RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Special Issue: 103-110 (November 2025)

# Cardioprotective Potential of Fucoidan, Vanillic Acid and its Combination in Lipopolysaccharide Stimulated Zebrafish using Notch Signaling Pathway

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10.18805/IJAR.B-5703


## ABSTRACT

**Background:** Centers for Disease Control and Prevention (CDC) in its 2022 report, has mentioned that nearly 6,97,000 death/year due to heart diseases in U.S. In low- and middle-income countries >75% deaths occur due to cardiovascular disease (CVDs). Also, natural derived compounds have emerged as promising cardioprotective agents due to their diverse mechanisms of action and potential to mitigate CVDs. Notch signalling pathway plays a key role in heart tissue repair and regeneration during or after CVDs situation mitigated by fucoidan (F), vanillic acid (VA) and its combination (FVA) were studied in this work.

**Methods:** In the present study, we investigated the cardioprotective properties of F, VA and its combination (FVA) in LPS-induced zebrafish embryo-larvae (ZFEL) through notch signaling pathway by determining the survival, heart rate (HR), nitric oxide (NO), immunofluorescence and protein expression.

**Result:** The LPS stimulated ZFEL showed increase in mortalities, reduction in HR, elevated in NO expression, upregulation of NICD, Notch signaling protein (Notch1, Delta1, Hey1 and Hes1) expression as compared to control group. The post treatment (PT) of F, VA and FVA showed remarkably improved LPS-induced survival rate, HR, NO, reduced NICD and downregulation of Notch signaling protein was reported in post treated group. In summary, our findings revealed that combination of FVA showed a promising cardioprotective potential *via* notch signaling pathway modulation in LPS stimulated ZFEL and can be utilized as alternative for synthetic drug treatment.


**Key words:** Cardiovascular diseases, Lipopolysaccharide, Notch signaling pathway, Zebrafish embryo-larvae.

## INTRODUCTION

In low- and middle-income countries >75% deaths occur due to CVD (WHO,2021). In India CVDs risk factors are prevalent in many places, including rural and underdeveloped areas and poorer states. Contrary to other populations, Indians are more likely to develop coronary artery disease (CAD. Strategies include evidence-based policy formation, strengthening of health systemsand a focus on prevention, early identificationand treatment are required to combat the epidemic (Ashavaid *et al*., 2012; Prabhakaran *et al*., 2018). In recent years, natural compounds have emerged as promising cardioprotective agents due to their diverse mechanisms of action and potential to mitigate cardiovascular diseases (CVDs). These compounds, derived from plants, fungiand natural sources, offers anti-inflammatory, lipid-modulating properties that contribute to cardiovascular health (Yadav *et al*., 2025; Lin *et al*., 2024; Sreedevi and Mavilavalappil, 2024). The exploration of these natural products is driven by the need for safer, cost-effective alternatives to conventional CVD treatments (Kaliyamurthi and Binesh, 2023). Nature derived compound like F sulphated polysaccharide from *Fucus vesiculosus* and VA phenolic compound have potential therapeutic properties against cancer, diabetes, renal ischemia, osteoarthritis, anticoagulant, bacterial/viral infections and neurodegenerative disease (Aysin *et al*., 2017; Sharma *et al*., 2020; Apostolova *et al*., 2020).

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**How to cite this article:** Piyushbhai, M.K., Binesh, A. and Venkatachalam, K. (2025). Cardioprotective Potential of Fucoidan, Vanillic Acid and its Combination in Lipopolysaccharide Stimulated Zebrafish using Notch Signaling Pathway. *Indian Journal of Animal Research*. **59 (Special Issue):** 103-110. doi: 10.18805/IJAR.B-5703.

