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Resveratrol suppresses angiogenesis by down-regulating *Vegf/Vegfr2* in Zebrafish *(Danio rerio)* embryos

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ABSTRACT

The anti-angiogenic activity of resveratrol, a stilbenoid found in grapes and peanuts was assessed using zebrafish model. Zebrafish embryos at 50% epiboly stage [5.25 hours post fertilization (hpf)] were treated with (10, 50 and 100μM) resveratrol to evaluate their effects on angiogenesis and also the toxicity effects. Morphological variations including angiogenic phenotypes, hatching, survival and heart rates were examined. The number of apoptotic cells during embryonic development was evaluated using acridine orange staining. Angiogenesis was measured by scoring the development of intersegmental vessel (ISV). *Role of vascular endothelial growth factor VEGF and its receptor VEGFR2 was evaluated by studying their gene expression using Reverse Transcriptase PCR. Resveratrol at 100μM could inhibit the formation of major blood vessels (ISVs) possibly by downregulating VEGF and its receptor VEGFR2. The toxicity assays revealed various morphological deformities including developmental delay, pericardial edema and tail bending, reduction in survival and hatching rates in embryos treated with different concentrations of resveratrol in a dose dependent manner. The downregulation of VEGF and its receptor VEGFR2, which are the primary determinants of growth and blood vessel formation suggest that resveratrol of micromolar (μM) concentration might potentiate its anti-angiogenic activity by targeting genes involved along the VEGF signaling pathway. This study, for the first time, identifies resveratrol to possess anti-angiogenic activity in vivo and suggests that it might have a great potential for future research and development as a therapeutic agent.*

Key words: Resveratrol, Angiogenesis, *VEGF*, Zebrafish.

INTRODUCTION

Angiogenesis, the formation of new blood vessels by sprouting from pre-existing endothelium, is a significant component of a wide variety of biological processes, including embryonic vascular development, differentiation, wound healing and organ regeneration [1, 2], and pathological processes, including tumor progression [1–13]. *VEGF* has been demonstrated to be the fundamental mediator of physiological and pathological angiogenesis [14], and acts through two tyrosine kinase receptors *VEGFR2* and *VEGFR1*. *VEGFR2 (KDR/Flk-1)* has a higher affinity for *VEGF* and is a major transducer of the *VEGF* signal in endothelial cells [15, 16]. There are several angiogenesis inhibitors undergoing phase I or phase II clinical trials, including Monoclonal antibodies targeted against *VEGF* or *VEGFRs* [17, 18], soluble decoy receptors that sequester ligands [19] and small molecule inhibitors that inhibit kinase activity [20]. Inhibition of vascular endothelial growth factor *(VEGF)* and its receptor *(VEGFR)* has been the focus of anti-angiogenic therapies [21], with several small molecular drugs now undergoing clinical trials [22]. Although there are a large number of reports documenting upregulation of *VEGF* mRNA and protein during angiogenesis, but only a little information is available on receptors and the signaling pathways *in vivo* and hence the present study.

Natural products, which include a variety of anti-angiogenic compounds, are given the advantage of proven safety in human diseases. Current knowledge regarding the anti-angiogenic potential of natural products has demonstrated

that flavonoid constituents in Gingko biloba and Genistein (a soy isoflavone) are considered to exert potent anti-angiogenic property [23, 24]. *VEGF* is viewed as an attractive therapeutic target for the development of novel antitumour agents [25]. Another area of intense research is to develop vascular-disrupting agents that can disrupt the existing tumour vasculature and causing tumor cell necrosis [26, 27]. Resveratrol (Res, trans-3, 5, 4'-trihydroxystilbene), a constituent of red wine and vegetables is known to possess antioxidant, antiplatelet-aggregation, anticancer, and anti-angiogenic activities [28]. Recently, resveratrol is reported to suppress angiogenesis by down-regulating *FGF-2* and *VEGF*-induced neovascularization *in vivo* [29]. Reports suggest that resveratrol could inhibit bovine aorta endothelial cell proliferation, migration and tube formation *in vitro* [30]. However the impact of resveratrol on developing embryos or fetus or the teratogenic effect of this compound is not known. In the present study, we have used zebrafish embryonic model to study the phenotypic changes induced by resveratrol when used in higher doses and also to evaluate its anti-angiogenic activity targeting receptor tyrosine kinase possibly by reducing the expression of *VEGF/VEGFR2* along the signaling cascade.

