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Rakesh P

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Sivasankar P

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Chrisolite B

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Magesh Kumar P

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Mohamed Mansoor M

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Selvamageswaran M

Department of Fish Pathology and
 Health Management, Fisheries College
 and Research Institute, Tamil Nadu
 Dr. J. Jayalithaa Fisheries
 University, Thoothukudi, Tamil
 Nadu, India

Ramesh P

College of Fishery Science, Andhra
 Pradesh Fisheries University,
 Muthukur, SPSR Nellore district,
 Andhra Pradesh, India

Vasanth K

College of Fishery Science, Andhra
 Pradesh Fisheries University,
 Muthukur, SPSR Nellore district,
 Andhra Pradesh, India

Corresponding Author:

Ramesh P

College of Fishery Science, Andhra
 Pradesh Fisheries University,
 Muthukur, SPSR Nellore district,
 Andhra Pradesh, India

Antiviral activity of aqueous leaves extract of herbal medicines against *Tilapia Lake Virus* (TiLV)

Rakesh P, Sivasankar P, Chrisolite B, Magesh Kumar P, Mohamed Mansoor M, Selvamageswaran M, Ramesh P and Vasanth K

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Abstract

Tilapia lake virus (TiLV) is a novel virus that cause large scale mortalities in Tilapia farming and is considered as a threat to the global tilapia industry. In the present study, the antiviral activity of herbal medicines against TiLV were investigated in both *invitro* and *invivo*. A total of 6 herbal medicines including Tulasi (*Ocimum sanctum*), Naval (*Syzygium cumini*), Guava (*Psidium guajava*), Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*), Papaya (*Carica papaya*) were used at different concentrations *viz.* 50, 100, 250, and 500 µl/ml of medium for screening the antiviral activities against TiLV. Among the 6 medicines, 2 medicines Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) inhibited the cytopathic effect caused by TiLV in SSN1 cell line. In this study, the concentration of herbal medicine at 500µl /ml of the medium showed better effect against TiLV in virus concentration of $10^{6.73}$ TCID₅₀ ml⁻¹ when compared to the control group. Our study has revealed that herbal medicines have potent antiviral effects in fish virus in cell culture. The finding suggests that herbal medicines have the potential as a therapeutic agent in the treatment of TiLV infection. This study was further performed using tilapia fingerlings against Tilapia Lake Virus (TiLV). The screened of two herbal medicines includes Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) against Tilapia Lake Virus in cell culture has antiviral effect. Out of these two herbal medicines, Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) has antiviral effect against TiLV on three different concentrations include 20ml, 40ml, 60ml/kg. There was recovery of tilapia fingerlings during this treatment. In this study, higher survival was occurred at 40ml dose and medium survival occurred at 60ml dose and least survival occurred at 20ml dose of medicine concentration. This was the first report of giving therapy against TiLV using herbal medicines.

Keywords: *Tilapia Lake Virus*, Herbal medicines, Cell culture, Experimental study, Antiviral activity, Herbal Treatment

Introduction

Aquaculture is the fastest developing sector in the world to satisfy the humans needs. Tilapia culture plays a major role in fisheries sector due to its fast growth, disease tolerance, marketable price and excellent meat quality Sgnaulin *et al.* (2020) [49]. Nile tilapia is the important cultivable species in tilapia farming sector because of its marketable value in many countries Watanabe *et al.* (2002) [56]. Tilapia is more resistant to pathogen and it can tolerate wide range of water quality parameters. Due to their improper culture practises, there will be chance of invading different pathogens Abowei *et al.* (2011) [1]. There was no report of viral diseases since 2009 and during summer, there was mass mortality observed in different parts of Israel and it was identified as tilapia lake virus Eyngor *et al.* (2014) [19]. Tilapia lake virus (TiLV) is an emerging orthomyxo- like virus which was found in many moribund fish in thailand Surachetpong *et al.* (2017) [52]. Later the virus was reported in Ecuador, Colombia, Egypt, Thailand, India, Tamilnadu Bacharach *et al.* (2016) [7]; Fathi *et al.* (2017) [21] Kembou Tsofack *et al.* (2016) [25]; Surachetpong *et al.* (2017) [52]; Behera *et al.* (2018) [9]; Saranya *et al.* (2020) [48]. TiLV belongs to the genus *Tilapinevirus*, family *Amnoonviridae* and it is a negative sense single-stranded RNA virus (-ssRNA virus), consist of 60–80 nm in size and 10 negative-sense RNA segments with ~ 10, 000 nt in length Al-Hussinee *et al.* (2018) [4]. Tilapia lake virus disease (TiLVD) is an emerging viral disease which affecting the tilapia producing countries in the world includes, Southeast Asia, Africa, and South and North America and it has been resulted in high mortalities leading to high economic losses

