IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 19, Issue 3 Ser.9 (March. 2020), PP 08-14
www.iosrjournals.org

Therapeutic potential of *Evolvulus alsinoides* an herbal extract on infectious diseases in Zebra fish model

Dinesh. S[1], Anil Kumar. H. V[2], Muralidhar.S. Talkad[3*]
1 Research Scholar, CMJ University, Shillong. India
2Professor of Sericulture, Laboratory of Applied Biological Sciences, DVS College of Arts and Science, Shimoga - 577201, Karnataka, India
*3*Professor, Herbal Research & Project Consultant –R&D, Natural Remedies Pvt Ltd. Hosur Road, Bangalore -560100, India*
**Corresponding Author*

Abstract: *Zebrafish models of bacterial infection can reveal key aspects of infection biology and provide fundamental advances in understanding the biology of cellular immunity. It is to be expected that the study of host–pathogen interactions using zebrafish will continue to illuminate the complexity that underlies bacterial infection in higher vertebrates, including humans.*
In the present study focused on the therapeutic potential of Evolvulus alsinoides an herbal extract on Pseudomonas aeruginosa induced infectious diseases in Zebra fish model, it has given a lead for post treatment protective effect of plant extracts
Evaluation of MIC for the Evolvulus alsinoides (EA) extract showed anti microbial response against E. coli, Pseudomonas aeruginosa and Candida albicans 6.25, 25 and 12.5 mg/ml, respectively. The in vitro cytotoxicity MTT assay was performed for the Evolvulus alsinoides plant extract showed CTC 50 value as 277.17 µg/ml against Human colorectal adenocarcinoma cells (MCF-7) cell lines
In the present study Histopathological observation also supported by gills inflammation as distorted gills slits, edematous and congestion in negative control fishes were reduced to normal in treated group 500mg/ml
In the present study identification of IRG1 gene in Zebra Fish, when they subjected for PCR amplification followed by agarose gel electrophoresis (PCR-AGE) were clearly evident that in Negative control gene is expressed in 750 bp due to severe infection, in treated with plant extract (EA) 500mg/ml were faded gene pattern observed could be due to reduced infection and inflammation drastically.

Date of Submission: 02-03-2020 Date of Acceptance: 17-03-2020

I. Introduction

A wide variety of pathogenic bacteria have been investigated using zebrafish models, providing unprecedented resolution of the cellular response to infection in vivo. The zebrafish also enables the characterization of gene function via overexpression, transient depletion, or genome editing1. The zebrafish genome has been fully sequenced, highlighting a remarkable similarity with humans2.

Zebrafish models have contributed to more understanding of cellular microbiology by providing an in vivo platform to study host–pathogen interactions from the single cell to whole animal level. In this present study we planned zebrafish infection with P. aeruginosa followed by post therapeutic approach in Evolvulus alsinoides (EA) extract could be a beneficial and novel therapies for humans in nearfuture

As in humans, the depletion of phagocytes can dramatically increase the susceptibility of Zebra fish larvae to P. aeruginosa3. In agreement with a key role for phagocytes in Pseudomonas control, both macrophages and neutrophils can rapidly engulf and kill systemically delivered bacteria

The high antioxidant capacity of Evolvulusalsinoides extracts were indicated its potential use in the treatment of diseases with reducing the excessive production of reactive oxygen species (ROS). The methanolic extracts of Evolvulusalsinoides exerted the most promising activities on the proliferation of human peripheral blood mononuclear cells (PBMC)4.

When human peripheral blood mononuclear cells (PBMC) treated with methanolic extract of Evolvulus alsinoides invitro showed lymphocytic proliferation at 250 µg/ml, which were significantly higher proliferation when compared to control4.

microorganisms replicate over time within a body site (e.g., lung, urinary tract, blood stream) releasing endotoxins and exotoxins that elicit the host inflammatory immune response. The persistence of infection in the form of total microbial load perpetuates systemic inflammation over time leading to host cellular injury and end- organ dysfunction. Animal models have shown that septic shock due to Escherichia coli peritonitis consistently occurs once a defined microbial load in the blood stream has been reached5.

