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genesis using CAM assay**

Effect of ginger extract on angiogenesis using CAM assay

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Abstract

Angiogenesis performs a critical role in the embryonic growth and several pathological conditions in cancer. This study evaluated the anti-angiogenic properties of the ginger extract using CAM assay. Ginger extract was prepared by methanol. Window was made after 5 days of incubation of eggs and the ginger extract was applied on day 6 at different doses (20, 50, 75 and 150 μ L). SPIP software was utilized to investigate the CAM region and diameter of blood vessels. Ginger extract proved anti-angiogenic consequence by decreasing the diameter of CAM of blood vessels. Comparable results were attained at dilution of 150 μ L. The anti-angiogenic activity of ginger extract implicates its possible application for diseases where inhibition of blood vessel formation is desired.

Introduction

Angiogenesis is important for the typical physiological activities such as cure from injury, menstrual cycle and embryo growth. It is also crucial to the pathogenesis of a number of diseases (Lu et al., 2016). Angiogenesis irregularity causes the initiation of several diseases and furthermore progression such as, age related macular degeneration and malignant tumor (Adair and Montani 2010). Malignant tumor needs excessive blood supply for their quick proliferation and development. Therefore, angiogenesis is significant for the start, development and metastasis of a malignant tumor (Lu et al., 2016). Eosinophil normalizes the angiogenesis while the response to hypoxia which is ordinarily observed in infectious regions (Efraim and Levi-Schaffer, 2013). The tumor associated macrophages discharge many pro-angiogenic factors. These are the basic fibroblast growth factor, vascular endothelial growth factor-A, adrenomedullin, thymidine phosphorylase, and urokinase-type plasminogen activator. These factors initiate the angiogenic mechanism switch (Riabov et al., 2014). Cancer cells need oxygen supply and proper nutrition for their development. Tumors without oxygen availability are not able to increase their size more than

1-2 mm. Immunoglobulin G stops the vascular endothelial growth factor-A receptor in the endothelial cells. Therefore, blocks the blood supply availability towards tumor. The naturally occurring health products have multifaceted organic compounds which have the great ability to stop angiogenesis process. These communicate with different pathways which influence cell signaling, the immune system and apoptotic and stops angiogenesis.

Ginger (*Zingiber officinale* Roscoe) belongs to family Zingiberaceae. Chemical investigation of ginger demonstrates that it consists of about 400 various complex compounds. The most significant ingredients are lipids (3-8%), terpenes and carbohydrates (50-70%) (Grzanna et al., 2005). Terpene ingredients present in the ginger consist of zingiberene, β -bisabolene, α -farnesene, β -sesquiphellandrene and α -curcumene, although phenolic ingredients consist of gingerol (23-25%), paradol, and shogaol (18-25%). Ginger also contains the amino acids, minerals and vitamins (Shukla and Singh 2007). Furthermore, ginger has been accounted concerning the illustration an ache easing to arthritis, muscle soreness and stomach pain cure. It is utilized as taste in foods and is usually recognized as



medicinal characteristics rhizomatous plant (Shukla and Singh 2007). Phytochemicals present in foods have beneficial characteristics as utilized complete food components in comparison with the only single ingredient (Liu, 2003). Therefore, adequate facts recommend that the collection of phytochemicals in food take work collectively throughout balancing and have a common characteristics system to reveal the most favorable cancer chemo-preventive and curative reward (Liu, 2004).

The study objective was to evaluate the effects of ginger extract on the angiogenesis by using chorioallantoic membrane assay.

Materials and Methods

Preparation of ginger extract

Fresh ginger was taken from the nearby local market. It was identified and placed in herbarium of the Institute for future reference. Washed it with distilled water and peeled out it in a laminar flow. Ginger was grated with the help of grater. Grated ginger was soaked in the methanol. Left it for 96 hours (4 day and night) to made the ginger extract. After 24 hours, the supernatant was collected every day. After 4 days, rota-vapor was used to concentrate the ginger extract. It was freeze dried by using lyophilizer to obtain powder type. The stock solution of ginger extract was prepared. The ginger extract solution was made by adding 100 mg/mL of dimethyl sulfoxide in powder form of ginger extract. Different suitable dilutions were prepared for use of CAM procedure. The complete research was performed with use of the single particular ginger extract to evade from dissimilarity and increase product fidelity.

