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Original Research Article

A NOVEL IMAGE PROBING SYSTEM (SPIP) FOR THE ASSESSMENT AND QUANTIFICATION OF STRUCTURAL CHANGES IN CHORIOALLANTOIC MEMBRANE (CAM) CAUSED BY THE STRAWBERRY JUICE

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ABSTRACT

The anticancer potential of berries has been related, at least in part, to a multitude of bioactive phytochemicals. Anticancer effects of berry bioactive are partially mediated through their abilities to counteract, reduce, and also repair damage resulting from oxidative stress and inflammation. To explore the role of angiogenesis we used SPIP (scanning probe image processor) for quantification of results. 0.7% concentration of strawberry juice showed a marked anti-angiogenic effect. There was decrease in diameter of primary, secondary, tertiary blood vessels and decrease in roughness parameters..

Key words: Strawberry juice, angiogenesis, Chorioallantoic membrane assay.

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INTRODUCTION

Angiogenesis, the growth of new blood vessels, is a fundamental physiological process required for development, reproduction, wound repair, and response to ischemia. Pathological angiogenesis, often referred to as neo-vascularization, is associated with disease conditions including retinopathies, arthritis, psoriasis and cancer (Folkman, 1995). Since angiogenesis is a process that is generally down regulated in healthy individuals, targeting of angiogenesis with safe anti-angiogenic compounds, that are selective against newly formed vessels while sparing existing ones, may not lead to side effects even after prolonged exposure. The anticancer

potential of berries has been related, at least in part, to a multitude of bioactive phytochemicals that these colorful fruits contain. Anticancer effects of berry bioactive are partially mediated through their abilities to counteract, reduce, and also repair damage resulting from oxidative stress and inflammation. In addition, berry bioactive also regulate carcinogen and xenobiotic metabolizing enzymes, various transcription and growth factors, inflammatory cytokines, and sub cellular signaling pathways of cancer cell proliferation, apoptosis, and tumor angiogenesis (Seeram, 2008). Strawberries are rich in anthocyanins which are member of flavonoid group of photochemical; chemically anthrocyanins are glycosides and are water soluble in nature. Major anthocyanins include cyanidin, pelargonidin, pentunidin. These are present in strawberries and their juices as well. Edible berry anthocyanins have been shown to inhibit cellular transformation and it demonstrates their potent inhibitory effect on inducible VEGF expression. Chicken chorioallantoic membrane (CAM) is an extra embryonic membrane formed on day 4 of incubation. It has very thick capillary network. Rapid capillary proliferation continues until day 11; the mitotic index then declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching (Ribatti et al. 2001). Chorioallantoic membrane (CAM) assay is a valuable model for evaluating angiogenesis and vasculogenesis and it has long been a favored system for the study of tumor angiogenesis and metastasis (Ribatti et al. 2001). However, its utility has been limited due to difficulty in measuring the angiogenic response to an experimental compound in an objective and quantifiable manner. By utilizing a novel approach to quantify angiogenesis (Ejaz et al. 2004), we have adapted the CAM assay to create an in ovo angiogenesis model system that is rigorously quantitative, amenable to high-throughput screening, and applicable for the testing of systemic and/or topical administration of experimental agents. The current experiment is designed to study the effects of strawberry juice on angiogenesis by using chicken chorioallantoic membrane assay (CAM assay).

MATERIALS AND METHODS

Forty fresh fertilized eggs, of similar weights were obtained from a local hatchery (Big Bird). All the eggs were sprayed with 70% ethanol to reduce contamination from egg surface and air dried. Eggs were then incubated at 37 ºC (humidity 55-60%) for 5 days. Fresh strawberry fruits were immediately, without storage, squeezed to juice. The edible portion of deep colored fruits were washed and chopped to squeeze fruit juice using a manual stainless steel screw squeezer. Juice was centrifuged at 10,000 rpm (4 ºC) for 30 min. (Lin et al. 2008). The supernatant was then collected and stored at -20 ºC for further use. On day 5 of incubation, eggs were windowed aseptically as described by (Ejaz et al., 2005) with some modifications. 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. The windows were then sealed with sterile Para- film tape and eggs were returned to the incubator at 37 ºC (humidity 55-60%) and incubated with the window upright till day 6 of incubation.

The 0.7% dilution was prepared using distilled water. The pH of this dilution was then determined with the help of pH meter and was adjusted in the range of 6.5-7.5. These dilutions were filtered through Sartorius syringe filters (0.2 µm) to reduce the risk of contamination. At day 6 of incubation windows were opened and 200 µl of each dilution was applied on developing CAM. Windows were sealed again with sterile Para-film tape and eggs were kept in incubator for further 24 hours. Eggs were then divided into two groups each having twenty eggs each.

Group A was kept as control which received distilled water only and group B received 0.7% dilution of strawberry juice. Image probing system SPIP (scanning probe image processor) was used for quantification of results. To ensure an objective 3D measurement of angiogenesis on CAMs, serial images with respective x and y dimensions were recorded, on day 7 of incubation, by Lebecca cam at 30 frames/s using a camera shutter speed of 1/2000s. By using Adobe Photoshop 6.0 (Adobe Systems Software- Ireland), all of the images were converted into grayscale and contrast was improved by black and white inversion, making every image possible to discern anatomical structures and to facilitate precise quantification of angiogenesis (Ejaz et al, 2004).

After image acquisition, all images were imported to scanning probe image processing software (IBM-Denmark) that works on specific algorithm (Garnaes et al. 2006). The diameter and length of various blood vessels was measured by using calibration and measurement command. 3D surface roughness (fourteen parameters), which is one of the main parameters in 3D image analysis, was also measured for precise quantification of angiogenesis on the surface of CAMs. Vascular area, abbot curve and angular spectrum of neovasculature on CAMs were calculated. Thus blood vessels of micrometer and/or nanometer scale were quantified to evaluate the in-depth effects of strawberry juice on angiogenesis (Ejaz et al, 2005)

All data was presented as mean ± SD. Analysis of variance (ANOVA) was performed to evaluate different parameters between controlled and treated samples; statistical significance was set at $P < 0.05$. Post hoc Student's *t*- test was also performed when significance was found $P < 0.05$ (Melkonian et al. 2002b).

