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ANTI-ANGIOGENIC ACTIVITY OF NATURAL FLAVONOID MYRICETIN ON CHICK CHORIOALLANTOIC MEMBRANE (CAM) IN-VIVO

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ABSTRACT

Myricetin is a natural flavonol, it possesses anti-oxidative, anti- proliferative and anti- inflammatory effects. The anti-angiogenic activity of Myricetin was studied using Chick Chorioallantoic Membrane (CAM) *in-vivo*. The Chick embryos were treated with Myricetin, upto 100µM of concentration there was no morphological changes. Higher than 100µM of concentration inhibited the formation of blood vessles but the concentration of 200µM caused lethality. Myricetin inhibited the neovascularisation in a dose-dependent manner in *in-vivo*. Histological cross-sections of Myricetin treated CAM revealed reduced large and small newly synthesized blood vessels. Treated embryos with Myricetin (100µM, 150µM and 200µM) significantly down- regulated the expression of VEGF-A with Real time PCR method. Our result showed that Myricetin inhibited the growth of newly formed blood vessels in chicken embryos and down regulated the expression of VEGF-A. This study reveals that the potential anti-angiogenic effect of Myricetin proves its role as a therapeutic agent against proliferative like cancer.

Key Words: Chicken Chorioallantoic membrane (CAM), Myricetin, Angiogenesis, Anti- angiogenesis, Vascular Endothelial Growth Factor (VEGF).

INTRODUCTION

The word angiogenesis is first named by Hertig in 1935 and the mechanism was coined by Folkman [1,2]. Angiogenesis, the formation of new blood vessels from pre-existing ones and plays a important role in both physiological and pathological processes such as embryonic development, wound healing, inflammation, tumor growth, and metastasis [3,4]. It involves signals to the adhesion, migration within the target endothelial cell. Angiogenesis occurs in the adult organisms, whereas vasculogenesis occurs during the embryonic development. Excessive angiogenesis is closely related to many human diseases, such as tumor growth. Tumor angiogenesis regulates the growth of blood vessels that penetrates into cancerous growth. One of the major molecules involved in the angiogenic process is the vascular endothelial growth factor (VEGF) family of proteins and their receptors. High expression of VEGF leads to abnormal angiogenesis and is associated with worse survival [5]. Anti-angiogenic therapeutic approaches have recently been shown to be effective for the treatment of certain cancers. Anti - angiogenesis is a form of targeted therapy to stop tumors from formation of new blood vessels. Anti-angiogenic therapeutic approaches have recently been shown to be effective for the treatment of certain cancers. To date the licensed angiogenesis inhibitors are bevacizumab, sorafenib, sunitinib and thalidomide.

Flavonoids are the most abundant polyphenols in human diets. Flavonoids suppress tumor growth via cell-cycle arrest and by induction of apoptosis [6]. Flavonoids possess anti-inflammatory, anti-oxidant,

anti-allergic, hepatoprotective, anti-thrombotic, anti-viral, and anti-carcinogenic [7].

Myricetin ($C_{15}H_{10}O_8$) is a pure compound, extract from the plant Myrica cerifera (Common name: Candle berry, Wax myrtle) medicinal plant. Myricetin is a naturally flavonol with hydroxyl substitutions at the 3, 5, 7, 3', 4',5 5',7' hexahydroxy flavones occurring in sweet potato leaves, parsley, currants (dried grapes), tea, berries, wines, onions [8,9]. It possesses anti-oxidative, anti-proliferative, and anti-inflammatory effects [10,11]. Some studies reported that flavonoids, including myricetin, are able to protect hippocampal cells against toxic effects induced by retenome [12]. It also had been indicated that myricetin possess neuroprotective effects on the Parkinson models *in-vivo* and *in-vitro* by anti-oxidation and anti-apoptosis activity [13]. Recent studies revealed that myricetin directly binds to MEK1 and JAK1 and thus inhibiting cell transformation.