Kembou Tsofack *et al.* (2017) ^[25], Mugimba *et al.* (2018) ^[34], Nicholson *et al.* (2017) ^[35], Surachetpong *et al.* (2017) ^[52], Surachetpong *et al.* (2020) ^[53]. Due to their necessity, the development of vaccines and biosecurity measures need to be standardised against TiLV infection Bergmann *et al.* (2017) ^[10]; Zhao *et al.* (2020) ^[58]. Several disinfectants and chemical reagent have been used for controlling TiLV infection Mai *et al.* (2022) ^[33].

Herbal plant extracts, plant-derived compounds (also known as phytoconstituents), extracts of particular plant parts (roots, stem, bark, flowers, fruits, and seeds), dietary supplements, and nutraceuticals all have a wide range of applications in the treatment of illnesses, from common to uncommon infectious and non-infectious diseases. Most of the disease treated compounds extracted from plants Rates *et al.* (2001) ^[45]. Various medical treatises have encouraged the use of herbal treatments, either alone or in combination with other formulations such leaf powder, pastes, decoctions, infusions, and tablets Lee *et al.* (1996) ^[30]. The worldwide has begun to examine at herbal medicine for the treatment of viral diseases due to the side effects of synthetic medicine, which are comparably more accessible and affordable and bear lesser risk of toxicity and resistance Akram *et al.* (2018) ^[2]. The aqueous extracts of herbal plants have shown antiviral activity against influenza virus and Herpes simplex virus He *et al.* (2011) ^[23]; Nolkemper *et al.* (2006) ^[39]. The herbal plant extract was incorporated into the feed to prevent fish disease, improve health status and decrease economic loss in aquaculture sector Rico *et al.* (2013) ^[46]. Dietary supplements are included into aquatic feed to promote fish health and improve the body systems in digesting and absorbing the nutrients obtained from the food they consume Dawood *et al.* (2021) ^[16]; Zheng *et al.* (2020) ^[59]; Alemayehu *et al.* (2018) ^[3]; Shekarabi *et al.* (2022) ^[50]. Several plant extracts had an antiviral property against fish and shrimp virus Kolkovski *et al.* (2011) ^[27]. With these advantages of herbal medicines, we have evaluated herbal medicines against Tilapia Lake virus for both *invitro* and *invivo* study.

Materials and Methods

Virus strain and Cell line

The virus TiLV isolated from the infected tilapia fish was used for the study. The SSN1 cell line derived from snakehead fish in the Department of Fish Pathology and Health Management of FCRI, Thoothukudi was used for virus isolation and propagation. The virus titer (TCID₅₀/ml) was determined by end-point dilution assay using 96 well plates and calculated according to the method of Spearman-Kärber Lei *et al.* (2021) ^[28].

Herbal medicines

In the present study, 6 herbal medicines including Tulasi (*Ocimum sanctum*), Naval (*Syzygium cumini*), Guava (*Psidium guajava*), Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*), Papaya (*Carica papaya*) are the herbal leaves extract used for evaluating antiviral activity. These were collected from FCRI, Thoothukudi. These were tested for their antiviral effect against TiLV infection in cell culture and experimentally infected tilapia fishes.

Determination of the cytotoxicity of the Herbal medicines

The cytotoxicity of the herbal medicines was determined in SSN1 cell cultures by cell viability. For this, medicines were added to the cell cultures in concentrations of 50, 100, 250, and 500 µl/ml and incubated for 7 days. The viability of the cells was determined by staining with a trypan blue solution (0.5%).

Treatment with herbal medicines in cell culture

The assay was performed in the full monolayer of SSN1 cell lines developed in cell culture plastic flask (25 cm²) (Thermo, Korea). When the monolayer became sufficiently confluent, the TiLV was inoculated with 50, 100, 250 and 500 µl of the stock (10^{6.73} TCID₅₀/ml) and incubated for 15 - 20 mins at room temperature. Followed by incubation, the virus solution was removed and herbal medicines was added with a concentration of 500 µl/ml of medium. After 72h of incubation, the result was observed Nefedchenko *et al.* (2015) ^[37].