CAM assay preparation

New treated 50 fertilized chicken eggs that were four days old taken from a nearby hatchery. Ethanol 70% was sprayed at each egg to minimize the chance of inflammation from the outer covering of egg. Eggs were dried out with air pressure. These eggs were incubated at the temperature of 37 °C and provide moisture 60-70% for 120 hours (5 days). Eggs were partitioned under two sets, one set remains untreated and used as control and second set was treated. There were more sub-sets 1, 2, 3 and 4 made by partitioning of the treated set of eggs. Ten eggs from every sub-set were in use. CAM assay was performed on each egg one by one in the laminar flow. Eggs were perforated carefully that there will no chance of contamination after incubation of 5 days. The perforated window should be little in size round about 2 cm in width. Window was prepared by eradicate the outer covering and the covering inside membrane at air-space location. Took a syringe and 4-5 mL white egg (albumin) was provided on the same day to permit the embryos growing in such a way that it

upgrades its quantification and manipulating characteristics. It allowed embryos to develop in such a way simple to evaluate. The windows were air tight with decontaminated parafilm. The eggs were taken back into an incubator at temperature 37°C and 55-60% moisture for the 6th day of incubation.

Administration of dose

After 6 days incubation, windows of every egg of sub-set was opened one by one to administer the different dilutions. 20 µL, 50 µL, 75 µL and 150 µL dilutions were administered to each subgroup simultaneously 1, 2, 3 and 4 into growing CAMs. Windows were air tight with decontaminated parafilm. Eggs were taken back into an incubator for one day (24 hour). Sealed Windows were unlocked on day 7. The shape and zone of CAM were assessed by getting images with a digital camera. Two drops of normal saline were added to separate the inner membrane from the CAM. An equal volume of acetone and methanol (1:1 volume) were provided to secure the blood vessels area. With the help of clamp and plain forceps, the CAM was separated. SPIP software was utilized for the quantification of outcomes about consequences. The entire pictures were changed into a gray scale for progress the complexity toward dark and white setback with Adobe Photoshop 6.0. Now each picture was observed with conceivable structures and to encourage the exact quantification about angiogenesis.

Image acquisition and quantification using SPIP software

Afterward the picture acquisition, SPIP (IBM, Denmark) was utilized to assess the picture that work at particular algorithm. The width and length size of various different blood vessels calculated by calibration and other calculating parameters. CAMs superficially angiogenesis was positively quantified by calculating the 3D surface irregularity, that is a critical factor in 3D picture investigation. The Abbot curve and the vascular area were calculated. Blood vessels were calculated and measure to micrometer size to examine the exact deep effect of curcumin derivatives extricate for angiogenesis. The fundamental parameters for 3D surface irregularity are as: Arithmetic mean height (Sa) is mean for supreme qualities for irregular profile. It is an area between irregular profile and mean line. This is a straight forward and proficient constraint which assists to demonstrate the surface irregularity. It is the standard length of the examined region. Four constraints used to illustrate the length of surface region. These partitioned into four sets: a) dispersion, b) extreme, c) the asymmetry of height distribution and d) height distribution sharpness. Root mean squared height (sq) is root mean square measurement of the surface inside sample region. It is scattering parameter. Maximum height (Sz) is that standard of the vital curvatures of summit inside the sampling inspecting

zone. While, the whole amount of curvatures from claiming the surface at perspective are equivalent to the whole from claiming central curvatures. Maximum peak height (Sp) is the proportion to increase the interfacial zone of outward over the sample zone. It exposes the mixture superficial possessions. The greater the Sp worth indicates the significance of either largeness or space or together. The efficient factors were detected in this learning is the essential liquefied preservation (Sz). Essential liquid preservation catalog is the proportion of void capacity of component specimen zone at the essential region above the root mean square deviance. Maximum valley depth (Sv) is the perpendicular distance between the peak of uppermost peak and lowest of the deepest valley within sample extent. It is the supreme of all the peak-to-valley principles.

Results

Ginger extract at different doses (20, 50, 75 and 150 μ L) was applied on eggs developing embryo to evaluate blood vessels at macroscopic stage anti-angiogenic action. Different doses showed different effects. These caused reduction of primary blood vessels (PBVs) and secondary blood vessels (SBVs). Tertiary blood vessels (TBVs) disappeared and decreased CAM area (Figure 1).

Blood vessels diameter and surface roughness was evaluated by SPIP software as given in Table I. This data showed that the blood vessels diameter (PBVs, SBVs and TBVs) was normal in control. There was a significant decreased in blood vessels diameter with different doses of ginger extract. Different values of blood vessels surface roughness was obtained with the different doses of ginger extract.

