of the fruit extract, which showed a perceptible reduction in the overall length and diameter of primary, secondary and tertiary blood vessels indicates a clear decline in the full vascular network of CAM. Out of these all treated group (0.1%, 0.3% and 0.5%) the most ample and vital antiangiogenic effect was observed with 0.5% dilution (Figure 2, 3, 4, Table 1).

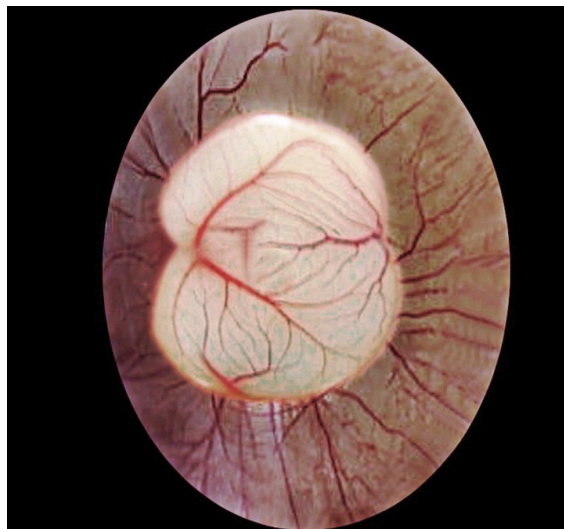
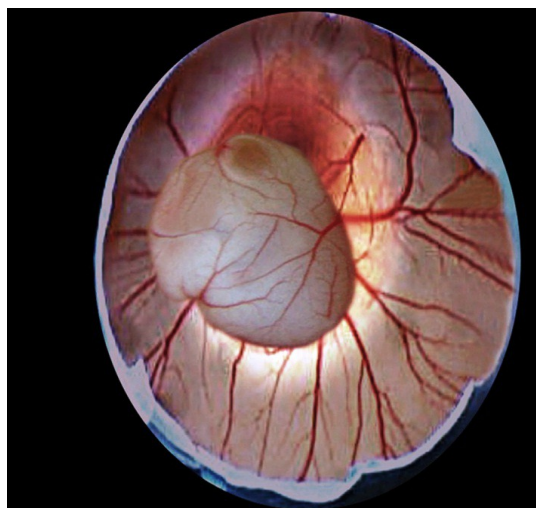
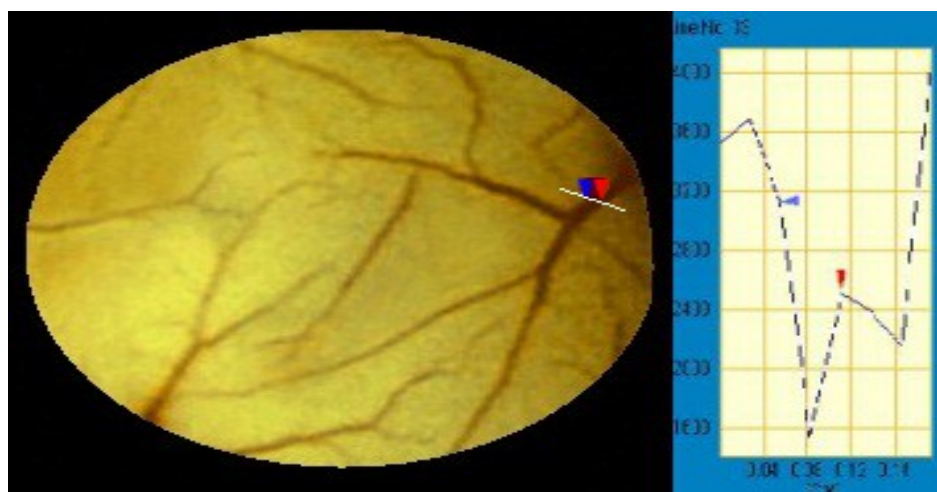
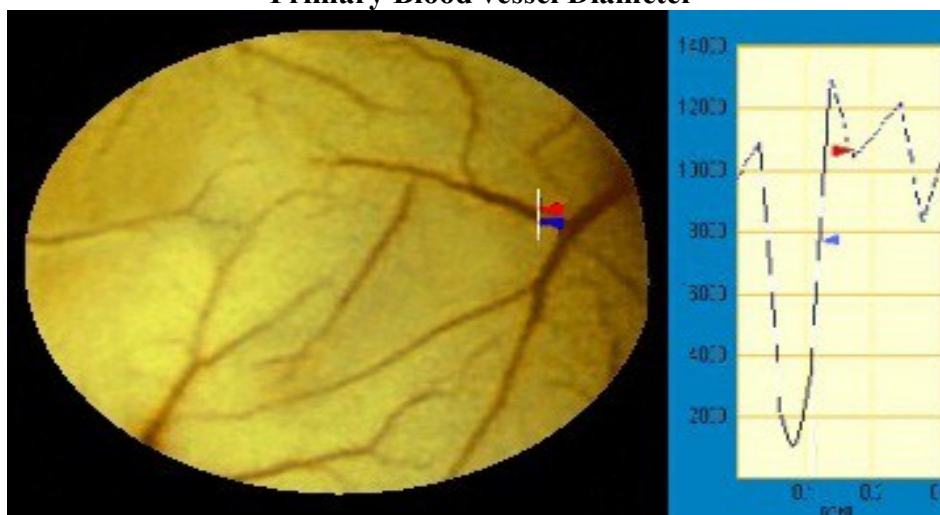
**CAM Treated with 0.3%****CAM Vascular structure of 0.3% Treatment****CAM Treated with 0.5%****CAM Vascular structure of 0.5% Treatment**

Figure1. Macroscopic assessment of CAM. Architecture of blood vessels was noted in control group and also in CAM treated with different concentrations of Pomegranate whole Fruit Extract.