The chick embryo chorioallantoic membrane (CAM) is an *in-vivo* model studying the inhibitors of angiogenesis [14]. The authors reported that around 72–96 hrs of incubation there was stimulation in the form of increased vessel density, with the vessels radially converging toward the center like spokes in a wheel [15]. Conversely, when an antiangiogenic compound was tested, the vessels become less dense and disappeared [16]. CAM assays have been widely used to study angiogenesis [17] tumor cell invasion and metastasis [18,19,20,21]. The CAM model has many advantages, such as (a) the highly vascularized nature of the CAM greatly promotes the efficiency of tumor cell grafting; (b) high reproducibility; (c) simplicity and cost effectiveness, and finally (d) as the CAM assay is a closed system. The CAM model may also be advantageous in the testing, screening and modifications of biosensors.

The present study revealed that Myricetin inhibited the growth of newly formed blood vessels in CAM of chick embryo, indicating the anti-angiogenic property and this property of Myricetin was checked *in-vivo* with vessel formation and expression analysis.

MATERIALS AND METHODS

Fertilized White leghorn chicken eggs (gallus gallus) were purchased from Tamil Nadu Veterinary and Animal Sciences University, Madhavaram, Chennai, India. Fertilized chicken eggs were incubated at 37°C in a humidified atmosphere (>60% relative humidity) [22] based on protocol for the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) method.

Dropping the CAM: At day 3 of post incubation, 2 ml of albumin was withdrawn, using a 21-gauge needle, through the large blunt edge of the egg in order to minimize adhesion of the shell membrane with CAM. A square window of 1 cm² was opened in the egg shell and sealed with paraffin film to prevent dehydration and the eggs were returned for incubation.

Drug administration: For the angiogenesis assay, Myricetin (SIGMA) dissolved in DMSO. The final concentration of DMSO was 0.1%. Chick embryos treated with 10µl of 1X PBS was used as drug carrier control. CAM was exposed to different concentrations of Myricetin from 25µM to 200µM for 48h. The window was closed with a transparent adhesive tape and the eggs were returned for further incubation for 48hrs at which vascularization potential of the CAM reaches its maximum. The developing vasculature on CAM was imaged with a digital camera and exported to a computer for image analysis. The same was confirmed with direct stereomicroscopic observations of well spread embryonic plates on glass slides of appropriate dimensions.

Histological preparation: For histological evaluation, the CAM tissues removed from chicken embryos were fixed in 10% buffered neutral formaldehyde with Calcium Acetate Formalin for 8 hrs. The membrane was immersed on increasing concentrations of ethanol for dehydration and was embedded in paraffin (melting point 58-60˚C). Serial sections (500µm) were cut in a plane parallel to the surface of the CAM and further processed for stained preparation of haematoxylin-eosin [23], which was observed under a light photomicroscope.

Analysis of VEGF mRNA expression by Real Time- polymerase chain reaction (RT- PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA) and reverse transcribed into cDNA using the SuperScript 3 First Strand cDNA Synthesis Kit (Invitrogen). The primer sequences for specific gene amplification are shown in Table 1. Real-time PCR was performed according to the standard protocol for the SYBR Premix Ex TaqTM Perfect Real Time system (Takara, Dalian, China) using an ABI 7300 detector (Applied BioSystems, CA, USA). 18s ribosomal RNA was used as an internal control.

RESULTS

Effects of Myricetin on neovascularization in CAM assay: The chick embryos were exposed to different concentrations of Myricetin from 25µM to 200µM for 48h (Fig.1). After Myricetin addition, the

embryos were closed with a transparent adhesive tape and the eggs were returned for further incubation for 48hrs. The pictures were captured under dissecting microscope. In (Fig.2), the vessels were confirmed with direct stereomicroscopic observations.

Haematoxylin-eosin (H&E) staining: Normal histology of CAM (H&E staining): The CAM consists of the ectoderm (interface with air/shell), stroma (loose connective tissue), and endoderm (interface with allantois) and is approximately 500µm thick in (Fig. 3), and quantification of vessel inhibition rate as shown in (Fig. 4).