Zebrafish (*Danio rerio*) is fast becoming a powerful model for drug discovery [31, 32]. Many cardiovascular, anti-angiogenic, and anticancer drugs elicit similar responses in zebrafish embryos as in mammalian systems [31]. Zebrafish is extensively used to screen developmental changes because of its small embryo size; large clutch size and permeability to small molecules, further it develops *ex utero*, offering visual access to most stages throughout development [33, 34] and the maternal effects due to small molecules will not affect the embryonic development. In particular, it is a perfect model for studying angiogenesis because the formation of blood vessels could be easily visualized and evaluation of blood flow is extremely easy to score in the zebrafish embryo, making it an attractive model. Circulation begins around 30 hpf and is present in the major blood vessels namely intersegmental vessel (ISV), cardinal vein (CV) and sub interstitial vessels (SIV). It is possible to study the effects of various chemicals on all aspects of vascular formation in a transparent animal [35] and these chemicals can be added directly to the fish water or injected into embryos and the toxicity evaluated.

With the view to evaluate the anti-angiogenic potential of resveratrol and the toxicity effects, we studied changes in the morphology of the embryos including the formation of blood vessels (ISVs) by varying the concentrations (10, 50 and 100µM). Interestingly blood vessels have been regressed and also many phenotypic abnormalities such as yolk sac enlargement, tail bending etc., have been observed at lower concentrations. Thus resveratrol at micromolar (µM) concentration could possibly exert its anti-angiogenic effect through inhibition of *VEGF* signaling and might be used for pharmacological intervention in angiogenic disorders.

EXPERIMENTAL SECTION

2.1. Maintenance of zebrafish and embryos

Zebrafish (wildtype) were obtained from local suppliers and maintained at 28^{o} C on a 14 hours light/10 hour dark cycle in 40 liters glass tanks with 4 females and 8 males in separate tanks. Embryos were collected by natural spawning with 2:1 male to female ratio [36] and staged according to Kimmel et al [37]. Embryo stage is denoted as hours post fertilization (hpf).

2.2. Drug treatment

Resveratrol (Sigma, USA) was dissolved in DMSO at stock concentrations of 5mM and then diluted with dose concentrations of 10µM, 50µM and 100 µM directly to the embryo media in a 6 well culture plate in which the synchronized embryos at 50% epiboly stage, were arranged by pipette, 20 embryos per well containing 2ml of embryo medium in each well. To study vessel formation and gene expression, different set of embryos were treated with resveratrol of dose concentrations 10, 50 and 100µM. Control embryos were treated with 2ml embryo medium with < 1% DMSO.

2.3. Morphology of zebrafish embryos

The embryos were maintained in individual wells of culture plates at 28˚ C until 72 hpf after drug treatment, and were visually inspected for viability, hatching rate, gross morphological defects and circulation. Phenotypic variations, survival rates and hatching rates of embryos with or without chorion under both normal and treated with resveratrol after 24, 48 and 72 hpf were examined using stereo-microscope (Euromax). Images were recorded and stored using a digital camera and image acquisition software attached to a computer.

2.4. Acridine orange staining

Acridine orange (AO) is a vital dye often used as a marker of apoptotic cells in zebrafish [38]. The occurrence of apoptotic cells during the first 4 days of zebrafish embryonic development has been studied and quantified previously by using acridine orange staining. The live embryos at 72 hpf were dechorionated and soaked in embryo medium containing 2 mg/ml acridine orange at 28ºC for 30 minutes. Embryos were washed with embryo medium

eight times 5 minutes each, anesthetized with tricaine, and fluorescent signals were detected using fluorescence microscope.

2.5. RBC staining

Staining of red blood cells (RBC) was performed by incubating embryos at 72 hpf in with o-dianisidine (Sigma, USA) after treating with resveratrol. Embryos were dechorionated with protease (1 mg/mL). Incubated for half an hour at 28°C and then washed using embryo medium for 3-4 times. Fixed in 4% paraformaldehyde for half an hour at RT and washed with PBS for 3-4 times. Fixed embryos were stained in dark using o-dianisidine (0.6 mg/mL), 0.01M sodium acetate (pH 4.5), 0.65% H_2O_2, and 40% (v/v) ethanol. After 30 min they were washed with PBS for 3-4 times and examined using compound microscope (Nikon Eclipse E200, USA) and photographed.