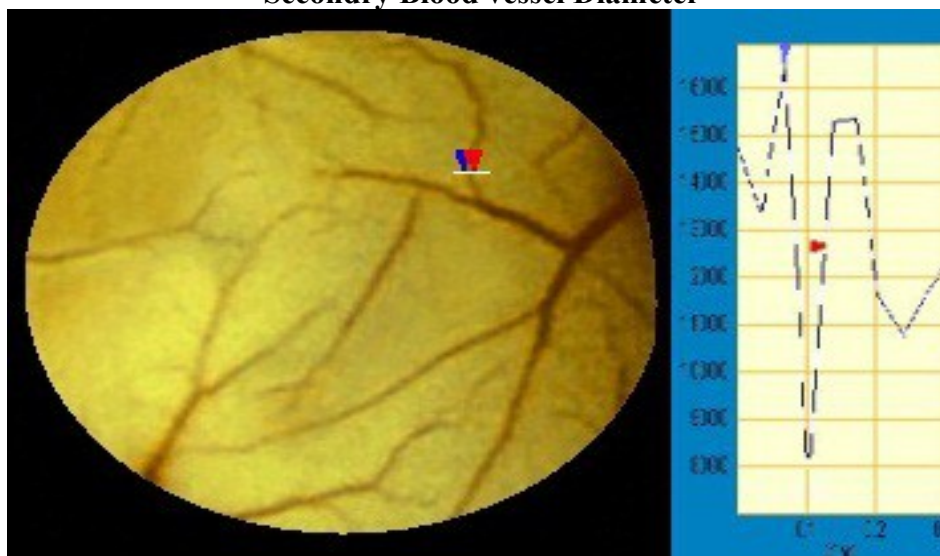
SPIP software was used for quantification of the diameter of blood vessels growing on CAM. Significant reductions in the diameter of primary blood vessels were seen among all treated groups. Total area of the CAM was also rigorously reduced among all groups treated with Pomegranate fruit extract (Figure 5, Table 1). 3D presentation of all Chorioallantoic membranes was studied in experiments, which is mandatory for the accurate quantification of the length, area and diameter of the CAM. 3D surface roughness parameters of CAMs were assessed to quantify the whole process of angiogenesis (Figure 6, 7, Table.2).



Primary Blood vessel Diameter



Secondary Blood vessel Diameter



Tertiary Blood vessel Diameter

Figure2. Evaluation of Diameter of CAM blood vessels.