Analysis of VEGF mRNA expression by Real time polymerase chain reaction: In (Fig. 5), treated embryos for 48hrs with Myricetin (150µM and 200µM) significantly decreased the expression of VEGF-A mRNA in a dose dependent manner. The data indicated that Myricetin exerted anti- angiogenic actions possibly via down- regulation of VEGF-A expression in CAM embryos**.**

DISCUSSION AND CONCULSION

The new pharmacological effect of Myricetin has been confirmed with the inhibition of angiogenesis on CAM in developing embryonated chicken eggs. In this study it is demonstrated that Myricetin inhibits the neovascularization in CAM of embryo. Some studies reported that flavonoids, including Myricetin, are able to protect hippocampal cells against toxic effects induced by retenome [12]. It has also been indicated that myricetin possess neuroprotective effects on the Parkinson models in *in-vivo* and *in-vitro* by anti-oxidation and anti-apoptotic activity [13]. There are scanty reports about anti-angiogenic activity of Myricetin and hence the present study evaluated the anti-angiogenic effect of Myricetin using *in-vivo* CAM angiogenesis model. Angiogenesis is controlled in normal human body and regulated by the system of angiogenesis growth inhibitors. Angiogenesis involves the degradation, proliferation, elongation and remodeling of the existing blood vessels. Several growth factors like VEGF and FGF-2 and also signaling pathways play an important role in regulation of angiogenesis [24,25,26,27]. From quantitative, macroscopic and microscopic analysis of CAM tissue, it was observed that Myricetin has anti-angiogenic potential. The chorioallantoic membrane (CAM), a specialized, highly vascularized tissue, serves as an indicator of the anti-angiogenic properties of compounds. The CAM is a useful tool to studying angiogenesis because 1) it is amenable to both intravascular and topical administration of study agents, 2) it is a relatively rapid assay, and 3) it can be adapted very easily to study angiogenesis-dependent processes, such as tumor growth. Its low cost, easy accessibility, anatomic features, physiologic and histological responses to manipulation and injury make it potential model for anti angiogenesis study. The CAM model is an *in vivo* system that can be used as the proven mammalian model. As the regulation of gene VEGF-A confers the normal angiogenesis process in the body so in this study the expression level of gene was studied with real time PCR and the statistical analysis revealed that there is highly significant difference between the expression of control gene 18 S ribosomal RNA and VEGF-A. So from the study it is concluded that there is down regulation of the VEGF-A gene indicating inhibition of formation of new blood vessels.

Our present study showed that Myricetin inhibited the formation of newly synthesized blood vessels in CAM of and down-regulated the expression of VEGF-A gene in CAM of developing embryonated chicken eggs.

ETHICS

The samples preparation and collections as per the approved standard protocols of Institutional Animal ethics and Biosafety committee **Lr.no: 1252/E/DFBS/IBSC/2011, Agenda-4.** The study was carried out during 2011 in the department of Animal Biotechnology, madras Veterinary College, Vepary.

ACKNOWLEDGEMENT

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Table 1: Primer Sequences:

Genes	Forward primers (5'⟶3')	Reverse primers (5'⟶3')
VEGF-A	AGAATGTGTCCCTGTGGATGTG	GCGCTATGTGCTGACTCTGATG
18s rRNA	CTCAACACGGGAAACCTCAC	CGGACATCTAAGGGCATCAC