Treatment using herbal medicines for challenged fishes against TiLV

Fishes and feed

A total of 160 fingerlings of Nile tilapia, *Oreochromis niloticus* (7.4cm in body length) were collected from fish farm, FC&RI, Thoothukudi. The fishes were maintained in 24 glass tanks with 100 L capacity. These fishes were acclimatized in a tank for 7 days period and feed two times per day of control diet between 9.00 am in the morning and 4.30 pm in the evening. About 40% of water was exchanged daily to maintain the water quality. These fishes were screened for the PCR to confirm the TiLV infection free fishes and it has been used for the experimental study. Two types of feed were used for this study, control feed and herbal complex feed. These were purchased from the market.

Feed preparation

Commercial feed was purchased from the market. The binder (sodium alginate) was added 1g in 100 ml of distilled water. The herbal medicines were purchased and incorporated into the feed for three different concentrations include 20 ml, 40 ml, 60 ml per kg of feed. 1kg of feed was weighed and 100ml of binder was added along with herbal medicines. The medicine with binder was added into the feed and mixed well for the proper incorporation of herbal medicines. Then it is air dried in a room temperature for 24 hrs and stored until use.

Challenge study

Two hundred and sixty-four tilapia fingerlings (weight 7 ± 0.5 g) were separated into two groups (duplicate/group) in a total of 24 tanks, 10 fish/tank. There was screened of 6 herbal medicines against TiLV in cell culture and two herbal medicines has showed antiviral property and these two herbal medicines were used for experimental study includes Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*). These medicines were administered into feed with three different concentrations of 20 ml/ kg of feed, 40 ml/ kg of feed, 60 ml/ kg of feed Lewandowski *et al.* (2019) ^[32]. The details of each group are given, negative control as group A, Virus control as group B, treatment with Gooseberry as group C (20 ml/ kg), D (40 ml/ kg), E (60 ml/ kg), treatment with Oregano as group F (20 ml/ kg), G (40 ml/ kg), H (60 ml/ kg), Oregano feed control as group I (40

ml/ kg), J (60 ml/ kg), Gooseberry feed control as group K (40 ml/ kg), L (60 ml/ kg) The control group were given intraperitoneal (IP) injection of L-15 medium at 100 μ L/fish. TiLV infected SSN1 cell line were given intraperitoneal (IP) injection at 100 μ L/fish with $1 \times 10^{6.5}$ TCID₅₀/ml. The herbal medicine mixed feed was given after the virus injection. The mortality and survival rate were monitored for 14 days period. Cumulative mortality and relative percent survival (RPS) from the different treatment groups were calculated as follows: Cumulative mortality (%) = [(total mortality in each treatment after challenge)/ (total number of fish challenged for some treatment)] $\times$ 100. Relative percent survival = 1- [(Mortality (%) in treated group)/ (mortality (%) in control group)] $\times$ 100 Amend *et al.* (1981) [5].

Results

Antiviral activity of herbal medicines in cell culture:

A total of six remedies of aqueous leaves extract of herbal were tested for their toxicity to SSN1 cell line. Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) medicines exhibited cell toxicity only at 1 ml/ml of medium by causing morphological changes and increasing dead cells. Further, these herbals were screened for detecting the antiviral effect against TiLV using the SSN1 cell line. Guava (*Psidium guajava*), Papaya (*Carica papaya*) leaves extract resulted in complete cell toxicity at 50 μ L/ml, 100 μ L/ml, 250 μ L/ml, 500 μ L/ml of concentration. Tulasi (*Ocimum sanctum*) has cell toxicity at 250 μ L/ml, 500 μ L/ml of concentration, The cells are good at 100 μ L/ml of

concentration but has no antiviral property against TiLV inoculated SSN1 cell line. Naval (*Syzygium cumini*) has showed cell toxicity at 100 μ L/ml, 250 μ L/ml, 500 μ L/ml of concentration and for 50 μ L/ml of concentration the cell are good and has no antiviral activity against TiLV. Out of six, two herbal leaves such as Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) showed the antiviral effect against TiLV by reducing the cytopathic effect. 100 μ L/ml, 250 μ L/ml, 500 μ L/ml, concentration of herbal leaves extract was evaluated and out of these three concentrations, 500 μ L/ml of herbal medicines shows antiviral activity. The potential antiviral effect of Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) were found as 70% and 50% in cell culture respectively (Fig.1, 2).

