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Anti-angiogenic_ activity_ of_ myricetin_ through_ VEGF-A down_ regulation in_ zebrafish (danio rerio)_*in-vivo*

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ABSTRACT

Myricetin is a natural flavonol; it possesses anti-oxidative, anti-proliferative, and anti-inflammatory effects. The molecular mechanisms responsible for its anti-angiogenic activity have not yet been elucidated. The anti-angiogenic properties of Myricetin were studied using Zebrafish embryo (Danio rerio) maintained *in-vivo*. The inhibition of newly synthesized blood vessels was evaluated by RBC- staining method and the Myricetin effect on the activity of VEGF-A was evaluated by RT- PCR analysis. Myricetin in Zebrafish embryos significantly inhibited the formation of new blood vessels in a dose-

dependent manner. Myricetin treatment attenuated the blood vessel formation documented through special staining. The inhibition was associated with down-regulation of VEGF-A through Real Time- PCR quantification of mRNA expression followed by the confirmation by Western blotting analysis. The results showed that Myricetin inhibited angiogenesis in the developing Zebrafish embryos through the blocking of newly synthesized blood vessels by down regulated expression of VEGF-A.

Keywords: *Zebrafish, Myricetin, Vascular Endothelial Growth Factor (VEGF), Angiogenesis, Anti angiogenesis*.

INTRODUCTION

Angiogenesis is a process of neovascularization, a complex, regulated, and multi-step physiological process which initiates a protease-mediated degradation of the basement membrane, loss of endothelial cell adhesion, proliferation and migration of endothelial cells into the surrounding stroma, and finally reassembly of endothelial cells to form the lumen of the new blood vessels (1). Excessive angiogenesis is closely related to many human diseases, such as tumor growth and metastasis, retinopathy and inflammation (2), (3). 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 has been observed in many cancers, and is associated with worse survival. VEGFR-1 is required for the recruitment

of hematopoietic stem cells and the migration of monocytes and macrophages while VEGFR-2 regulates vascular endothelial proliferation, migration and invasion and VEGFR-3 mediates lymph angiogenesis (4). VEGF and VEGF receptors have been implicated in the angiogenesis that occurs in many solid tumors like breast cancer (5), colon cancer (6), hepatoma (7), bladder cancer (8), gastric cancer (9) and prostate cancer (10). Several strategies have been developed for targeting the VEGF-signaling pathway as a part of anticancer therapy (11). Over expression of angiogenic factors in particular VEGF and bFGF in most hematologic malignancies result in increased angiogenesis and show poor prognosis as well as decreased overall survival (12). Anti-angiogenic therapeutic

approaches have recently been shown to be effective for the treatment of certain cancers.

Anti-angiogenesis agents are reported to cause reduction in the blood flow or vascular permeability, in many types of cancer. Anti-angiogenic therapy falls into three main categories: (1) monoclonal antibodies directed against specific angiogenesis growth factors and / or their receptors; (2) small molecule tyrosine kinase inhibitors; (3) agents with other mechanisms of action, including the inhibitor of mTOR (mammalian target of the rapamycin). Anti-angiogenic drugs exert therapeutical effects by blocking certain specific receptors. The application of anti-angiogenic compounds in cancer treatment is relatively new.

SU5416, a selective inhibitor of the tyrosine kinase activity of the vascular endothelial growth factor (VEGF) receptor Flk-1/KDR, is currently in Phase III clinical trials for the treatment of advanced malignancies, used as a positive control. SU5416 is a small molecule drug prevents formation of new blood vessels required for tumor growth by blocking the Flk1/KDR (13).

Several polyphenolic compounds are recognized as a potent cancer chemopreventive agent. Flavonoids are the most abundant polyphenols in human diets. Many research on flavonoids proved their anti-cancer activity on various cancer. Flavonoids suppress tumor cell growth via cell-cycle arrest and by induction of apoptosis in several tumor cell lines (14). Flavonoids possess anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, anti-

thrombotic, anti-viral, anti-carcinogenic and anti-angiogenic activity (15).

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 (16),(17). It possesses anti-oxidative, anti-proliferative, and anti-inflammatory effects (18),(19). Some studies reported that flavonoids, including myricetin, are able to protect hippocampal cells against toxic effects induced by retenome (20). It had also been indicated that myricetin possess neuro protective effects on the Parkinson models *in-vivo* and *in-*

vitro by anti-oxidation and anti-apoptosis activity (21). Recent studies revealed that myricetin directly binds to MEK1 and JAK1 and thus inhibiting cell transformation.