2.6. Total RNA extraction and reverse transcription

Total RNA was extracted from 120 zebrafish embryos (72 hpf) of each treatment group using the Tri reagent (Sigma, USA) in accordance with the manufacturer's instructions. RNA was reverse transcribed to single-strand cDNA using MMuLV Reverse transcriptase (50µg/µl) [NEB, Hitchin, United Kingdom]. PCR was carried out using Amplicon Master Mix kit. Primers sequences used in the study are given in Table 1.

Table 1: Primers used in the study

Primers	Forward primer	Reverse primer
VEGF-A	5'-ctcctccatctgtctgctgtaaag- 3'	5'-ctctctgagcaaggctcacag-3'
VEGFR2	5'-ggtgaagaaggacgatgagg- 3'	5'-acaggaatgttgctgctgct-3'
β-Actin	5'-ttcaccaccacagccgaaaga-3'	5'-taccgcaagattccataccca-3'

RESULTS

3.1. Phenotypic abnormalities

Zebrafish embryos treated with resveratrol of various concentrations (10, 50 and 100µM) generated a series of phenotypic variants with developmental delay, the most prominent being delayed hatching, pericardial edema and tail bending. Control embryos hatched normally after 48 hpf; whereas embryos treated with 100µM concentration did not hatch even after 72 hpf (Figure 1 H and L). Tail bending was another prominent phenotype; 50µM treated embryos possessed tail bending and tail kinking (Figure 1 G and K respectively). Enlargement of pericardial sac was observed in few embryos at 10µM concentration (Figure 1 J).

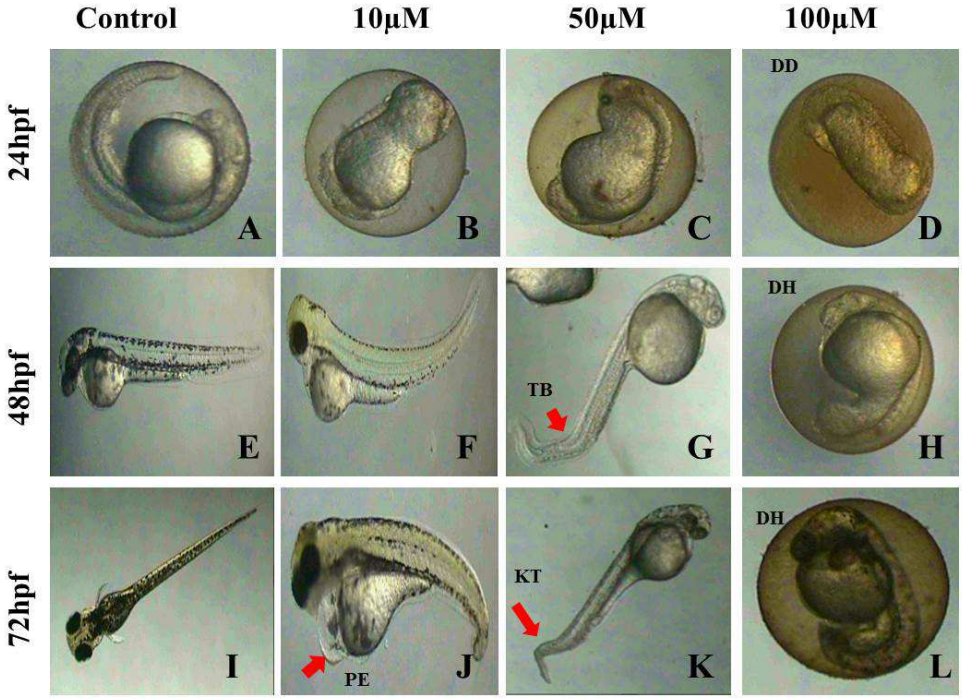


Figure 1: Morphological features of resveratrol treated embryos at 24, 48 and 72 hpf.
(A, E and I) control group, (B, C and D) developmental delay, (F) Whole body curvature, (G and K) Tail bending and Tail kinking respectively (arrow), (H, L) Delayed hatching, (J) Severe pericardial edema (arrow).

