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RESEARCH ARTICLE

SCREENING OF PHYTOCHEMICALS & BIOACTIVE ANTIBACTERIAL ACTIVITY IN *SPIROGYRA SP.*

Prarthana J[1] and Maruthi KR[2].

1. Research Scholar, PRIST University, Tamilnadu 613 403, working at Dept of PG Studies & Research in Biotechnology, SDM (Autonomous college) Ujire 574240,Karnataka
2. Research Supervisor, PRIST University ,Tamilnadu 613 403.

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Abstract

Algae are very important component of aquatic ecosystem, known for producing several 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 *Spirogyra sp*. Dried algal powder after identification were cold extracted & screened for glycosides,alkaloids,saponins,flavanoids,tannins,terpenoids,phenolics, anthraquinones,cardiac glycosides, etc. The extracts showing good phenolic content were tested for antibacterial activity against pure cultures of fish pathogens namely *Aeromonashydrophila*,. The GC-MS analysis of the extracts revealed the presence of several antimicrobial bioactive constituents in the extracts.

Introduction:-

Aquatic organisms are a rich source of structurally novel & biologically active metabolites[8].Many microscopic forms of algae are known to grow in fresh water as slimy 'blanket weed'. One such familiar algae is *Spirogyra* a filamentous algae, its cells form each filament consists of an extensive chain of identical cells. *Spirogyra* is the most common genus of Zygnemataceae and member of the freshwater algae. It exhibits the greatest diversity of the 12 to 13 genera recognized in this family of green algae (Transeau 1951, Kadlubowska 1972).

Seasonal variation in pH, temperature & dissolved oxygen play an important role in the multiplication of pathogens leading to disease in fish [73].*Aeromonashydrophil*a is an opportunistic pathogen known to cause disease during stressed condition.Symptoms of the disease may range from sudden death to lack of appetite, swimming abnormalities, pale gills & skin ulceration. Current treatment involves use of antibiotics such as Tetramycin®, an oxytetracycline, and Remet-30®. Potential problems associated with antibiotic include inadequate dosage, overdosing, drug resistance by bacteria[74]

The use of algae for therapeutic purpose has a long history & systemic examination of algae for biologically active substances. The aqueous & solvent extracts from algae were tested against gram positive & gram negative bacteria [7-10].The first investigation of the antibiotic activity of algae was carried out by (Pratt et al., 1944).Evidence of phytochemical and pharmacological studies on algae is available in the literature with special references to terpenoids and steroids (Parameswaran et al. 1944; Patterson et al 1968). Secondary and primary metabolites

Corresponding Author:-Prarthana J.
AddressDepartment of P.G. Studies & Research in Biotechnology S.D.M. (Autonomous) College, Ujire-574 240 Karnataka, India:-.

produced by these organisms may be potential bioactive compounds of interest in pharmaceutical industry. There are a number of reports on the evaluation of antioxidant activity in microalgae and cyanobacteria belonging to the genera *Botryococcus* (Rao et al. 2006), *Chlorella* (Wu et al. 2005; Goh et al.2010), *Dunaliella*(Herrero et al. 2006), *Nostoc* (Li et al.2007), *Phaeodactylum*(Guzman et al. 2001), *Spirulina* (Miranda et al. 1998; Jaime et al. 2005; Mendiola et al.2007), *Haematococcus*(Cerón et al. 2007) and*Chaetoceros* (Goh et al. 2010). These studies concluded that several microalgal genera contain potent antioxidants, both from lipophilic and hydrophilic nature. Justella et al (2011)proved antimicrobial property of ethanolic extracts of *Spirogyra grantiana*against*E.coli*. Antimicrobial & antioxidants activity of *Euglena tuba, Oscillatoriaagardhii&Anabenasphaerica ,Cosmariumsp, Chlorococcumhumicola* (Dipankar et al 2014;Abd El-Aty et al2014; Challouf et al2012;Bhagavathy et al 2011)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.* GC-MS analysis of the *Spirogyra* Diethyl ether,Acetone& Ethyl acetate extracts reported several bioactive components with antimicrobial property.

Material and method:-

Sample preparation & extraction:-

Samples were collected from Arasinamakki, 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. (Gonzalez del val*et.al*, 2001.)