Among these two medicines, Gooseberry leaves extract showed antiviral activity against TiLV infected SSN1 cell line. Oregano leaves extract showed less antiviral effect when compared with gooseberry. Upon infection, the virus inoculated SSN1 cell line monolayer exhibited typical cytopathic effect with the appearance of small foci with cell lysis and rounding up of cells at the edge of foci. No cytopathic effect was found in mock inoculated. The cells were started to regenerated in TiLV inoculated culture flask. Gooseberry leaves extract resulted in the appearance of full monolayer after the TiLV infected SSN1 cell line for 7 days period of incubation. Oregano leaves has showed more amount of cell lysis when compared with other herbal medicines for the period of 7 days. This study proves that the aqueous leaves extract of herbal has effective against fish virus in cell culture.

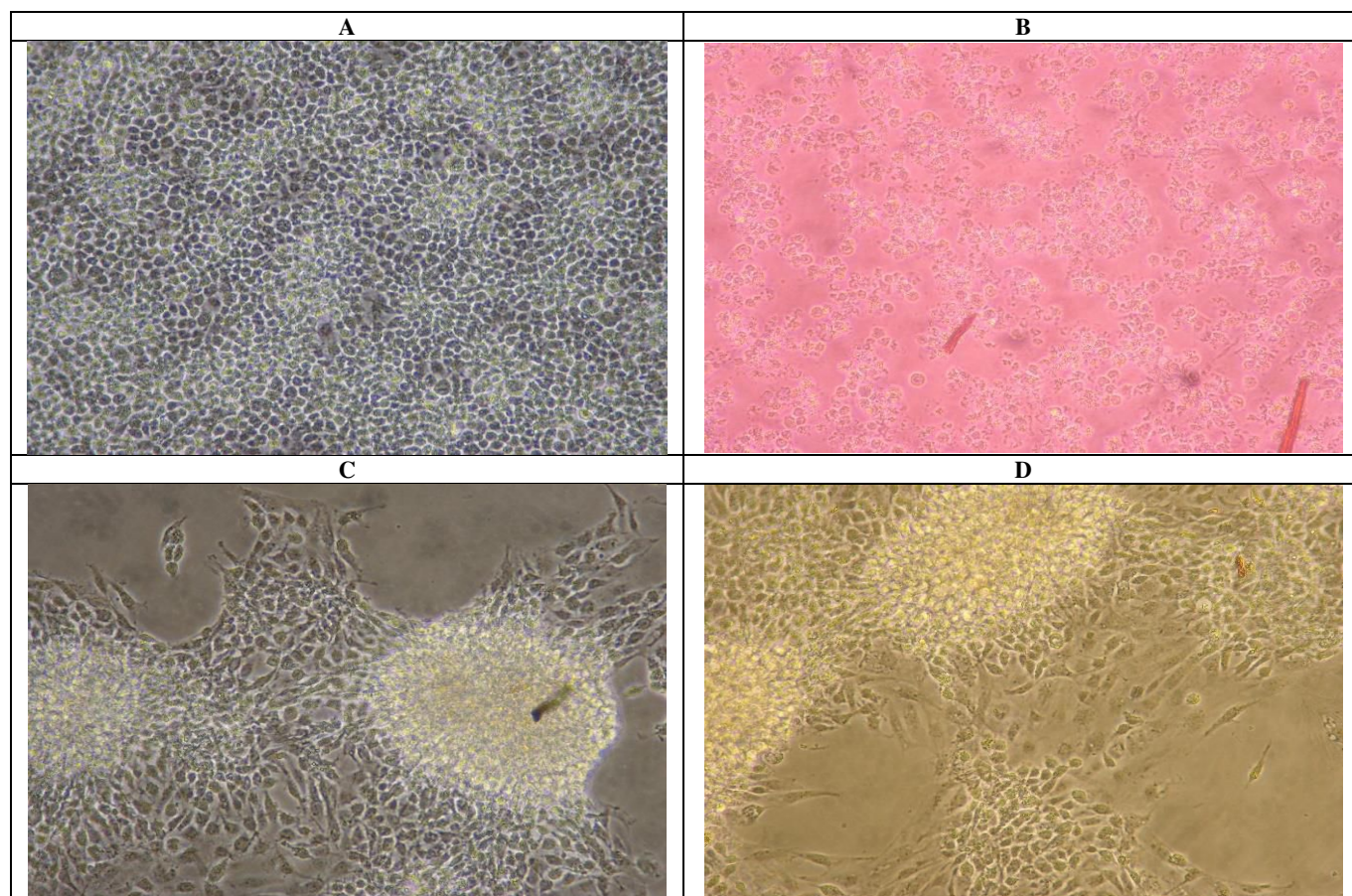


Fig 1: Cytopathic effect induced by TiLV in SSN1 cells. (A) Uninfected control SSN1 cells (B) TiLV (TCID₅₀/ml) infected cells (C) TiLV (TCID₅₀/ml) infected SSN1 cells treated with Gooseberry (*Phyllanthus acidus*) (D) TiLV (TCID₅₀/ml) infected SSN1 cells treated with Oregano (*Coleus amboinicus*).