Zebrafish (Danio rerio) has emerged as a valuable model organism in drug discovery processes, including target identification, disease modeling, lead discovery, and compound toxicology. The major advantages of a zebra fish based assay are obvious: (1) hundreds of compounds can be tested simultaneously using a microplate format, (2) the assay is relatively cost effective, fast, truly quantitative and suitable for large- scale screening, and (3) embryo maintenance, compound addition and embryo assessment are technically simple (22), (23), (24). It has a high genetic homology with humans over 85% as well as important parallels in

organogenesis and functional mechanisms (25). During the zebrafish embryonic development, the intersegmental vessels (ISVs) are formed from the main artery, the dorsal aorta (DA), through an angiogenesis mechanism between 16–19 h postfertilization (hpf) (22), (26). Vasculogenesis in zebrafish, as in other vertebrates, involves the differentiation of hemangioblasts from mesoderm, with subsequent differentiation of angioblasts and endothelial cells (27).

The present study employs developing zebrafish (Danio rerio) embryos as an *in-vivo* vertebrate model for studying inhibitors of angiogenesis. Our result showed that Myricetin, as a potent inhibitor of angiogenesis by down-regulating VEGF-A in zebra fish embryos.

MATERIALS AND METHODS

Embryo collection

Embryos were generated by natural pair wise mating, as described by (28) and staged according to (29). The embryos were maintained in embryo medium at 28° C until the somite stage. Healthy embryos were then dechorionated at 24 hours post fertilization (hpf) immediately prior to drug treatment by incubating with 1 mg/ml protease at room temperature for 3 min. Dechorionated embryos were then used for further analysis. Embryo stages were given as hours post fertilization (hpf).

Drug administration:

SU5416 Treatment

SU5416 (obtained from Gerald McMahon of SUGEN, Inc., South San Francisco, California, USA) (30). Embryos were exposed to different

concentrations of SU5416 from 0.1μM to 2μM for 48 h at 28˚C were used as a positive control.

Myricetin Treatment

For the angiogenesis assay, 99% pure Myricetin (Sigma) was used. Twenty four hour post- fertilization the embryos were exposed to different concentrations of Myricetin, which was added directly to embryo water from 10μM to 25μM. Zebrafish embryos treated with 0.1% DMSO (Dimethylsulphoxide) were used as drug carrier control.

RBC Staining

o-dianisidine staining was used to study the expression of globin (31). On 3th day during the development, embryos were fixed in 4% paraformaldehyde for half an hour and washed

with PBS (Phosphate Buffer Saline) for 3-4 times. Dechorionated embryos were stained for 30 min in the dark in o-dianisidine 0.6mg/ml, 0.01 M sodium acetate (pH 4.5), 0.65% Hydrogen peroxide, and 40% (v/v) ethanol. After 30 min it is washed with PBS for 3-4 times and examined under the microscope and micro photographed (Nikon).

Analysis of VEGF mRNA expression by Real Time Polymerase Chain Reaction

RT-PCR was carried out in a MX3000p PCR system (Stratagene, Europe). Reaction was performed using Kapa sybr green fast qPCR master mix kit (It contains all the PCR components along with SyBR green dye).The more sensitive and reproducible method of real-time PCR measures the fluorescence at each cycle

as the amplification progresses. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase have started to have an effect on the efficiency of amplification. Template, primers, SyBR Green dye and RNase free water were thawed and placed on ice. The reaction mixture was prepared as follows 5µl Master mix, 2µl forward and reverse primer, 2.5µl Nuclease free water and 0.5µl Template. Reaction mixture was mixed by pipetting up and down for few times. All reactions were performed in triplicate along with no template control (NTC). Reaction mixture was placed in the thermal cycler, which was programmed as followed by 40 cycles (95°C for 30sec, 58°C for 30sec, 72°C for 45sec). Real- time

Polymerase chain reaction was done for target genes (VEGF) and internal control (β-Actinin).