Figure 2 describes that Abbott curve height of blood vessels decreased with increased the dose of ginger extract. The decreased height than control showed the anti-angiogenic activity.

Surface roughness was different with the change in concentration of ginger extract. These values were decreased than the control. Abbott curve reveals that blood vessels were reduced in treated CAMs and control values were not decreased. The blood vessel diameter of treated eggs was also decreased. These showed that ginger extract is a strong inhibitor of angiogenesis.

Discussion

Many plants have been studied for their anti-angiogenic activity e.g. *Teucrium stocksianum* (Tabassum et al., 2016), *Argassum wightii* (Shim et al., 2003), and *Pleurotuseous* (Xu et al., 2015). The characteristics of ginger its ingredients are linked with anti-inflammatory, anti-mutagenic and antioxidant activities (Govindarajan and Connell 1983). Angiogenesis is an energetic propagation and differentiation procedure, which have need of endothelial propagation, passage and tube development (Chen et al., 2005). Tumors with physically powerful angiogenic action are associated with a lesser patient survival speed (Giatromanolaki et al., 1996).

The combination of this technique and new strategies in medication discovery has large prospective in medication of the future. Anti-angiogenic medicines have fewer side effects than other procedures for curing cancer. Therefore, the thought of joining these techniques with other cure procedure is very attractive. Ginger extract was used to investigate the anti-angiogenic activity by using CAM assay in the laboratory. The CAM region and standard surface

Table I

Diameter and surface roughness of blood vessels at different concentrations of ginger extract

	Diameter (mm)				
	Control	20 μ L	50 μ L	75 μ L	150 μ L
Blood vessels					
Primary blood vessels	77.2	70	52	17.5	11.4
Secondary blood vessels	44	42	39	6.7	3.5
Tertiary blood vessels	35	33	15.6	2.5	0.5
Surface roughness of blood vessels					
Arithmetic mean height (Sa)	10.2	11.3	7.1	9.3	8.7
Root mean squared height (Sq)	12.20	13.5	9.8	12.4	10.8
Maximum height (Sz)	59.7	63.0	71.2	74.9	60
Maximum valley depth (SV)	32.6	23.3	27.0	36.0	30.9
Maximum peak height (Sp)	27.1	39.2	44.2	38.9	29.6

