Original Article

The Possible Anti - Angiogenic Activity of Zeaxanthin in Ex Vivo and in Vivo in Animal's Study

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Abstract

Background: Creation of new capillary blood vessels from pre-existing vessels is known as angiogenesis. The restoration of normal blood flow and, by extension, the outflow of gases, nutrients, and growth hormones makes angiogenesis crucial in a wide range of physiological and pathological situations, including wound healing and bone repair and regeneration.

Tissue engineering and regenerative medicine rely heavily on angiogenesis because it controls the survival, proliferation, and differentiation of newly generated tissue structures. On the other hand, tumor cells with an angiogenic character have more proangiogenic pathways than downregulating mechanisms. Because of this, endothelial cells experience a period of rapid proliferation that fosters the development of a tumor microenvironment that is abundant in oxygen and nutrients and promotes the tumor's metastasis to new locations. In order to treat persistent diseases or cancerous tumors, doctors have begun using anti-angiogenesis-based therapies, which involve giving patients compounds that block the development of new blood vessels.

Objective: To identify the anti-angiogenic activity of Zeaxanthin and Melatonin and their combination in *ex vivo* rat aorta anti-angiogenic assay and in vivo chorioallantoic membrane (CAM) animals' study.

Methods: 12-14 weeks-old Albino male rats were used for the study. The tested substances zeaxanthin and melatonin were serially diluted. *An ex vivo* rat aorta ring experiment has been used to examine zeaxanthin potential antiangiogenic properties; the zeaxanthin-induced zone of blood vessel inhibition was measured in CAM assay *in vivo* chorioallantoic membrane**.**

Results: The result of this study showed that there is a significant antiangiogenic activity of zeaxanthin and melatonin and there is an additive antiangiogenic effect of Z and M combination in ex vivo rat aorta ring assay

Each Z and M showed significant anti-angiogenesis activity in the *in vivo* assay and for the combination, it was discovered that melatonin and zeaxanthin each produced a significant inhibition zone of blood vessels in the CAM assay.

Conclusion: Zeaxanthin and melatonin and their combination exerted significant antiangiogenic effect on *ex vivo* rat aortic ring, Zeaxanthin and melatonin and their combination showed significant antiangiogenic effect on *in vivo* chick chorioallantoic membrane model.

Keywords: Possible Anti, angiogenic Activity, Zeaxanthin, Ex Vivo.

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INTRODUCTION

The cardiovascular system is the first functional organ system to emerge during embryonic development. Vasculogenesis and angiogenesis are the two main processes that contribute to the creation of blood vessels *(Kolte et al., 2016)*.Developing new blood vessels, or vasculogenesis, involves the differentiation of mesodermal cells into either endothelial precursor cells or angioblasts. This leads to the development of capillary plexuses, which are uniformly simple vascular structures that will eventually give rise to the more complex, hierarchically organized arteries, veins, and capillaries *(Adair and Montani, 2010)*.In contrast angiogenesis involves the development of new blood vessels from preexisting ones. First, capillaries are formed as a result of angiogenesis, which causes the primary capillary plexuses to grow angiogenically. The vascular tree then develops concurrently with the physiological growth of the neighboring tissues *(Bikfalvi, 2004)*. Moreover, angiogenesis regulates the maintenance, multiplication, and specialization of newly generated tissue structures, making it essential in tissue engineering and regenerative medicine *(Mastrullo et al., 2020)*. However, when tumor cells have an angiogenic character, proangiogenic pathways outnumber down regulating mechanisms. Endothelial cells go through a rapid growth phase as a result, which helps to create a tumor

microenvironment that is rich in oxygen and nutrients and encourages the spread of the tumor to distant places *(Maishi and Hida, 2017)*.Thus, there has been a lot of interest in anti-angiogenesis based therapies as very effective anticancer medications, which entail administering compounds to treat chronic illnesses or malignant tumors in order to inhibit the formation of blood vessels *(Maj et al., 2016)*.