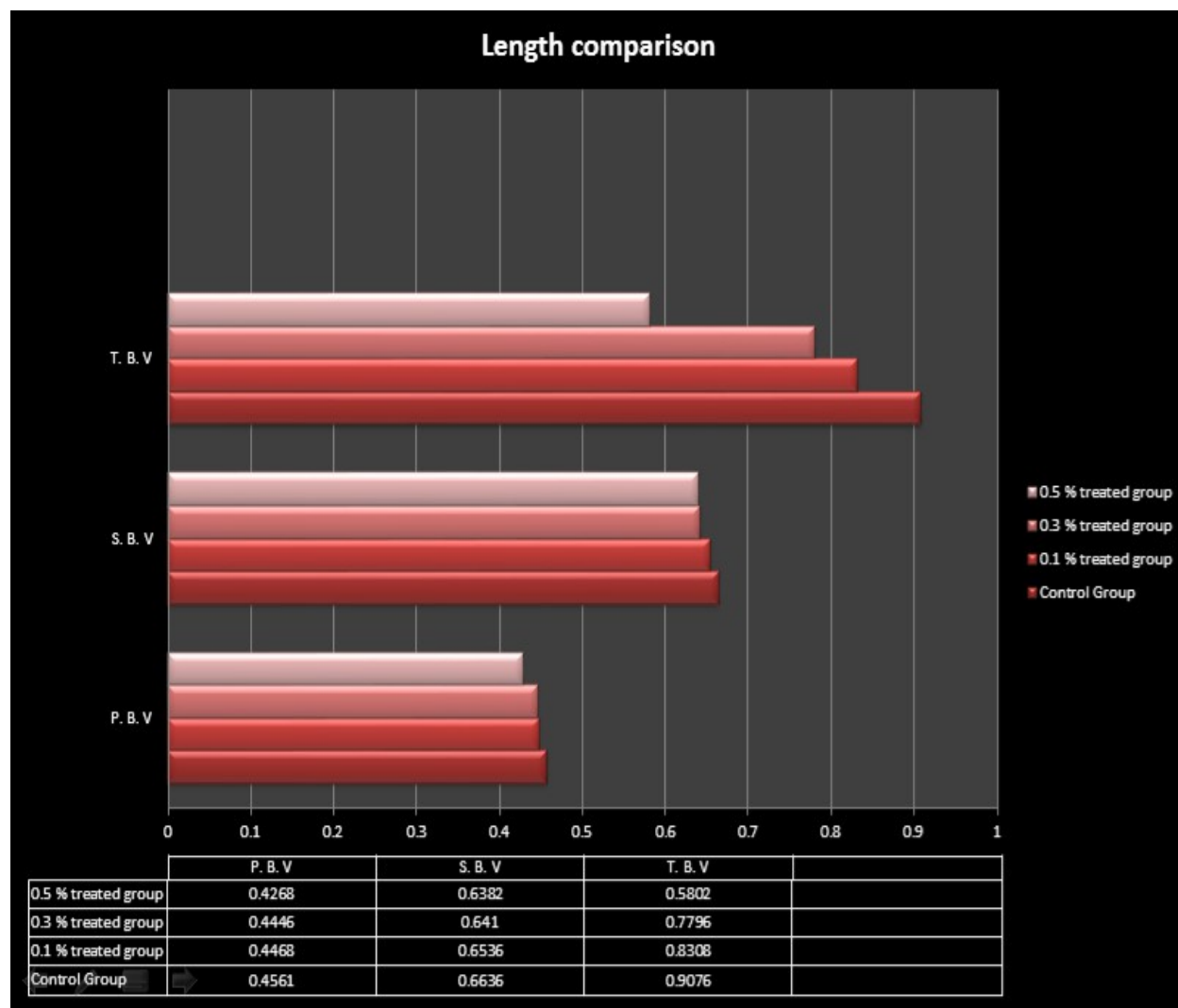


Figure3. The Comparison of length of blood vessels of Controlled Group and Treated groups (P.B.V= Primary Blood Vessel) (S.B.V= Secondary Blood Vessel) (T.B.V= Tertiary Blood Vessel) in Cm

Table1. Length, diameter of blood Vessels (B.V) and area of CAM in Control group (A), 0.1% (B), 0.3% (C) and 0.5% (D) dilution treatment groups in 10^{-2} meter.

Parameter (cm)	Group A	Group B	Group C	Group D
Length of Primary blood vessels	0.4561	0.4468	0.4446	0.4268
Length of secondary blood vessels	0.6636	0.6536	0.6413	0.6382
Length of tertiary blood vessels	0.9076	0.8308	0.7796	0.5802
Diameter of primary blood vessels	0.0313	0.0310	0.0308	0.0308
Diameter of secondary blood vessels	0.0183	0.0175	0.0170	0.0163
Diameter of tertiary blood vessels	0.0075	0.0072	0.0060	0.0038
Area of CAM (cm ²)	1.816	1.6181	1.3141	1.1312