Western Blotting Analysis

Equal amount of total protein was mixed with 2X sample buffer and boiled for 5 min. The protein was separated on 10 % SDS-PAGE and electrotransfered onto a PVDF membrane (Bio-Rad, USA). The blots were blocked with 5% blocking buffer for 4 hours. After blocking, membrane was incubated with respective Zebrafish monoclonal antibodies in 1:1000 dilution overnight at 4˚C. Then the membranes were washed thrice with T-TBS for 10 minutes each, followed by conjugated secondary antibody (1:10,000 dilutions) incubation for 45 min at room temperature. Finally, signals were visualized using Enhanced Chemiluminescent System

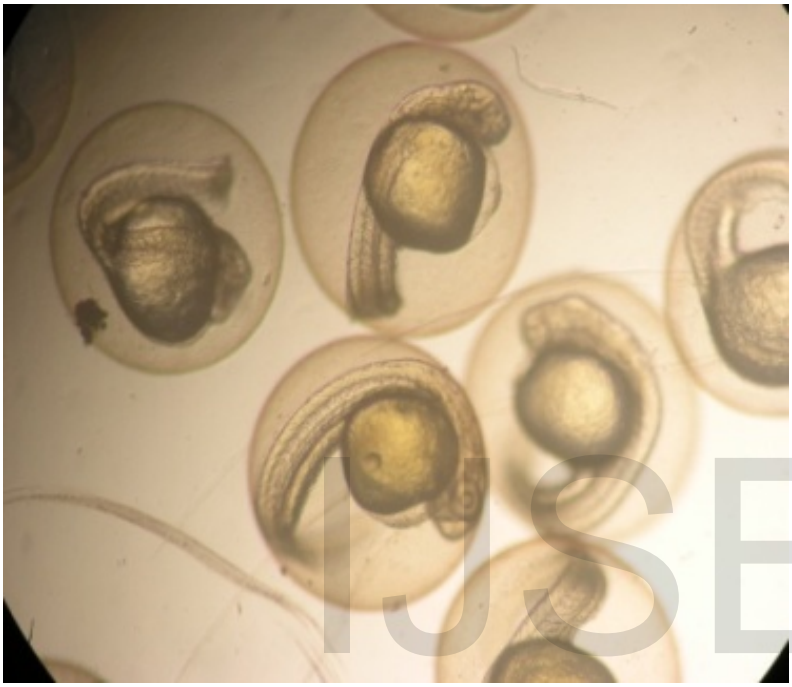
(Pierce Biotechnology Inc, USA) and the signals were captured by Chemi Doc XRS system (Bio Rad, USA) and the intensity of the bands were quantified by Quantity One software (Bio Rad, USA). β -actin is used as the internal control.

RESULT

Morphological Screening

Zebrafish embryos were exposed to different concentrations of Myricetin from 10µM to 25µM for 48h. After Myricetin addition, the embryos were maintained in individual wells of microtitre plates at 28˚C until 72 hpf. After 24 and 48h of Myricetin addition to the wells, embryos were visually inspected for viability, gross morphological defects, heart and circulation. There were no morphological changes observed in the embryos treated with Myricetin upto 10µM concentration but higher than 25µM concentration caused lethality. To study the anti-angiogenic effects of Myricetin, the embryos were dechorionated by protease (1mg/ml) at 24 hpf as shown in Fig.1.

A. Before protease treatment



B. After protease treatment

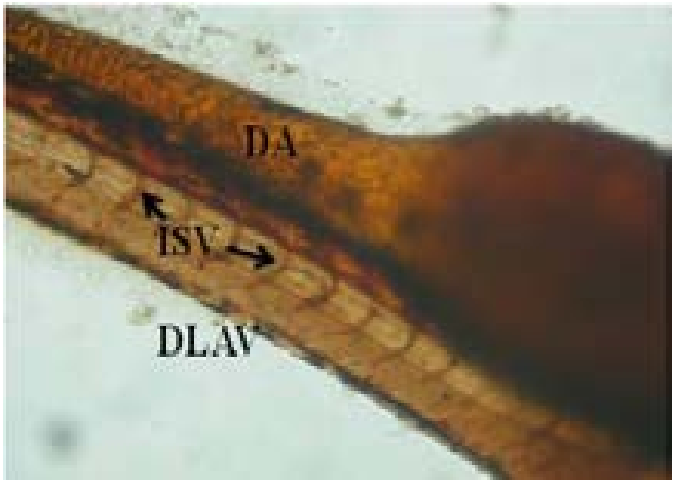
Fig. 1: Dechorionation of zebrafish embryos: At 24 hpf, embryos were treated with protease (1mg/ml) to obtain embryos out of their chorions.