Type of Angiogenesis

Both sprouting angiogenesis and intussusceptive angiogenesis occur throughout pregnancy and later in life. In the 1980s, researchers discovered fibroblast growth factor, the first growth factor, and determined that it triggered the process of angiogenesis in stem cells. Many cells, including macrophages and tumor cells, produce FGF, which is responsible for triggering the primary phases of the angiogenesis process *(Murakami and Simons, 2008)*

Melatonin, also known as N-acetyl-5-methoxytryptamine or simply M, is a naturally occurring hormone that is produced by the pineal gland and other organs, including the skin, the gastrointestinal tract, and the retina, in humans and other mammals during the night *(Kvetnoy et al., 2022)*. Melatonin regulates angiogenesis in pathological conditions like cancer *(Bielenberg and Zetter, 2015)*, Bone defects *(Zheng et al., 2022)*, *Skin incision wound (Pugazhenthi et al., 2008)*, Atherosclerosis *(Ma et al., 2018)*.

Zeaxanthin Xanthophyll carotenoids, like zeaxanthin (,-Carotene-3,3′-diol), are a type of carotenoid. The ionone rings and polyene chain both feature this number of conjugated bonds. A hydroxyl group in the ionone rings facilitates the esterification of fatty acids *(Bernstein et al., 2016)*. It is important that they are present in the eyes because they prevent AMD and cataracts. Zeaxanthin is reported to have a concentration of (ng/g) of 591 in human liver, 90 in human lungs, 14 in human breasts, 35 in human prostates, 32 in human colons, and 6 in human skin. *(Mozaffarieh et al., 2003)*.

METHODS

Rat aorta ring assay (RAR assay in ex vivo)

This assay was performed according to *(Brown et al., 1996)* protocol with a minor modifications accomplished by *(Sahib, 2017)*.

It is started by taking five Albino male rats (2 months old) which authenticated via cervical dislocation. The thoracic aorta is then extirpated, and cross-sectioned into 1 mm thickness rings.The assay was performed in a 48-well tissue culture plate. 300 µl of (3mg/ml fibrinogen with 5mg/ml of Aprotinin in serum free medium [M199]) is added to each well. Each ring tissue is placed in the center of the well. To each well 10 µl of thrombin; prepared at 50 NIH U/mL in 0.15 M NaCl was added and then was incubated for 10 min at 37°C in a humidified 5% CO_2 incubator.Then 0.3 ml/well of Medium M199 supplemented with 20% of heat inactivated fetal bovine serum (HIFBS), 0.1% E-aminocaproic acid, 1% L-glutamine and 0.6% gentamycin was added. The test substance is added to the complete growth medium at concentration of 100µg/mL and each treatment was performed in six replicates. The test substance preparation is done by dissolving it in dimethyl sulfoxide (DMSO) and diluting in M199 growth medium to make the final DMSO concentration1%.The culture plates then returned to 5% CO2 humidified incubator at 37°c for five days. the angiogenesis response and the stability of neo vessels are enhanced due to accumulation of endogenous growth factors in the system.The DMSO (1% v/v) and melatonin (100µg/mL) were used as negative and positive controls respectively.

Vessel growth was quantified manually under (40x) magnification on day 5, with aid of camera and software package.The percentage of blood vessels inhibition was determined according to the formula: **Blood vessels inhibition = 1- (A_0/A) ×100** Where:

A_0= distance of blood vessels growth for the test substance in mm.

A= distance of blood vessels growth in the control in mm.

In vivo chorioallantoic membrane (CAM) animals' study

The assay was performed by using a fertilized chicken eggs from the poultry field and kept in a humidified incubator at a constant humidity of 60 % and at a temperature of 37°C for 72 hours where eggs were placed in horizontal position. On Day 4 a small pinpoint hole is created at the apex of the eggs and 2-3 ml of albumin is removed, The eggs were incubated for further 24 hours.

On Day 5 a small square window (3-4 cm) of the shell was made and the test samples that were soaked, then eggs were incubated again for another 72 hours. Finally, the inhibition zone photographed and calculated. The test samples were prepared as 10mg/ml and 50µl (final dose was 0.5mg/disc) of zeaxanthin and melatonin and 25µl of each substance was taken to make the combination (final dose was 0.25mg for each one/disc) placed on the filter paper discs and left to dry before being transferred to the CAM.

Statistical Analysis

Continuous data presented as mean ± standard deviation, the comparison between two groups carried out using independent t-test, while between three groups using ANOVA, if significant difference observed Tukey Post-hoc test. Level of significance was 0.05, all analysis carried out using GraphPad Prism version 9.4.1

RESULTS

Anti-angiogenic activity of zeaxanthin measured in an ex vivo rat aortic ring test

Zeaxanthin exerts a significant inhibition effect on rat aorta ring, in which as the concentration of Zeaxanthin increased there was significant increase in the inhibition rate, as

illustrated in table 1 and figure 1.