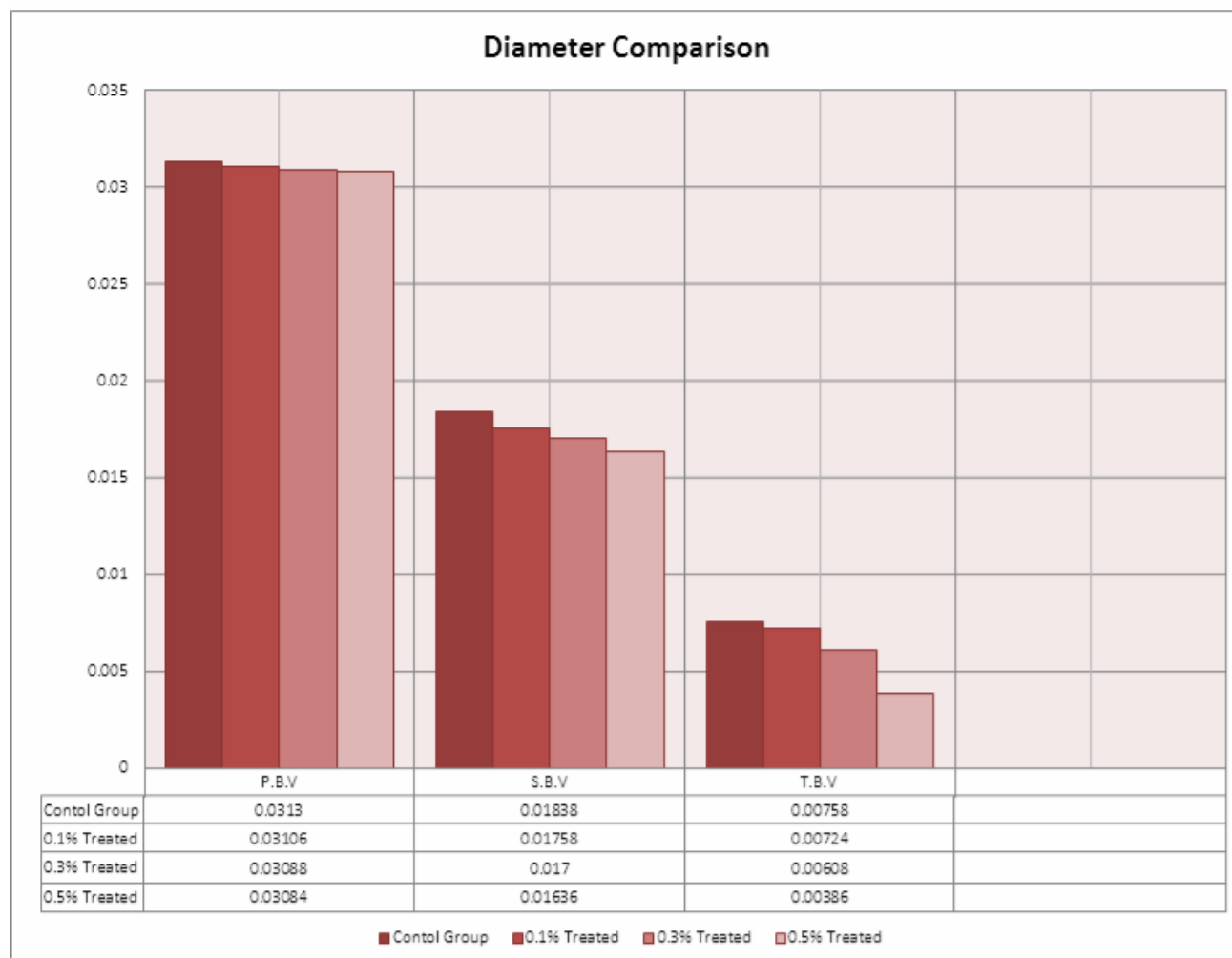


Figure4. The Comparison of diameter of blood vessels of Controlled Group and Treated groups (P.B.V= Primary Blood Vessel) (S.B.V= Secondary Blood Vessel) (T.B.V= Tertiary Blood Vessel) in Cm

The surface roughness, showing neo-vascularization, of control CAMs was considerably larger than that of treated CAMs. The average roughness values of control and treated (0.1%, 0.3%, 0.5%) CAMs were $3.222 \pm 6 \times 10^{-6}$, $3.068 \pm 6 \times 10^{-6}$, $2.84 \pm 6 \times 10^{-6}$ and $2.71 \pm 6 \times 10^{-6}$ meter respectively (Table 2). Image histogram and Abbot Curve of neo-vasculature was also obtained and thoroughly observed as described by [19] the detailed assessment of image histograms of controlled group and treated groups CAMs of B, C and D showed clear differences with the range of -12.5 to 17.5 μm , -10.0 to 9.0 μm , -9.0 to 8.0 μm , -4.0 to 5.0 μm respectively, which indicates a sequential decrease in the height of blood vessels on the surface of CAM as compared to controlled CAM (Fig. 8, 9).



Figure5. The Comparison of Area of Cam in cm^2

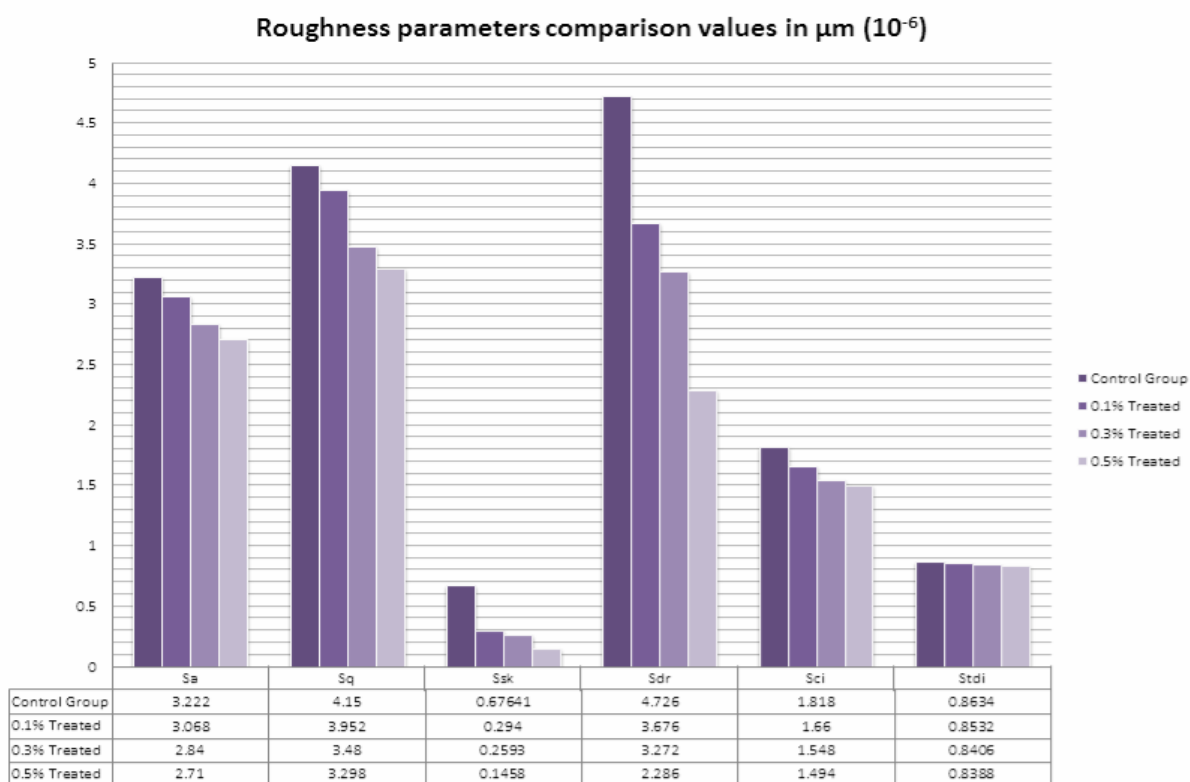


Figure6. Stdi (texture index), Sci (ratio of void volume of the unit sampling area at core zone over root mean square deviation), Sdr (ratio of increment of interfacial area of a surface over sampling area), Ssk (Surface deviation), Sq (root mean Square), Sa (average Surface roughness).