**Submitted:** 13-10-2025 **Accepted:** 24-11-2025 **Online:** 28-11-2025

Notch signaling play a key role in self-renewal, dedifferentiation of cells, regulate dynamic transformation, homeostasis by temporal as well as spatial expression (*notch* ligand, receptors and cell enriched transcription factors) of this pathway (Zong *et al*., 2009). This signaling pathway regulated by various mechanism such as glycosylation (inhibit pathway), receptor trafficking and other regulatory mechanisms (transcription of AKT, RUNX1, SIRT6, CBFB, DEC1, microRNA-26a,26b,153,182 and 34ap; NO) (Wang *et al*., 2017; Chen *et al*., 2017; Binesh

*et al*., 2019). At the time of heart wall formation (differentiation of cardiomyocytes to non-cardiomyocytes through myogenesis inhibition), EMT development, heart valve formation, atrioventricular canal remodeling as well as maturation were regulated by Notch signaling pathway (High *et al*., 2008, Luxán *et al*., 2016). In induced vascular injury, several components of notch pathway expression modulated such as Notch 1/3, Jag 1/2 as well as Hey 1/2 and forms thick layer of vascular smooth muscle cells (neointima) due to significant downregulation of Hey 2 in mice (Sakata *et al*., 2004).

In recent years, *Danio rerio* (Zebrafish) was widely used as non-mammalian model organism due to its unique characteristics such as faster reproduction, easy handling, 200-300 embryo production, rapid morphogenesis, lower cost, ease of genetic manipulation, the ability to regenerate their heart after injury, 70% of genomic similarity of human genome, large scale mutagenesis and high-throughput drug screening (Huang *et al*., 2021 and Quyoom *et al*., 2025) makes ideal model for screening of bioactive compounds to develop novel drugs to treat/prevent non-communicable disease (diabetes; CVDs) (Jamir *et al*., 2024), respiratory, liver and kidney disease. Previously, Jeong *et al*. (2017) and Yu *et al*. (2020) utilized LPS induced cardiac abnormalities in ZFEL to elucidate the therapeutic potential of nature derived compounds. Hence, in present study F, VA and its combination cardioprotective activity was elucidated in LPS stimulated ZFEL.

## MATERIALS AND METHODS

Adult wild-type AB strains of zebrafish male and females (*Danio rerio*, Hamilton-Buchanan,1822 were maintained as per standard protocol (Piyushbhai *et al*., 2024). Prior approval of IAEC (10/SA/Fish/IAEC/IFPGS/2024) of Institute of Fisheries Postgraduate Studies, TNJFU were obtained for the zebrafish experiments and conducted during 2022-24. Two males and one female were used for pairwise mating for the embryos collection.

LPS from *E. coli* O111:B4 purified by phenol extraction (SIGMA) was diluted with Millipore water for preparing the 1mg/mL stock solution by heating at 70-80°C and vortexed. F from *Fucus vesiculosus* was dissolved in Millipore water for 1 mg/mL stock solution preparation. From F stocks solution varying concentration (50, 100, 600, 900 and 1200 µg/ml) and VA (100,200,300,400, 500 and 600 µg/ml) were prepared in in 24-well plate for $LC_{50}$ investigation. 24 hours post fertilization (hpf) we exposed ZFEL to LPS induction for 1 hr and post treated F and VA concentration given in (Table 1) for 1 hr to evaluate cardioprotective efficacy.

After fixing the optimal induction of LPS, PT of F and VA in ZFEL. We determined how PT of F and VA attenuate cardiac impairment induced by LPS as well as the ZFEL survival shown in a Kaplan-Meier survival. For each treatment given in triplicate (n=10) and ZFEL maintained at room temperature, scored daily for survival, without changing the egg water until 96 hpf. At 96 hpf, LPS induced and treatment groups (F, VA and FVA) 10 larvae heart beating rate of atrium and ventricle were measured method described by Chang *et al*. (2021). At the end of experimental trail, 30 ZFEL of control and treatment groups were anesthetize using MS222 and homogenized using plastic pestle in 100mMTris-HCL buffer (pH 7.4); centrifuged at 12,000 rcf (4°C) for 15 min to obtain the supernatant and

**Table 1:** Different experimental groups (F, VA, LPS and FVA combination and exposure time.

| Groups | Dosage concentration (µg/ml) |
|---|---|
| Control | No treatment |
| F | 80 µg/ml |
| | 100 µg/ml |
| | 120 µg/ml |
| VA | 10 µg/ml |
| | 25 µg/ml |
| | 45 µg/ml |
| LPS | 5 µg/ml for 1 hr |
| LPS induction and PT of F | 5 µg/ml + 80 µg/ml for 1 hr |
| | 5 µg/ml + 100 µg/ml for 1 hr |
| | 5 µg/ml + 120 µg/ml for 1 hr |
| LPS induction and PT of VA | 5 µg/ml + 10 µg/ml for 1 hr |
| | 5 µg/ml + 25 µg/ml for 1 hr |
| | 5 µg/ml + 45 µg/ml for 1 hr |
| LPS induction and PT of FVA | 5 µg/ml + (80 µg/ml+ 45 µg/ml) for 1 hr |