Survival rates also decreased at higher concentrations (30% decrease at 100µM concentration) (Figure 2) suggestive of toxicity effects produced by resveratrol. Hatching rates of embryos treated with 100µM resveratrol were significantly lower (90%) than control (Figure 3). Heart rates decreased with increasing concentration of resveratrol, control had 185 beats/min at 72 hpf whereas 100µM treated embryos had only 155 beats/min. Resveratrol significantly affected the heart rates in a concentration dependent manner.

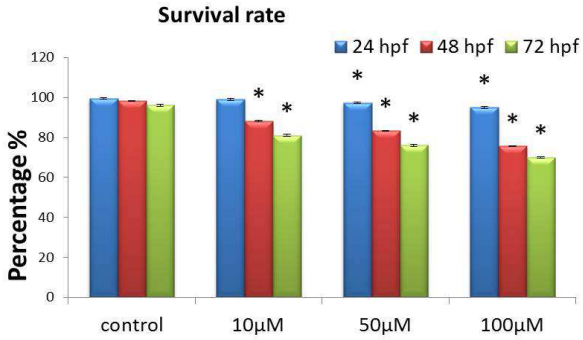


Figure 2: Embryos (at 50% epiboly stage) were incubated in various concentrations of resveratrol, and the survival rates were recorded at 24, 48 and 72hpf. Survival rate of embryos decreased in concentration dependent manner. The death of an embryo was defined as no visual heart beat. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus compounds treatment groups at p<0.05 level using Student's-Newman-Keul's test.

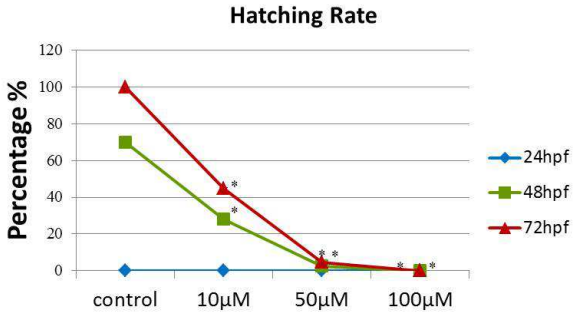


Figure 3: Embryos (at 50% epiboly stage) were incubated in various concentrations of resveratrol, and the hatching rates were recorded at 24, 48 and 72hpf. Hatching rate of embryos decreased with increasing concentration of resveratrol. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus compounds treatment groups at p<0.05 level using Student's-Newman-Keul's test.

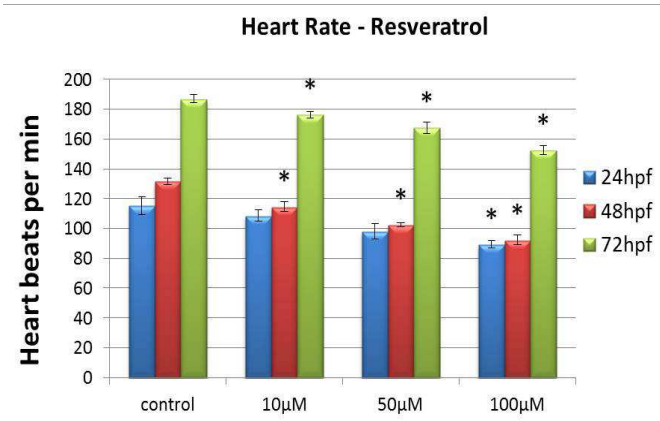


Figure 4: Embryos (at 50% epiboly stage) were incubated in various concentrations of resveratrol, and the heart beats/min were recorded at 24, 48 and 72hpf. Heart rate of embryos decreased in concentration dependent manner. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus compound treatment groups at p<0.05 level using Student's-Newman-Keul's test.

Our study also manifested that inhibition of *VEGF/VEGFR2* in zebrafish embryos could result in severe developmental defects. Resveratrol affected the development of zebrafish in a highly significant manner. The areas primarily affected were tail, heart, yolk sac. In our previous studies using zebrafish, theophylline, a methylxanthine induced several abnormal phenotypes with bent tail, malformed eyes or no eye development; developmental delay with complete absence of vasculature was also observed [39] and Genistein, a naturally occurring isoflavonoid in soybeans is also found to inhibit the growth of intersegmental vessels in zebrafish demonstrating its anti-angiogenic activity [40].

