World Journal of Pharmaceutical research

Volume 3, Issue 1, 498-507. Research Article ISSN 2277 – 7105

PRELIMINARY PHYTOCHEMICAL SCREENING AND EFFECT OF *AEGLE MARMELOS* EXTRACTS AGAINST BACTERIAL COLD WATER DISEASE CAUSING ORGANISM

***Kumar Prasanna[1] and Devaraj Raghunathan[2]**

[1]Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Chinna Kolambakkam, Maduranthagam Tk, Kancheepuram-603 308.

[2]Department of Microbiology, School of Life Sciences, Hindustan College of Arts and Science, Padur, Kelambakkam, Chennai

Article Received on
28 September 2013

Revised on 30 October 2013,
Accepted on 06 December 2013

***Correspondence for Author:**
Kumar Prasanna
Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Chinna Kolambakkam, Kancheepuram, India.
prasanna_naveenmadhu@yahoo.com

ABSTRACT

Bacterial cold water disease is common in the fresh water streams, ponds, reservoirs and commercial farming ponds which are caused by *Flavobaterium psychrophilum* of family Flavobacteriaceae. Increasing in sight of antibiotic resistance strains of the fish pathogen the effect of newly developed chemical constituents are required to prevent the infection or disease it causes. The Indian system of utilizing medicinal plants for treating the pathogen with sacred tree of Indian temples Vilvam bark *Aegle marmelos* is effectively studied for the effectiveness over *Flavobacterium psychrophilum*. The standard well diffusion method was followed with varying concentration from 50-250 mg/ml and standard protocol of Harbone's phytochemical analysis to carry out phytochemical studies. It has shown sensitivity to the bark extracts of hexane and aqueous 15mm each extract at 250mg/ml. The screening of secondary metabolites revealed flavonid, steroid and terpenoids which has made Vilvam bark as a good source of antibacterial agent. *Aegle marmelos* could be used to treat or prevent the infection related to bacterial cold water disease and higher specification on effective studies need to carry for development of new chemical constituent or drug.

Key words: bacterial cold water disease, medicinal plant, well diffusion, sensitivity.

INTRODUCTION

Medicinal plants are believed to treat disease and it has been in usage since ancient days has a primary health care. But the effect of medicinal plant in other aspects like environmental conditions, aquatic conditions are need to be scientifically validated and their effectiveness over other factors has to be tabulated. In the effect to study the aquatic treatment of bacterial cold water disease causing organism against Indian sacred tree of Shiva temple Vilvam Bark (*Aegle marmelos*).

Aegle marmelos

Aegle marmelos (Rutaceae) found all over India which is originated from Eastern Ghats and central India. Mostly found in lord Shiva temple because it is considered as a sacred tree. It is indigenous to Indian sub continent and mainly found in tropical and subtropical regions. Vernacular name of *Aegle marmelos* has been called different in different regions in India, Bael-Hindi, Bilva-Sanskrit, Bilvaphal-Gujarati, Belo-oriya, and Vilvam-Tamil.

Aegle marmelos is a slow growing medium sized tree upto 15m tall with spiny branches with bark flake and spreading. Leaves are alternate, deciduous borne singly composed of 3-5 oval pointed toothed leaflets with long petiole. Fruits are yellowish green with small dots on the outer surface. Pulp is yellow in color and seeds are embedded in it with white, cotton like hairs on their surface. Flowers are greenish white in color, bisexual and hypogenous stalk. The flowers are borne in lateral; panicles from leaf axils.

Traditional uses

Aegle marmelos has been extensively used in treatment for disease has primary health care in traditional medicine. Leaves of *Aegle marmelos* are used in the treatment of stomach ailments and gastroenteritis. The extract of leaves is beneficial in the treatment of leucorrhea and conjunctivitis. Fruits are carminative and astringent and find good utilities in thyroid related disorder. The bark is used in the treatment of diarrhea, bowel irritation and as a stimulant. The entire plant is used for primary health care in ancient medicine and can be scientifically validate to develop new pharmacological drug [1]. The effect of environmental conditions is still to be evaluated for its effectiveness. The usages of medicine in humans are extensively given and the effect over human illness causing effect is studied earlier.

The effect of the bark against the bacterial cold water disease causing organism of the family Flavobateriaceae in the present study for the scientific validation for environmental crisis.

Bacterial cold water disease

Bacterial cold water disease is common in fresh water fishes and cultivable pond fisheries. It was first described by Borg [2] (1948) that the causative agent is a gram negative rod shaped bacterium that cause acute septicemic infections and lesions. In 1956 Wood and Yasutake [3] found septicemic infection in salmonids and other species which caused lesions and peduncle in the fishes. Later studied and revealed that it was a case of bacterial cold water disease. It has synonyms of Rainbow trout fry syndrome, Bacterial fly anemia, Peduncle disease and Tail rot.

