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Phytochemical analysis of antimicrobial bioactive constituents from *Oscillatoria sp.*

KEYWORDS: Algae, *Oscillatoriasp* ,phytochemical, antibacterial activity, GC-MS, bioactive

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ABSTRACT Fresh water algae are potential sources of biologically active compounds with antiviral, antibacterial, antifungal, and anticancer activities. In the present investigation, the effect of different solvents, including methanol, acetone, petroleum ether, benzene, chloroform, hexane, diethyl ether, ethanol, ethyl acetate were done on Oscillatoriasp isolated from Arasinamakki, near Shishila. Dried algal powder after identification were cold extracted & screened for glycosides,alkaloids, saponins,flavonoids, tannins,terpenoids,phenolics, anthraquinones, cardiac glycosides, etc. The extracts showing good phenolic content were tested for antibacterial activity against fish pathogens namely Aeromonashydrophila.

1. Introduction:

Cyanobacteria are found in a variety of habitats; live in terrestrial, fresh, brackish, or marine water. They usually are too small to be seen individually, but sometimes can form visible colonies (Mankiewics et al., 2003; Briand et al., 2003; Mazur et al., 2003). Algae are now drawing a greater interest following the increase in demand for biodiversity in the screening programs as therapeutic drugs from natural products. Their ubiquitous distribution throughout the biosphere, (Tandeau-de-Marsac, 1993); allow them to grow essentially under all environmental conditions , ranging from freshwater to extreme salinity, and can survive in moist, black earth and even desert sands and they have as well been found in clouds, being in addition essential components of coral reefs. This wide spectrum of ecosystems contributes to the myriad of chemical compounds that they are able to synthesize, thus accounting for their unique potential as antimicrobial agents. In the present study dried algal extracts were dissolved in different solvents with the increasing order of polarity, different extracts were tested for the presence of chemical constituents. The extracts showing good phenolic content were estimated &analyzed for antimicrobial activity against fish pathogens *Aeromonashydrophila.*

2. Material & method:

2.1 Sample preparation & extraction:

Samples were collected fromArasinamakki, near Shishila. Dakshinakannada, India .Algae samples were cleaned all epiphytes and necrotic parts were removed. Samples were rinsed with sterile water to remove any associated debris. Sample was kept under sunshade for 7 days. After drying the sample, it was ground thoroughly to powder form. The powdered samples were extracted with at room temperature with different solvents. The extraction is carried out for 10 days, after that extracts were filtered ,the filtrate is dried using rot evaporator and concentration is determined & subjected to analysis. (Gonzalezdelval et.al, 2001.)

2.2 Phytochemical analysis:

The extracts were subjected to phytochemical tests for presence of following biomolecules by using the standard qualitative procedures as described in literature [Guzman et al.,2001].

1.) Test for Glycosides :10 ml of 50% H2SO4 was added to the 1 ml of extract in a boiling tube. The mixture was heated in boiling water bath for 5 min. 10 ml of Fehling's solution (5 ml of each solution A and B) was added and boiled. A brick red precipitate indicated the presence of glycosides.

2.) Test for Alkaloids: 1 ml of 1% HCl was added to the 3 ml of extract in a test tube and was treated with few drops of Meyer's reagent. A creamy white precipitate indicated the presence of alkaloids.

3.) Test for saponins
5 ml of extract was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observedfor the formation of emulsion, which indicated the presence of saponins.

4.) Test for flavonoids
A few drops of 1% NH3 solution was added to the 2 ml of extract in a test tube. A yellow coloration was observed for the presence of flavonoids.

5.) Test for tannins
To 0.5 ml of extract solution, 1 ml of distilled water and 1-2 drops of ferric chloride solution were added and observed for brownish green or a blue black coloration.

6.) Test for terpenoids
5 ml of extract was mixed with 2 ml of CHCl3 in a test tube. 3 ml of concentrated H2SO4 was carefully added along the wall of the test tube to form a layer. An interface with a reddish brown coloration was confirmed the presence of terpenoids.

7.) Test for cardiac glycosides
5 ml of extract was mixed with 2 ml of glacial acetic acid containing 1 drop of FeCl3.The above mixture was carefully added to the 1 ml of concentrated H2SO4. Presence of cardiac glycosides was detected by the formation of a brown ring.

8.) Test for phlobatannins
10 ml of extract was boiled with 1% HCl in a boiling tube. Deposition of a red precipitate indicated the presence of phlobatannins.

9.) Test for Anthraquinones
Extract was mixed well with benzene, and then half of its own volume of 10% ammonia solution was added. Presence of a pink, red or violet coloration in the ammonial phase indicated the anthraquinones.

10.)Test for Phenols
3 mL of 10% lead acetate solution were added to 5mL of plant extract. A bulky white precipitates indicated the presence of phenols.

11.)Test for Resins:Extract few amount of 95% ethanol add few amount of 4% HCl turbidity indicates presence of resins

12.)Test for sterols:Extract treated with choloroform& sulphuric acid ,development of green then red colour indicates sterol positive.