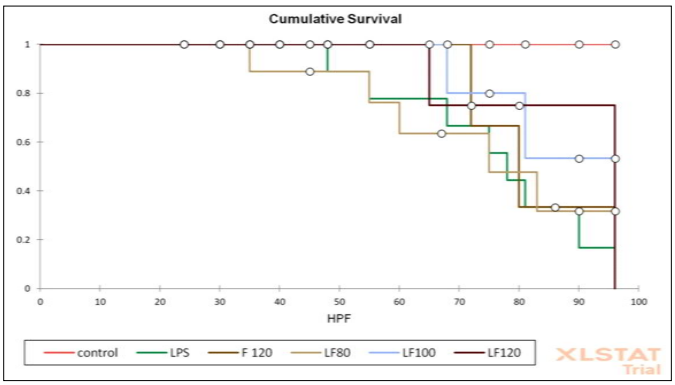



**Fig 1:** Cumulative survival determined using kalpan-meier for LPS, F and PT of F in LPS induced ZFEL.

NO was determined using Griess reagent (SRL) method described by Haridevamuthu *et al*. (2022). 60 ZFEL from each exposure groups and control were protein isolation and immunoblotting as described by Binesh *et al*. (2018) and Piyushbhai *et al*. (2024). 10 ZFEL from each exposure groups and control were anesthetize using MS222 and fixed with 4% paraformaldehyde in PBS overnight at 4°C. The whole mount immunohistochemistry was performed method described by Santos *et al*. (2023). The data presented in this manuscript were analysed using SPSS 26.0.

## RESULTS AND DISCUSSION

In present study, the cumulative survival evaluated for the effective concentration F and VA and PT groups to determine the cumulative survival. In LPS induced group nearly 80-90% larvae died and higher concentration of F (120 µg/mL)-30%, VA (45 µg/mL) -20% mortalities. On other hand, LPS induced ZFEL post treated with different concentration of F showed 80% survival in 100 and 120 µg/mL respectively except 50% in 80 µg/mL group shown in Fig 1. The PT of VA increased survival in dose dependent manner higher survival was observed in VA 45 µg/mL treatment group compared to LPS induced group shown in Fig 2. The LPS induction to ZFEL showed increased mortalities as compared with control whereas PT of F (100 and 120 µg/mL) when compared to 80 µg/mL. which indicate that fucoidan have the therapeutic potential to attenuate the LPS induced harmful effect and improved cumulative survival percentage *via* activating various signaling pathways. In context to that, Jeong *et al*. (2017) pretreated ZFEL using fucoidan 100 µg/mL to inhibit LPS induced inflammation. They found that fucoidan reduced NO as well as ROS levels in LPS induced ZFEL and showed anti-inflammatory effect *via* reduction in migration of neutrophils and macrophages. Also, VA higher concentration does not cause significant mortality in VA treated group as compared to control. The PT of VA to LPS induced ZFEL significantly reduced the mortality caused by LPS and increased survival reported in LV 25 and 45 µg/mL VA treated group.

ZFEL widely used for cardiovascular disease model due to transparent body potentially help to identify abnormal HR observing through stereomicroscope (Kithcart and MacRae,2017). The LPS induction leads to reduction in HR upto 108±4.33 heart beat (HB)/min as compared to control 159±3.21 HB/min. PT of F in LPS stimulated ZFEL shown in the Fig 3. PT of VA to LPS induced ZFEL shown enhancement in LVA1, LVA2 and LVA3 treatment groups respectively shown in Fig 4. Overall, the PT of combination of FVA showed the cardioprotection via bringing heart rate 156±0.88 HB/ min shown in Fig 4. The reduction in heart