Distribution

It was initially isolated in Washington in 1948 by Borg and this bacterium has been found in many salmonids producing regions. Not only in western hemisphere it also identified in England, Australia, Canada, German and European countries.

Flavobacterium psychrophilum

Flavobacterium psychrophilum is a gram negative, long, thin rod bacteria which can be isolated from affected fishes and bacterial colonies grow at 18-25˚C and individual colonies in yellow in color. The disease caused by this organism is a hit in salmonids producing regions where the infections cause in the parts of gill, tail and muscle tissues. It is also reported for eel, carp, and tench [4, 5]. In younger fishes the severity range is higher compare to the adult fishes. The infection has set of lesions with yellow color edges to caudal peduncle regions [6]. These lesions may cause muscle tissue errodement and fin erosion. In highly infected fish, the bacteria *Flavobacterium psychrophilum* can be easily found in the parts of liver, intestine, peritoneum and spleen [3].

Pathogenesis

The infection of *Flavobacterium Psychrophilum* most likely in fresh water and aquatic environment conditions. The fish in which dead are likely to contain more number of bacterial colonies [7]. The fish samples has recovered and collected has bacteria infected at skin and inflicted injuries [8]. The bacteria can strongly inhibit or suppresses the non specific humoral defense mechanism of which infection at higher rate [9]. The effect of histological and necrosis factor of the internal organs of infected fish has been observed [3, 10, 11, 12, 13, 14, 15]. *Flavobacterium psychrophilum* associates with phagocytes in kidney, spleen with particularly affected with hemosiderosis, necrosis and hemorrhage [10, 11, 13, 16]. The virulence nature of

the bacteria is determined for severity of Bacterial cold water disease and strains of *Flavobacterium psychrophilum* with high virulence [14, 17, 18].

Treatment for bacterial cold water disease

The chemical treatment and stress management were used to treat Bacterial cold water disease at controlled condition. However, the antibiotics like sulfonamides, nitro furans and nifurpirinol are used against *Flavobacterium psychrophilum* which showed effective, but these antibiotics are of carcinogenic and not registered or licensed for use in treating Bacterial cold water disease. Because these fishes are consumed and used for commercial purpose [19, 20, 21, 22]. The other chemical drugs like Oxytetracycline, Amoxylin and Oxolinic acid were used and former showing resistance and developing the resistance to strains and United States approving for Florfemicol for prevention and therapeutic agent [16, 23, 24, 25, 26, 27]. However the bacteria developing resistance to antibiotics and quality of the effective parameters have to be reassessed and sensitive chemical drug should be produced to prevent future infection.

MATERIALS AND METHODS

Plant collection

Bark sample was collected from nearby lord Shiva temple and bark is cut into small pieces. Then the bark sample is kept for shadow drying for 3 weeks and powdered with electric blender.

Extraction

The powdered sample was weighed 25g and packed in the sox-let apparatus with 250 ml of solvent which is 1:5 ratio. The solvent system is used of high polar gradient from hexane-chloroform and distilled water. Then extraction was carried out and the residues were air dried and then the residues were collected and stored in 4˚C for future use.

Concentration of the extract

The residue of extracted sample was taken and weighed 1g and dissolved in 100 ml of mother solvent and then the varying concentration was taken for analyzing antibacterial activity.

Bacterial species

The fish pathogen *Flavobacterium psychrophilum* MTCC NO: 2495 has been procured from Microbial type culture collection centre, IMTECH, Chandigarh, India. The bacterial culture of *Flavobacterium psychrophilum* was sub cultured in the modified Wakimoto medium slants

at 30˚C and stored at 4˚C for future use. For the antibacterial screening of the organism, it was sub cultured in the nutrient broth and tested in nutrient agar at 30˚C.

Media components

The media compounds were purchased from Micro fine chemicals.

Composition of Wakimoto Medium:

Ingredients	Gm/lit
Ca(NO3).4H2O	0.5
Na2HPO4.12H2O	2.0
Peptone	5.0
sucrose	15.0
FeSO4.7H2O	0.5
Agar	15.0

Composition of Nutrient agar:

Ingredients	Gm/lit
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0
Agar	15.0

Composition of Nutrient broth:

Ingredients	Gm/lit
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0

Phytochemical analysis

The preliminary phytochemical studies were carried out by standard protocols [28].