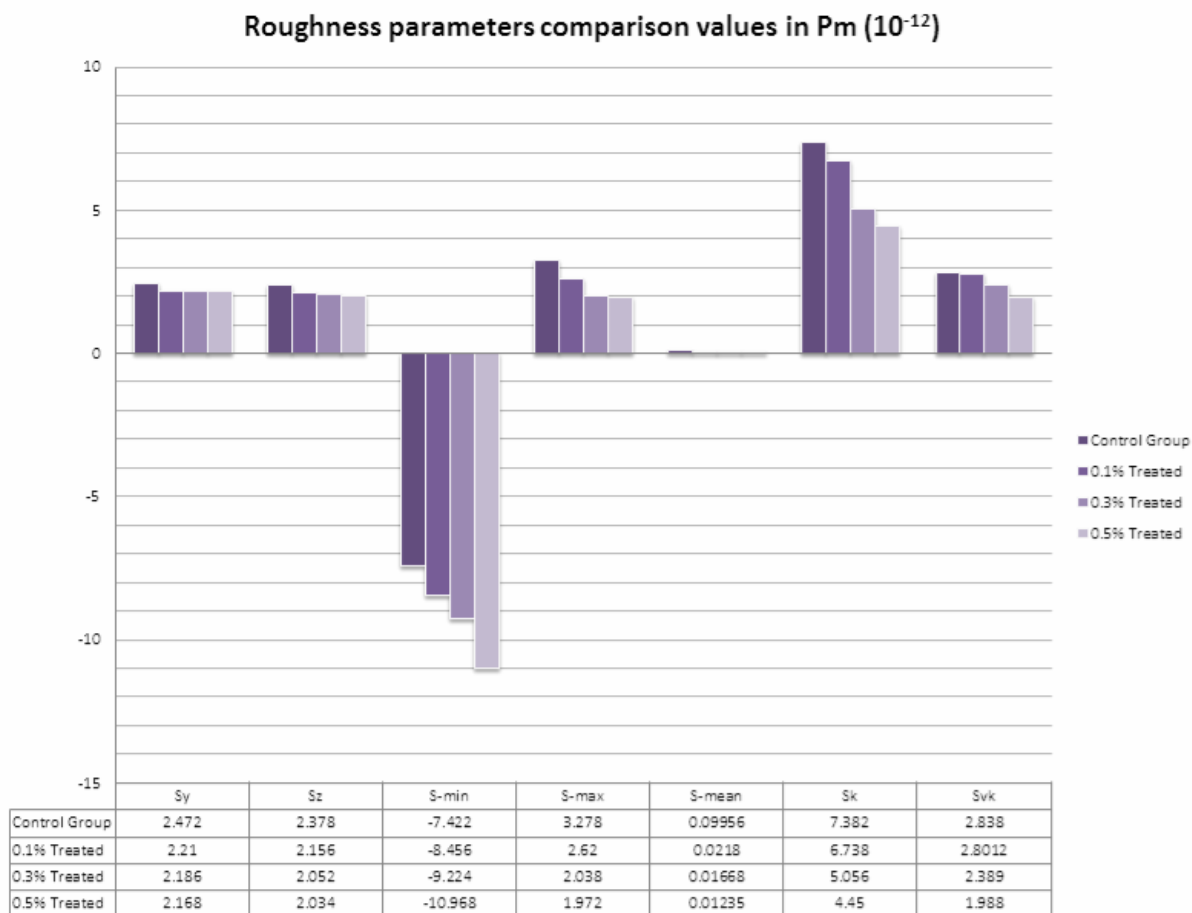
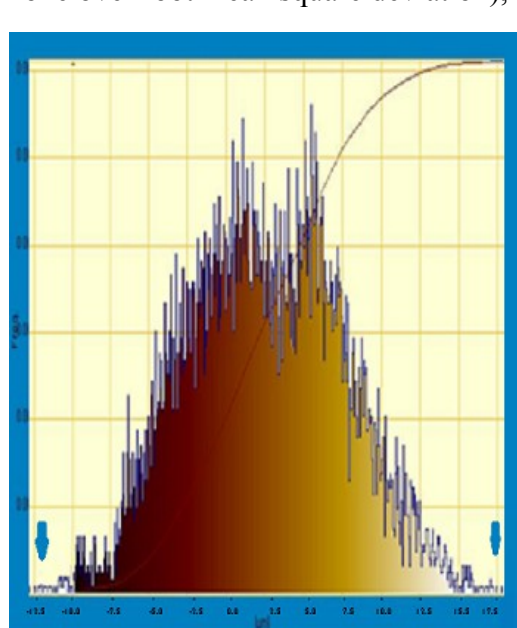


Figure7. Sy (lowest valley), Sz (average absolute height), Smin (minimum height), Smax (maximum height), Smean (mean height), Sk (Core roughness depth), Svk(Reduce Valley depth).