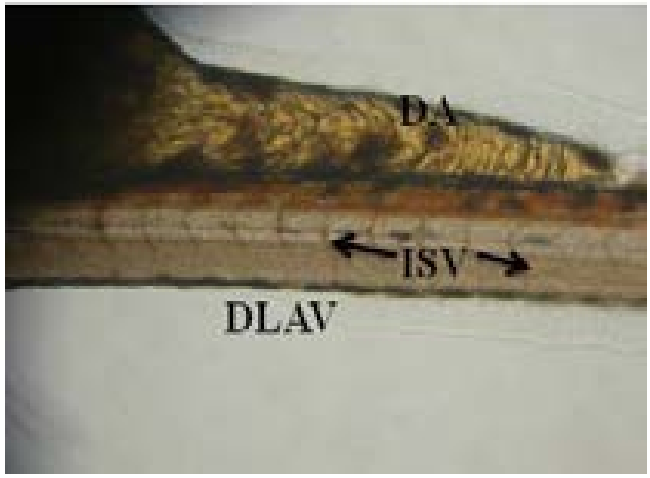
RBC staining of intersegmental vessel (ISV)

Embryos were then exposed to different concentrations of SU5416 (0.1µM to 2µM) and Myricetin (10µM, 15µM, 20µM, 25µM) were stained at 72 hpf with RBC staining as shown in

Fig.2 and Fig.3. RBCs in dorsal longitudinal anastomotic vessel (DLAV), dorsal aorta (DA) and intersegmental vessel (ISV) were clearly seen in control embryos (0.1% DMSO). In SU5416 treated embryos ISV region is completely inhibited in 2μM but in Myricetin treated embryos, RBCs were observed only in DLAV, DA and but not in the ISV region at a concentration of 20μM, which indicates that ISV formation is inhibited by Myricetin. This clearly demonstrates the anti-angiogenic property of Myricetin.

A) Control B) 0.1% DMSO





C) 2μM

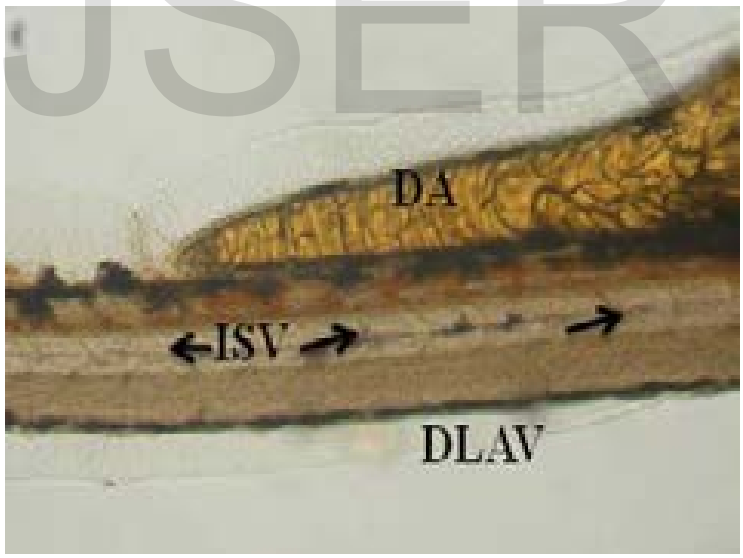
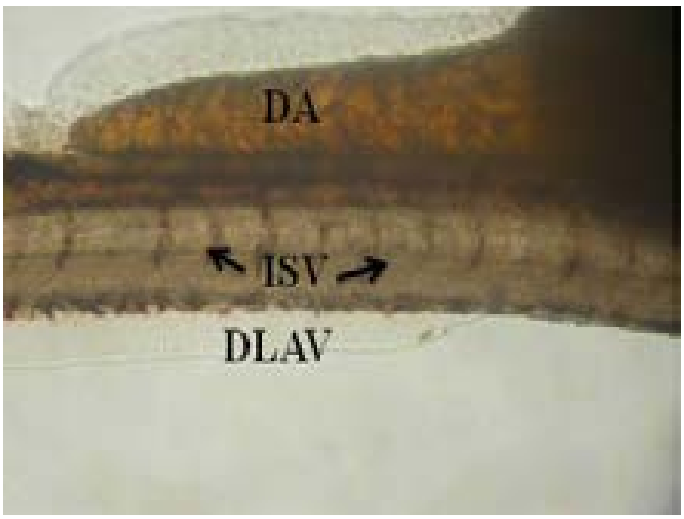


