



Original Research Article

EVALUATION OF PHARMACOLOGICAL EFFECT OF MORUS NIGRA EXTRACT ON ANGIOGENESIS USING CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

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ABSTRACT

Angiogenesis is the process of formation of new blood vessels from the pre-existing blood vessels controlled by various growth regulators. Disturbances of this regulatory process, by different physiological and pathological disorders results in angiogenesis which leads to the progression of tumor formation. The present study was aimed to explore the effect of *Morus nigra* (Berry), on angiogenesis by using CAM assay on chicken embryo model. Fertilized eggs were incubated on the 5th day at 37 °C and dose of different dilutions 0.03%, 0.05%, 0.1%, and 0.5% of this plant extract was applied on 6th day. Evaluation of PBVs, SBVs and TBVs, 3D roughness parameters, angular spectrum and abbot curve of treated CAMs was made on 7th day by image probing system (SPIP). *Morus nigra* showed antiangiogenic effect by reducing the diameter of CAM blood vessels by applying the dilutions of 0.03%, 0.05%, 0.1%, and 0.5% while significant results were obtained at dilution of 0.5%.

Key Words: Angiogenesis, *Morus nigra*, CAM assay.

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INTRODUCTION;

Formation of new blood vessels from prevailing small capillary blood vessels is continuous process which is termed as "angiogenesis" [1]. Angiogenesis is a physiological process occurring in bone fracture, wound healing, the female menstrual cycle, embryogenesis as well as in normal tissues in which nutrient-deplete or hypoxia occurs [2,3]. In addition to this, angiogenesis is essential for the growth of malignant tumors so angiogenesis is an important target for tumor therapy [4] regulated by proangiogenic and antiangiogenic factors like vascular endothelial growth factor (VEGF) [5]. Because of their increased permeability, increased interstitial space leakage, limited diffusion of oxygen [6], changes in genes expression are obvious for angiogenic vascular endothelial growth factor (VEGF), transferrin receptors, erythropoietin and the other proteins responsible for effective oxygen delivery which are controlled by Hypoxia-Inducible Factor 1 alpha (HIF-1 α) [7,8] and VEGF stimulated by hypoxia is the most important angiogenic stimulator, effective in migration and proliferation of endothelial cells and development of new capillary blood vessels [9,10]. It is proposed that HIF-1 α is responsible for angiogenesis through the stimulation of VEGF but also promotes the flow of blood to tumor cells through complex mechanisms by target genes. Thereby, angiogenesis play important role in cancer progression by activating the oncogenic protein [11]. Numerous antiangiogenic factors progress the modility of cancer treatments by decresing the responsible angiogenic factors activity and survival factors for endothelial activity and by increasing the activation of naturally present anti-angiogenic substances. Various medicinal plants effectively treat angiogenesis such as Black Raspberry [12] Green tea, Bananas [13], Liquorice [14], Reishi mushroom [15]. Endocrine diseases such as diabetes mellitus (DM) and its type II is controlled by *Morus nigra* L. [16]. Treatment for hepatitis, jaundice and certain bacterial infections by *Morus nigra* have also reported for about 3500 species against *Streptococcus faecalis*, *Staphylococcus aureus*, *Micrococcus flavus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Salmonella abony* [17,18]. The

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CAM (chorioallantoic membrane) is an extraembryonic membrane which is developed on 4th day of incubation, having thick capillaries network. CAM assay is a valuable model for evaluating vasculogenesis and angiogenesis which has long been a favored system for the study of metastasis and tumor vascularization [19,20]. Current investigation was designed to evaluate the effect of *Morus nigra* extract on angiogenesis by using the CAM assay.

MATERIALS AND METHODS;

Plant collection and extraction processing

The fresh fruits of *Morus nigra* were collected from District Sargodha (Pakistan) and was identified and authenticated by Dr. Fateh Muhammad, Professor of Botany, Government Postgraduate College, and Jauhirabad. Aqueous methanolic (70:30) extract of *Morus nigra*, was prepared by using the cold maceration process [21]. Then prepared Four dilutions (0.03%, 0.05%, 0.1%, and 0.5%) of *Morus nigra* with sterilized distilled water and neutralized with 0.1N NaOH adjusted in range 6.5-7.5. After that all dilutions were filtered by using syringe filter (0.2 µm) for complete sterilization [22].

Preparation of Chorioallantoic membranes

Fifty fresh fertilized chicken eggs of 4th day were obtained from a local hatchery (Big Bird). All the eggs were sprayed with 70% ethanol to reduce contamination from eggs surface and were air dried. Then Eggs were incubated at 37 °C at humidity 60-70% for 5 days [23].

Chorioallantoic membrane assay

Eggs were divided into two groups. One was control (C) and second was treated group. Treated group was further divided into 1, 2, 3 and 4 sub-groups. Ten eggs per each group were taken. Then CAM assay will be performed in the laminar flow hood on each chicken egg one by one. On day 5 of incubation, eggs were windowed aseptically as described by [24]. Briefly, a small window (approximately 2cm in diameter) was made by removing the shell and inner shell membrane from the air-space site. On the same day, 4 -5 ml of albumin was aspirated with a sterile syringe to allow the embryos to develop in a way accessible to quantification and enhance its manipulating properties and let the embryo to grow in a way easy for assessment. The windows were then sealed with sterile Para film and eggs were returned to the incubator at 37 °C (humidity 55-60%) till day 6 of incubation. At day 6 of incubation windows of eggs of each group were opened and 150 µl of dilution was applied on treated developing CAMs. Windows were sealed again with sterile parafilm and chicken eggs were returned to the incubator for 24 hrs.

Image acquisition and image probing system

On 7th day windows were opened and pattern of CAMs and CAMs area were evaluated by taking images with digital Lebecca cam at 30 frames/s using a camera shutter speed of 1/2 000s. SPIP (scanning probe image processor) is Image probing system that was used for quantification of results. All images were converted into grayscale for improving the contrast by black and white inversion with the help of Adobe Photoshop 6.0 (Adobe Systems Software- Ireland) so that every image possible to discern anatomical structures and to facilitate precise quantification of angiogenesis. After the image acquisition, SPIP (IBM-Denmark) was used to evaluate the images which work on the specific algorithm [25]. The length and diameter of different blood vessels was measured through the calibration and measurement command. The CAMs surface angiogenesis was precisely quantified by measuring the 3D surface roughness (fourteen parameters) which is important parameter in the 3D image analysis. Vascular area, angular spectrum and abbot curve of CAMs were measured. The blood vessels were quantified in micrometer scale to evaluate the indepth effects of *Morus nigra* extract on angiogenesis.