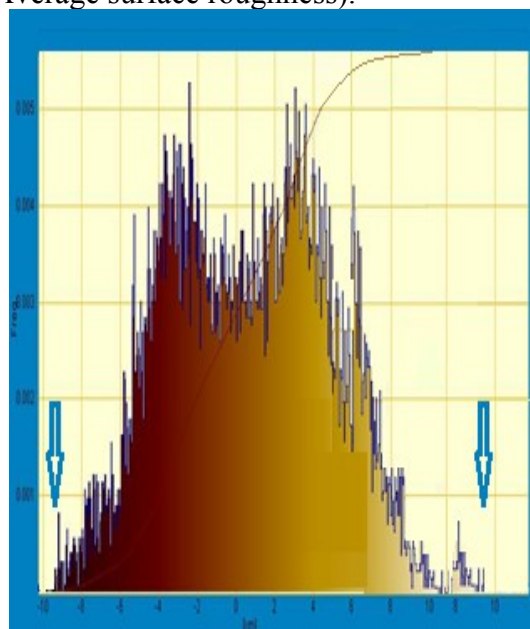
Table2. Roughness parameters of Control and treated CAMs

Sr #	Parameter (μm) 10^{-6}	Control	0.1%	0.3%	0.5%
1	Sa	3.222 \pm 6	3.068 \pm 6	2.84 \pm 6	2.71 \pm 6
2	Sq	4.15 \pm 6	3.952 \pm 6	3.48 \pm 6	3.298 \pm 6
3	Ssk	0.67614	0.294	0.2593	0.1458
4	Sy(Pm) 10^{-12}	2.472 \pm 7	2.21 \pm 7	2.186 \pm 7	2.168 \pm 7
5	Sz(Pm) 10^{-12}	2.378 \pm 7	2.156 \pm 7	2.052 \pm 7	2.034 \pm 7
6	Smin(Pm) 10^{-12}	-7.422 \pm 6	-8.456 \pm 5.2	-9.224 \pm 6.2	-10.968 \pm 6.6
7	Smax(Pm) 10^{-12}	3.278 \pm 7	2.62 \pm 7	2.038 \pm 7	1.972 \pm 7
8	Smean(Pm) 10^{-12}	0.09956	0.0218	0.01668	0.01235
9	Sdr Ratio	4.726 \pm 11	3.676 \pm 10	3.272 \pm 4.8	2.286 \pm 9
10	Sci Ratio	1.818	1.66	1.548	1.494
11	Sk(Pm) 10^{-12}	7.382 \pm 6.2	6.738 \pm 6.2	5.056 \pm 6.2	4.45 \pm 6.2
12	Svk(Pm) 10^{-12}	2.838 \pm 5	2.8012 \pm 5	2.389 \pm 5	1.988 \pm 5
13	Std	0.8634	0.8532	0.8406	0.8388

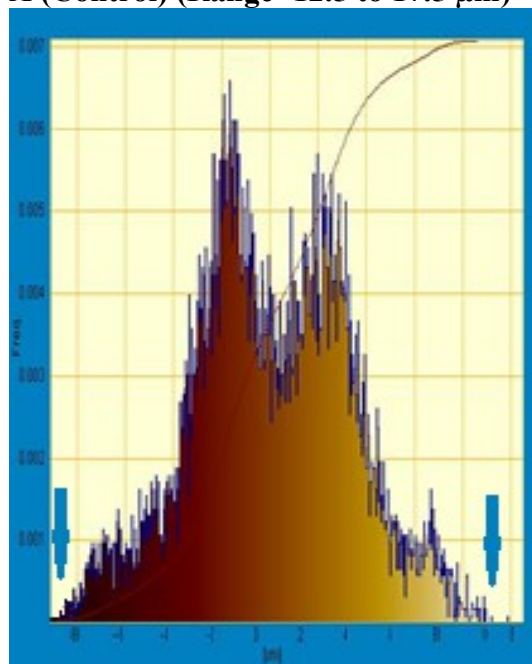
Sz (Average absolute height of 5 most peaks and 5 deepest pits), Sy (Lowest/Deepest valley), Svk (Reduce valley depth from maximum to minimum), Stdi (Texture index of the whole surface), Ssk (Skewness of Surface), Ssc (Arithmetic mean summit), Sq (Root mean Square deviation), Smin (Minimum height of the valley), Smean (Mean height of the peaks), Smax (Maximum height of the peak), Sk (Core roughness depth), Sdr (Ratio of increment of interfacial area of a surface over sampling area), Sci (Ratio of void volume of the unit sampling area at core zone over root mean square deviation), Sa (Average surface roughness).



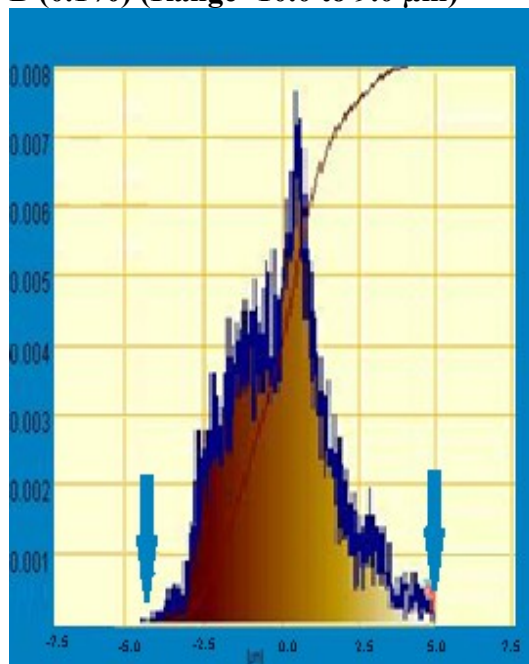
A (Control) (Range -12.5 to 17.5 μm)



B (0.1%) (Range -10.0 to 9.0 μm)



C (0.3%) (Range -9.0 to 8.0 μm)



D (0.5%) (Range -4.0 to 5.0 μm)

Figure 8. Image Histogram of CAM in treated A (Control), B (0.1%), C (0.3%) And D (0.5%)