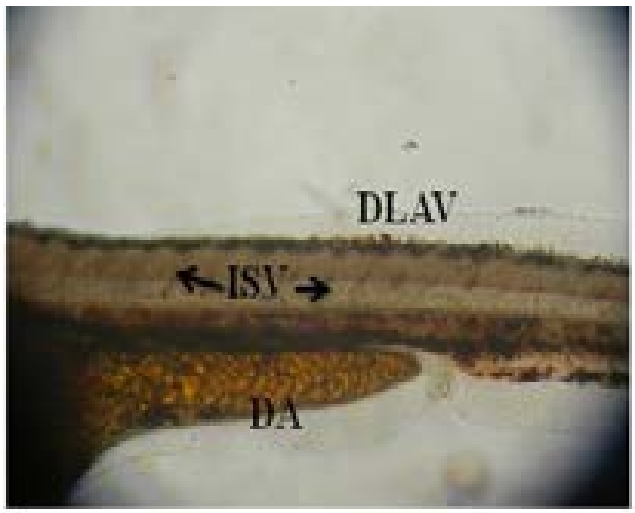
Fig. 2: SU5416 blocks angiogenic vessel formation in zebrafish embryos. Lateral view of RBC stained

embryos at 72 h pf. (A & B) treatment with control and 0.1% DMSO had no effect on vessel formation as indicated by arrows. (C) Treatment with a 2µM concentration of SU5416 caused a reduction of the ISV formation (indicated by the arrow). The SU5416 was a positive control.

Anti-angiogenic activity of Myricetin in Zebrafish Embryo (Danio rerio)

D) 10µM E) 15µM





F) 20µM G) 25µM

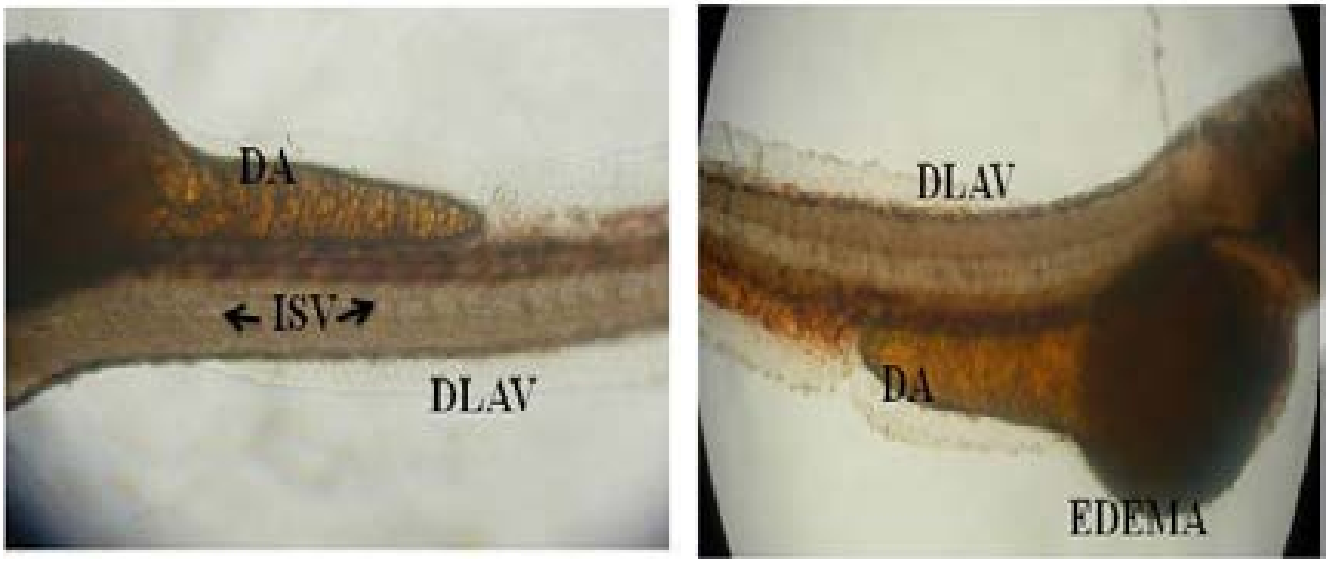


Fig. 3: Anti-angiogenic activity of Myricetin using zebrafish embryo model. Zebrafish embryos were treated with Myricetin concentrations D) 10µM, E) 15µM, F) 20µM, G) 25µM. RBCs in dorsal longitudinal anastomotic vessel (DLAV), dorsal aorta (DA) and intersegmental vessel (ISV) were clearly seen in control embryos as well as 0.1% DMSO (A&B) indicated by arrow. The dorsal longitudinal anastomotic vessel (DLAV), dorsal aorta (DA) and intersegmental vessel (ISV) of

zebrafish embryos are seen in (D & E). F) 20µM treated embryos showed highly inhibition of blood vessels formation (indicated by arrow). G) 25µM Myricetin treated zebrafish embryos showing pericardial edema.