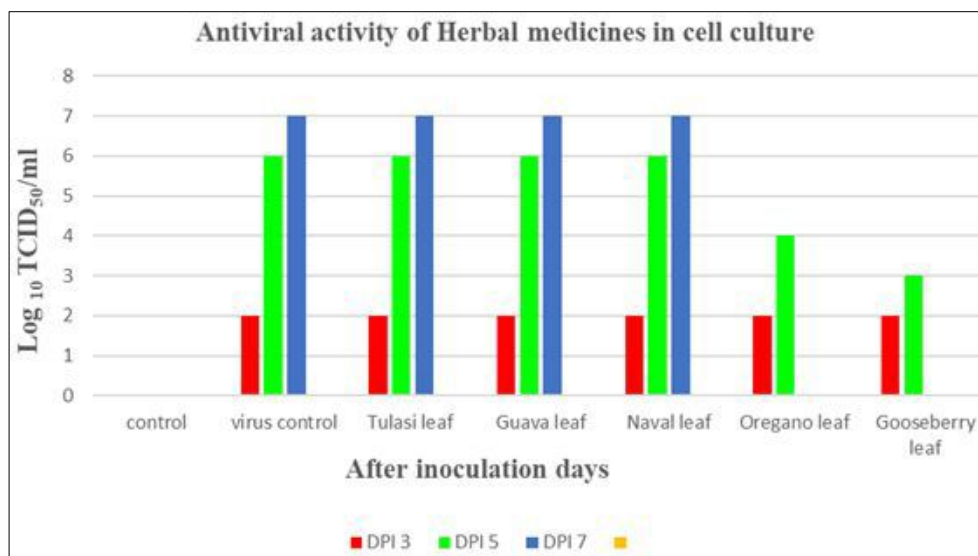


Fig 2: Antiviral effect of aqueous leaves extract in SSN1 cell line against TiLV for the period of 3, 5, 7 days interval.

Antiviral activity of herbal medicines in experimentally infected fishes

In this study herbal extract has been incorporating into feed for the analysis of antiviral effect against Tilapia Lake virus. In this study the herbal extract includes Gooseberry (*Phyllanthus acidus*), Oregano (*Coleus amboinicus*) have been treated using herbal incorporated feed. Tilapia fishes were injected at 100 µl/fish via intraperitoneal route. In this experiment, the mortality started at 3dpi in virus control and the cumulative mortality was occurred at 13dpi (Figure 3). This study was performed for 14 days period using tilapia fingerlings. During the experimental study, the fishes were resulted in certain symptoms include Lethargy, Abdomen swelling, Exophthalmia, Anorexia, Pale skin, haemorrhages, Fluid accumulation in the internal organs during dissection. Most of the fishes were died with the symptoms of abdominal swelling. There was no toxicity were observed on using herbal mixed feed for the negative control fishes. Gooseberry and Oregano mixed feed was given at 40ml and 60ml concentration of leaves extract for negative control

fishes and none of the concentration shows mortality in experimental tank. In this experimental study, Relative percentage survival (RPS) of virus infected tilapia fingerlings was 10%, 35%, 30% in 20ml, 40ml, 60ml concentration of gooseberry leaves mixed feed and for oregano leaves was 5%, 25%, 20% in 20ml, 40ml, 60ml concentration feed as mentioned (Table 1 and 2). The antiviral property of leaves extract was varied at different concentration of medicines. This shows that the aqueous leaves extract of herbal plants has antiviral property against TiLV (figure 4, 5). The highest antiviral effect was observed at 40ml concentration per kg of feed in gooseberry leaves when compared with other treatment. The medium antiviral effect was observed at 60ml in gooseberry, 40ml and 60ml concentration in oregano. The least antiviral effect was observed at 20ml concentration for both the herbal leaves extract against TiLV. There was recovered of fishes against TiLV and it showed that these herbal leaves have some potential against fish virus.