Clinical experience similarly demonstrates that site cultures may remain positive for a variety of infections for days or longer despite initiation of appropriate antimicrobial therapy. The microbiologic paradigm holds that the inflammatory response of severe sepsis and septic shock cannot be overcome unless the underlying infection has been effectivelyeradicated6

In critically ill patients with Pseudomonas infections, extended infusion of piperacillin-tazobactam (3.375 g over 4 h every 8 h) has been associated with improved clinical outcomes. Pseudomonas aeruginosa, a broad host range pathogen, capable of infecting plants, invertebrates, and vertebrates, was lethal after injection into the yolk circulation7.

In the present study focused on MIC, MTT, histopathological observation of gills for inflammation and to identify inflammatory IRG1 gene in Zebra Fish by PCR amplification followed by agarose gel electrophoresis (PCR-AGE)

II. Material and Methods

Plant extract

Fresh mature whole plant Evolvulus alsinoides was collected from natural source in rural parts of South Karnataka regions of India. Routine pharmacognostic investigations were carried out to confirm authenticity of this material. The identification was carried out by the botanist in our R&D. The whole plant was dried and cut into small pieces and crushed to a coarse powder using blender. Coarse powder was subjected to extraction in Sox let apparatus (in ratio of 1:5 to the quantity of raw material) for 6 h under reflux condenser at 45o C using thermostat.

This extract was cooled to room temperature and evaporated using condenser then filtered through filter cloth (#100 mesh size), to get methanolic extract of Evolvulus alsinoides plant. The resulting extracts were stored in well-packed container at room temperature for future use. The yield of dried extract was approximately 40% and a homogenate was made in 50% ethanol. Three different concentrations of the extract wereprepared

Test organism used

Adult Zebra fish (Danio rerio) procured from a pet store in a local market, Bangalore. Adult zebrafish with an average standard length of (2.00 ± 0.20 cm) and an average weight of (0.40 ± 0.18 g) was used.

Test protocol

The Zebra fish were acclimatized to laboratory conditions at 12 light and 12 dark photo period. The fishes were maintained in dechlorinated tap water contained in beakers. The water was oxygenated uniformly using an air bubbler8. The fishes were divided into 6 groups of 10 each, the fishes were challenged with Pseudomonas aeruginosa 100 CFU/ml for infectious disease induction except Normal control, rest of the groups i.e., Negative Control (Pseudomonas aeruginosa induced), Standard Control Levofloxacin 25mg /ml, Evolvulus alsinoides (EA) methanolic extract at the dose of 100, 250 and 500mg/ml for 15 days. the fishes were observed continuously for behavior and mortality, on day 16, fishes euthanized in ice then fixed in 10% Buffered Neutral formalin and subjected for histopathologyevaluation

The study was conducted by scientists and technicians in compliance with the guidelines laid down by the Institutional Animal Ethics Committee (IAEC). (vide SB/04/19) All applicable for the care and use of animals were followed.

he fishes fixed in 10% buffered formalin (pH 7.4) and routinely processed in paraffin. Sections of 4µm thickness were made using Microtome (Leica RM 2125, Leica Microsystems GmbH, Germany) placed on glass slides and stained with hematoxylin and eosin method and then mounted in DPX. The slides were examined and photographed under Olympus microscope.

For MIC-test organism were E. coli, Pseudomonas aeruginosa, Candida albicans, Muller-Hinton broth, Dimethyl sulphoxide, Levofloxacin and Amphotericin B antibiotics (Himedia, India)

MCF-7 (Human Breast Cancer) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 □ g/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO2 at 370C until confluent. The cells were dissociated with TPVG solution (0.2% Trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm2 culture flasks and all experiments were carried out in 96 well microtiter plates (Tarsons India Pvt. Ltd., Kolkata,India).