Analysis of VEGF mRNA and protein expression by Real time polymerase chain reaction and Western Blotting

In Fig. 4, treated embryos for 48hrs with Myricetin (20µM and 25µM) significantly decreased the expression of VEGF-A mRNA in a dose dependent manner. In fig.5, the down regulated VEGF- A was confirmed by blotting analysis. The data indicated that Myricetin exerted anti- angiogenic actions possibly via down- regulation of VEGF-A expression in Zebrafish embryos.

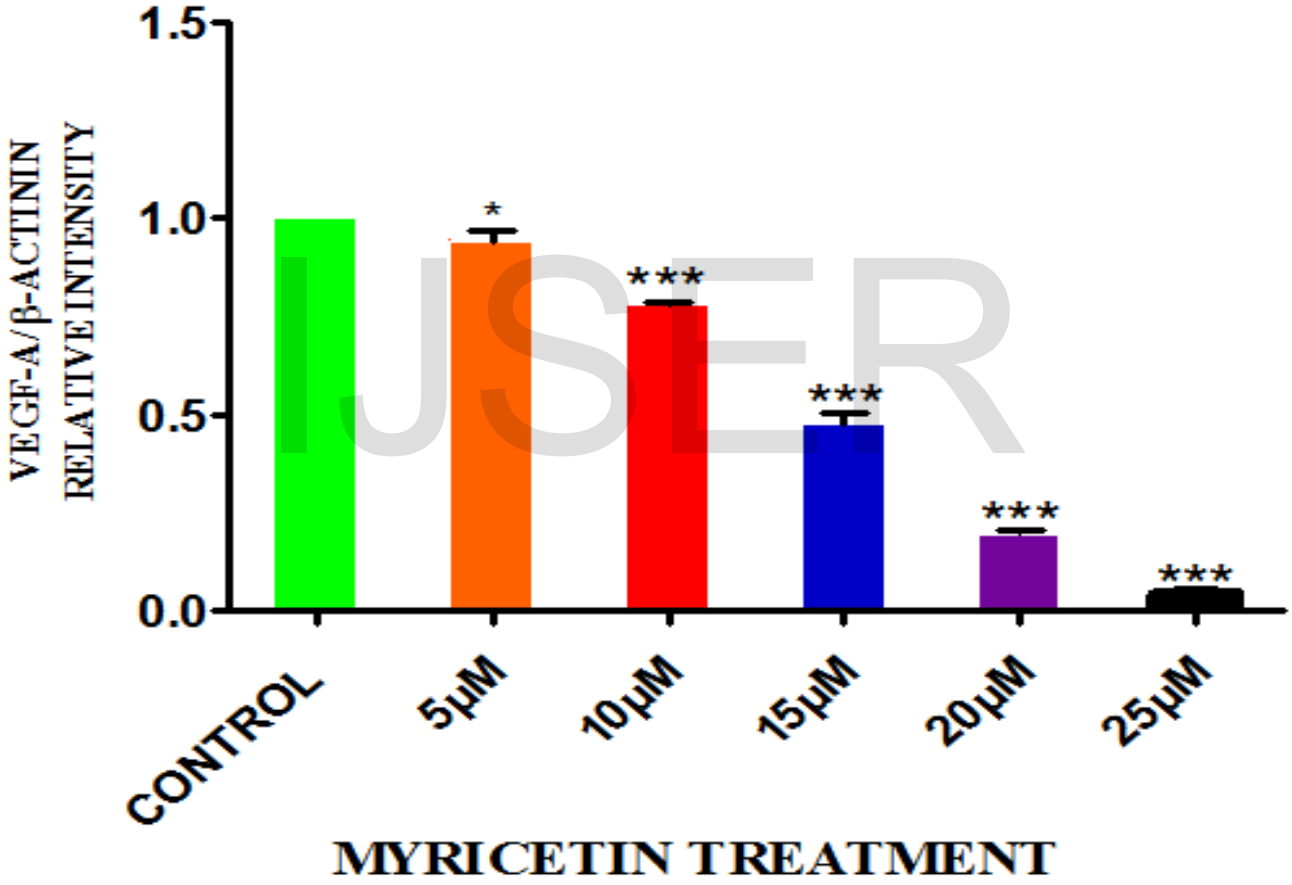
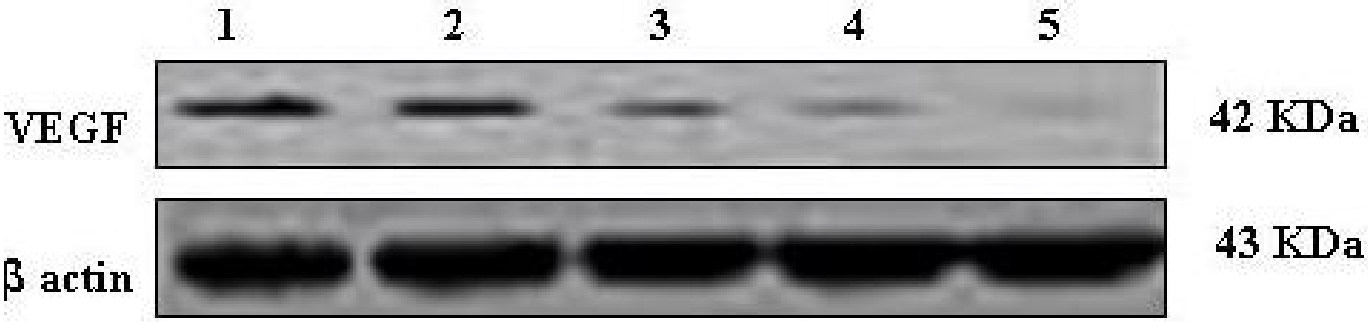