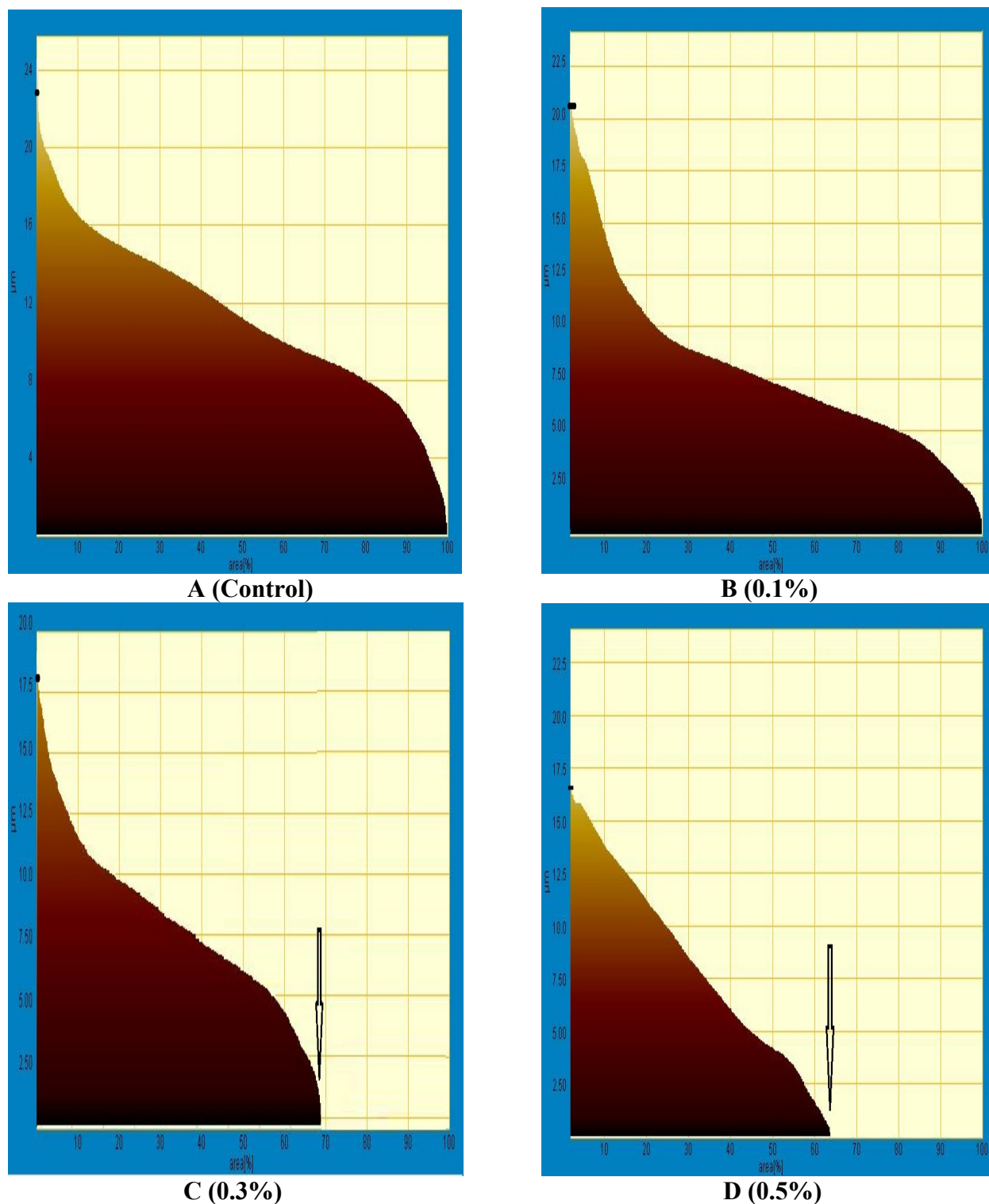


Figure9. Abbott curve of blood vessels in group A (Control) as well as B (0.1%), C (0.3%) And D (0.5%) dilution treated Eggs.