Test for alkaloid: one ml of extract with 2 ml of Mayer's reagent [1.36gm of Mercuric chloride and 5.0gm of Potassium iodide in 100 ml of water] which gives dull white or creamy precipitate.

Test for flavonid: one ml of the extract with 1 ml of ferric chloride which gives brown color.

Test for phenolic compound: one ml of the extract with 3 ml of 5% ferric chloride which gives dark blue or bluish black color.

Test for steroid: one ml of the extract with 1 ml of chloroform and equal volume of concentrated sulphuric acid added along the sides of the test tube, where upper layer turns red and sulphuric acid layer turns yellow with green florescence.

Test for phytosterols: one ml of the extract with 2ml of chloroform and added 10 drops of acetic anhydride and further added 5 drops of concentrated sulphuric acid gives change in red color through blue to green.

Test for terpenoids: one ml of the extract with 3 ml of warm acetic acid and drop of concentrated sulphuric acid gives red color and changes to blue color.

Test for saponnins: one ml of the extract with 20 ml of distilled water and shaken for 15 min and observed formation of the foam.

Test for volatile oils: one ml of the extract was added in china dish and heated it and residues gives aromatic smell.

Test for glycoside: one ml of the extract with 3 ml of chloroform and shaken and add 10% ammonium solution which gives pink color.

Antibacterial assay

Agar well diffusion method was followed for the antibacterial study against varying concentration of the bark extracts ranging from 50mg/ml, 100 mg/ml, 150 mg/ml, 200mg/ml and 250 mg/ml. and compared with commercial antibiotic of ampicillin with varying concentration. The Petri plates were swabbed with *Flavobacterium psychrophilum* from the broth culture and the wells were made by 6mm of sterile cork borer at minimum distance to the each well. Then the plant extracts has been added to it for the screening of antibacterial effectiveness.

RESULTS

The following result was obtained for the phytochemical analysis of vilvam bark and effect of antibacterial activity against *Flavobacterium psychrophilum*.

Phytochemical analysis

The presence of secondary metabolites in the bark of Aegle marmelos extracts has relieved [Table 1], validated and it shows the presence of flavonid, steroid and terpenoids contents.

Table 1: Phytochemical analysis of bark of Agele marmelos.

S.NO.	Compounds	Hexane extract	Chloroform Extract	Aqueous extract
1	Alkaloid	-	-	-
2	Flavonid	+	+	+
3	Steroid	+	+	+
4	Phytosterols	-	+	-
5	Terpenoids	-	+	+
6	Saponnins	-	-	+
7	Volatility	+	+	+
8	Glycoside	-	-	-
9	Phenol	-	-	-

+ = positive

- = negative

Antibacterial activity

The well diffusion method has shown the extracts effectiveness against bacterial pathogen with varying concentration with respective inhibiting zones [Table 2]. The extracts actively diffused for the growth microorganism which has inhibited their growth and resulted in positive note.

Table 2: Effect of bark extract against bacterial pathogen and their inhibition zones.

S.NO:	Species	Con. of the well mg/ml	Hexane extract In mm	Chloroform extract In mm	Aqueous extract In mm	Standard (Ampicillin) In mm
1.	*Flavobacterium psychrophilum*	50	-	-	-	8
		100	-	-	9	10
		150	9	-	11	12
		200	12	-	13	15
		250	15	-	15	20

DISCUSSION

The extracts of hexane with 15 mm and aqueous extract showing 15 mm at their higher concentration of 250 mg/ml. The chloroform extract does not show any inhibition on the microorganism. The antibacterial effectiveness might be due to the presence of secondary metabolites like steroids, terpenoids and flavonid. Thus the relative sensitivity leaves another option for preventing bacterial cold water disease with higher knowledge and other specific studies related to which compound responsible for the inhibition for the growth of *Flavobacterium psychrophilum* and it might help in the developing new chemical drug for the effective treatment of Bacterial cold water disease.