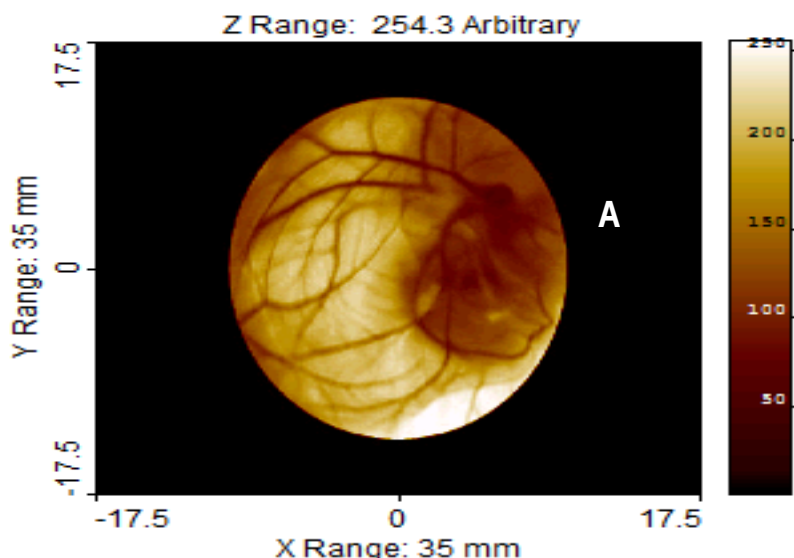
Statistical analysis;

All data was presented as mean $\pm$ SD. Analysis of variance (ANOVA) was performed to evaluate different parameters between controlled and treated samples; statistical significance was set at $P < 0.05$ [26].

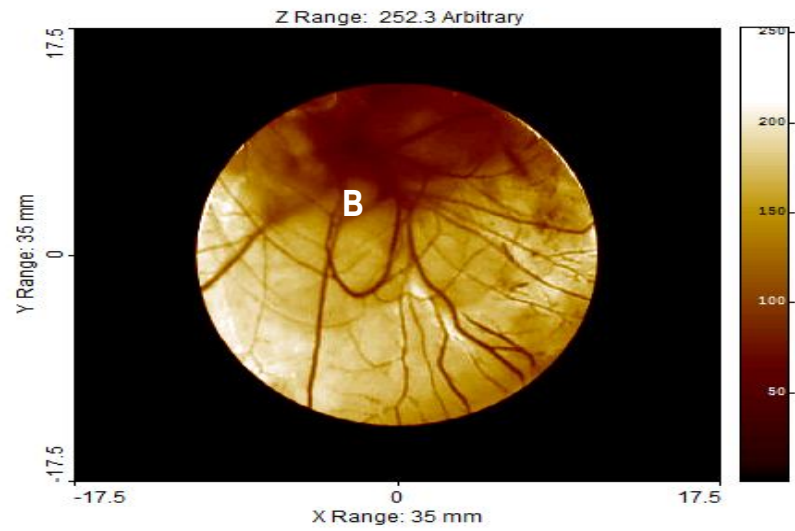
RESULTS;

A significant reduction in blood vessel formation by the application of various dilutions of *Morus nigra* extract was observed with reference to control group. In control groups, all chicken egg CAMs area was increased and blood vasculature was well developed. The network of blood vessels was started from "Y" shaped branch which was originated from umbilical cord of chicken embryo. Further this "Y" shaped branch was divided into primary blood vessels (PBVs), secondary blood vessels (SBVs) and tertiary blood vessels (TBVs). When chicken embryos were treated with *Morus nigra* dilutions (0.03%, 0.05%, 0.1%, 0.5%) then it caused decrease in blood vessels diameter and CAM area. Most pronounced reduction was seen at 0.5%. Control and treated chicken embryos were quantified by a novel scanning probe image processor (SPIP). It was observed that treated CAMs showed reduced diameter of blood vasculature development than control. A significant reduction in diameter of PBVs, SBVs and TBVs was evident among all treated groups as compared to control group. A significant reduction in the diameter of primary, secondary and tertiary blood vessels was recorded among *Morus nigra* treated groups.