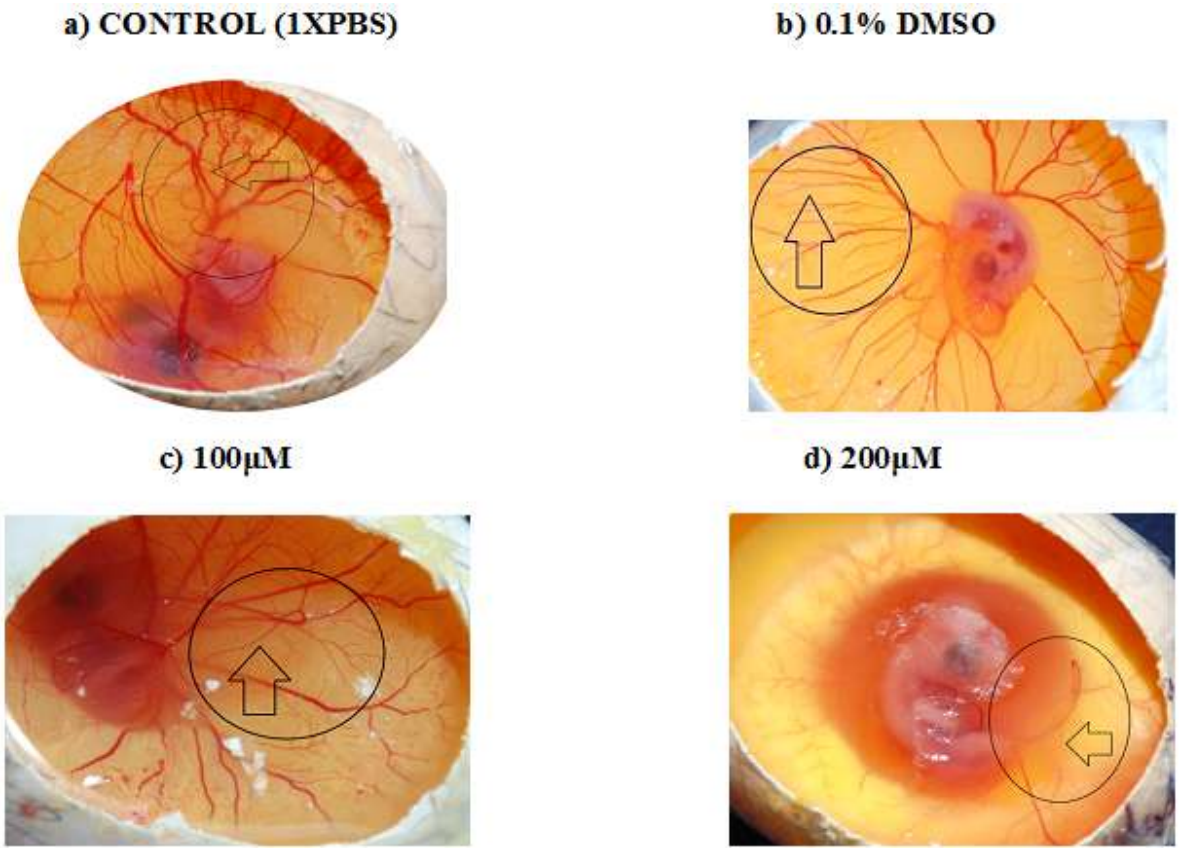

Fig.1: Macroscopic Observation of CAM treated with Myricetin along with control.

It shows that Anti-angiogenic activity of Myricetin using CAM embryo model. The chick embryos were treated for 48hrs with Myricetin (A) 1X PBS, (B) 100µM, (C) 200µM. The embryos treated with Myricetin upto 100µM concentration were no morphological changes but higher than 200µM concentration caused lethality. Myricetin inhibited the neovascularisation in a dose-dependent manner. Arrows indicate the inhibition of neovascularization in CAM. The developing vasculature on CAM was imaged with a digital camera. In **(Fig.2)**, the vessels were confirmed with direct stereomicroscopic observations.