RESULTS AND DISCUSSION

In this study we have examined the effect of strawberry juice from fresh strawberry fruits on angiogenesis using chicken chorioallantoic membrane (CAM) assay. We observed a significant reduction in blood vessel formation by the application of single dilution of strawberry juice with reference to control group. The following results were obtained

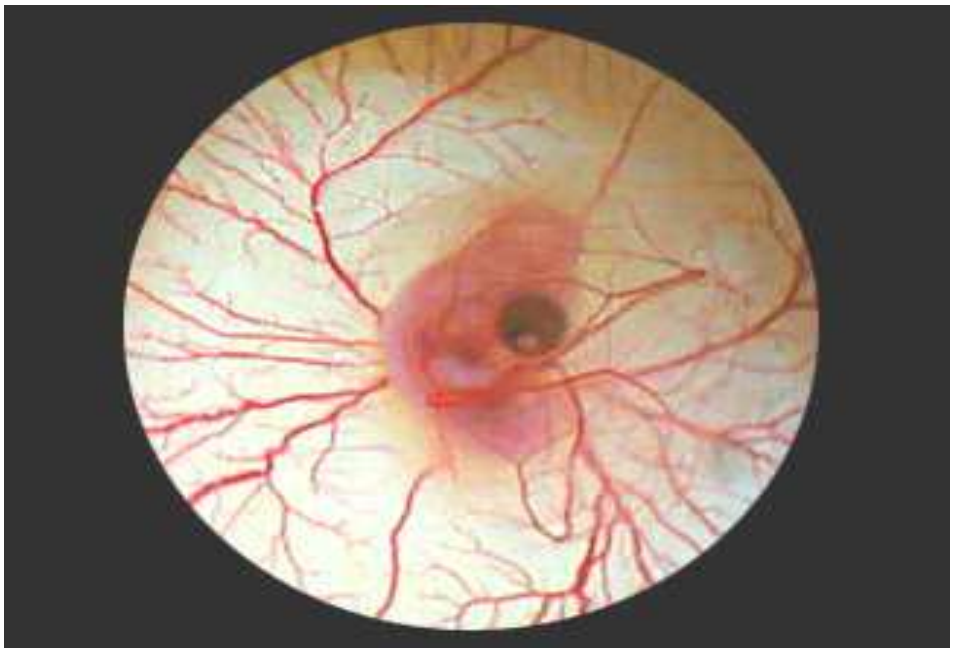
In control samples, the blood vessels were tremendously arranged in a tree like branching pattern with equivalent distribution, covering the whole area of the CAM. The vascular architecture of the CAM appeared originating from the main "Y" branch of the blood vessel, which was further differentiated into primary, secondary and tertiary branches (Fig. 1A)

Application of the 0.7% dilution of strawberry juice caused marked changes in vascular architecture of the CAMs. Anti-angiogenic activities were observed after application of dilution of strawberry juice, which resulted in thinning of primary and secondary blood vessels, and fading of tertiary blood vessels. This shows a marked reduction in the complete vascular network of CAM (Fig.1B).

A novel image probing system (IPS) was utilized for the assessment and quantification of structural changes in CAMs caused by the application of dilution of strawberry juice.

SPIP was utilized for computerized quantification of the diameter of CAM vasculature. A significant reduction in diameter of primary, secondary and tertiary blood vessels 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 strawberry juice treated groups (Fig. 3).

A)