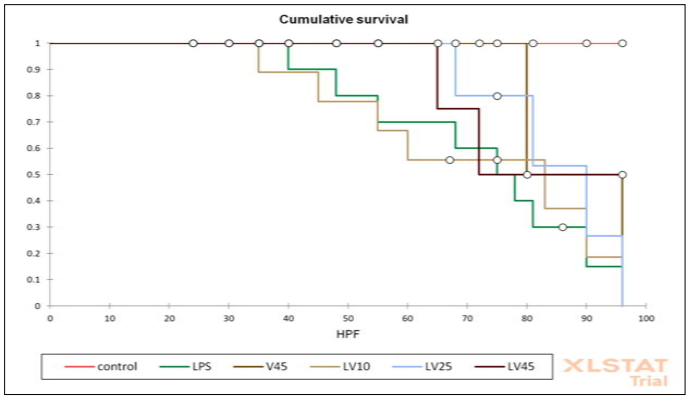



**Fig 2:** Cumulative survival determined using Kalpan-Meier for LPS and PT of VA in LPS induced ZFEL.

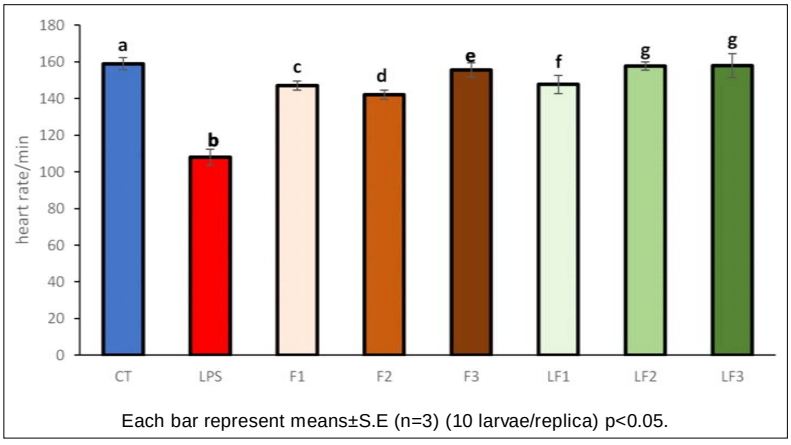



**Fig 3:** The ZFEL larvae heart rate/min in control, LPS, F and PT of F in LPS induced ZFEL.

rate in LPS treated group due to the cardiac edema (harmful effect of LPS on cardiac cell membrane) resulting into elongation of heart chamber and might be alters the hemodynamics of ZFEL (Zakaria *et al*., 2018). The 80 and 100 µg/mL of F and higher dosages of VA slightly reduced the heart rate without causing any cardiac abnormalities as compared to control. The PT of F, VA and FVA different significantly attenuated LPS induced cardiac rhythm alteration *via* cellular and molecular mechanism. In contrast, Lee *et al*. (2013); Sanjeewa *et al*. (2019) and Wang *et al*. (2021) reported that F isolated from *Ecklonia cava*; *Sargassum honeri* and *S. fusiforme* pretreatment to various