Evaluation of MIC for the Evolvulus alsinoides extract:

The determination of MIC in plant extracts (EA) with 100mg /ml the broth micro-dilution method was performed. The inoculums of the tested isolates were prepared using the colony suspension method. Ninety-six-well culture plates were used, and serial two-fold dilutions of the extracts were dispensed into the plate wells.

The plant extract concentration being 100, 50, 25, 12.5, 6.25, 3.12, 1.56 mg/ml, Standards + DMSO+ broth+ test organism (T.O) is positive control, Sterility control DMSO + broth is maintained, Negative control test organism + DMSO + broth is maintained, the plates were incubated at 37 °C for 24h

The bacterial and fungal activity in the test wells was detected by adding 40µL of 0.2 mg/ml of 2-(4- Iodo phenyl)-3-(4-nitro phenyl) 5- phenyl tetrazolium chloride (I.N.T.) (Himedia, India) solution dissolved in sterile distilled water to each well. The plates were incubated for further 30 min and estimated visually for any change in color to pink indicating reduction of the dye due to bacterial growth. The lowest concentration (highest dilution) of the plant extract required to inhibit visible growth of the tested microorganism was designated as the MIC. Standard Antibiotics Levofloxacin for bacterial and Amphotericin B for fungal assay were used

Evaluation of MTT assay of MCF-7 cell line using plant extract:

For cytotoxicity studies, 100µl of test drug was separately suspended and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 10% v/v concentration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtiter plate, 0.1 ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer washed once with medium and 100 µl of different test concentrations of test drugs was added on to the partial monolayer in microtiter plates. The plates were then incubated at 37o C for 72 h in 5% CO2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval.

After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37o C in 5% CO2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan.

The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the Standard formula and concentration of test drug needed to inhibit cell growth by 50% (CTC50) values was generated from the dose-response curves for each cell line.

RNA isolation and PCR amplification followed by agarose gel electrophoresis (PCR-AGE) analysis:

RNA can be purified with the kit from a variety of tissues and cells, including difficult-to-lyse tissues. miRNeasy Kits offer superior yields to alternative methods of miRNA purification, such as using TRIzol Reagent.

RNA prepared using the miRNeasy Mini Kit is highly pure and ready for use in sensitive downstream applications, this specialized protocol, enabling enrichment of miRNAs and other small RNAs (<200 nt), the RNeasy MinElute Cleanup Kit is required.

The miRNeasy Mini Kit (cat. no. 217004) can be stored dry at room temperature (15–25°C) for at least 9 months if not otherwise stated on label. QIAzol® Lysis Reagent. Added 700 µl QIAzol Lysis Reagent to the sample and disrupt and homogenize using an appropriate method. Incubate the entire fish homogenate at room temperature (15–25°C) for 5 min., add 140 µl chloroform and cap tube securely, vigorously shake for15s. Incubated at room temperature for 2–3 min, followed by Centrifuged for 15 min at 12,000 x g at 4°C. Transferred the upper aqueous phase to a new collection tube. Avoided transferring any interphase. Added 1.5 volumes (usually 525 µl) of 100% ethanol and mix thoroughly by pipetting. Pipetted up to 700 µl sample, including any precipitate, into a RNeasy® Mini column in a 2 ml collection tube. Closed the lid and centrifuge at ≥8000 x g for 15 s at room temperature. Discarded the flow-through. If expected RNA yield is >30 µg. Subjected for PCR amplification followed by agarose gel electrophoresis (PCR-AGE) and identification of IRG1 gene in Zebra Fish (mol.wt. approx., 750bp)

Statistical Analysis

All experiments were carried out in triplicate and repeated three times. Statistical analysis was performed using SPSS software (SPSS 22.0 for Windows, Chicago, IL, USA), and all data were expressed as the mean ± SD of triplicate cultures.

Analysis of variance (ANOVA) and t-tests were used to evaluate differences between the mean value of negative control-treated and extracted samples. Differences with p values of less than 0.05 were considered statistically significant.