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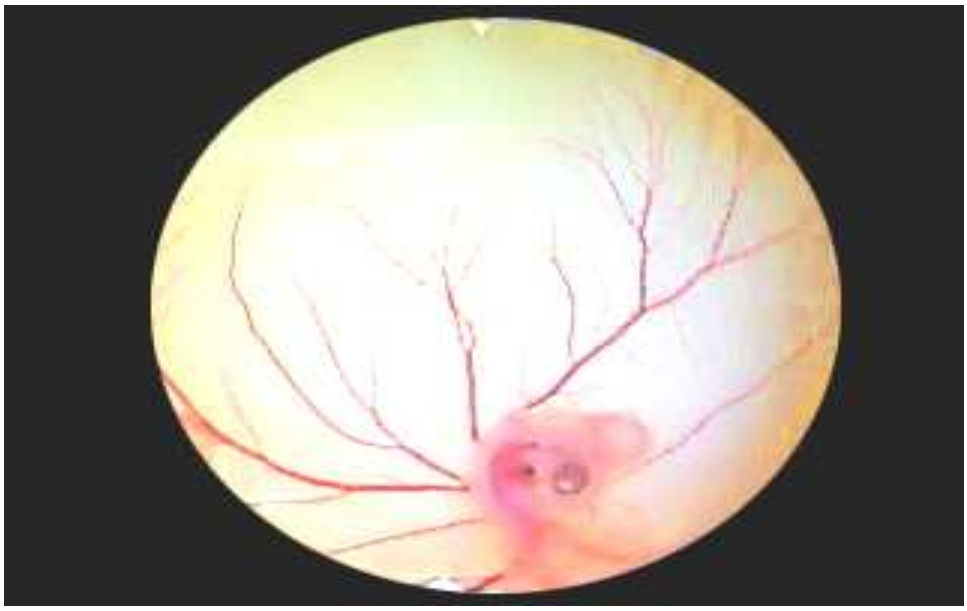
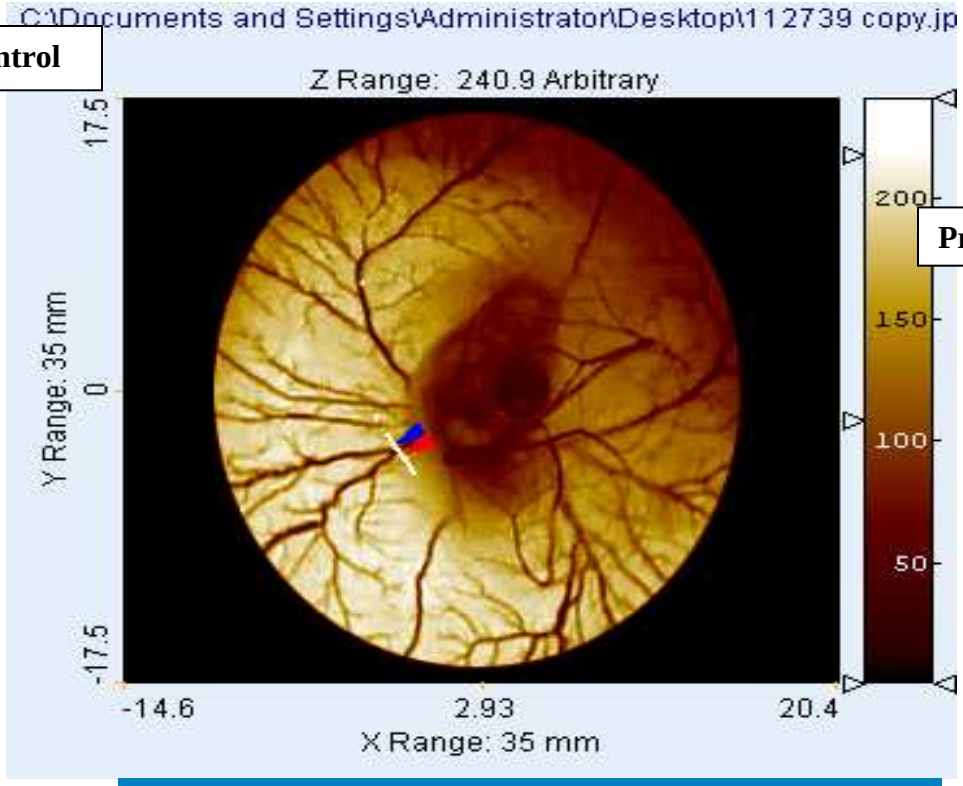
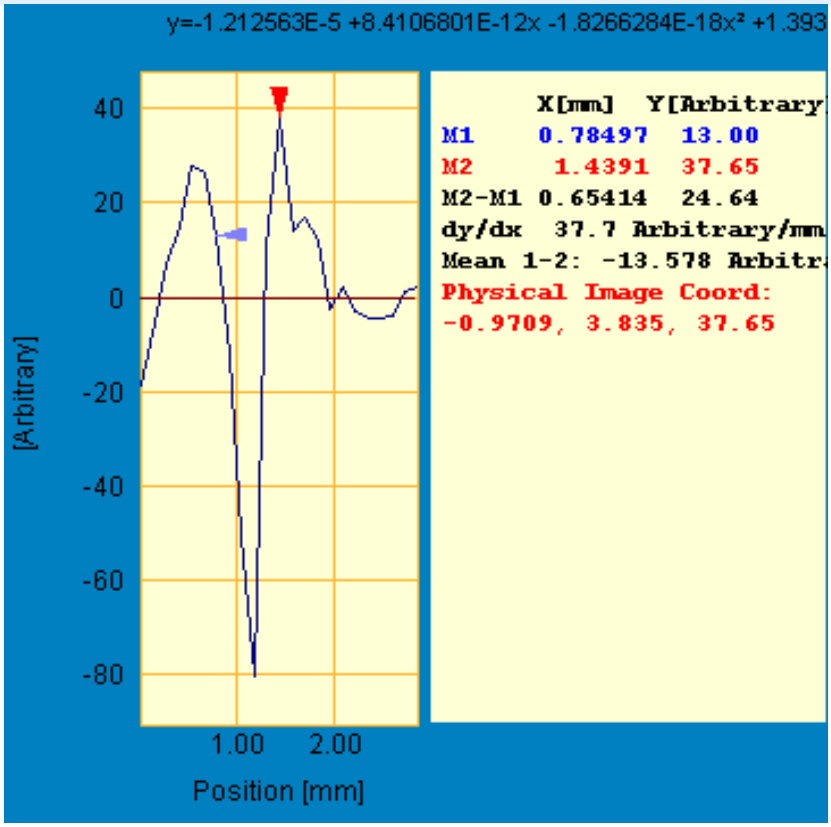