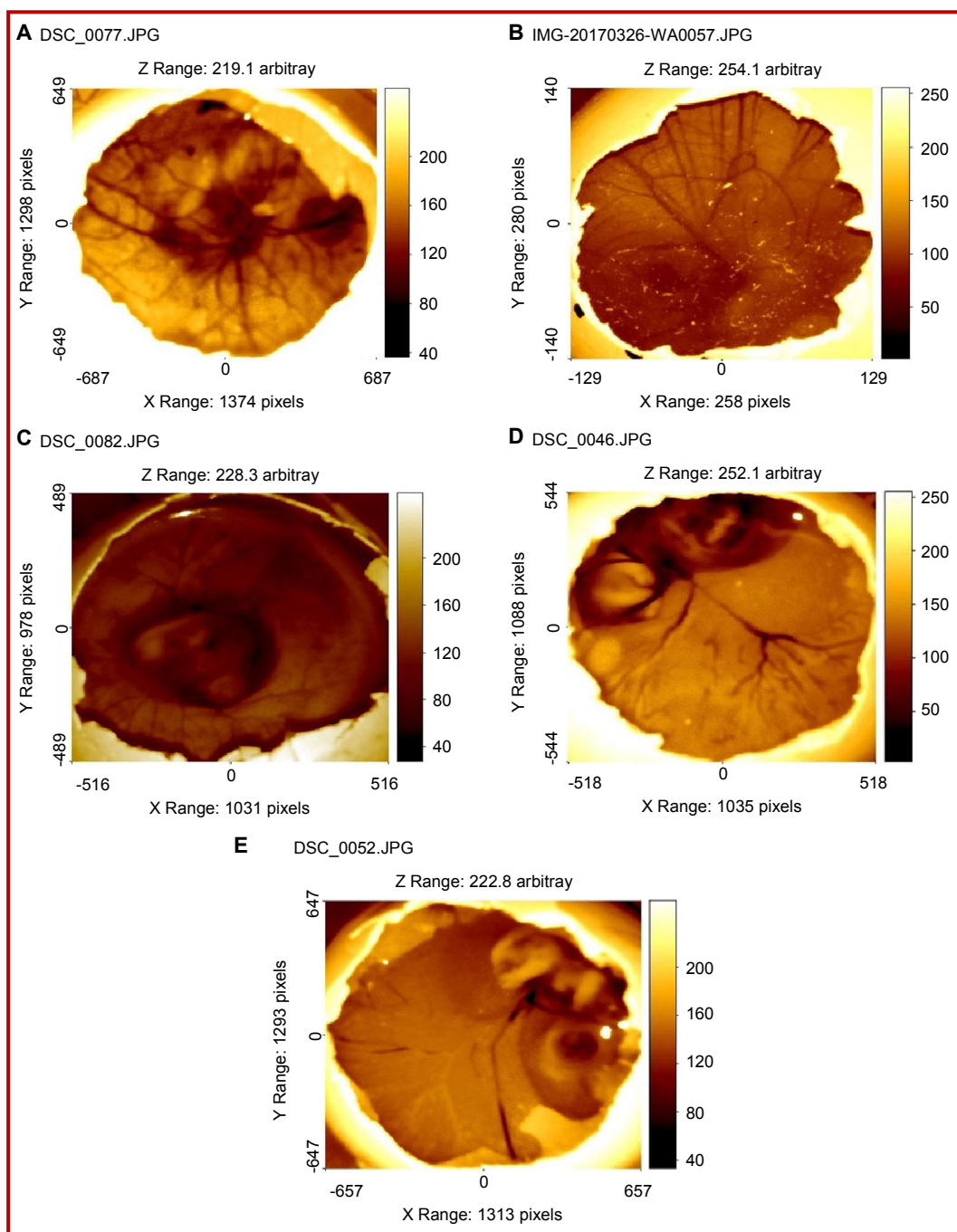


Figure 1: Macroscopic assessment of CAM. Clearly visible CAM vessels i.e. PBVs, SBVs, TBVs and clearly develop CAM region in (A) control normal vessels development. Decreased in CAM region revealed anti-angiogenic actions in treated dilutions of ginger extract i.e. (B) 20 μ L, (C) 50 μ L, (D) 75 μ L, (E) 150 μ L

roughness factor were measure to find out the anti-angiogenic action of ginger extract. The CAM regions were reduced in treated eggs by using ginger extract. The diameter of treated eggs blood vessels and the length of Abbott curve and standard surface roughness was also reduced in the eggs which were treated. The control eggs which were not treated, their embryo developed normally. The data obtained by analysis from the different groups which were treated revealed

that the significant results were obtained at the 150 μ L dose of ginger extract.

Amount of angiogenesis and CAM region has straight association i.e. raised in angiogenesis enhanced the CAM region and decreased in angiogenesis reduced the CAM region. CAM is extremely well vascularized region. This is because of angiogenesis. The compound revealed anti-angiogenic activity due to decrease in