Melatonin exerts a significant inhibition effect on rat aorta ring, in which as the concentration of Melatonin increased there was significant increase in the inhibition rate as illustrated in table 1 and figure 2.

Both drugs demonstrated a significant dose-dependent inhibition of blood vessel growth in comparison to the control group, which consisted of one percent DMSO; this result was statistically significant (P<0.001).

Table 1: comparison between Melatonin and Zeaxanthin for their effect pm angiogenesis for Ex vivo rat aorta

Concentration	Zeaxanthin	Melatonin	p-value
6.25 mcg	38.6% ± 4.3	63.6% ± 1.9	0.328
12.5 mcg	51.1% ± 4.6	77.2% ± 1.6	0.205
25 mcg	64.9% ± 6.0	87.7% ± 2.3	0.261
50 mcg	77.9% ± 6.3	93.4% ± 2.4	0.247
100 mcg	90.6% ± 6.9	96.7% ± 2.6	0.249
Data presented as mean ± standard deviation			

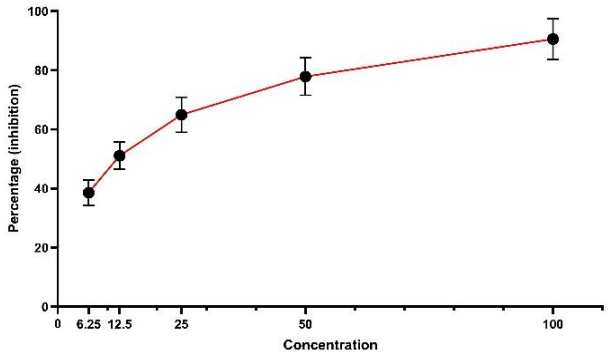

Figure1: Dose response curve of Zeaxanthin in rat aortic rings model (represent concentrations vs. percentage inhibition)

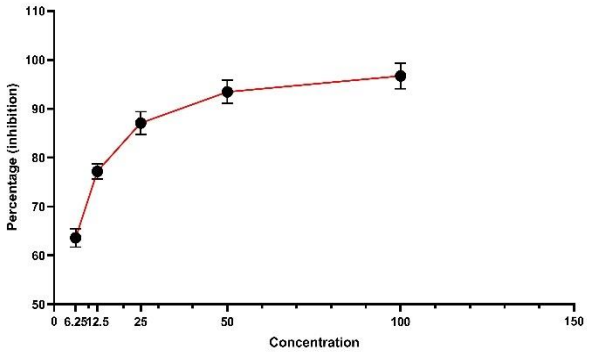

Figure 2: Dose response curve of Melatonin in rat aortic rings model (represent concentrations vs. percentage inhibition)

The anti-angiogenic effect of Melatonin and Zeaxanthin combination on Ex vivo rat aorta ring

Table 2: Assessment of percentages of inhibition for combination of melatonin and zeaxanthin at 50 mcg/ml

Substances	Percentage of inhibition
Melatonin 50 mcg/ml	93.4%
Zeaxanthin 50 mcg/ml	77.9%
Combination 50 mcg/ml [a]	86.0%
[a] Based on relative IC_{50} proportion (11.435: 3.588; 3.19: 1; for zeaxanthin/ melatonin ratio); 38.1: 11.9 mcg/ml	

Based on previous study by *(Chou, 2006)*:

$$CI = \frac{D_1}{(D_{m)_1}[\frac{f_a}{(1-f_a)}]^{m_1^{-1}})} + \frac{D_2}{(D_{m)_2}[\frac{f_a}{(1-f_a)}]^{m_2^{-1}})}$$

CI: combination index, D_1: concentration of Drug 1, D_2: concentration of drug 2, D_m: IC_{50}, f_a: fraction affected. Based on this equation, the *CI* was found to be 1.042 (which indicate a additive effect).