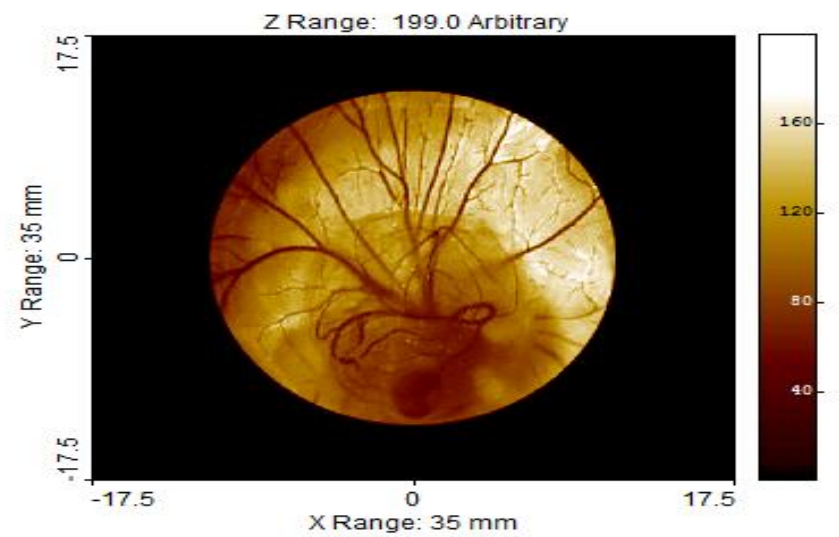
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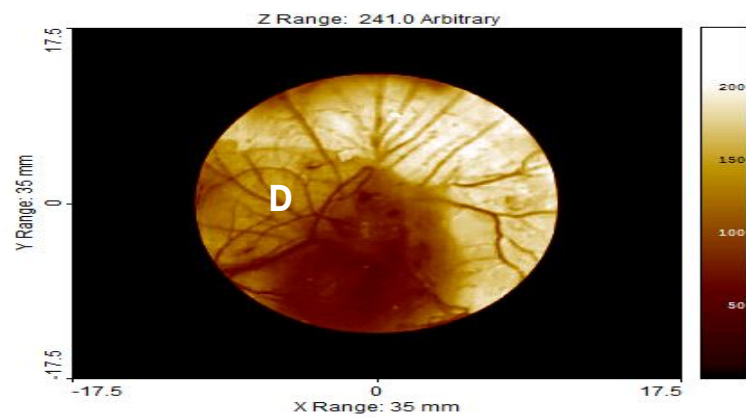
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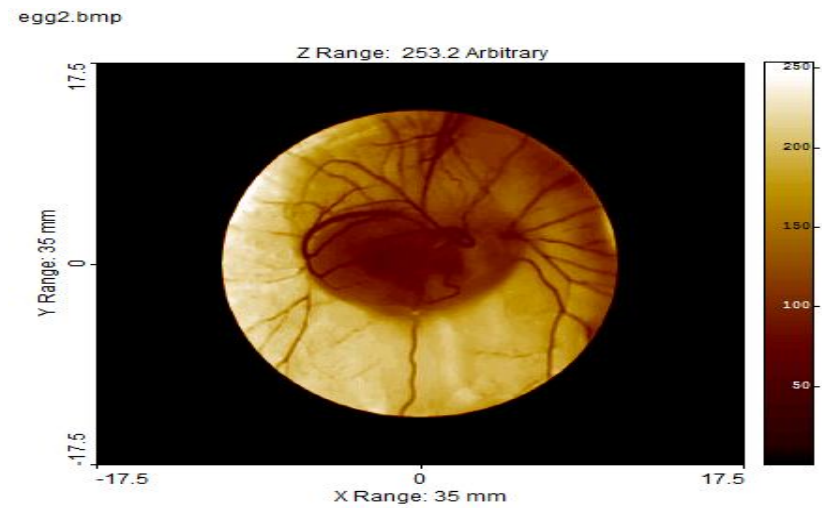
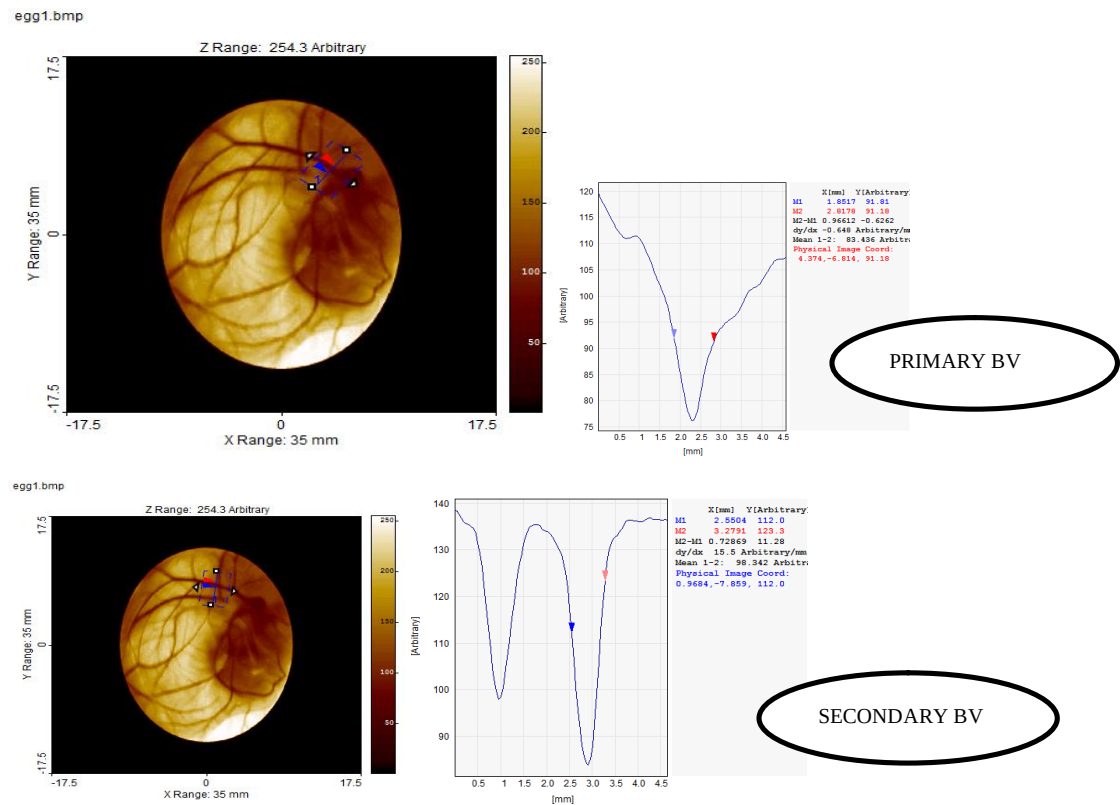


Figure 1: Macroscopic evaluation of Chicken CAM. Note well defined vascular architecture of CAM vessels i.e. PBVs, SBVs, TBVs and well develop CAM area in (A) control while extensive deterioration of CAM vessels and reduction in CAM area representing antiangiogenic activities in treated dilutions of *Morusnigra* i.e. (B) 0.03% (C) 0.05% (D) 0.1% (E) 0.5%