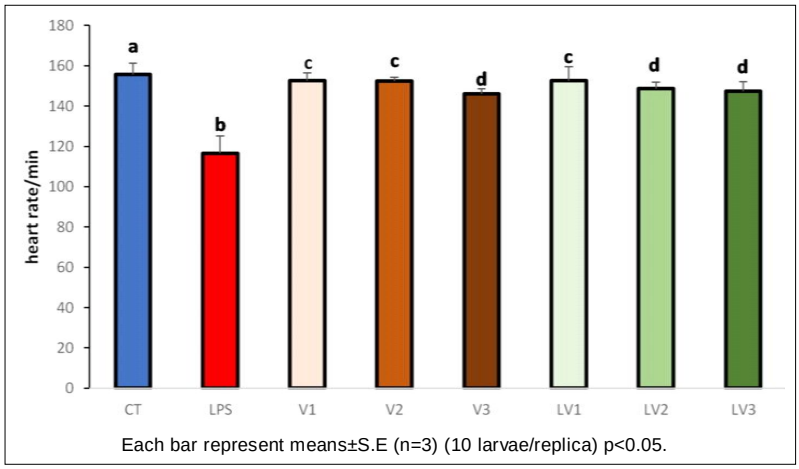



**Fig 4:** The ZFEL larvae heart rate/min in control, LPS, VA and PT of VA in LPS induced ZFEL.

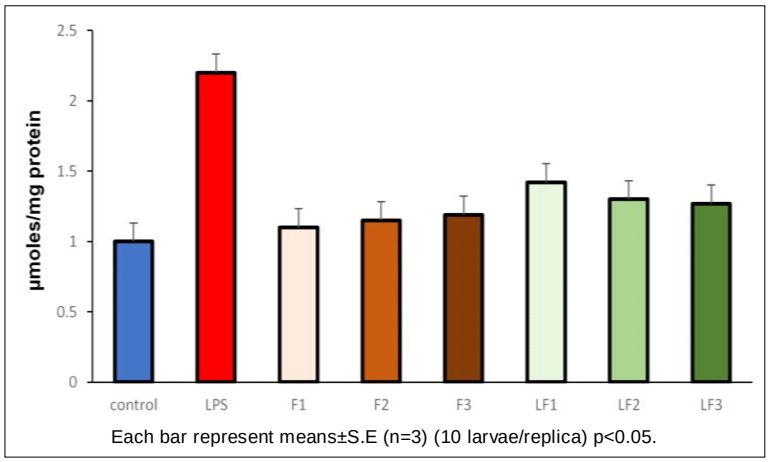



**Fig 5:** Effect of F on the NO levels in LPS induced ZFEL.

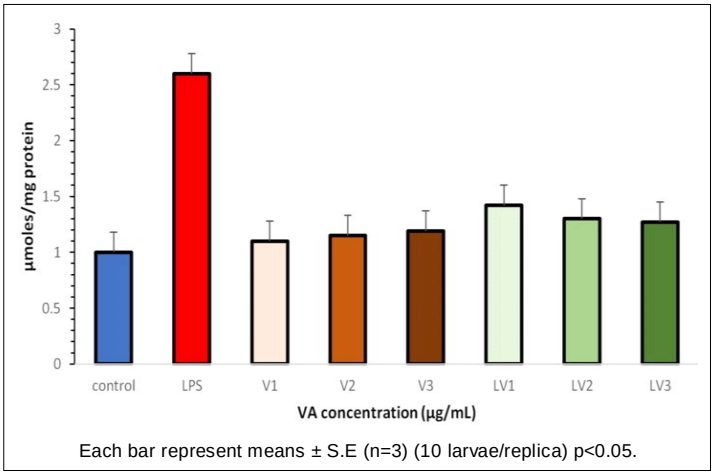
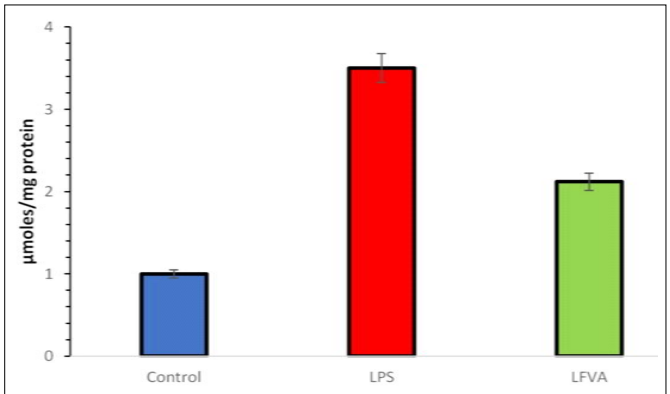