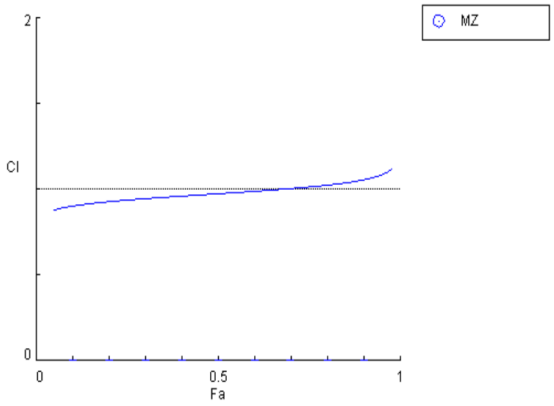

Figure 3: In vivo chick chorioallantoic membrane (CAM) assay of melatonin, zeaxanthin, and their combination

It was discovered that melatonin and zeaxanthin each produced a significant inhibition zone of blood vessels in the CAM. The scoring for this zone was (++) and (+++) respectively, As shown in table 3 and figure 4 for melatonin, zeaxanthin and for the combination.

Table 3: The results of the inhibitory zone of blood vessel growth in in vivo (CAM) assay for melatonin, zeaxanthin and combination were as follows:

Eggs number	Zone of inhibition Area (mm) / Scoring for melatonin	Zone of inhibition Area (mm) / Scoring for zeaxanthin	Zone of inhibition Area (mm) / Scoring for combination
1	(5) +	(8) ++	(11) +++
2	(9) ++	(7) ++	(8) ++
3	(11) +++	(4) +	(11) +++
4	(12) +++	(11) +++	(8) ++
5	(8) ++	(7) ++	(12) +++
6	(4) +	(8) ++	(7) ++

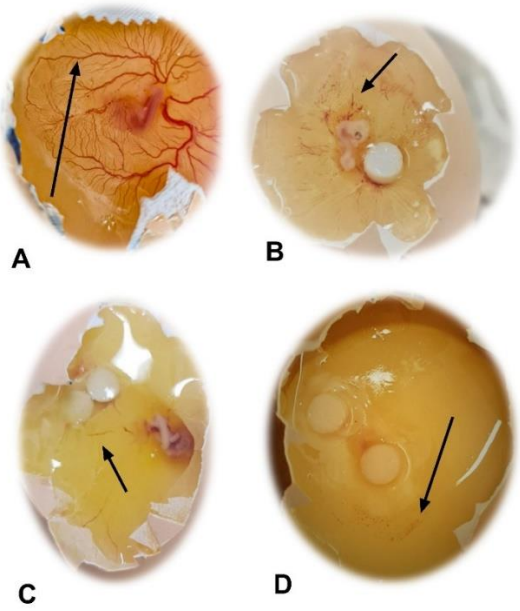

Figure 4: A) negative control, B) zeaxanthin, C) Melatonin, and D) combination

DISCUSSION

Ex vivo replication of the key angiogenic stages, such as endothelial migration, proliferation, proteolytic breakdown of the extracellular matrix, creation of capillary tubes, pericyte recruitment, and vascular regression, is possible using the aortic ring assay *(Aplin et al., 2008)*. The angiogenic response of the aortic explants, which is similar to angiogenesis *in vivo* aside from the absence of blood flow, is controlled by paracrine interactions between endothelial cells, macrophages, fibrocytes, and pericytes involving a variety of angiogenic factors and inflammatory cytokines/chemokines *(Nicosia, 2009)*.

Ex vivo aortic ring models combine the benefits of *in vitro* and *in vivo* tests. Neovessel growth happens in a specific environment, and the culture system is simple to modify to suit various experimental setups. The explants' native endothelium still functions as endothelial cells do in living tissue and has not been altered by multiple passages in culture. Endothelial cells that are correctly polarized and their accompanying pericytes make up the microvessels *(Nicosia and Villaschi, 1995)*. Fibroblasts can be seen in the interstitium between outgrowing microvessels. There are no circulating leukocytes due to the lack of blood flow, but resident macrophages and dendritic cells migrate out of the aortic adventitia and actively take part in the angiogenic process *(Aplin et al., 2006)*.

In the current study melatonin was considered as a positive control, it exerted significant antiangiogenic effect on *Ex vivo* rat aorta ring model in a dose dependent manner, it effect was significant compared to negative control (DMSO), with an IC_{50} = 3.588 and its behaviour follow sigmoid curve.