3.2. Acridine orange staining

To clarify whether the reduction of cell viability and anti-angiogenesis at high concentrations of resveratrol is related to apoptosis, we stained the embryos with acridine orange. Embryos were subjected to various concentrations of resveratrol (10, 50 and 100µM) for 72 hpf and subjected to acridine orange staining. The developmental defects observed in brain, eyes, tail etc., could be due to several possible mechanisms such as increased cell death, reduced cell proliferation and endothelial cell migration. Control embryos showed no changes or less apoptotic cells (Figure 5 A and E) whereas minimal changes were recorded with 10µM resveratrol (Figure 5 B and F), but intense staining was observed towards the yolk sac in many embryos when treated with 50µM resveratrol (Figure 5 G). Excessive

staining was observed in the tail region especially towards the caudal fin in embryos treated with 100µM resveratrol at 72 hpf (Figure 5 D and H).

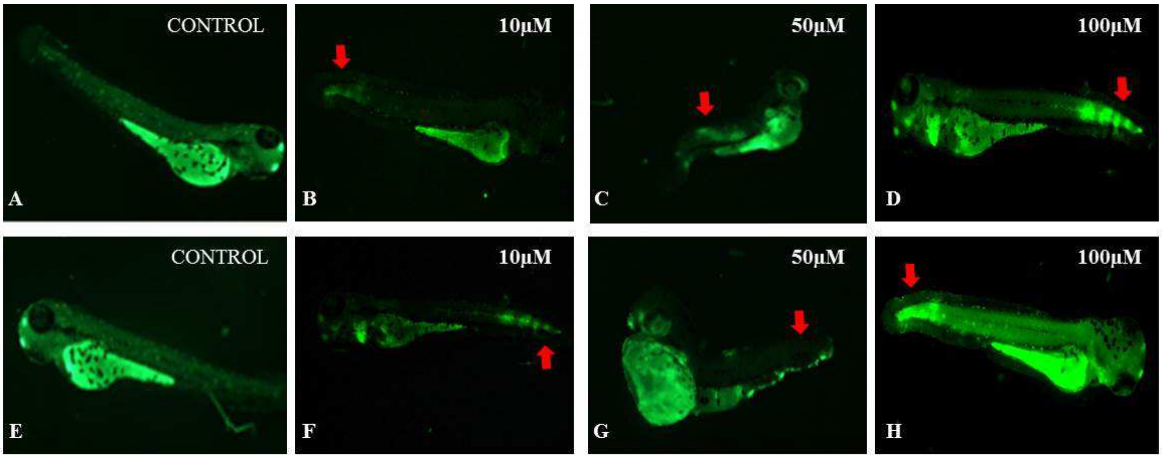
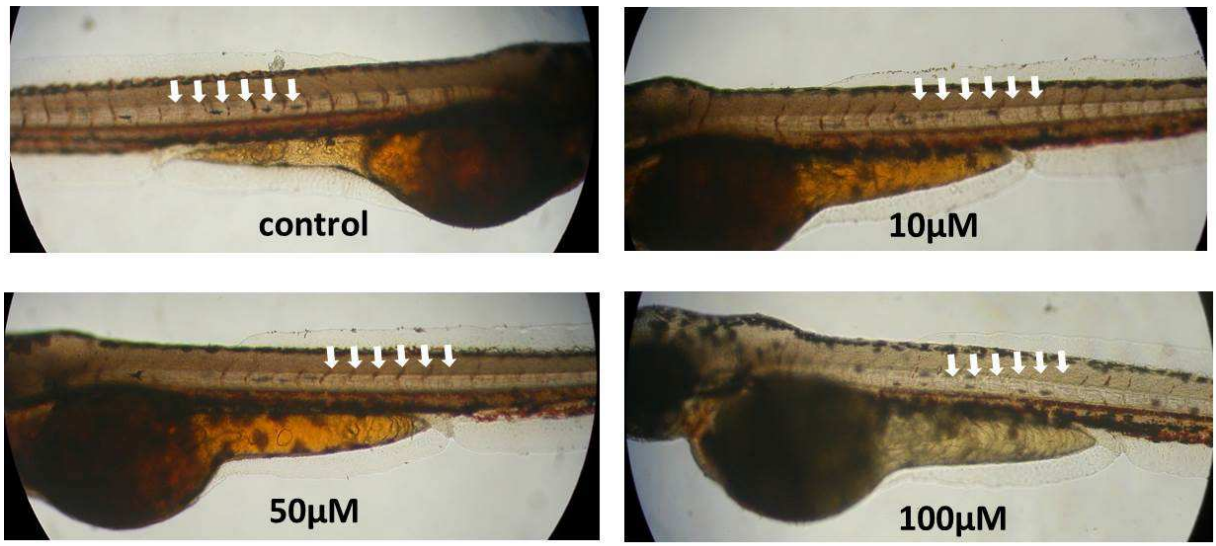