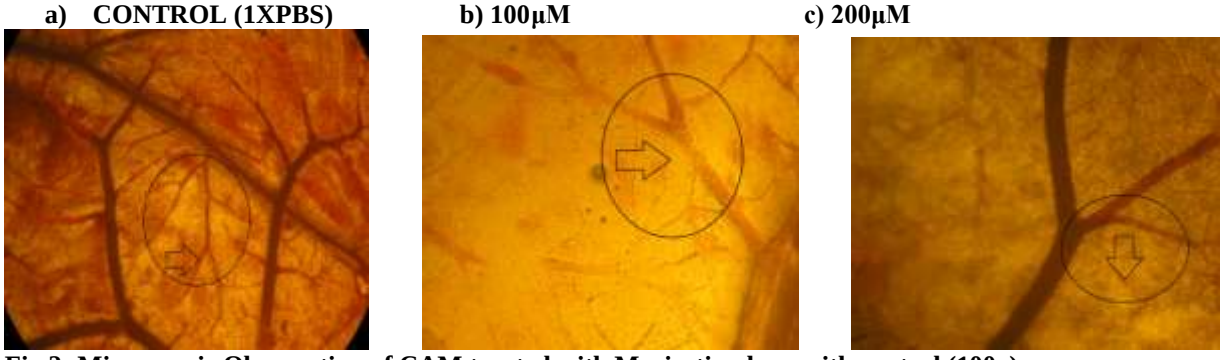

Fig.2: Microscopic Observation of CAM treated with Myricetin along with control (100x)

It shows that the reduced formation blood vessles in the CAM treated with 100µM concentration of myricetin and also still reduction in blood vessels at 200µM concentration.