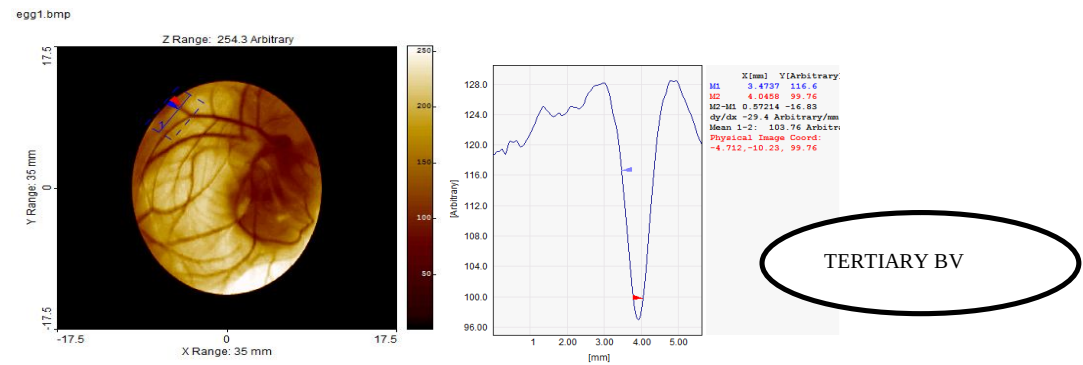
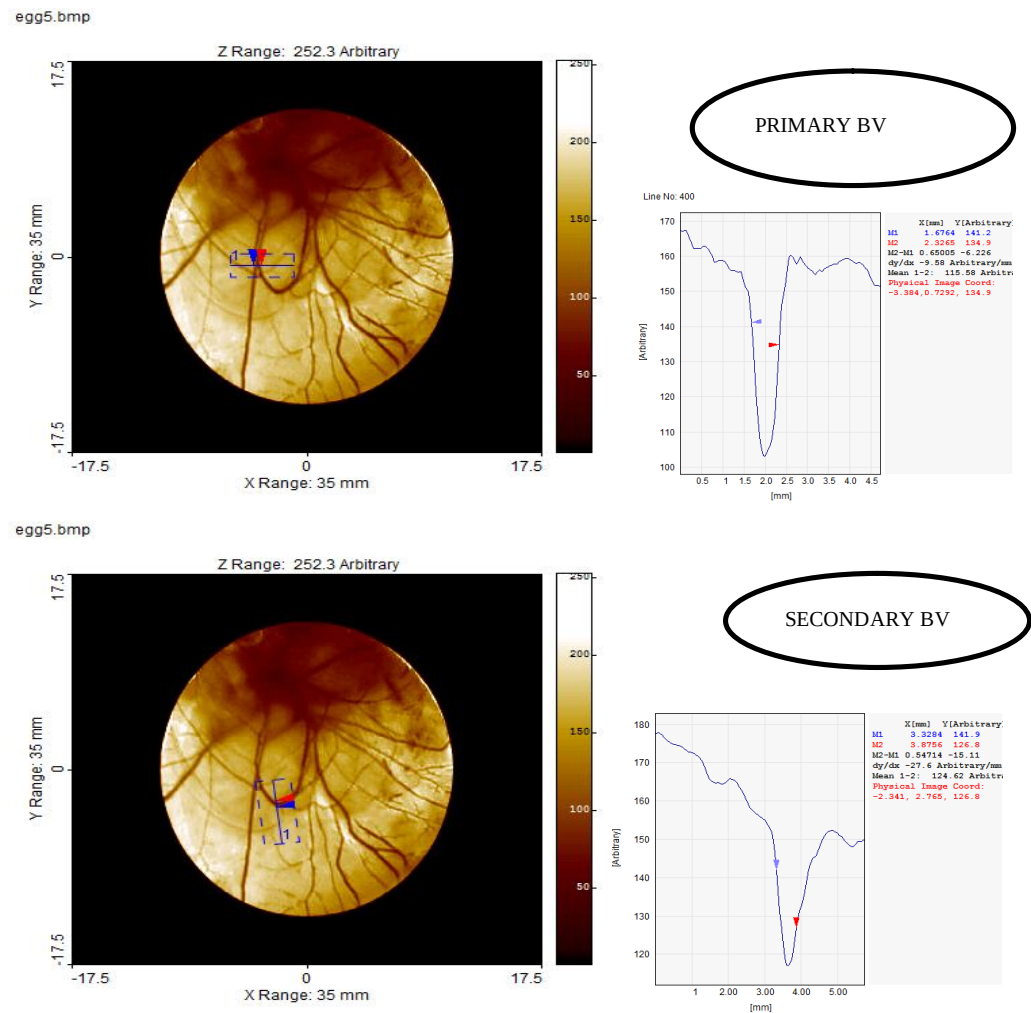


Figure 2: Comparative evaluation of diameter of control CAM blood vessels.



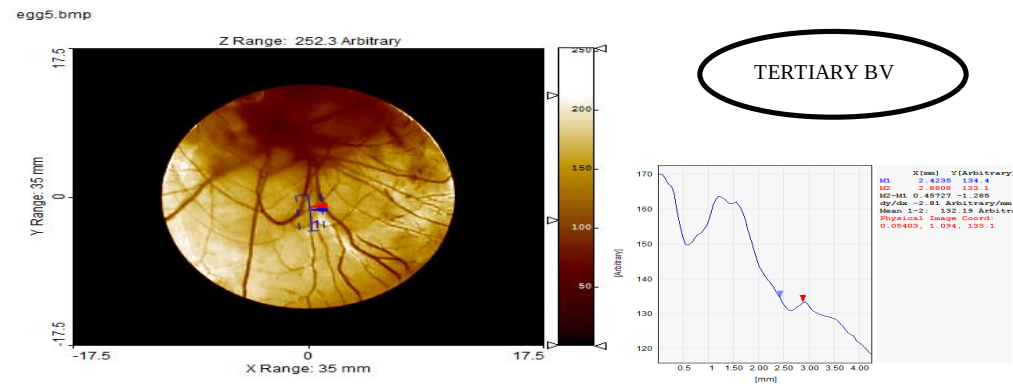
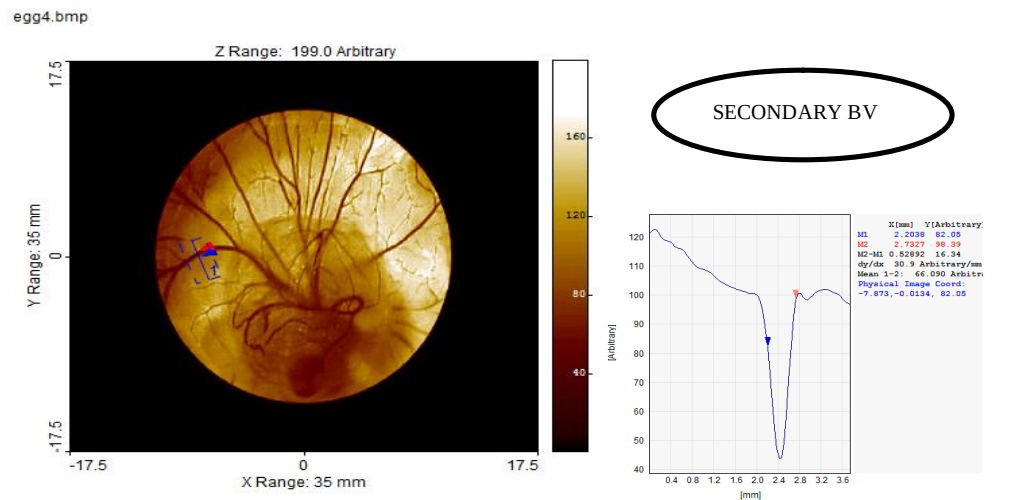
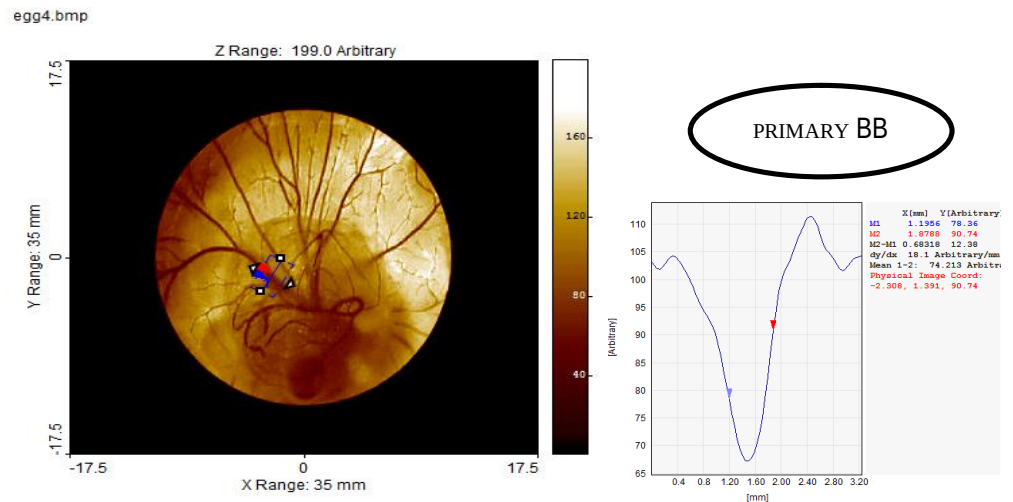