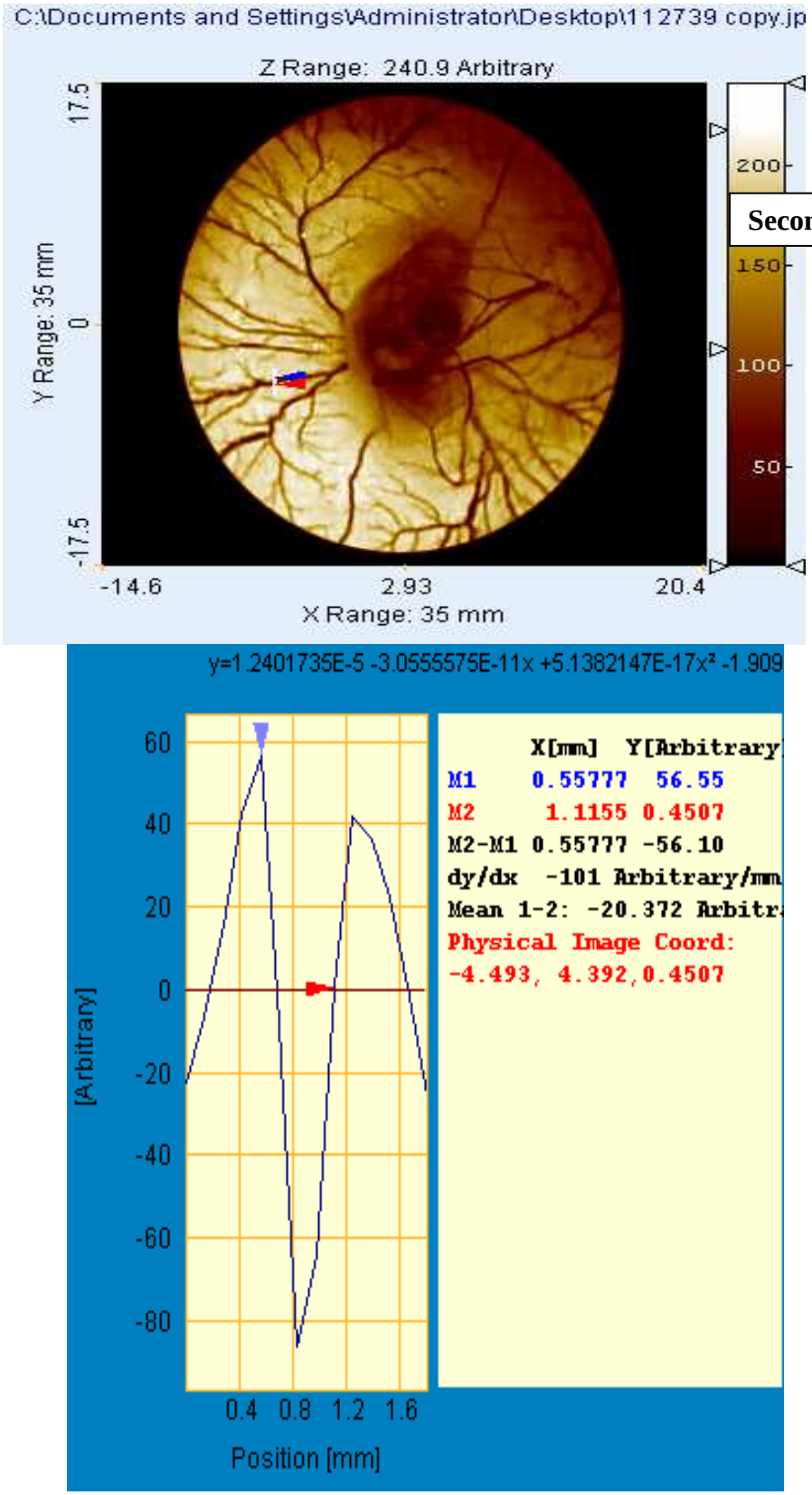
Figure 1. Macroscopic evaluation of chicken Chorioallantoic membrane (CAM) at day 6 of incubation. Note the well defined architecture of CAM blood vessels consisting of primary, secondary and tertiary blood vessels in control group with well developed area of CAM (A), while CAM treated with strawberry juice resulted in extensive decrease in CAM blood vessels and reduction in total area of CAM representing extensive antiangiogenic B = 0.7% dilution.