Figure 5: Control embryos showing no or less apoptotic cells, whereas 50µM resveratrol treated embryos shows few apoptotic cells in tail and yolk region and 100µM treated embryos shows increased apoptosis in tail.(red arrow)

Acridine orange (AO) exhibits metachromatic fluorescence that is sensitive to DNA conformation, making it useful for detecting apoptotic cells [41]. AO is a cell-permeant nucleic acid intercalating dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. Because the intact membrane of live cells excludes charged dyes such as propidium iodide (PI), short incubation with this dye results in selective labeling of dead cells, while live cells show minimal uptake [42]. In our study, acridine orange staining revealed the apoptotic potential of resveratrol in which the tail region was mostly affected when compared to control group.



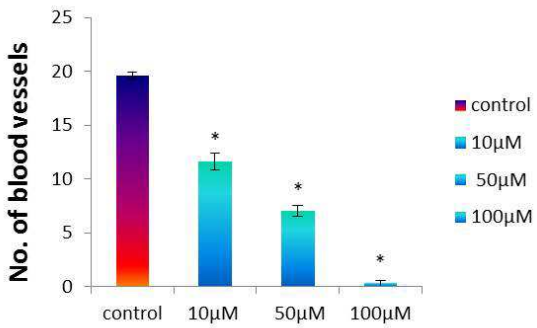


Figure 6: RBC staining: angiogenesis is compromised when resveratrol concentration is increased. A) Control embryos showing normal vascular development (arrows), B) Intensity of ISVs is slightly reduced, C) ISVs sprouting is irregular and reduced and D) Complete absence of ISVs indicating reduced angiogenesis at 100µM. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus compound treatment groups at $p<0.05$ level using Student's-Newman-Keul's test.

3.3. Anti-angiogenic effect of resveratrol in zebrafish embryos

We used RBC staining to evaluate the effects of resveratrol on the blood vessel patterning in zebrafish model. Zebrafish embryos were treated with different concentrations of resveratrol (10, 50 and 100 µM) at 50% epiboly stage and maintained till 72 hpf. Figure 6 illustrates the formation of intersegmental vessels (ISV; shown with arrows) in zebrafish. At 72 hpf, a significant reduction in the number of complete ISVs and angiogenic sprouts was observed in resveratrol treated groups when compared to control group (Figure 5 A), with the moderate reduction at 10 and 50 µM resveratrol groups (Figure 5 B and C) and greatest reduction at 100 µM resveratrol groups (Figure 5 D). These results demonstrate that resveratrol has the ability to inhibit ISVs but have to be further confirmed with transgenic model.

Our study provides novel insight into the mechanisms of action of resveratrol in a living organism. Therefore, we further explored their anti-angiogenic effects and found that resveratrol could disrupt major blood vessels *in vivo* through RBC staining. It is interesting to note that the observed regression in the blood vessels of the embryos was towards the outer edges of the ISVs, indicating perhaps that newly formed blood vessels are the targets of resveratrol. ISVs reduced to nearly 90% when treated with 100µM resveratrol treated embryos. The RBC staining assay allowed a more sensitive detection of vascular defects [43, 44] and by this means, we performed, to our knowledge, the largest forward genetic screen for blood vessels involved in angiogenesis. Taken together, our in-depth study on resveratrol in zebrafish embryos suggests that this flavanoid has the potential to inhibit angiogenesis which further lead to deformities in developing embryos at micromolar (µM) concentration. Results were similar to a previous study in which resveratrol showed anti-angiogenic and vascular-targeting activities using *in vivo* models [45] and also Myricetin, a flavonol inhibited ISV formation and down-regulated *VEGF-A* expression in zebrafish embryos [46].