Figure 3: Comparative evaluation of diameter of 0.03% treated *Morus nigra* CAM blood vessels



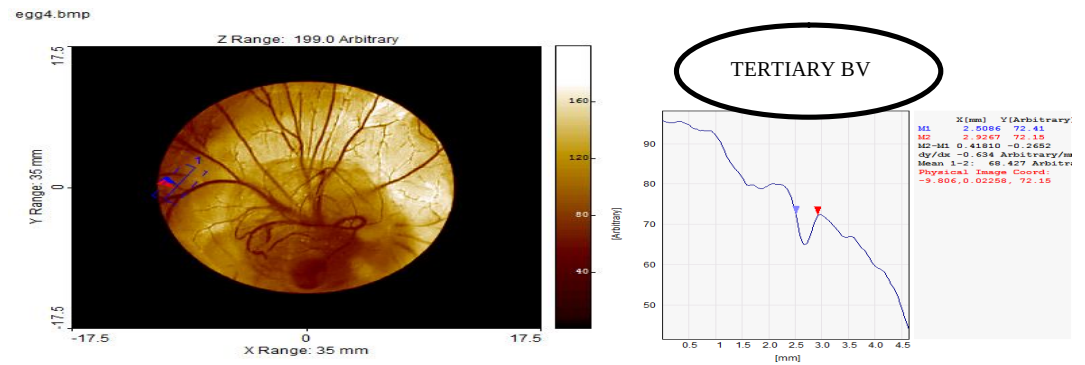
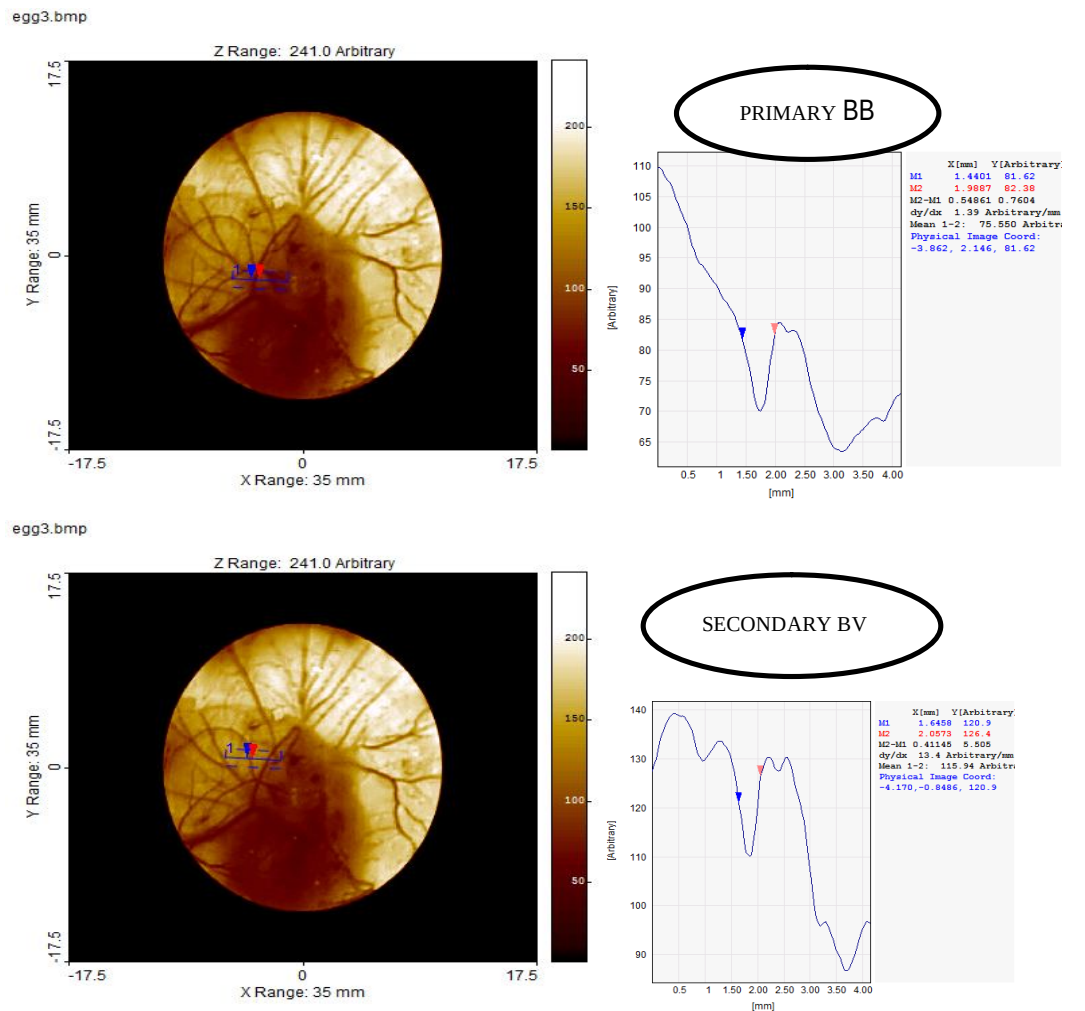


Figure 4: Comparative evaluation of diameter of 0.05% treated *Morus nigra* CAM blood vessels.