Control



Primary BV

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17.5
Y Range: 35 mm
0
-17.5
-14.6
2.93
20.4
X Range: 35 mm
200
150
100
50
Secondary
y=1.2401735E-5 -3.0555575E-11x +5.1382147E-17x² -1.909
60
40
20
0
-20
-40
-60
-80
[Arbitrary]
0.4 0.8 1.2 1.6
Position [mm]
X[mm] Y[Arbitrary
M1 0.55777 56.55
M2 1.1155 0.4507
M2-M1 0.55777 -56.10
dy/dx -101 Arbitrary/mm
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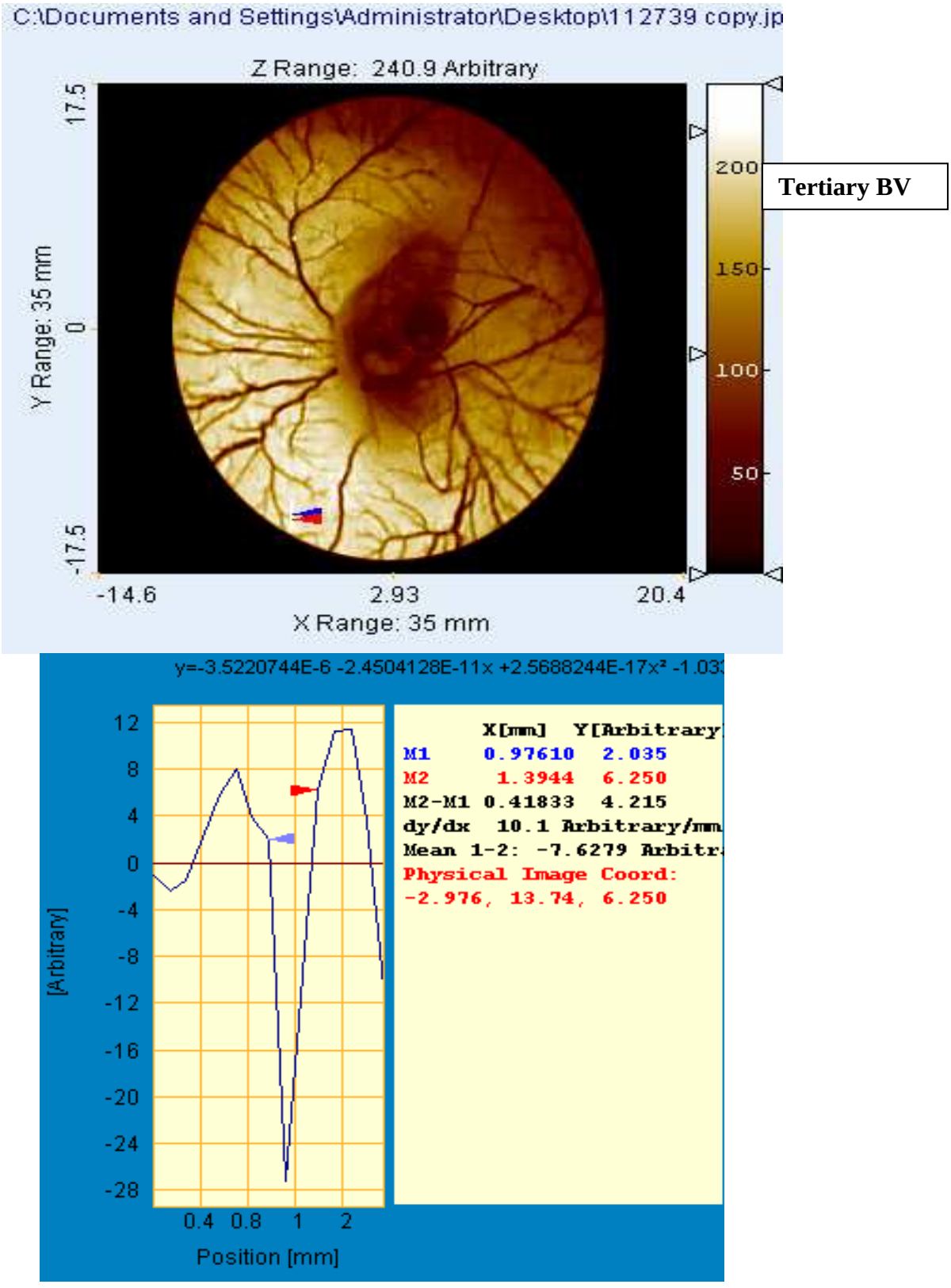
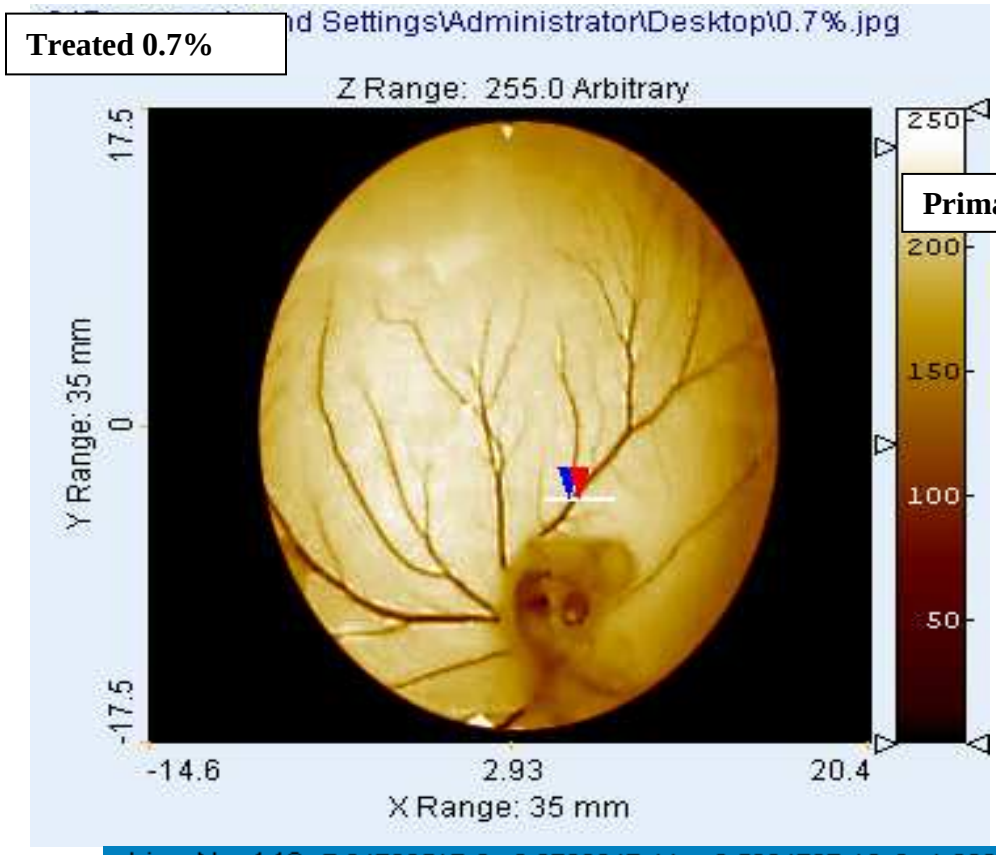