REFERENCES

1. Sharma PC, Bhatia V, Bansal N, Sharma A. A Review on Bael tree IndJ.Nat.Prod. Resource 2007; 6(2):171-8.
2. Borg A.F. Studies on myxobacteria associated with diseases in salmonid fishes. Doctoral dissertation. (1948) University of Washington, Seattle.
3. Wood E.M. & Yasutake W.T. Histopathology of fish III-peduncle (“cold water”) disease. *Progressive Fish Culturist* (1956) 18, 58-61.
4. Lehmann J., Mock D., Sturenberg F.J. & Bernardet J.F. (1991) First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. *Diseases of Aquatic Organisms* 10, 217-220.
5. Wakabayashi H., Toyama T. & Iida T. A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathology* (1994). 29, 101-104.
6. Holt R.A., Rohovec J.S. & Fryer J.L. (1993) Bacterial cold-water disease. In: *Bacterial Diseases of Fish* (ed. by V. Inglis, R.J. Roberts & N.R. Brombage), pp. 3-22. Blackwell Scientific Publications, Oxford.
7. Madetoja J, Nyman P, Wiklund T. *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss. Dis Aquat Organ* 2000; 43: 27-38.
8. Miwa S, Nakayasu C. Pathogenesis of experimentally induced bacterial cold water disease in ayu *Plecoglossus altivelis. Dis Aquat Organ* 2005; 67: 93-104.
9. Siwicki AK, Pozet F, Morand M, Kazu K, Gqbski E, Trapkowska S. Atypical bacterial gill disease in rainbow trout (*Oncorhynchus mykiss*): influence on non specific humoral defence mechanisms. Archives of Polish Fish 2004; 12: 5-12.

10. Bruno DW. *Cytophaga psychrophila* (*Flexibacter psycrophilus*) (borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the UK. *B Eur Assoc Fish Pat* 1992; 12: 215-6.
11. Decostere A, D'Haese E, Lammens M, Nells H, Haesebrouck F. *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. *J Fish Dis* 2001; 24: 481-7.
12. Ekman E, Norrgren L. Pathology and immunohistochemistry in three species of salmonids after experimental infection with *Flavobacterium psychrophilum*. J Fish Dis 2003; 26: 529-38.
13. Ekman E. Natural and experimental infections with F*lavobacterium psychrophilum* in salmonid fish. Ph.D. Thesis. Swedish University of Agricultural Sciences, ppsala 2008.
14. Lorenzen E, Dalsgaard I, From J, *et al*. Preliminary investigation of fry mortality syndrome in rainbow trout. *Bull Eur Assoc Fish Pathol* 1991; 11: 77-9.
15. Obach A, Baudin-Laurencin A. Vaccination of rainbow trout *Oncorhynchus mykiss* against the visceral form of coldwater disease. *Dis Aquat Organ* 1991; 12: 13-5.
16. Dalsgaard I, Madsen L. Bacterial pathogens in rainbow trout *Oncorhynchusmykiss* (Walbaum), reared at Danish freshwater farms. *J Fish Dis* 2000; 23: 199-209.
17. Nicolas P, Mondot S, Achaz G, Couchenot C, Bernardet J, Duchaud E. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum. Appl environ Microbiol* 2008; 74: 3702-9.
18. Stenholm AR, Dalsgaard I, Middelboe M. Isolation and characterization of bacteriophages infecting the fish pathogen *Flavobacterium psychrophilum*. *J Appl Microbiol* 2008; 74: 4070-8.
19. Amend DF. In: Snieszko SF Ed. A Symposium on Diseases of Fishes and Shellfishes Maryland: *Am Fish Soc* 1970; 5: 258-65.
20. Cipriano RC, Holt RA. *Flavobacterium psychrophilum,* cause of bacterial cold-water disease and rainbow trout fry syndrome. United States Department of Interior, U.S. Geological Service, National Fish Health Research Laboratory 2005; Fish Disease Leaflet No. 86.
21. Post GP. Textbook of fish health. New Jersey; T.F.H. Publications 1987.
22. Wood JW. Diseases of Pacific salmon their prevention and treatment, 3rd edition. lympia: Washington Department of Fisheries 1979.

23. Bruun MS, Madsen L, Dalsgaard I. Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities. Aquaculture 2003; 215: 11-20.
24. Bruun MS, Schmidt AS, Madsen I, Dalsgaard I. Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 2000; 187: 201-12.
25. Chen YC, Davis MA, LaPatra SE, Cain KD, Snekvik KR, Call DR.Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, O*ncorhynchus mykiss* (*Walbaum*), and spawning coho salmon, *O. kisutch* (Walbaum). *J Fish Dis* 2008; 31: 765-73.
26. Gultepe N, Tanrikul TT. Treatment methods of *Flavobacterium psychrophilum*: cause of rainbow trout fry syndrome (RFTS) and bacterial coldwater disease (BCWD) in Turkey. *J Fish Int* 2006; 1:102-5.
27. Hadidi S, Glenney GW, Welch TJ, Silverstein JT, Wiens GD. Spleen size predicts resistance of rainbow trout to Flavobacterium psychrophilum challenge. *J Immunol* 2008; 180: 4156-65.
28. Harbone J.B., (1998) 'phytochemical methods'. A guide to modern techniques of plant analysis 3rd edn. Chapman and Hall, New York, 1-150.