III. Result

Evaluation of MIC for the Evolvulus alsinoides (EA) extract

Anti-Bacterial Assay			
Sl. No	Test group	*E. coli* MIC in mg/ml	*Pseudomonas aeruginosa* MIC in mg/ml
1.	EA+ T.O +DMSO + Broth	6.25	25
2.	Std+ T.O +DMSO + Broth	1.56	6.25
3.	DMSO+ Broth	No growth	No growth
Anti-Fungal Assay			
4	EA+ T.O +DMSO + Broth	12.5	*Candida albicans* MIC in mg/ml
5	Std+ T.O +DMSO + Broth	3.12	
6	DMSO+ Broth	No growth	

Evaluation of MTT assay of MCF-7 cell line using plant extract:

Sl.No	Name of the test compound	Concentration in µg/ml	% of inhibition	CTC_{50}(µg/ml)
1	*Evolvulus alsinoides*(EA) methanolic extract	1000	79.33±0.25	277.17
		500	68.49±0.18	
		250	50.68±0.21	
		125	43.19±0.19	
		62.5	34.17±0.14	

Histopathological observation of Zebra fish target organs: magnification (X100)

Fig: 1, Normal control: gills showing normal Morphology

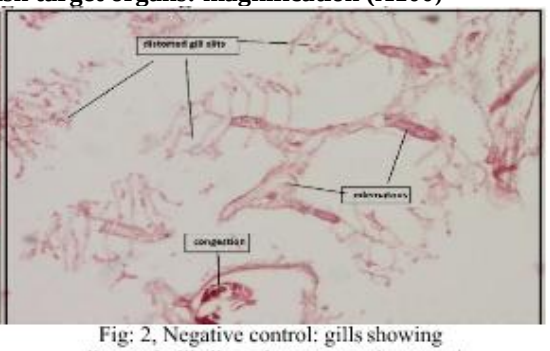


Fig: 2, Negative control: gills showing distorted gill slits, edematous and congestion