Fig.4: Dose-response of Myricetin on the expression of VEGF in zebrafish embryos. β–actin

was used as internal control. The expression levels are first normalized to β-actin and then expressed as the percentage of control. The 25μM 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.

Fig. 5: Western Blotting analysis



1) Control; 2) 10µM; 3) 15µM; 4) 20µM; 5) 25µM

Fig. 5: The down regulated VEGF- A was confirmed by blotting analysis. The data indicated that Myricetin exerted anti- angiogenic actions possibly via down- regulation of VEGF-A expression in Zebrafish embryos.

Fig. 6: Quantification of Protein

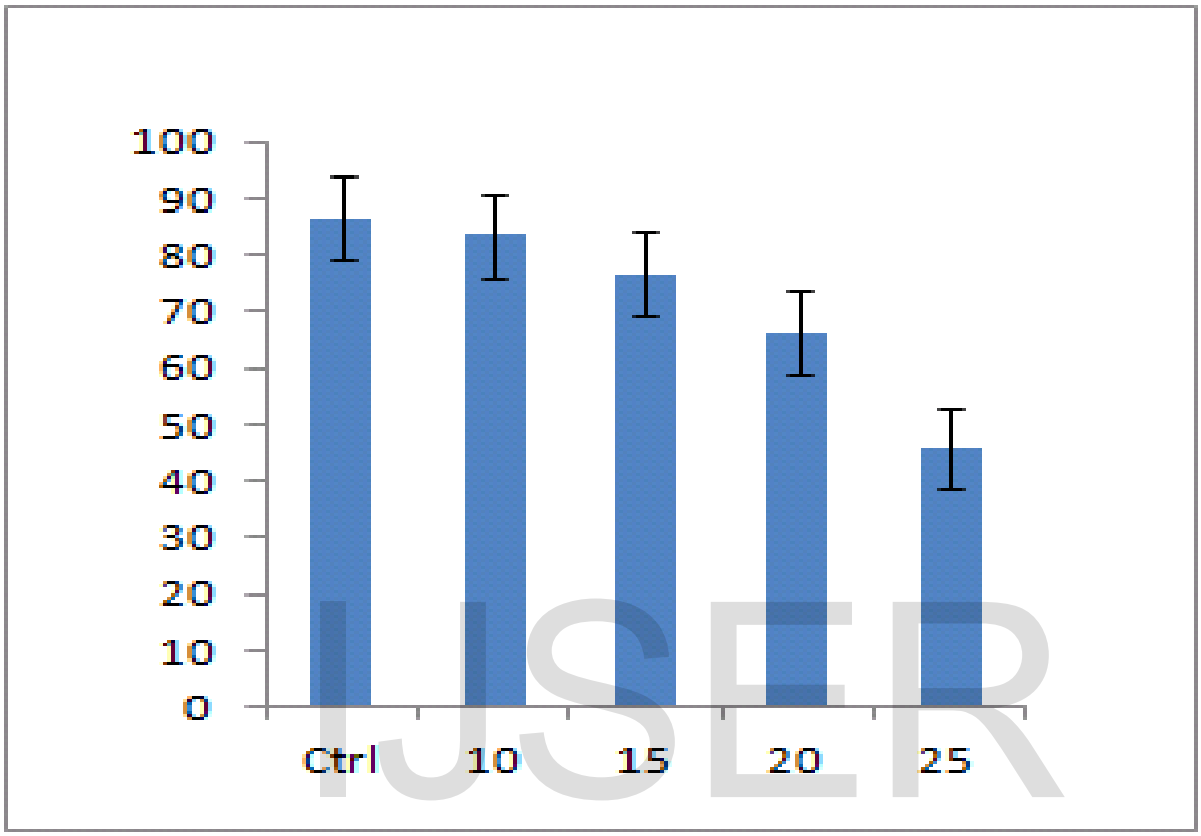


Fig. 6: Dose-response of Myricetin on the protein expression of VEGF in zebrafish embryos. β–actin was used as internal control. The expression levels are first normalized to β-actin and then expressed as the percentage of control. The 25μM of Myricetin treated embryos down regulated the VEGF-A formation compared to that of control.

Table 1: Primer sequences

S. No	Genes	Forward primers (5′⟶3′)	Reverse primers (5′⟶3′)	Temperature (˚C)	Base pair (bp)
1.	VEGF-A	GAGAGCC AGCGACT CACCGCA ACAC	GTTCGCT CGATCA TCATCTT GGC	67.8	623
2.	β-actin	TCCCCTTG TTCACAAT AACC	TCTGTTG GCTTTGG GATTC	53	383

DISCUSSION:

In this study, we have shown that Myricetin inhibits the intersegmental vessel formation in zebrafish embryo. Some studies reported that flavonoids, including myricetin, are able to protect hippocampal cells against toxic effects induced by retenome (20). It also had been indicated that myricetin possess neuroprotective effects on the Parkinson models in *in-vivo* and *in-vitro* by anti-oxidation and anti-apoptotic activity (21). Recent studies revealed that myricetin directly binds to MEK1 and JAK1 and thus inhibiting cell transformation. From these previous findings, it is clear that Myricetin can be used as anti-cancer drug. In this study, the anti-angiogenic effect of Myricetin was evaluated in the zebrafish embryo.

Zebrafish embryos have been widely used as an *in-vivo* vertebrate animal model for