2.3 Estimation of Phenolic content

Volume - 7 | Issue - 2 | February - 2017 | ISSN - 2249-555X | IF : 3.919 | IC Value : 79.96

The amount of total phenolics in methanol extract was determined with Folin– Ciocalteu reagent according to the method of Singleton and Rossi with Gallic acid as the standard [Herrero et al.,2006]. Briefly standard stock solution of 10 mg/10 ml of gallic acid was prepared in distilled water. From this, various concentrations ranging from 200-1000 µg / ml were prepared. To this 1 ml Folin and Ciacalteau reagents (1:2 with water) was added and kept at room temperature for 5 min and then 1 ml of 7% sodium carbonate solution was added to the reaction mixture and incubated at room temperature for 90 minutes. The colour developed was read at 750 nm. A 100 µl of methanol extract of sample was mixed with the same reagents. Gallic acid was used as the reference standard and the results are expressed as milligram gallic acid equivalent (mg / g dry weight of *Oscillatoria sp.*

2.4 Antibacterial activity

Pure cultures of *Aeromonashydrophila,* was used as test microorganisms. Different solvent extracts were checked for antibacterial activity against the lawn cultures by agar well diffusion method. In each respective solvent is chosen as in the form of control.

2.5 Gas chromatography and mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was performed using an BR-5MS(5%Diphenyl/95% Dimethyl poly siloxane)capillary column (length 30 m × diameter 0.25 mm × film thickness 0.25 µm) with helium at 1 ml for 1 min as a carrier gas. The mass spectrometer was operated in the electron impact (El) mode at 70 eV in the scan range of 50–500 m/z. The split ratio was adjusted to 1:10, and the injected volume was 2 µl. The injector temperature was 280^{0}C, and the oven temperature was kept at 110^{0}C for 3.5 min, rose to 280^{0}C at 5^{0}C min−1 (total run time 37.50 min). Peak identification of crude Oscillatoriaextracts were performed by comparison with retention times of standards, and the mass spectra obtained were compared with those available in NIST libraries (NIST 11 – Mass Spectral Library, 2011 version) with an acceptance criterion of a match above a critical factor of 80% according to Srinivasan et al 2013.

3. Results & Discussion:

3.2.2Phytochemical analysis

Important phytochemicals, such as alkaloids, triterpenoids, steroids, tannin, saponin, coumarins, terpenoids, quinine, phytosteroids, phlobatannins and flavonoids were screened for their presence and presented in Table 1

Table1; Phytochemical constituents of Oscillatoria extracts

Oscillatoria sp.	Ethanol	Acetone	Methanol	DiEthylEther	Benzene	Chloroform	Hexane	Petroleum ether	Ethyl Acetate
Glycosides	--	--	--	--	--	--	--	--	--
Alkaloids:	--	--	--	--	--	--	--	--	--
Saponins	++	++	++	++	++	--	--	++	++
Flavonoids	++	++	++	--	--	++	--	--	
Tannins	--	--	--	++	+	++	--	--	++
Terpenoids	--	++	++		++	++	--	--	++
cardiac glycosides	++	++	++	++	++	++	++		++
Phlobatannins	--	--	--	--	--	--	--	--	--
Anthraquinones	--	--	--	--	--	--	--	--	--
Phenols	+	+	+	--	--	--	--	--	--
Sterols	--	--	--	--	--	--	--	--	--
Resins	--	--	--	--	+	--	--	--	--

3.2.3Estmation of Phenolic content

Diethyl ether, Acetone & Ethyl acetate Oscillatorias extracts shown positive for tannins,flavonoids,terpenoids are analyzed .Highest phenolic content shown in ethyl acetate extract Table 2

Table2: Assay of Phenolic content of OscillatoriaAcetone, Diethyl ether & Ethyl acetate extracts

Sl.No	StdConc	OD 750nm	Sample extract(0.1ml)	OD at 750nm
1	0.2mg	0.24	Methanol	0.41
2	0.4	0.41	Chloroform	0.07
3	0.6	0.90	Acetone	0.17
4	0.8	1.22	Benzene	0.10
5	1.0	1.95	DEE	0.12
6			Ethyl acetate	0.51

3.2.4Antibacterial activity

The antibacterial activity of *Oscillatoria* extracts of Diethyl ether, Acetone & Ethyl acetate on *Aeromonashydrophila* presented in Table 3.The agar well diffusion method was used to evaluate the antibacterial activity by measuring the zone of inhibition. Among three extracts *Oscillatoria* ethyl acetate extract was found to be superior controlling growth of all three pathogens.