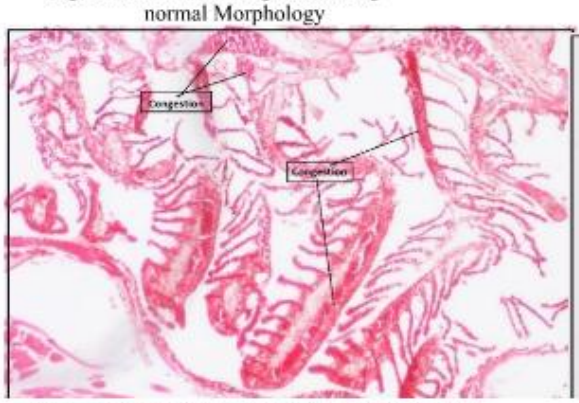


Fig: 3, Standard control: gills showing congestion

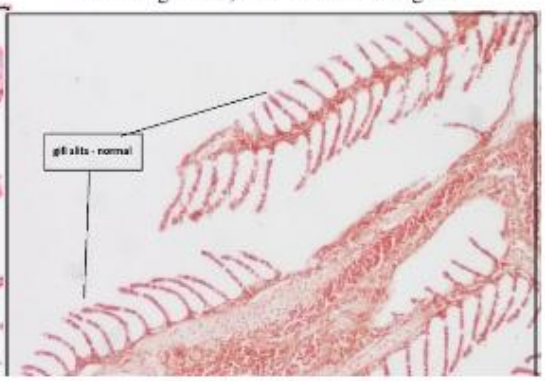
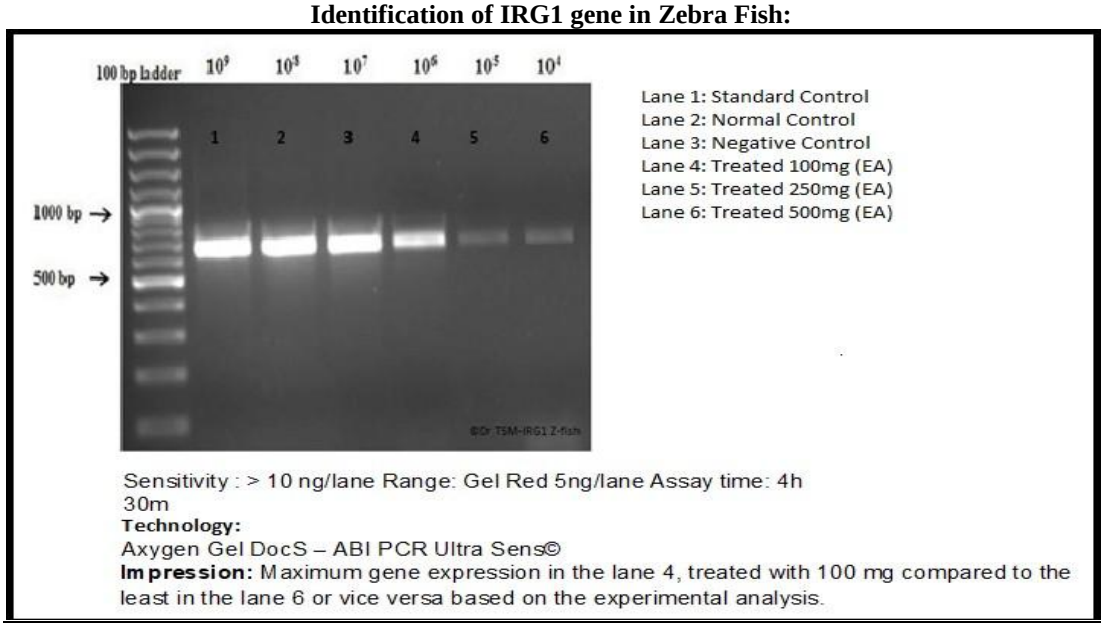


Fig: 4, PA induced & Treated 500mg/ml: gills were normal - NAD+

Identification of IRG1 gene in Zebra Fish:



(Fig-5):PCR amplification followed by agarose gel electrophoresis (PCR-AGE)

IV. Discussion

The study of host–pathogen interactions has illuminated fundamental research avenues in both infection and cell biology. Zebrafish (Danio rerio) larvae are genetically tractable, optically accessible, and present a fully functional innate immune system with macrophages and neutrophils that mimic their mammalian counterparts.

A wide variety of pathogenic bacteria have been investigated using zebrafish models, providing unprecedented resolution of the cellular response to infection in vivo.

Evaluation of MIC for the Evolvulus alsinoides (EA) extract showed against E. coli, Pseudomonas aeruginosa and Candida albicans 6.25, 25 and 12.5 mg/ml, respectively

The in vitro cytotoxicity MTT assay was performed for the Evolvulus alsinoides plant extract showed CTC 50 value as 277.17 µg/ml against Human colorectal adenocarcinoma cells (MCF-7) cell lines

Overall, the zebrafish has helped to elucidate mechanisms of pathogenesis underlying opportunistic Pseudomonas infection and serves as a platform to reveal bacterial effectors required for escape from innate immunity. It can also be used to suggest novel therapies aimed at boosting innate immune function to control opportunistic bacterial infection in humans

Pseudomonas aeruginosa is a significant cause of mortality in patients with cystic fibrosis (CF), the interaction of the CF isolate P. aeruginosa PASS1 with the innate immune response studied and with iron and phosphate acquisition genes were upregulated in PASS1 cells in the zebrafish. Transcriptional changes in the host immune response genes highlighted phagocytosis as a key response mechanism to PASS1 infection.

Transcriptional regulators of neutrophil and macrophage phagocytosis were upregulated alongside transcriptional regulators governing response to tissue injury, infection, and inflammation. The zebrafish host showed significant downregulation of the ribosomal RNAs and other genes involved in translation, suggesting that protein translation in the host is affected by PASS1 infection

The host gene expression during P. aeruginosa PASS1 infection shows upregulation of an array of genes including transcriptional regulators, toll-like receptors and chemokines to be involved in the initiation, and process of phagocytosis. Decreased expression levels of ribosomal RNAs and translation proteins suggested that protein translation in the host is impacted by bacterialinfection9.