Table 1: Relative percentage survival of Gooseberry treated tilapia fishes against TiLV

	Survival%	Mortality%	RPS value
Negative control	100	0	100
Virus control	0	100	0
Gooseberry 20ml/kg	10	90	10
Gooseberry 40ml/kg	35	65	35
Gooseberry 60ml/kg	30	70	30

Table 2: Relative percentage survival of Oregano treated tilapia fishes against TiLV

	Survival%	Mortality%	RPS
Negative control	100	0	100
Virus control	0	100	0
Oregano 20ml/kg	5	95	5
Oregano 40ml/kg	25	75	25
Oregano 60ml/kg	20	80	20

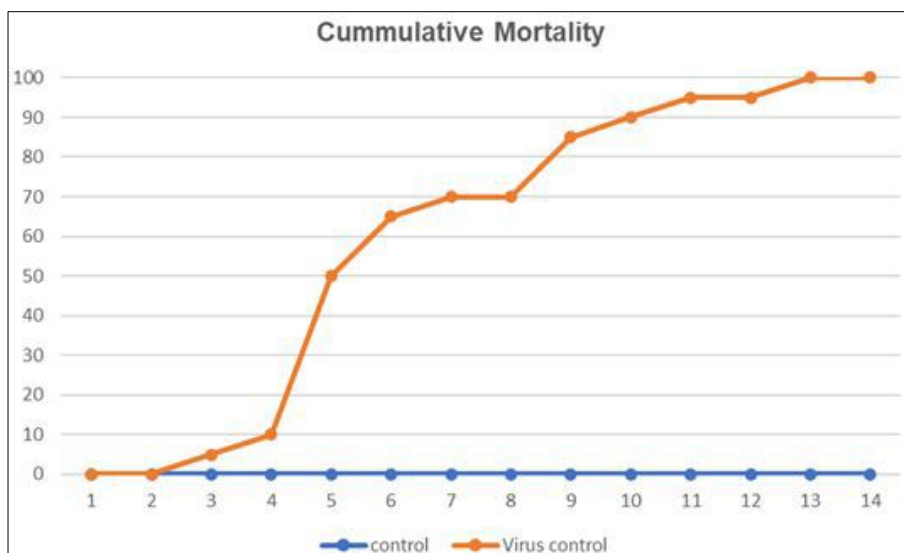


Fig 3: Cumulative mortality occurred at 14 days period of time against TiLV infected fingerlings.

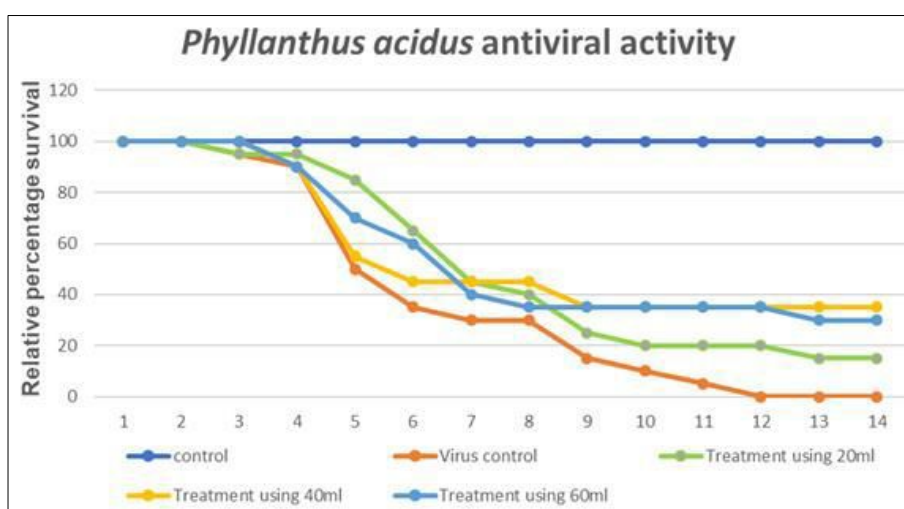


Fig 4: *Phyllanthus acidus* inhibitory effect against TiLV infected fingerlings.