Table 3: Antibacterial activity of extracts depicted through zone of inhibition

Sl.No		Zone of inhibition in mm				
	Sample Extract	*Aeromonashydrophila*				
		10µl	*25 µl*	*50 µl*	*100 µl*	*C µl*
1	Eth Ace	10µ0.21	14µ0.16	16µ0.11	18µ0.13	08µ0.21
2	Methanol			12µ0.33		10µ0.31
3	Ethanol				11µ0.18	10µ0.12

Values are mean inhibition zone (mm) ± S.D of three replicate

MIC for Ethyl acetate 1.75mg/ml, MIC for Methanol extract 4.25mg/ml, MIC Ethanol 2.15mg/ml

3.2.5 GC-MS analysis

The GC–MS analysis of the crude *Oscillatoria* Benzene extract ,Table 4 revealed eleven components of that the main chemical constituent was the Dibutylphthalate Retention time(RT)15.53min ,16.91. Ethanolextract,Table 5 revealed fourteen peaks of that main peaks were Hexadecanoc acid ethyl ester RT 16.00 & 34.00%&n-Hexadecanoic acid RT 15.82 &Ethyl acetate extract Table 6 revealed six peaks ,Bis(2-ethylhexyl)phthalate RT23.85 & 98.40% ,Methanol extract revealed five peaks, n-Hexadecanoic acid RT 15.59 & 51.20%,Bis(2-ethylhexyl)phthalate RT23.81 & 43.80%.

Table 4: GC MS analysis of Oscillatoria Benzene extracts

Name of the compound	RT	Mol.Wt	Peak %
Nonane	5.39	128	0.40
Tetradecane,2,6,10-trimethyl-	6.99	240	0.66
Decane2,3,5,-tetrametthyl-	8.49	198	0.06
Tert-hexadecanethiol	9.88	258	0.90
10,12-Octadecadiyonic acid	14.12	276	2.65
Dibutyl phthalate	15.53	278	16.91
Phthalicacid,butyltetradecyl ester	15.77	418	2.27
1,2 –Benzenedicarbolic acid ,butyl octyl ester	16.83	334	1.17
Bis(2-ethylhexyl)phthalate	23.83	390	0.06
Bisphenol,bis(tert-butyldimethylsilyl)ether	26.23	456	0.70
Androstane-11,17-dione,3-[(trimethylsilyl)oxy]-17-[O-(phenylmethyl)oxime],-(3α,5α)	34.71	481	023

Table 5: GC MS analysis of Oscillatoria Ethanol extracts

Name of the compound	RT	Mol.Wt	Peak %
Phenol,2,4-bis(1,1-dimethylethyl)-	10.04	206	0.69
Ingol 12-acetate	10.43	408	0.35
Diethyl phthalate	11.04	222	4.72
1-Dodecanol,3,7,11-trimethyl-	12.34	228	0.96
4-Methyldocosane	12.85	324	0.35
2-Pentadecanone,6,10,14-trimethyl-	14.05	268	2.38

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9-t-Butyltricyclo[42.1.1(2,5])decane-9,10-diol	14.30	324	0.39
n-Hexadecanoicn acid	15.59	256	5.26
Hexadecanoc acid ethyl ester	16.00	284	34.00
Octadecanoic acid,17-methhyl-,methyl ester	18.85	312	1.48
Bis(2-ethylhexyl)phthalate	23.81	390	28.94
Bispheol,bis(tert-butyldimethylsilyl)ether	26.21	456	4.03
Androstane-11,17-dione,3-[(trimethylsilyl)oxy]-,17-[O-(phenylmethyl)oxime],(3 α,5α)	34.72	481	0.49
2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	14.91	324	0.39

Table 6: GC MS analysis of Oscillatoria Ethyl acetate extracts

Name of the compound	RT	Mol.Wt	Peak %
Diethyl phthalate	11.04	222	0.13
2H-Pyran,2-(7-dodecynyloxy)tetrahydro-	14.04	266	0.03
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	14.91	276	0.15
n-Hexadecanoic acid	15.54	256	0.68
Hexadecanoic acid, ethyl ester	16.01	284	0.60
Bis(2-ethylhexyl)phthalate	23.85	390	98.40

Table 7: GC MS analysis of Oscillatoria Methanol extracts

Name of the compound	RT	Mol.Wt	Peak %
Diethyl phthalate	11.05	222	3.70
9-t-Butyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	14.29	224	0.64
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-,9-diene-2,8-dione	14.92	276	0.66
n-Hexadecanoic acid	15.59	256	51.20
Bis(2-ethylhexyl)phthalate	23.81	390	43.80

RT: Retention time, Molwt: Molecular weight

4. Conclusion:

Aeromonas hydrophila have the potential of causing zoonotic disease, a disease spreads from animal to human being during accidental cases. Currently antibiotic treatment is preferred to resolve disease. But, use of antibiotics has potential problem of being include inadequate dosage, overdosing, drug resistance by bacteria. Hence in the present study natural antimicrobial substance as a substitute for synthetic antibiotics is analyzed. Oscillatoria extracts are prepared in different solvents with increasing order of polarity. Extracts are subjected to phytochemical tests for Glycosides, alkaloids, saponins,flavonoids,tannins,phenols,cardiac glycosides, sterols, resins etc. Extracts showing positive for phenol, tannins, flavonoids were estimated for phenolic content & tested for antimicrobial activity against pure cultures of *Aeromonashydrophila* . Crude extracts subjected to GC –MS analysis reported many several bioactive compound showing antimicrobial, anti oxidant&anti fungal property. The cold extraction procedure adopted helped in the accountability of lipidous& hydrocarbon molecule. Such natural antimicrobial substance showing broad spectrum activity can be used to replace synthetic antibiotics more effectively ,less toxicity also can development of antibiotic resistant strains can be curtailed.

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