Expression of cyp1a was most strongly induced by P. aeruginosa. Cyp1a is known to be induced by toxic chemicals in vascular endothelium, but also in the epithelium of the gills10-11. P. aeruginosa PAO1 and PA14 are known to secrete large amounts of toxins and protein virulence factors12-15.

In the present study histopathological observation also supported by gills inflammation as distorted gills slits, edematous and congestion in negative control fishes (Fig.2) were reduced to normal in treated group 500mg/ml(Fig-4)

Since cyp1a belongs to the cytochrome P450 family, its induction might be involved in a detoxification response. The observation mentioned that many of the genes regulated by P. aeruginosa are associated with the GO term “response to stress”, and the lack of enrichment of genes with the GO-term “immune system process”

is consistent with a response to toxins rather than an immune response to systemic infection with immunoresponsive gene 1 (irg1) irg1l was also highly up regulated after P. aeruginosa and E. coli exposure

In the present study identification of IRG1 gene in Zebra Fish (mol.wt. approx., 750 bp), when they subjected for PCR amplification followed by agarose gel electrophoresis (PCR-AGE) were clearly noticed that in lane 3, Negative control sample, gene is expressed in 750 bp due to severe infection, in lane 5 and 6, treated with plant extract (EA) faded gene pattern could be due to reduced infection and inflammation drastically (Fig.5)

Some chemokines are inducible by physiological stress and recruit leukocytes to sites of injury, whereas others are constitutive in nature and are responsible for basal trafficking of leukocytes. All chemokines are tightly regulated by feedback mechanisms because of their potential for severely damaging host tissues through uncontrolled persistent expression. A variety of chemokines has been identified inzebrafish16

Overall, the zebrafish has helped to elucidate mechanisms of pathogenesis underlying opportunistic Pseudomonas infection and serves as a platform to reveal bacterial effectors required for escape from innate immunity. It can also be used to suggest novel therapies aimed at boosting innate immune function to control opportunistic bacterial infection in humans17.

Therapeutic potential of Evolvulus alsinoides an herbal extract on infectious diseases in Zebra fish models, in MIC, MTT, histopathological and gene expression studies were established beneficial effect of Evolvulus alsinoides an herbal extract for human infectious diseases

The preliminary phytochemical screening of E.alsinoides contains some secondary metabolites such as glycosides, alkaloids, poly phenols, carbohydrates, amino acids and proteins, saponins, volatile oil, flavonoids and tannins18.

These plant metabolites have shown significant in vitro antioxidant activity. Therefore, Evolvulus alsinoides may hold great potential in preventing clinical deterioration in stress induced oxidative load and related disorders in our study since rich flavonoids component is Quercetin in plant extract showed 0.400 µg/ml4.

V. Conclusion

Therapeutic potential of Evolvulus alsinoides, an herbal extract on infectious diseases in Zebra fish models were clearly established the beneficial effects of Evolvulus alsinoides plant extracts could be ideal for the human bacterial infections and to control present day infectious diseases

Acknowledgment

The authors are extremely grateful to Mr. Aamir and Shrishti Tiwari, Chromgene Biotech Pvt Ltd, Bangalore-97, Mr. Pavan Kumar and Mrs. Sushma Sun Biologicals, K.R Road, Shastri Nagar, Bangalore-560028, INDIA, for their immense guidance and support for this project.

Competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1]. Varshney, G.K. et al. Understanding and editing the zebra fish genome. Adv. Genet. 2015;92: 1–52
[2]. Howe, K. et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496: 498–503
[3]. Brannon,M.K.etal.Pseudomonas aeruginosa Type III secretion system interacts with phagocytes to modulate systemic infection
[4]. of zebrafish embryos. Cell Microbiol. 2009; 11: 755–768
[5]. Dinesh S, Anil Kumar. H. V and Muralidhar. S. Talkad. 'Evaluation of Herbal Extract Evolvulus alsinoides on Lymphocyte Proliferation Assay, In Vitro', International Journal of Current Advanced Research. 2020; 09(01): pp.21051-21055.
[6]. Kumar A, Haery C, Paladugu B, Kumar A, Symeoneides S, Taiberg L, et al. The duration of hypotension before the initiation of antibiotic treatment is a critical determinant of survival in a murine model of Escherichia coli septic shock: association with serum lactate and inflammatory cytokine levels. J Infect Dis. 2006; 193:251–8.
[7]. Stephen Y. Liang and Anand Kumar, Empiric Antimicrobial Therapy in Severe Sepsis and Septic Shock: Optimizing Pathogen Clearance Curr Infect Dis Rep. 2015 July; 17(7): 493.
[8]. Lodise TP Jr, Lomaestro B, Drusano GL. Piperacillin-tazobactam for Pseudomonas aeruginosa infection: clinical implications of an extended-infusion dosing strategy. Clin Infect Dis. 2007; 44:357–63.
[9]. Prashanth D'souza and Dr. Muralidhar TS. Effect of Phytocee™ (a Phytogenic feed additive) on survivability of Zebra fish subjected to ammonia stress. International Journal of Fisheries and Aquatic Studies 2019; 7(5):459-462
[10]. Kumar SS, Tandberg JI, Penesyan A, Elbourne LDH, Suarez-Bosche N, Don E, Skadberg E, Fenaroli F, Cole N, Winther-Larsen HC and Paulsen IT. Dual Transcriptomics of Host-Pathogen Interaction of Cystic Fibrosis Isolate Pseudomonas aeruginosa PASS1 With Zebrafish. Front. Cell. Infect. Microbiol. 2018;8:406.
[11]. Guiney PD, Smolowitz RM, Peterson RE, Stegeman JJ: Correlation of 2,3,7,8 tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. Toxicol Appl Pharmacol 1997, 143:256-273.
[12]. Jönsson ME, Brunström B, Brandt I: The zebrafish gill model: Induction of CYP1A, EROD and PAH adduct formation. AquatToxicol. 2009; 91:62-70.
[13]. Darby C, Cosma CL, Thomas JH, Manoil C: Lethal paralysis of Caenorhabditis elegans by Pseudomonas aeruginosa. Proc Natl

Acad Sci USA. 1999;96:15202-15207.

[14]. Galloway DR: Pseudomonas aeruginosa elastase and elastolysis revisited: recent developments. Mol Microbiol. 1991; 5:2315-2321. [14]. PetersJE,Galloway DR: Purification and characterization of an active fragment of the LasA protein from Pseudomonas aeruginosa: enhancement of elastase activity. J Bacteriol. 1990; 172:2236-2240.

[15]. Wick MJ, Frank DW, Storey DG, Iglewski BH: Structure, function, and regulation of Pseudomonas Aeruginosa exotoxin A. Ann Rev Microbiol. 1990;44:335-363.

[16]. Baoprasertkul P, He C, Peatman E, Zhang S, Li P, Liu Z. Constitutive expression of three novel catfish CXC chemokines: homeostatic chemokines in teleost fish. Molecular Immunology. 2005; 42:1355e66.

[17]. Vincenzo Torraca1 and Serge Mostowy. Zebrafish Infection: From Pathogenesis to Cell Biology. Trends in Cell Biology. February 2018; Vol. 28, No. 2:143-156.

[18]. Omogbai B. A. and Ze. F. A. 2011. Phytochemical screening and susceptibility of bacteria pathogens to extracts of Evolvulus alsinoides. Sci. World J; 6:5-8.

Muralidhar.S.Talkad, et al. "Therapeutic potential of Evolvulus alsinoides an herbal extract on infectious diseases in Zebra fish model." *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 19(3), 2020, pp. 08-14.