FIG 2. Diameter of the blood vessel on CAM of treated control egg.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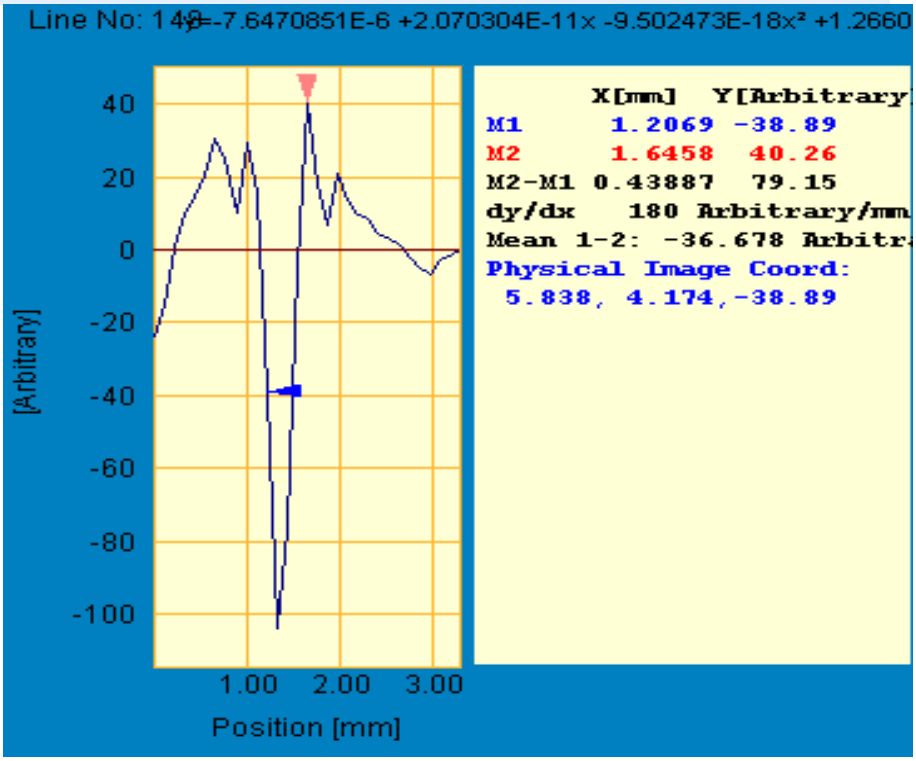
X Range: 35 mm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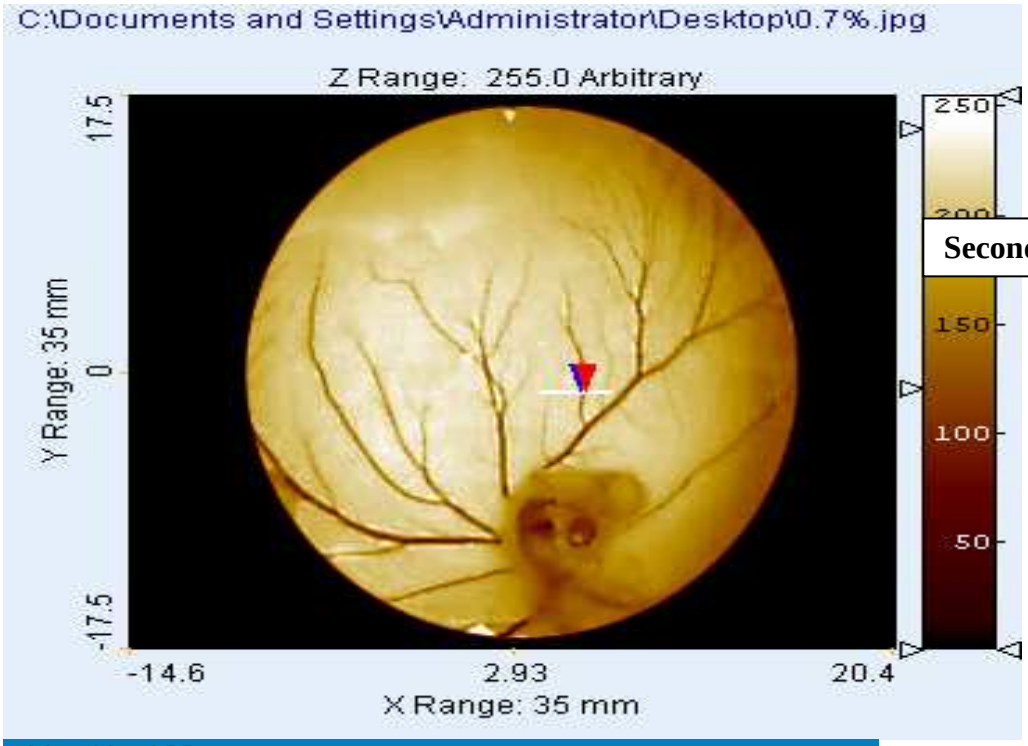
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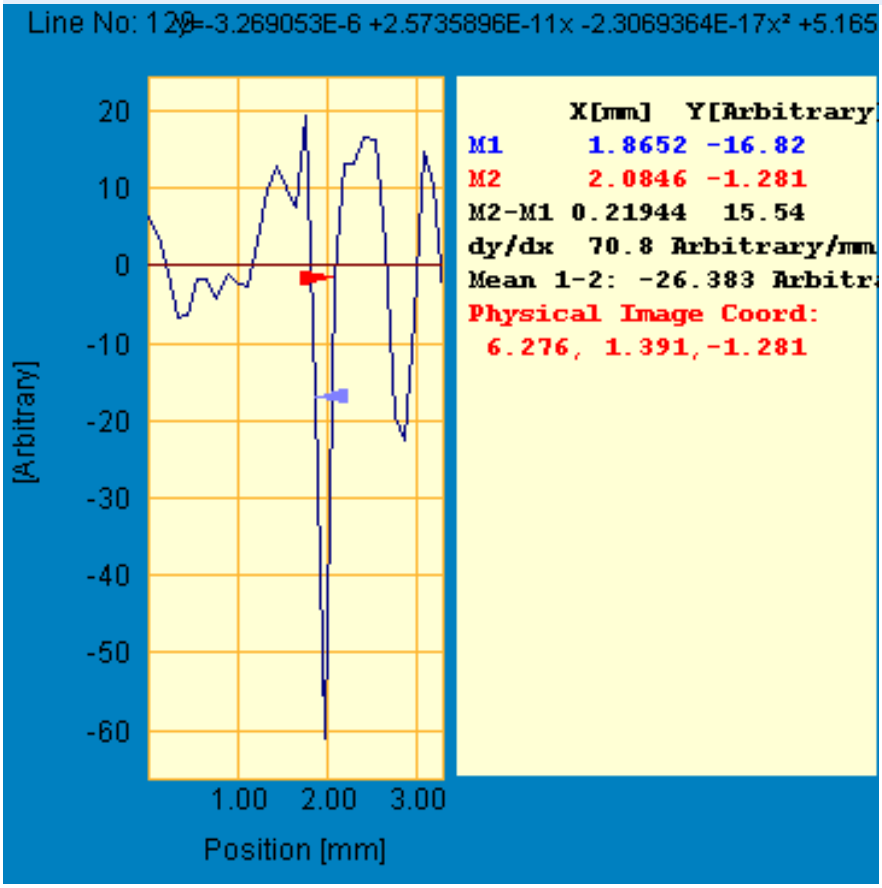
Secondary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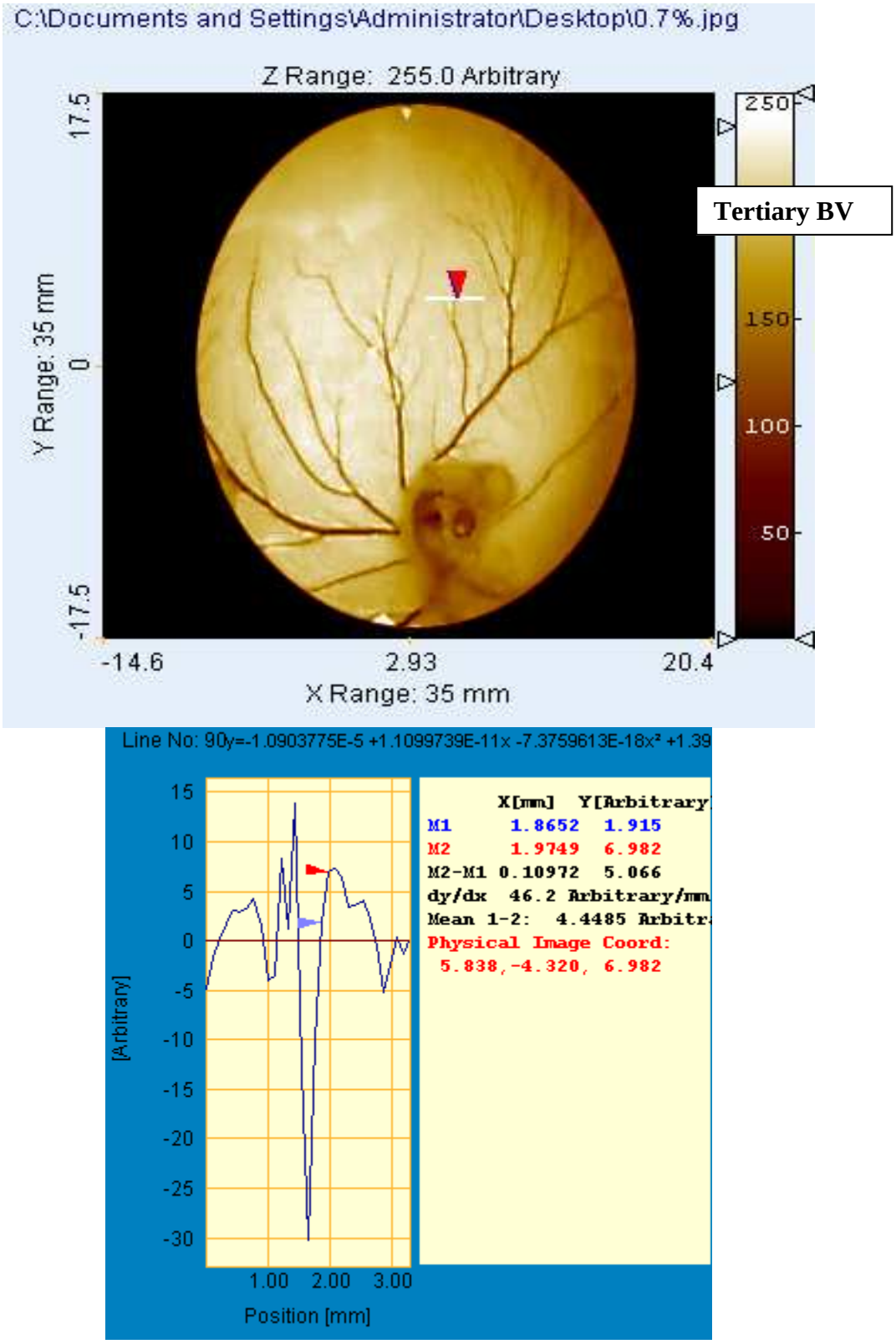