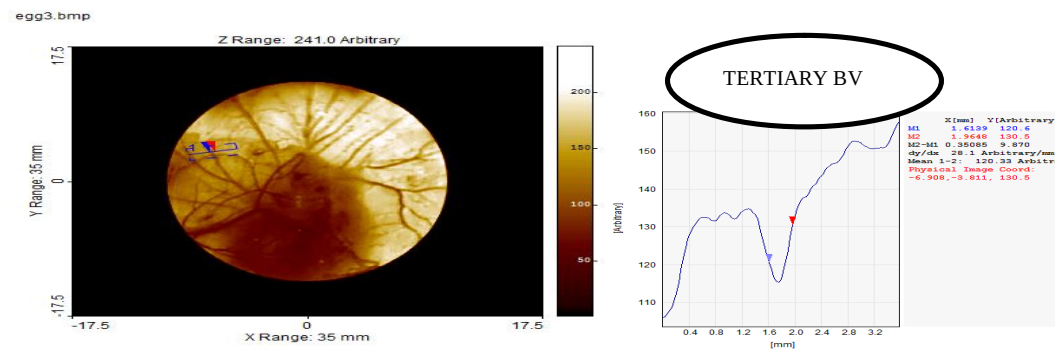
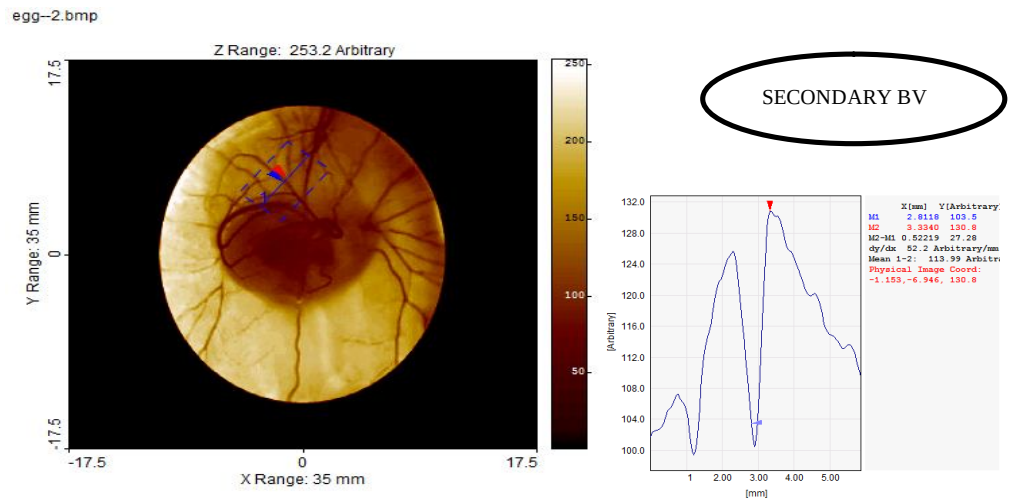
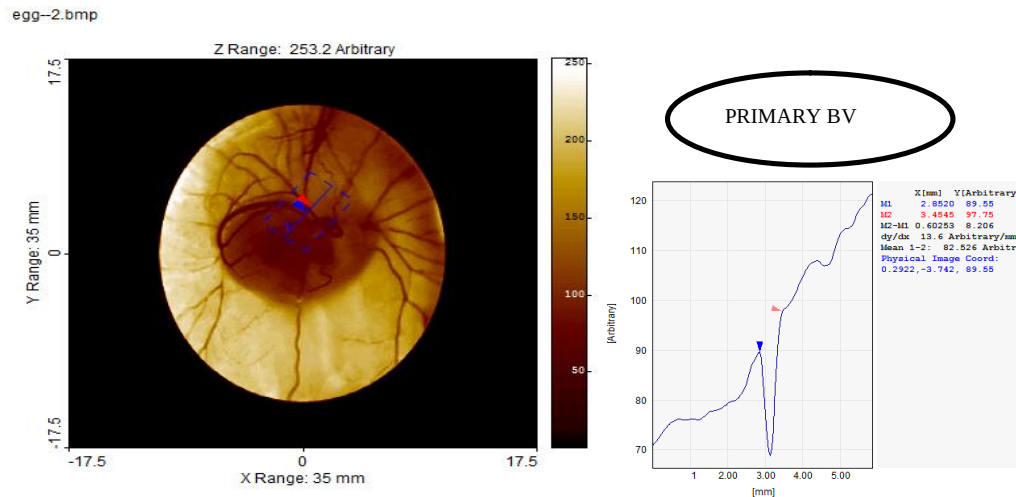


Figure 5: Comparative evaluation of diameter of 0.1% treated *Morus nigra* CAM blood vessels.



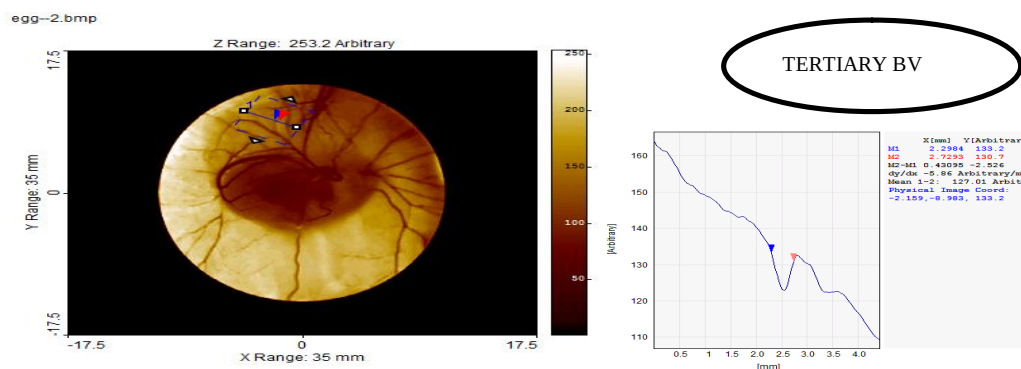
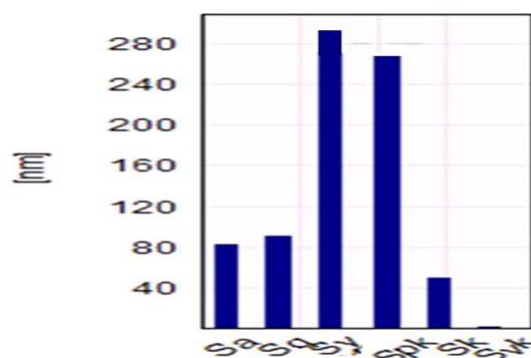


Figure 6: Comparative evaluation of diameter of 0.5% treated *Morus nigra* CAM blood vessels.