In the current study zeaxanthin was chosen as the investigated drug to assess its potential antiangiogenic properties, it exerted significant antiangiogenic effect on *Ex vivo* rat aorta ring model in a dose dependent manner, it effect was significant compared to negative control (DMSO), with an IC_{50} = 11.435 and its behaviour also follow sigmoid curve. Additionally, the effect of combining both M and Z was found to be additive in nature with combination index (CI) equal to 1 as indicated by *(Chou, 2006)*.

In a study by Huang et al. on the antiangiogenic effects of carotenoids in rat aortic rings, the authors found that 7 days of incubation with VEGF (50 ng/ml) dramatically increased the development of new blood vessels by 211 percent as compared to the vehicle control group. Later, it was discovered that carotenoids dramatically reduced the development of new vasculature caused by VEGF, with an inhibition of between 25 and 56 percent (depending on the carotenoids involved), in comparison to the group that had been treated with VEGF *(Huang et al., 2018)*.

In a rat aortic model, "Lycopene," a different member of the carotenoids family, was evaluated for its antiangiogenic properties. As opposed to the control group, they discovered that treatment with lycopene resulted in a considerable and concentration-dependent suppression of the sprouting of arteries from rat aortic rings, with an inhibition of 51% *(Chen et al., 2012)*.

Siphonaxanthin, a specific keto-carotenoid discovered in green algae, was the subject of another investigation. Ex vivo microvessel development was inhibited by siphonaxanthin in a dose-dependent manner. Even at very low concentrations, significant inhibition was seen *(Ganesan et al., 2010)*.

In an ex vivo angiogenesis study employing a rat aortic ring, fucoxanthin and deacetylated product fucoxanthinol were evaluated for their antiangiogenic properties. Both inhibited microvessel development in a dose-dependent way *(Sugawara et al., 2006)*.

Zeaxanthin's ability to act as an antiangiogenic agent in an ex vivo rat aorta assay has never been studied before, which highlights how new the findings of the present study are. This approach is frequently employed as an effective one to assess antiangiogenic compounds in a complex system where endothelial cells, fibroblasts, pericytes, and smooth muscle cells interact.

In vivo chick chorioallantoic membrane (CAM) assay of melatonin, zeaxanthin, and their combination.

Utilizing CAM assays, a great deal of research has been done on angiogenesis, tumor cell invasion, and metastasis *(*Deryugina *and Quigley, 2008)* The CAM model has many benefits, such as (a) the highly vascularized nature of the CAM greatly enhances the efficacy of tumor cell grafting; (b) high reproducibility; (c) simplicity and cost effectiveness; and (d) because the CAM assay is a closed system, the half-life of many experimental molecules, such as small peptides, tends to be much longer compared to animal models, allowing experimental study of potential anti-metastatic compounds *(Cimpean et al., 2008,)*. Fibronectin, laminin,

collagen type I, and integrin v3 are all examples of proteins found in the extracellular matrix (ECM) that are also present in the CAM. These extracellular matrix proteins serve as a carbon copy of the cancer cell's native environment *(Giannopoulou et al., 2001)*.

In the present study both zeaxanthin and its combination with melatonin exerted a pronounce effect on CAM assay, in which they produced a significant zone of inhibition in most of the samples.

Our results were consistent with earlier research by Kuhnen et al., who investigated the influence of a xanthophyll-rich maize seed extract on the development of blood vessels (maize seeds contain zeaxanthin). In the CAM assay, maize seeds inhibited the process of vascularization (28–50% inhibition) in a dose-dependent manner as compared to the control group *(Kuhnen et al., 2009)*.

Lycopene treatment of fertilized eggs dramatically reduced the number of blood vessel points, according to a study by Chen et al. using the in vivo CAM test to measure lycopene activities. Lycopene's inhibitory effect grew more potent with dosage, reaching a 38 percent reduction in inhibition compared to the control group *(Chen et al., 2012)*.

In the study by Huang et al, the antiangiogenic impact of lycopene was investigated using an ex vivo CAM test. Compared to the solvent control, lycopene considerably reduced the number of blood vessels (by 43%) and significantly reduced neovascularization *(Huang et al., 2013)*.

Conclusions

1. Zeaxanthin and melatonin exerted significant antiangiogenic effect on *ex vivo* rat aortic ring, additionally their combination exerted additive effect in this model.
2. Zeaxanthin and melatonin exerted significant antiangiogenic effect on *In vivo* chick chorioallantoic membrane model.

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