**Fig 6:** The NO levels in ZFEL larvae control, LPS, VA and PT of VA in LPS induced ZFEL.

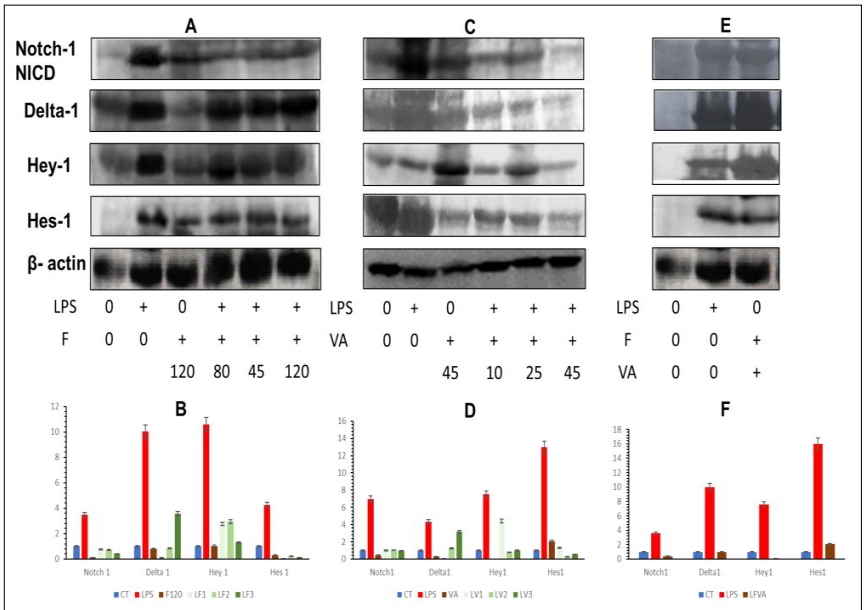
concentrations of F (12.5, 25, 50 and 100 µg/mL) helps to ameliorate the LPS induced abnormal heart beating rate *via* reducing it. The male albino Wistar rats induced with L-NAME via drinking water and co-treated with VA. They reported that co-treatment of VA significantly modified cardiac marker enzymes, NOx levels, left ventricular function and elevated eNOS mRNA expression to normalize L-NAME induced hypertensive effect in rats (Kumar *et al*., 2014). Baniahmad *et al*. (2020) reported that pretreatment of VA (10,20 and 40 mg/kg) for two weeks significantly increased systolic blood pressure and reversed the HR near to normal in 20 and 40 mg/kg dose in doxorubicin induced rat.

The ZFEL induced with the LPS showed significantly 2.2-fold increase the NO production as compared to control, F and VA treated group. Correspondingly, ZFEL post treated with different concentration of F, VA and FVA effectively reduced the LPS induced NO production shown in Fig 5-7 respectively. The LPS treated ZFEL showed significant increase in NO level when compared to control and other groups. Several researchers used LPS induced ZFEL model for evaluating anti-inflammatory as well as oxidative stress properties of F isolated from various seaweeds (*S. horneri/fusiforme*, *E cava, F vesiculosus*) (Lee *et al*., 2013; Kim *et al*., 2014; Jeong *et al*., 2017; Wang *et al*., 2020; Sanjeewa *et al*., 2021). Lunderg and Weitzberg, (2022) mentioned that induction of proinflammatory cytokines/ bacterial product to immune cells/ epithelial cells leads to generation of increased amount of iNOS for prolonged




Each bar represent means±S.E (n=3) (10 larvae/replica) p<0.05.

**Fig 7:** The NO levels in ZFEL larvae control, LPS, PT of F and VA combination in LPS induced ZFEL.




(A-B) fucoidan, (C-D) Vanillic acid and (E-F) fucoidan and vanillic acid combination effect on Notch signaling pathway (Notch1, Delta1, Hey1 and Hes1) protein expression and β-actin as internal control. data are presented as mean±S.E. *p<0.05 relative to control.

**Fig 8:** Notch signaling pathway protein expression analysis in experimental ZFEL: Effect of F, VA and FVA combination on Notch1, Delta1, Hey1 and Hes1 protein expression in LPS induced ZFEL at 96 hpf.

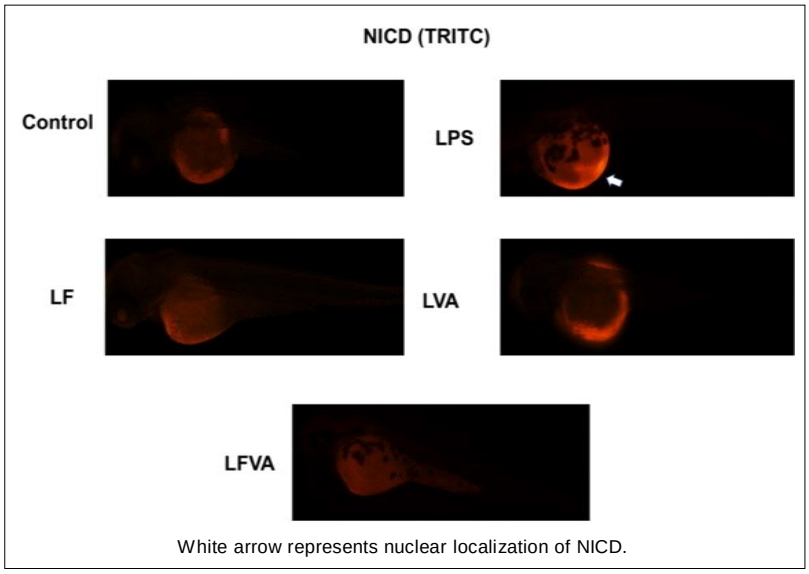



**Fig 9:** Immunofluorescence analysis of NICD expression: Representative photomicrograph of ZFEL in all group stained with NICD (TRITC-red).