Table 1: Diameters of blood vessels (*Morus nigra*)

VESSELS	DIAMETER(mm)				
	CONTROL	0.03% Conc.	0.05% Conc.	0.1% Conc.	0.5%Co
y	0.966	0.650	0.683	0.548	0.603
lary	0.728	0.547	0.528	0.411	0.522
/	0.573	0.457	0.418	0.350	0.433

3D surface roughness parameters of control and treated CAMs were measured to obtain the more accuracy. They were evaluated to quantify angiogenesis (Table: 3.2). The neo-vascularization is representing by surface roughness of control was significantly greater than treated CAMs. The value of average surface roughness of control and treated CAMs were 81.10 ± 1.90 nm and 69.01 ± 0.58 nm respectively. Similarly, the graphical representation by Abbott curve (Fig.3.8) was used to evaluate even minute differences in the blood vessels height on CAMs surface. The heights of Abbott curves for control and treated CAMs were 280 and 240 respectively which showed that height of control was greater than treated CAMs. Entire data of 3D roughness parameters was obtained in mean $\pm$ SD. One way ANOVA was performed by using SPSS (ver. 16) to assess all parameters between control and treated groups; statistical significance was $P < 0.05$.



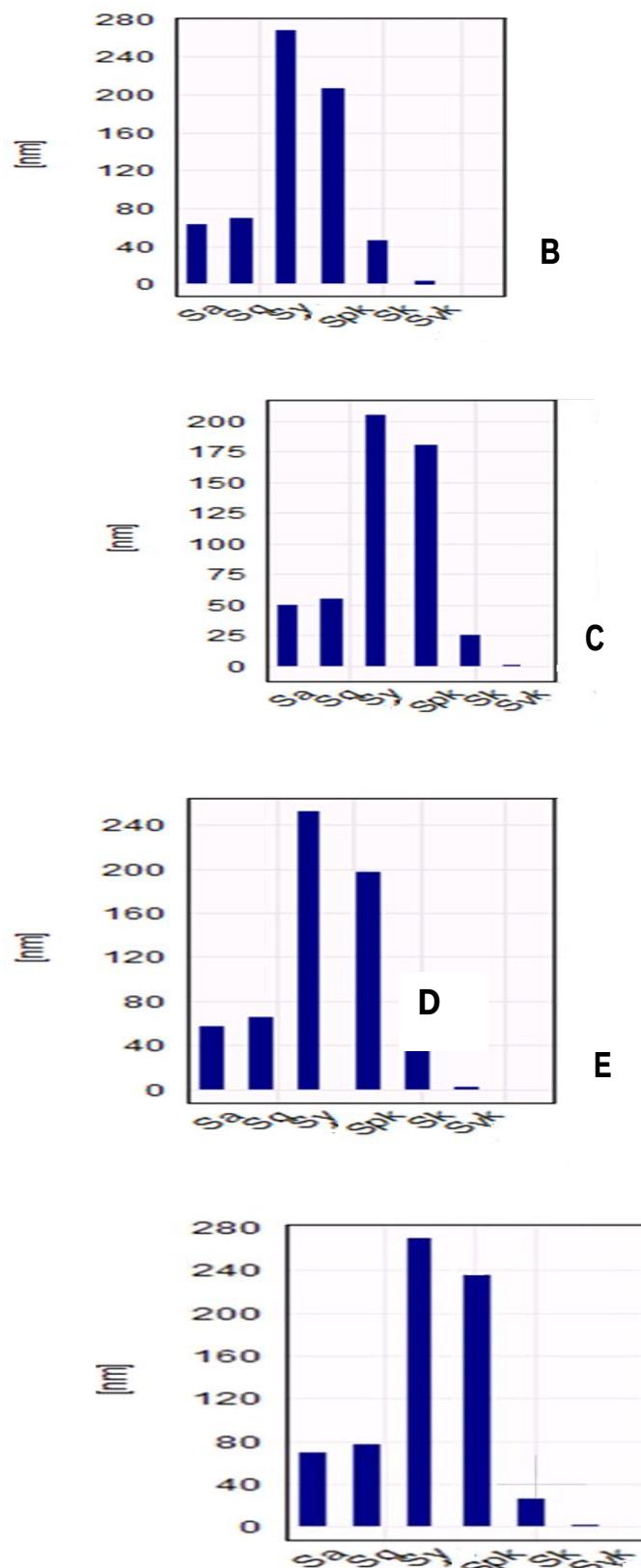


Figure 7: The graphical representation of various surface roughness parameters A) Control CAM, B) 0.03%, C) 0.05% D) 0.1% E) 0.5% treated *Morus nigra* CAMs.