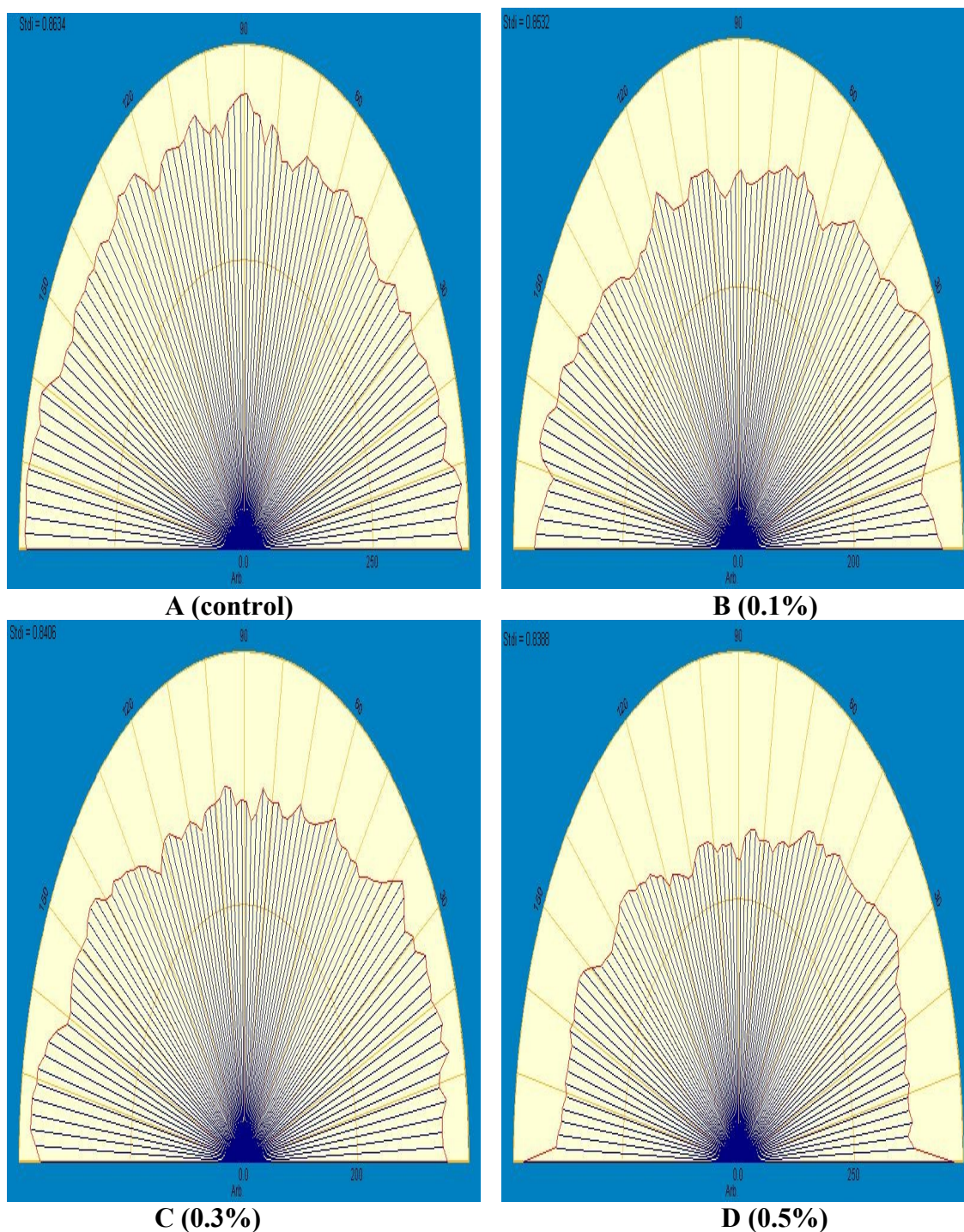


Figure10. Angular spectrum of blood in group A (Control) as well as B (0.1%), C (0.3%) And D (0.5%) dilution treated Eggs.

The angular spectrum which denotes the bounty of regular variations of intensity with angle [25] was found significantly less in treated CAM whereas more in control CAM (Fig. 10).

When ANOVA was applied on all parameters, P-value was found to be lesser than 0.05 ($P < 0.05$).

DISCUSSIONS

Pakistan has a rich medicinal plants heritage. Medicinal plants contain different substances which can be used to prevent, cure or treat various diseases. Since the previous four decades, there have been astonishingly sound revivals of concern in medicinal plants study [7]. In the study of medicinal plants, this new world wide interest has led to the categorization of new molecules and segregation of active chemical compounds from the plants of curative nature [8]. Among commonly and popularly used natural fruits, pomegranates are widely well-known for their potential health benefits. The various diseases against which pomegranate extract is useful for prevention are prostate cancer, Acquired immune deficiency syndrome [26], prostatic hyperplasia, diabetes [27], rhinovirus infection, common cold, and oxidative stress in diabetic hemodialysis, cardiovascular protection [28] and atherosclerosis [10].

However still not a single study has been conducted to check the effects of pomegranate fruit extract on angiogenesis with the help of CAM assay. The basic objective of this study was to test the hypothesis that pomegranate fruit extract obtained from fresh fruits may or may not decrease the process of angiogenesis. We examined the effect of pomegranate fruit extract with its various dilutions on the process of physiological angiogenesis (As this is not a diseased condition) with the help of CAM assay. Various dilutions of pomegranate fruit extract (0.1%, 0.3% and 0.5%) were prepared and then introduced on developing CAMs at day 6 and their final effects on angiogenesis were observed on day 7. Different dilutions of Pomegranate fruit extract produces varied effects on their respective CAMs and the final effect observed was antiangiogenic. The angiogenesis parameters were assessed. 3D surface roughness, length and diameters of primary, secondary and tertiary blood vessels, area of CAMs showed noticeable reduction in their values by all three dilutions of pomegranate fruit extract as compared to control samples.

All the parameters evaluated demonstrate that pomegranate fruit extract inhibits formation of blood vessels in Chicken chorioallantoic membrane. The mechanism responsible for this process may be the inhibition of VEGF expression, which is the main regulator of the process of angiogenesis. This study proved that pomegranate fruit extract inhibits angiogenesis which showed that pomegranate fruit extract may be used for cancer treatments as an antiangiogenic agent which is according to other studies conducted by scientists [29, 30]. Therefore future studies should be designed to investigate the potential of pomegranate fruit extract for the prevention and treatment of cancer diseases in human subjects.

CONCLUSION

It was concluded that pomegranate fruit extract possess antiangiogenic properties. So, it can be included and considered in further studies for the development of drugs to treat cancer as antiangiogenic agent.

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