3.4. Down-regulation of VEGF/VEGFR-2 mRNA in zebrafish embryos

We examined the mRNA expression of *VEGF* and *VEGFR2* in embryos treated with resveratrol of different concentrations using RT-PCR. Distinct differences in the expression of both *VEGF* and *VEGFR2* mRNAs between resveratrol treated groups (10, 50 and 100 µM) and the control group was observed. Interestingly both these genes were down-regulated (Figure 7) when treated with resveratrol of 100µM with a significant reduction in the mRNA levels even at lower concentration of 50 µM. There was less reduction in *VEGF* and *VEGFR2* at 10µM concentration almost similar to that obtained with control.

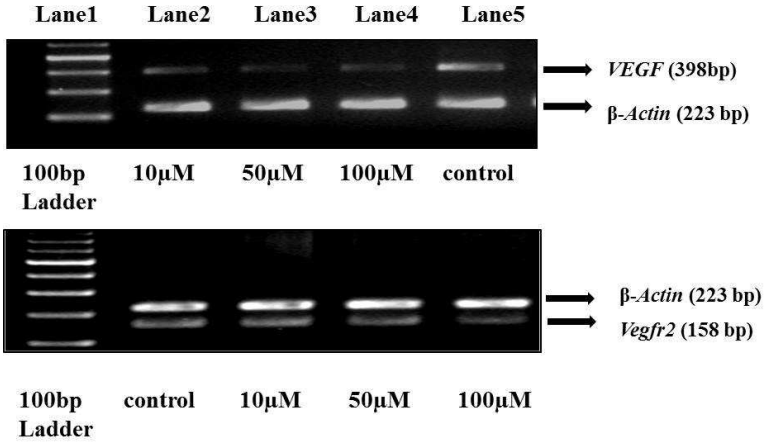


Figure 7: *VEGF and VEGFR2* band intensity decreased with increasing concentration of resveratrol at 72hpf, with 100µM showing reduced expression.

The data indicates that resveratrol suppressed the expression of *VEGF* and *VEGFR2* in embryos in a concentration-dependent manner. Thus resveratrol could inhibit the formation of major blood vessels probably by down-regulating *VEGF/VEGFR2* mRNA expression. Our research intensively focuses on the inhibitory effects of resveratrol on ISVs development in response to *VEGF/VEGFR2* gene expression. Phenotypic changes of angiogenesis always involve in angiogenesis- related signaling pathways. *VEGF* is a potent pro-angiogenic factor that stimulates endothelial cell proliferation, migration and tube formation, some key events of the angiogenic process [47]. The biologically relevant *VEGF* signaling events are mainly mediated by *VEGFR2* [47–50]. Strong evidences are there to demonstrate that blocking the activity of *VEGFR2* can limit the ability of angiogenesis [48], and *VEGFR* inhibitors are a promising class of angiogenesis treatment drugs [49]. In the present study, we investigated whether resveratrol, a natural polyphenol compound found in various plants including grapes and their related products, could inhibit blood vessel formation in embryonic zebrafish model and act as an angiogenesis inhibitor. We found that a half-

maximum inhibitory concentration of 100µM resveratrol significantly blocked *VEGF* and *VEGFR2* gene expression suggesting resveratrol to be a potent *VEGF* and *VEGFR2* inhibitor [51].

CONCLUSION

In this study, we report the novel biological functions of resveratrol as an inhibitor of angiogenesis in zebrafish embryonic model. Altogether, our study elucidates the mechanism of the anti-angiogenic activity of resveratrol at least in part. We have shown that resveratrol could inhibit angiogenesis through down-regulation of *VEGF/VEGFR2* gene expression at 100µM resveratrol, suppressing *VEGF* mediated signaling pathway which plays multiple roles in regulating neovascularization and inducing apoptosis in developing zebrafish embryos. Hence, our findings provide inspiration for further development of plant based compounds such as resveratrol and flavanoids as a novel *VEGFR2* inhibitor for the treatment of angiogenesis-related disorders.

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