TABLE 2: CONTROL AND THE TREATED MORUS NIGRA CAMs ROUGHNESS PARAMETERS

Sr. No.	Parameters	Control	MORUS NIGRA (CONCENTRATIONS)			
			0.03%	0.05%	0.1%	0.5%
1	Sa	81.10±1.90	63.20±2.09 ^{* *}	52.83±2.51 [*]	57.93±1.52 ^{* *}	69.01±0.58 ^{* *}
2	Sq	86.13±2.96	67.43±2.64 ^{* *}	54.93±2 ^{* *}	67.60±2 [*]	79.11±2.08 ^{* *}
3	Ssk	1.03±0.029	0.817±0.016 [*]	0.800±0.01 [*]	0.97±0.043 ^{* *}	0.88±0.016 ^{* *}
4	Sku	2.394±0.040	3.110±0.16 ^{* *}	3.029±0.009 ^{* *}	3.384±0.058 ^{* *}	3.149±0.038 ^{* *}
5	Sy	282.46±2.28	268.14±2.51 ^{* *}	208.10±3.21 ^{* *}	250.83±1 [*]	270.86±2.09 ^{* *}
6	Sz	282.46±2.28	268.14±2.51 [*]	208.10±3.21 ^{* *}	250.83±1 [*]	270.86±2.09 ^{* *}
7	Ssc	1.98E-09±1.06E-11	1.85E-09±1E-10 [*]	1.16E-09±5.72E-11 [*]	1.21E-09±1.15E-11 [*]	1.34E-09±4.05E-11 [*]
8	Sdq	0.000191±7.69E-06	0.00013±0.000005 ^{* *}	0.000104±1E-06 [*]	9.51E-05±1.53E-07 ^{* *}	0.000124±1.01E-06 [*]
9	Sdr	7.04E-07±9.25E-10	6.14E-07±5.48E-08 [*]	5.88E-07±5.77E-08 [*]	4.9E-07±5.63E-08 ^{* *}	6.53E-07±5.9E-08 [*]
10	Sci	1.983±0.009	1.31±0.01 ^{* *}	1.26±0.06 [*]	1.026±0.01 ^{* *}	1.876±0.02 ^{* *}
11	Spk	266.14±1.24	210.73±5.03 [*]	179.20±1.52 ^{* *}	196.74±2 [*]	237.13±2.09 ^{* *}
12	Stdi	0.869±0.012	0.681313±0.07 ^{* *}	0.670±0.1 [*]	0.733±0.01 [*]	0.746±0.06 [*]
13	Sk	42.61±1.61	29.17±3.60 ^{* *}	25.90±3.60 [*]	31.08±2 [*]	32.51±1.52 ^{* *}
14	Svk	1.813±0.007	0.355407±0.027 ^{* *}	1.182±0.068 ^{* *}	1.369±0.1 [*]	1.033±0.001 [*]

Sa, average roughness; **Sq**, root mean square deviation; **Ssk**, skewness of the surface; **Sku**, kurtosis of the surface; **Sy**, lowest valley; **Sz**, maximum height of the surface; **Ssc**, arithmetic mean summit; **Sdq**, root mean square slope; **Sdr**, developed surface area ratio; **Sci**, core fluid retention; **Spk**, reduce summit height; **Stdi**, texture index; **Sk**, core roughness depth; **Svk**, reduce valley depth. 'E' Stand for Exponent

^{*} " Significant (P < 0.05) ^{* *} " Highly significant (P < 0.01)

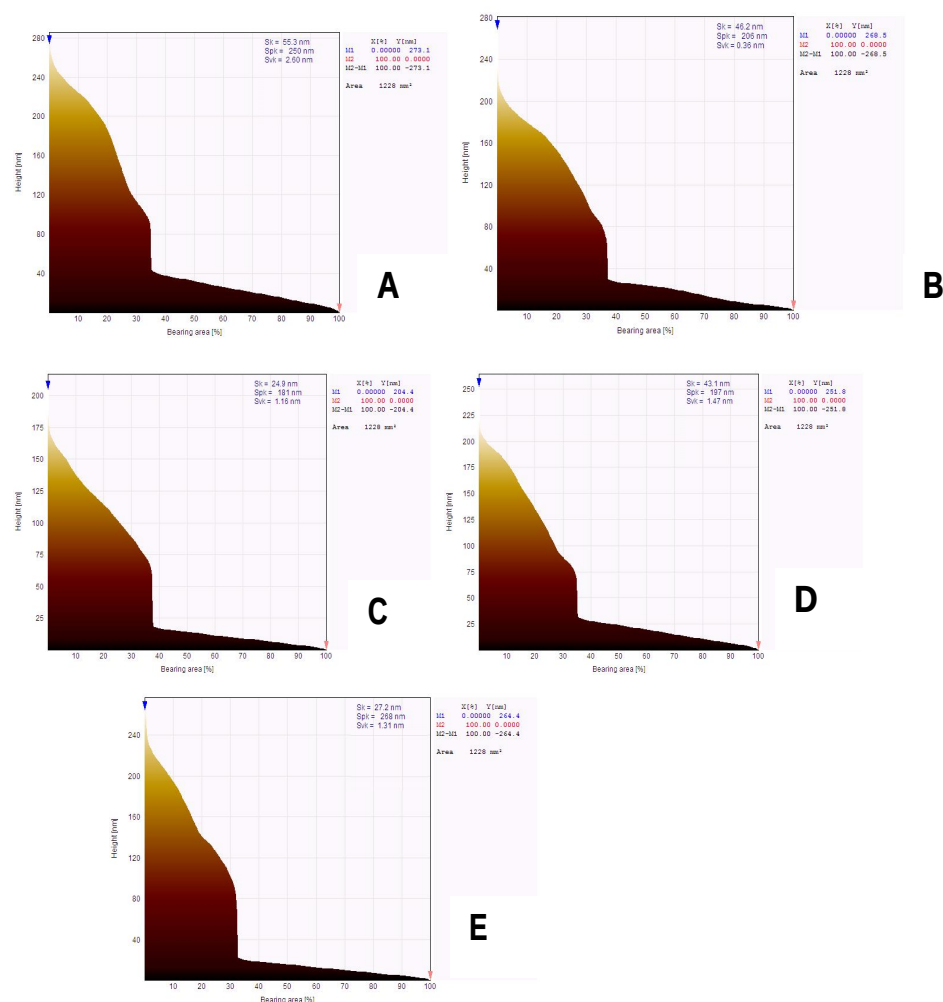


Figure 8: Abbott curve of CAM blood vessels A) Control CAM, B) 0.03% , C) 0.05% D) 0.1% E) 0.5% treated *Morus nigra* CAMs

DISCUSSION;

Plants are being used in treatment of various diseases caused by different pathological conditions like angiogenesis [27, 28]. In the present study the indigenous plant *Morus nigra* was studied to evaluate the effect on angiogenesis by using in vivo CAM assay in fertilized chicken eggs. It was observed that *Morus nigra* extract showed antiangiogenic effect by reducing diameters of PBVs, SBVs, and TBVs. The values of 3D surface roughness parameters; Sa, Sq, Ssk, Sku, Sdr, Sci, Sy, Sz, Ssc, Sdq, Spk, Svk, Std and Sk, Abbott Curve, angular spectrum were also decreased as compare to control values. Previous study reported that flavonoids possess antioxidant activity [29, 30]. In another study it was reported that antioxidant potential of flavonoids prevented angiogenesis by inhibiting PDGF and EGF [31, 32]. It was also reported that *Morus nigra* contain flavonoids so its antiangiogenic effect could be due to the presence of chemical constituent flavonoids.

CONCLUSION;

The finding of the present study indicated the significance of *Morus nigra*. However, further studies are required to isolate the active antiangiogenic constituents from this plant and evaluate the exact mechanism of action development of new techniques for prevention and therapy of various angiogenesis dependent diseases.

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