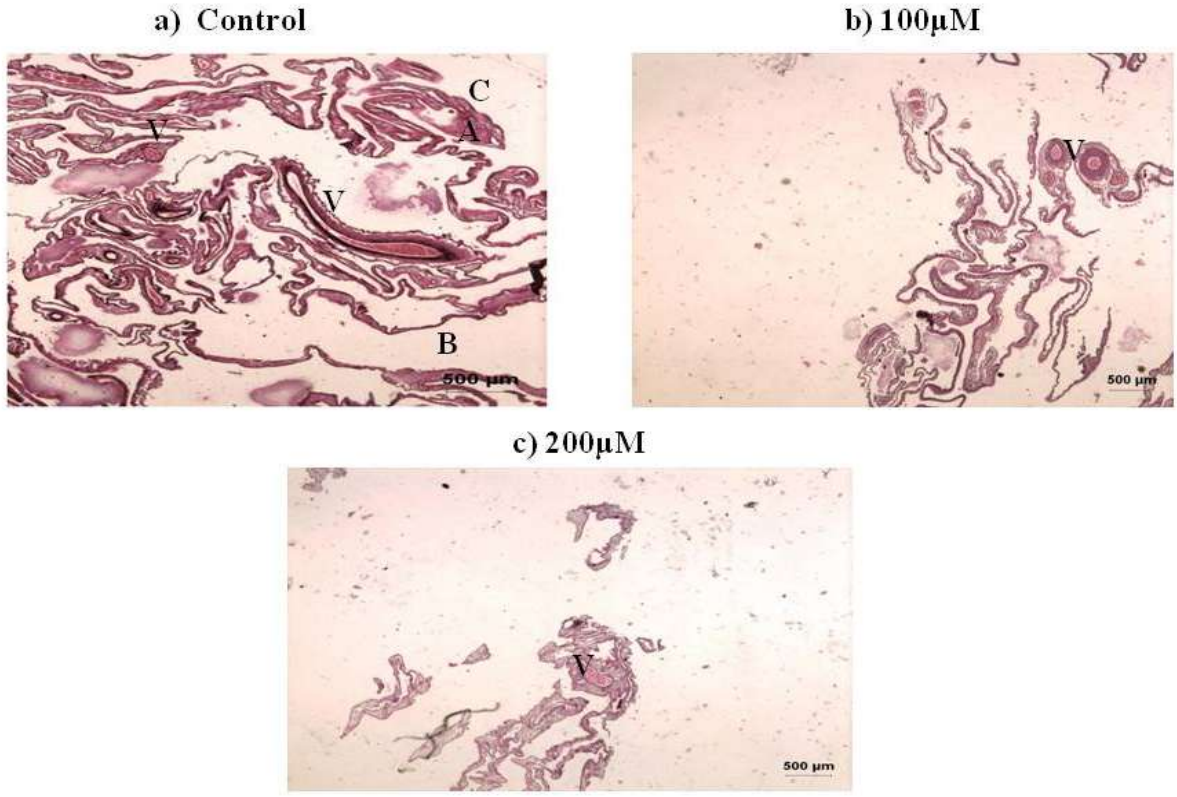

Fig.3: Histological Cross section of CAM by H&E staining method

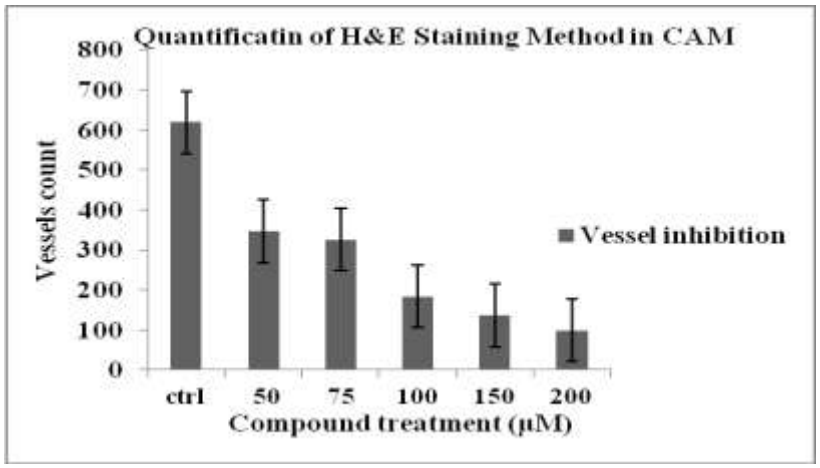

Fig. 4: Quantification of Vessel inhibition rate in H&E staining Method

Fig. 3 & 4: Chick chorioallantoic membrane (CAM). Histologic cross-section of mature chick CAM demonstrating large and small blood vessels (V), primary stratum (A), thin stratum (B), and inner shell membrane (ISM) (C). A capillary plexus/blood sinus exists between the primary (A) and thin (B) layers. Some erythrocytes in this plexus/sinus are faintly visible as circular bulges around the thin stratum (B). Histological cross-section of Myricetin treated chick chorioallantoic membrane (CAM) reduced the large and small newly synthesized blood vessels at 200µM concentration.

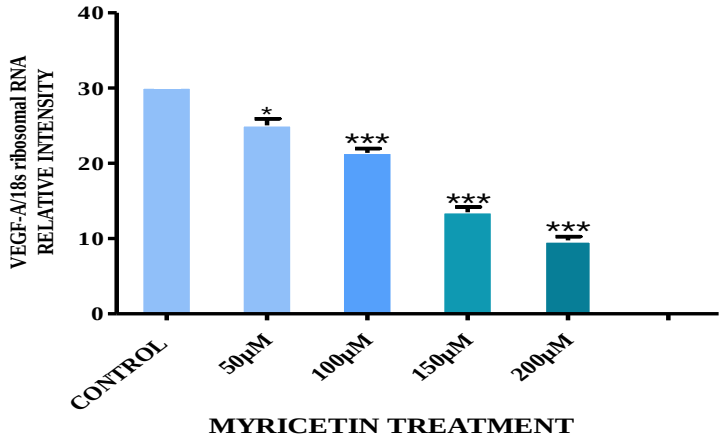

Fig.5: VEGF m-RNA expression Analysis

It shows that Dose-response of Myricetin on the expression of VEGF-A in chick embryo. 18s ribosomal RNA was used as internal control. The expression levels are first normalized to internal control and then expressed as the percentage of control. The 200µM of Myricetin treated embryos down regulated the VEGF-A formation compared to that of control. Each value represents the mean ± S.E.M (n=3) from a representative experiment. The data were analysed by one-way ANOVA followed by the Student Newman–Keul's test, *** indicate highly significant and * denotes significance p>0.05.

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