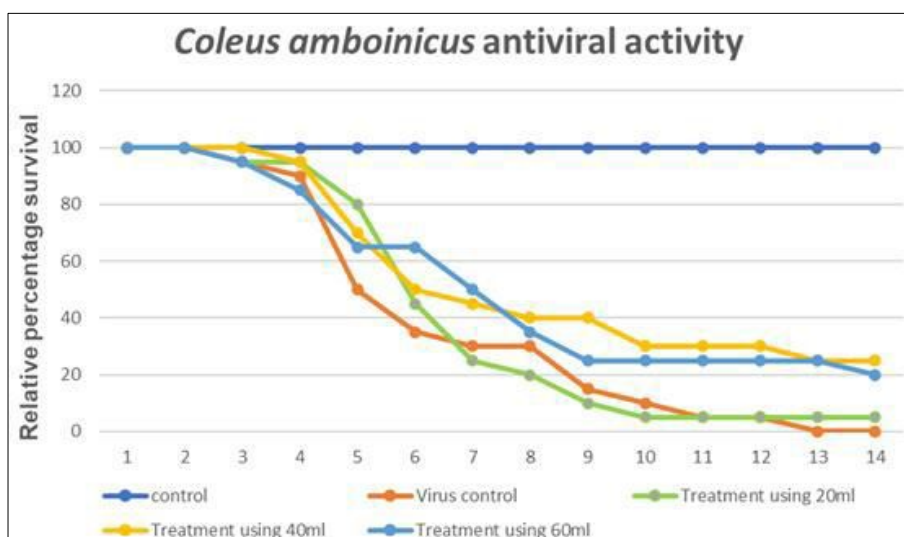


Fig 5: *Coleus amboinicus* inhibitory effect against TiLV infected fingerlings

Discussion

This study shows that the herbal leaves extract has an anti-viral activity against fish virus. Till date there was no proper antiviral drugs or treatment against Tilapia Lake virus and for the prevention of this virus certain studies should be

done on developing vaccines or antiviral compounds (Lertwanakarn *et al.* 2021) [31]. In this study the antiviral compounds have been screened using fish cell line against Tilapia Lake virus. Numerous cell lines have been found to be suitable for TiLV cell culture through research Eyngor *et*

al. (2014) ^[19]; Kembou Tsofack *et al.* (2017) ^[25]. Use of herbal medicines in the feed is promising for treating large batches of fish that are suffering from bacterial infections and to prevent loss. Oral treatment has been producing remarkable survival rates in fish with bacterial sickness Valladão *et al.* (2015) ^[54]. Due to their efficiency, safety, environmental friendliness, and reduced drug resistance, herbs and their extracts have become more and more promising supplements and alternatives Zhang *et al.* (2022) ^[57]. *Eclipta alba* aqueous leaf extracts have non-specific immune response and disease resistance against experimentally infected *Oreochromis mossambicus* against *A. hydrophila* Christyapita *et al.* (2007) ^[15]. Aqueous extracts of *P. guajava* leaves has antiviral activity against Newcastle Disease Virus in chicken Chollom *et al.* (2012) ^[14]. In this study we have screened the antiviral efficacy of herbal medicines in SSN1 cell line and then used of herbal diet for experimentally infected tilapia fishes against TiLV. *P. acidus* leaves and roots are used as antidotes to viper venom Anjaria *et al.* (2002) ^[6] and to ameliorate hypertension in humans Leeya *et al.* (2010) ^[30] and it acts as an antiviral against TiLV in cell culture. The methanolic extract of pulp of *P. acidus* resulted as antibacterial, hypoglycemic, antidiarrheal, anaesthetic and analgesic compounds in experimental mice Afrin *et al.* (2016) ^[20]. The acetone extract of fruit of *P. acidus* was evaluated for anticancer and anti-inflammatory activity in cancer cell line Priya *et al.* (2015) ^[43]. *P. acidus* and *P. guajava* extracts showed plaque reduction against IHN and IPNV, while *P. acidus* alone exhibited 100% plaque reduction for IPNV and OMV Direkbusarakom *et al.* (1996) ^[17]. *P. guajava* aqueous leaves extract has resulted cell toxicity in SSN1 cell line at different concentration of medicine. *P. acidus* and *P. guajava* aqueous leaves extract acts as an immunostimulant for Nile tilapia infected *Streptococcus agalactiae* and herbal treatment using commercial feed diet has positive results for fish diseases Kamble *et al.* (2018) ^[24]. *C. amboinicus* leaves extracted oil act as strong antibacterial activity against enteric pathogens in human Velasco *et al.* (2009) ^[55] and aqueous extract of *C. amboinicus* leaves resulted in 50% cell survival against TiLV infected SSN1 cell line. *C. amboinicus* leaves extract acts as a nano particle using green synthesized method which resulted in antibacterial activity against human pathogens Ramya *et al.* (2022) ^[44]. Oregano (*Coleus amboinicus*) aqueous and methanolic leaves extract act as an anti-asthmatic effect in mice model Robles *et al.* (2017) ^[47]. The aqueous extract of Tulsi (*Ocimum sanctum*) plant had a low inhibitory effect on paramyxovirus but a significant inhibitory effect on orthomyxovirus in animal virus Patil *et al.* (2008) ^[41] and the aqueous leaves extract also showed no antiviral activity for TiLV in cell culture. The aqueous leaf extract of *O. sanctum* used for the synthesis of silver nanoparticles and acts as an antibacterial activity Brahmachari *et al.* (2014) ^[12]. Tulsi water extract resulted in antioxidant property in human hepatoma cell line Shivananjappa *et al.* (2012) ^[51]. The seeds of *Carica papaya* extract using petroleum-ether against *I. multifiliis* were evaluated against infected gold fishes Ekanem *et al.* (2004) ^[18]. The leaves extract of *Carica papaya* has been screened for the growth, feed conversion efficiency and disease resistance on juveniles *Clarias gariepinus* against *Aeromonas hydrophila* Olusola *et al.* (2018) ^[40]. *Carica papaya* has resulted in cell toxicity even at low concentration in SSN1 cell line against TiLV.