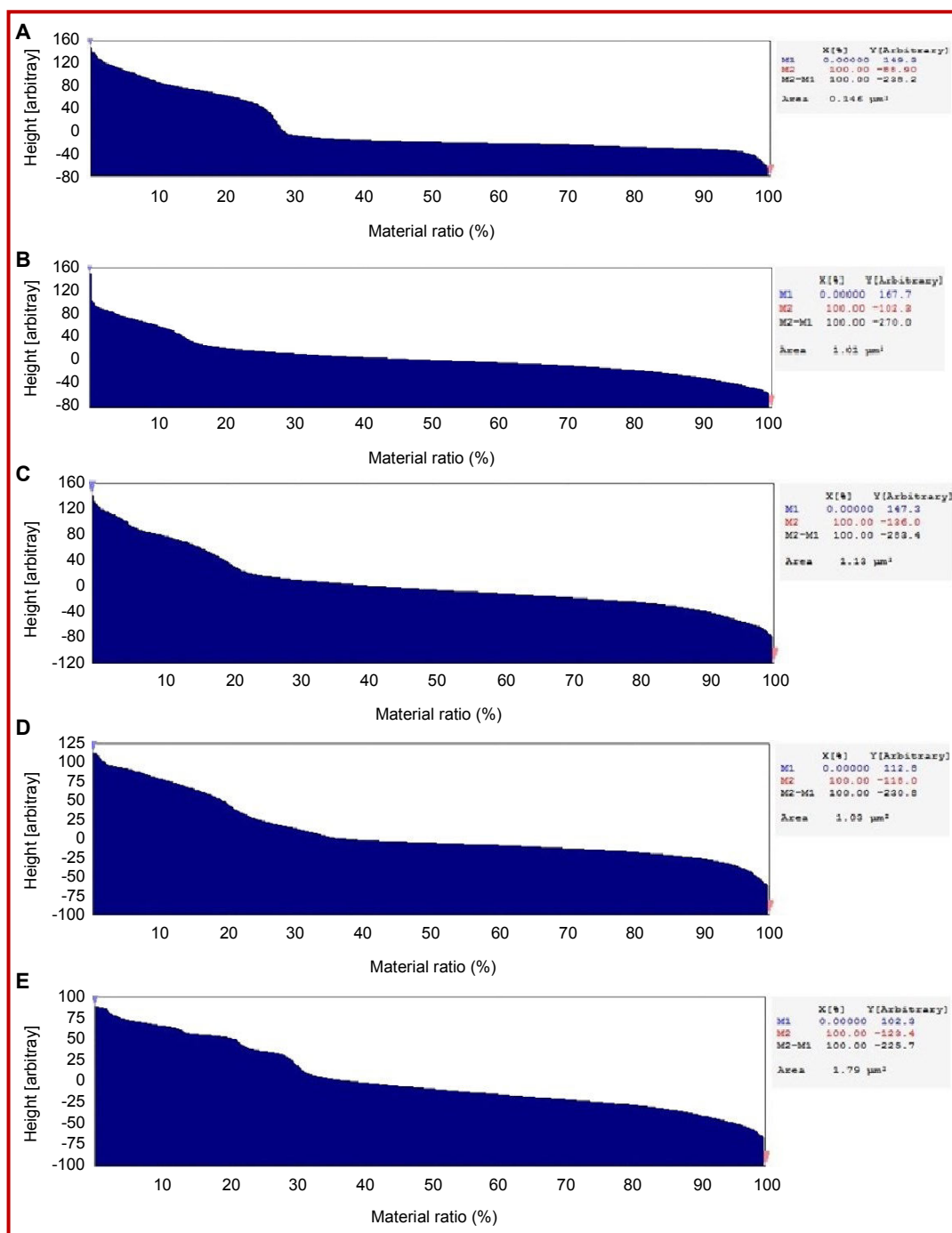


Figure 2: CAM assay Abbott curve of blood vessels, control (A), 20 μL (B), 50 μL (C), 75 μL (D) and 150 μL (E)

CAM region, blood vessels diameter and the standard surface roughness of CAM. These are investigative constraints of anti-angiogenic possessions of ginger extract.

Ginger extract possessed anti-angiogenic action. This might be utilized as an adjuvant treatment with other anti-neoplastic medication to stop tumor development. Many anti-angiogenic medicines techniques can be planned to investigate the effects of anti-angiogenic medicine on tumor development and morphology with

pharmacokinetics investigation of particular medicines.

Aminopeptidase-N is an important membrane bound protease. It is a metallo-reliant (Zn^{2+}) enzyme (Antczak et al., 2001). This enzyme relates to the family M1, which is the family of MA of peptidases (Rawlings and Barrett 1999), it is also known as gluzincins (Hooper 1994). It has 967 numbers of amino acids and less N-terminal cytoplasmic area. It has a particular transmembrane and a more cellular ectodomain consist of active

site (Olsen et al., 1988). Pflleiderer and Colliers separated this enzyme from kidney of pig (Pflleiderer and Celliers 1963). Aminopeptidase-N (APN) is assumed a key part for cancer intrusion and angiogenesis (Look et al., 1989). It seems that ginger contains some constituents that inhibit aminopeptidase-N leading to anti-angiogenic properties.

There was a substitute technique vascular normalization which utilized for moderately decreased in cancer blood vessels quantity and encouraged maturation of blood vessels during the anti-angiogenic cure. It is a curative technique during the angiogenesis cure through inhibitors for making balance between pro and anti-angiogenic.

Most favorable anti-angiogenic treatment might need complex explanation solution. Choosing suitable grouping of numerous curative approaches (counting surgery, chemotherapy, radiation therapy, anti-angiogenic therapy, and vascular encouragement) with practical arrange might progress patient recovery. Discover new molecular targets and improved important prognostic biomarkers used for vascular normalization might make the angiogenesis inhibitors for cancer therapy mediator progress on in the upcoming days.

Conclusion

Ginger extract proved anti-angiogenic consequence by decreasing the diameter of CAM of blood vessels

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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