periods of timing produces NO and its different free radicals and causes toxicity to cells/organism (Piyushbhai *et al*., 2024). Many researchers reported that pretreatment of F significantly reduced the NO production whereas in the present study, PT of F different concentration might reduce the production of peroxynitrite ($ONOO^-$) which play key role in oxidative stress, protein nitration and help to cellular detoxification process due to fucoidan inhibit production of iNOS (Lind *et al*., 2017; Piyushbhai *et al*., 2023). Whereas in present study, the PT of F, VA and FVA significantly lowered the NO levels in dose dependent manner in LPS induced ZFEL.

The overexpression of Notch leads EC inflammatory response *via* upregulation of adhesion molecules of endothelial cells, pro-inflammatory chemokines and Notch receptors upregulation reported in atherosclerotic lesions (Binesh *et al*., 2018). The protein expression profile of various Notch signaling protein like Notch1, Delta1, NICD, Hey1, Hes1 (3.46,10,10.6, 4.26 fold respectively) were upregulated in LPS induced group whereas PT of F, VA and FVA inhibited expression of notch proteins shown in a Fig 8. The LPS induction causes alteration of notch protein overexpression might affect the normal epithelial-mesenchymal transition (Valizadeh *et al*., 2022). The PT of 80,100 and 120 µg/mL of F showed the dose dependent inhibition of Notch-1, Delta-1, Hey-1, Hes-1protein expression. The PT of F suppresses the notch pathway leads to positively regulation of differentiation; vascular tip formation and vessel bifurcation helps to ameliorate LPS induced cardiomyopathy in ZFEL (Gridley,2007). The PT of VA significantly downregulated the notch signaling. The phenolic compounds like curcumin, resveratrol showed the vascular smooth muscle cell phenotypic switching leads to release of blocked NICD and enhances contractile phenotype via the cavelolin-1/Notch1/myocardin pathway (Zhang *et al*., 2014; Sun *et al*., 2017; Sharma *et al*., 2024b). Which help us to conclude that LFVA might have therapeutic potential against CVDs via inhibiting Notch signaling protein mainly associated during CVDs pathophysiology.

An intense and prominent expression of NICD was observed in yolk sack as well as pericardial area of LPS induced group shown in Fig 9. The PT of F, VA and FVA of remarkably reduced the immunoreactivity of the NICD in LPS induced group. The LPS induction caused increase NICD nuclear translocation leads to hyperactivation of Notch pathway leads to impedes CM proliferation and induces scarring (Binesh *et al*., 2020; Valizadeh *et al*., 2022). Immunofluorescence analysis of NICD showed intense nuclear expression and translocation in cardiac as well as yolk sac region of LPS induced ZFEL. The F treatment inhibited the expression of NICD expression near to control ZFEL. Hence, the inhibition of NICD expression might attenuate LPS induced cardiac abnormalities via regulating vascular cell proliferation, apoptosis of monocytes, macrophages and vascular smooth muscle cells (Sharma *et al*., 2024a). Whereas the PT of LFVA significantly reduces the intensity of NICD suggest that LFVA might have cardioprotective activity.

## CONCLUSION

The LPS induction to ZFEL mimics pathophysiological events occurs in human during the CVDs beginning to the very late stage. The PT of F, VA and FVA combination attenuated the LPS induced CVDs dysfunction in ZFEL. Among this FVA combination possess the ability to attenuates pericardial effusion via notch signaling expression. Hence, this nature derived compound and their combination may be used for the management of CVDs due to its multi-targeted action. Further research needs to be done how different pathway may involve in cardioprotection.

## ACKNOWLEDGEMENT

This study was supported by the grant from the Science and Engineering Research Board (SERB), Department of Science and Technology (DST), (CRG/2022/003847), Govt. of India.

### Disclaimers

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### Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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