The methanolic extract of papaya leaves extract has good result in embryonic development, egg hatchability and good survival in larval Asian sea bass Haser *et al.* (2022) ^[22]. Naval (*Syzygium cumini*) leaves extract acts as a growth and immunomodulatory potential of *Vibrio parahaemolyticus* on the juveniles of *Litopenaeus vannamei* Prabu *et al.* (2018) ^[42]. *Syzygium cumini* dried fruit extract have been evaluated using dietary supplementation for hybrid catfish and analysed of growth and physiological parameters Munglue *et al.* (2019) ^[36]. The seeds of medicinal plants of *Syzygium cumini* was incorporated into feed diet for the early juveniles of *Macrobrachium malcolmsonii* Bhavan *et al.* (2014) ^[11]. *Syzygium cumini* fruit extract acts as a growth inhibitor and induces apoptosis in cervical cancer cell lines in humans Barh *et al.* (2008) ^[8]. The ethanolic extract of *Syzygium cumini* fruit has an anti-cancer agent in cancer cell lines Khodavirdipour *et al.* (2021) ^[26]. The leaves extract of *Syzygium cumini* has an antidiabetic and anti-oxidant property for both *invitro* and *invivo* study in humans Chagas *et al.* (2018) ^[13]. Aqueous leaves extract of *Syzygium cumini* resulted in antiviral property of SSN1 fish cell lines. This is the first report of using these six herbal leaves extract against *Tilapia Lake virus* for both *invitro* and *invivo* study. This show that the herbal leaves extract has antiviral potential against TiLV for both *invitro* and *invivo* study. Results of antiviral effect of leaves of gooseberry and oregano has confirm the inhibition of TiLV at minimal level through feed incorporation. *P. acidus* leaves has more inhibitory effect at 40ml concentration of feed when compared with other treatment against TiLV. *C. amboinicus* leaves has better survival at 40 and 60ml concentration of feed. *P. acidus* and *C. amboinicus* leaves has shown low survival at 20ml concentration of feed. These aqueous leaves extract has shown antiviral property but the survival rate was minimum in tilapia fingerlings against challenged with TiLV. Further screening of certain herbal treatment will pave the way for the inhibition of virus in fishes. RPS valued should be above 60% for the treatment against disease. This experimental study has RPS value of 60% below which clearly showed that the above screened herbal medicine has better survival against TiLV.

Conclusion

An aqueous leaves extract of herbal medicine has antiviral potential against tilapia lake virus for both *invitro* and *invivo* study. This study presents the first evidence of the application of herbal mixed diet against TiLV challenged fishes. This study has minimum survival rate against experimentally infected tilapia fishes. We have demonstrated that the herbal medicine acts as a therapeutic agent against fish virus. In this study, the treatment was done using medicated feed. This new information could be used in future research for the screening of certain herbal remedies against fish diseases.

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Authors' Contribution

Rakesh P: Formal analysis and writing original draft of the manuscript; Sivasankar P & Chrisolite B: conceptualized,

methodology, designed and supervision of the study; Magesh Kumar P, Mohamed Mansoor M & Selvamageswaran M: reviewed the draft of the manuscript; Ramesh P & Vasanth K: data analysis and interpretation.

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