studying inhibitors of angiogenesis. The major advantages of a zebrafish-based assay compared with existing assays are: (1) hundreds of compounds can be tested simultaneously using a micro-plate format (2) the assay is fast, approximately 3 days from addition of compound to assay results (3) embryo maintenance is easy during assay procedures and (4) compound addition and embryo assessment is straight forward due to ease of access. More recently, its value as a model organism for drug target discovery, target validation, drug discovery strategies and toxicological studies has begun to be recognized (32),(33). It has s high genetic homology with humans over 85% as well as important equivalents in organogenesis and functional mechanisms (25). Different growth factors have been identified as positive regulators

of angiogenesis and are secreted by cancer cells to stimulated normal endothelial cell growth through paracrine mechanisms (34). VEGF is a potent and specific mitogen for endothelial cells that activates the angiogenic switch *in-vivo* and enhances vascular permeability (35). Angiogenesis is stimulated by several protein growth factors. Among these, vascular endothelial growth factor (VEGF) family plays a major role. Enhanced expression of VEGF has been observed in human cancers including colorectal, breast, non-small cell lung and ovarian cancers which are directly correlated with increased neovascularization (36).

CONCLUSION:

Zebrafish embryos at 24 hpf treated with Myricetin showed a dose-dependent inhibition of ISV formation compared to vehicle control. For

reference, embryos treated with 2µM SU5416 served as positive control, which showed similar ISV regression. Our present study revealed that Myricetin, a flavonols inhibited ISV formation and down-regulated VEGF-A expression in zebrafish embryos. In addition to drug screening zebrafish embryos are an attractive model for identification of novel therapeutic agents. Myricetin was demonstrated to have potent anti-angiogenic activity *in-vivo* zebrafish embryos. To test for the ISV formation which is formed by angiogenic process, sprouting from dorsal longitudinal anastomotic vessel, RBC staining was performed. They revealed RBCs in no ISV region at the concentration of 20µM of myricetin at 72 hpf embryos through the blocking of newly synthesized blood vessels by down regulated expression of VEGF-A.

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