Figure 3. Diameter of the blood vessel on CAM of treated (0.7%) egg.

Table. 1. Roughness of control and treated CAMs

Sr. No.	Parameters	Control	0.7% Dilution
1	Sa	101.2 ± 2.11 nm	70.32 ± 3.250 nm
2	Sq	137.6 ± 1.66 nm	101.3 ± 3.55 nm
3	Ssk	1.66 ± 0.152	1.39 ± 0.0085
4	Sku	1.966 ± 0.137	2.67 ± 0.099
5	Sy	255 ± 1.300 nm	240 ± 1.67 nm
6	Sz	255 ± 1.300 nm	240 ± 1.67 nm
7	Ssc	1.296 ± 0.014 nm	1.089 ± 0.008 nm
8	Sdq	0.00019± 0.00006 nm	0.00013 ± 0.00006 nm
9	Sdr	1.935 ± 0.010	1.134 ± 0.046
10	Sci	1.307 ± 0.199	0.859 ± 0.0028
11	Spk	359.7 ± 5.65 nm	250.8 ± 14.92 nm
12	Stdi	0.70 ± 0.0036	0.61 ± 0.0076

Sa, average roughness; Sq, root mean square deviation; Ssk, skewness of the surface; Sku, kurtosis of the surface; Sdr, developed surface area ratio; Sci, core fluid retention; Sy, lowest valley; Sz, maximum height of the surface; Ssc, arithmetic mean summit; Sdq, root mean square slope; Spk, reduce summit height; Sti, texture index.

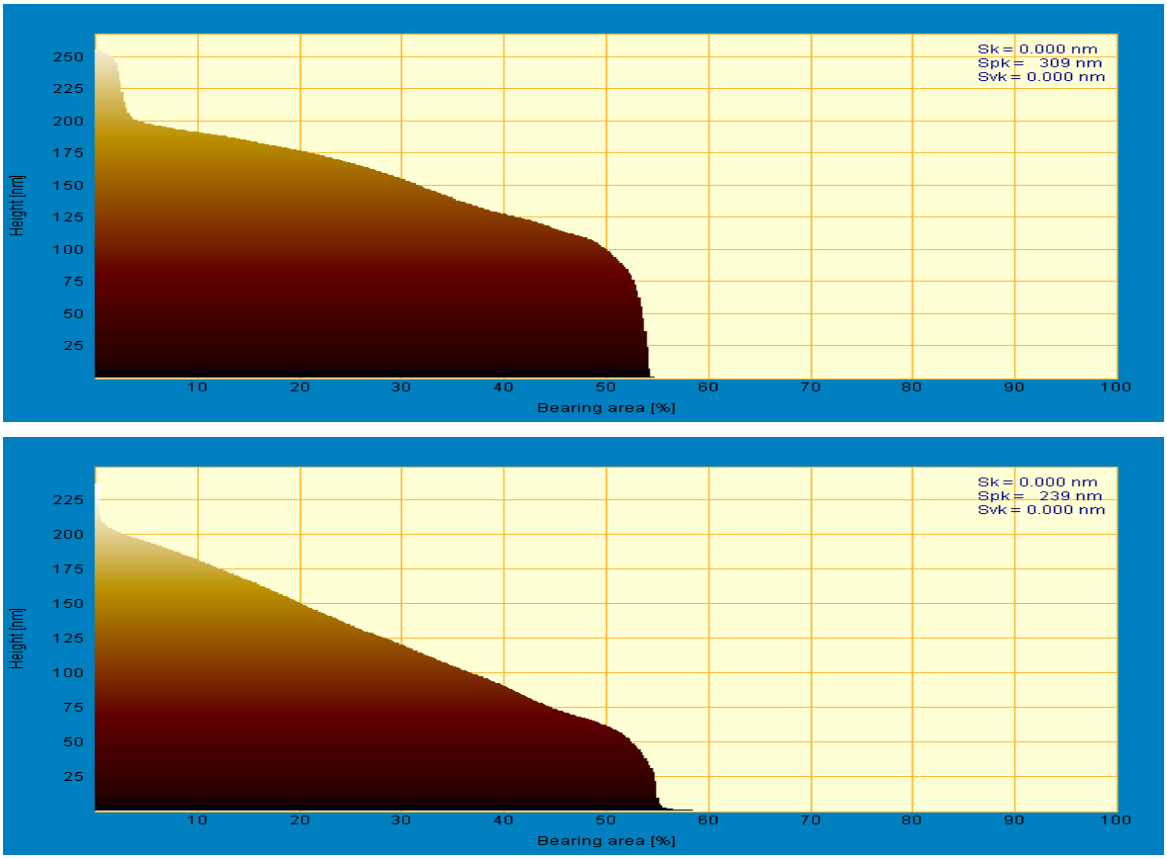

Fig. 4. Abbot curve of the blood vessels on CAM of control (A) and treated (B) eggs showing less height of blood vessels on the CAM of treated sample (B) than control (A).

For more accuracy, the 3D surface roughness of normal and treated CAMs was measured. Parameters of 3D surface roughness of CAMs were evaluated to quantify angiogenesis (Table. 1). The surface roughness, representing neo-vascularization, of control CAMs was significantly greater than that of treated CAMs. The average roughness values of control and treated CAMs were 101.2 $\pm$ 2.11 and 70.32 $\pm$ 3.250 nm respectively.

Moreover, the Abbott curve, a graphic representation of roughness, was also measured to evaluate even minute differences in the height of blood vessels on the surface of CAMs. The heights of Abbott curves for control and treated CAMs were 255 and 210 respectively (Fig. 4), showing that the height of blood vessels on control cams was greater than that of treated CAMs. When ANOVA is applied on all parameters, P-value was found to be than 0.05 ($P < 0.05$).

CONCLUSION:

All the parameters evaluated demonstrate the anti-angiogenic effect of strawberry juice in chicken Chorioallantoic membrane. Our results showed that inhibition of angiogenesis by strawberry juice may be due to suppression of endothelial cell spreading, migration and angiogenesis. It is recommended that this area of research for strawberry juice may be continued on tumor models to find out its efficacy against that particular condition

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REFERENES

Ejaz, S; K.B. Seok and L. C. Woong (2004). A novel image probing system for precise quantification of angiogenesis. Tumori. 90: 611-617.

Ejaz, S; K. B. Seok and L.C. Woong (2005). Toxicological effects of mainstream whole smoke solutions on embryonic movements of the developing embryo. Drug Chem. Toxicol. 28: 1-14.

Folkman, J (1995). Angiogenesis in cancer, vascular, rheumatoid and other diseases. Nat.Med. 1: 27-31.

Folkman, J (2001). A new family of mediators of tumor angiogenesis. Cancer Invest. 19:754755.

Garnaes, J; P.E. Hansen, N. Agersnap, J. Holm, F. Borsetto and A. Kuhle (2006). Profiles of a high-aspect- ratio grating determined by spectroscopic and atomic-force microscopy.Appl. Opt. 45: 3201-3212.

Lin, J. Y and C. Y. Tang (2008). Strawberry, loquat, mulberry and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation using murine peritoneal macrophages. Food Chemistry. 107: 1587-1596.

Melkonian, G; L. Chering, R. Marr, C. Tong and P. Talbot (2002a). Mainstream and sidestream cigarette smoke inhibit growth and angiogenesis in the day five chick chorioallantoic membrane. Toxicol. Sci. 68: 237-248.

Ribatti, D; B. Nico, A. Vacca, L. Roncali, P.H. Burri and V. Djonov (2001). Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis in vivo. Anat. Rec. 264: 317-324.

Ribatti, D; A. Vacca, L. Roncali and F. Dammacco (1996). The chick Chorioallantoic membrane as a model for in vivo research on angiogenesis. Int J Dev Biol. 40(6): 1189-1197.

Seeram, N.P; R. A. Momin, M.G. Nair and C.D. Bourquin (2001). Cyclooxygenase Inhibitory and antioxidant cyaniding glycosides in cherries and berries. Phytomedicine. 8(5): 362-369.

Seeram, N. P (2008). Berry fruits for cancer prevention: current status and future prospects. J. Agric. Food Chem. 56(3): 630-635.