Phytochemical Analysis:-

The extracts were subjected to phytochemical tests for presence of following biomolecules byUsing the standard qualitative procedures as described in literature [15].

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 AandB) 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 observed for 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 ora blue black coloration.
6. Test for terpenoids: 5 ml of extract was mixed with 2 ml of CHCl3in 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 glacialacetic acid containing 1 drop of FeCl3.Theabove mixture was carefully added to the 1ml of concentrated H2SO4. Presence of cardiacglycosides 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.

Estimation of Phenolic Content:-

The amount of total phenolics[1] in methanol extract was determined with Folin– Ciocalteu reagent according to the method of Singleton and Rossi with Gallic acid as the standard **[23]**. 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 each 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 *Spirogyra sp).*

Antibacterial Activity:-

Pure cultures of *Aeromonashydrophila*, was used as test micro-organisms. 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.

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 °C, and the oven temperature was kept at 110 °C for 3.5 min, rose to 280 °C at 5 °C min^{-1} (total run time 37.50 min). Peak identification of crude spirogyra extracts 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 2016

Results and Discussion:-

Phytochemical 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 Spirogyra extracts.

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

++ Copiously present, + moderately present, - absent

Estmation of Phenolic content:-

Diethyl ether, Acetone & Ethyl acetate spirogyra 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 Spirogyra Acetone,Diethyl ether & Ethyl acetate extracts

Sl.No	StdConc	OD 750nm	Sample extract(0.1ml)	OD at 750nm
1	0.2mg	0.24	Acetone	0.63
2	0.4	0.41	DEE	0.04
3	0.6	0.90	Eth Ace	1.09
4	0.8	1.22		
5	1.0	1.95		

Antibacterial activity:-

The antibacterial activity of *Spirogyra* 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 *Spirogyra* 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				
1	Sample Extract	*Aeromonashydrophila*				
		10µl	*25 µl*	*50 µl*	*100 µl*	*C µl*
2	DiEthylEther	--	--	10±0.11	11±0.32	08±0.43
3	Acetone	--	11±0.21	12±0.33	12±0.55	10±0.54
4	Ethyl Acetate	15±0.56	18±0.41	23±0.33	26±0.57	13±0.62

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

MIC for Diethyl ether 5.54mg/ml, MIC for Acetone extract 3.75mg/ml, MIC Ethyl acetate 2.15mg/ml

GC-MS Analysis:-

The GC–MS analysis of the crude *Spirogyra* Diethyl ether extract ,Table 4 revealed seven components of that the main chemical constituent was the Bus(2-ethylhexyl)phthalate Retention time(RT)23.94min ,92.54%&n-Hexadecanooic acid RT 15.61 & 2.35%. Acetone extract,Table 5 revealed twenty four peaks of that main peaks were n-Hexadecanoic acid RT 15.82 & 29.02% ,Bis(2-ethylhexyl)phthalate RT23.88 & 21.60% , Tetradecanoic acid RT13.13 &16.46%&Octadecenoic acidRT18.50 & 11.17 %. Ethylacetateextracts Table 6showed thirty six peaks of that main peaks were Bis(2-ethylhexyl)phthalate RT 23.97 & 65.86%, n-Hexadecanoic acid RT 15.88 & 10.81%.

Table 4:- GC MS analysis of Spirogyra Diethyl ether extracts

Name of the compound	RT	Mol.Wt	Peak Area%	
ButylatedHyydroxytoluene	9.98	220	1.36	Antioxidant Antimutagenic, anticarcinogenic,inactivation of virus[28]
Methoprene	10.54	310	0.08	Insectisidal[29]
Tetradecanoic acid	13.08	228	1.93	Antibacterial & antifungal[30]
Dibutyl phthalate	15.55	278	1.56	Antimicrobial,Disruptor of estrogen activity [31]
n-Hexadecanooic acid	15.62	256	2.35	Antioxidant, hypocholesterolemic, anti-inflammatory , antibacterial[32]
Octadecanoic acid	18.44	284	0.19	Cancer preventive Insectifuge*
Bis(2-ethylhexyl)phthalate	23.94	390	92.54	Antibacterial & antifungal[33]

Table 5:- GC MS analysis of Spirogyra Acetone extracts

Name of the compound	RT	Mol. Wt	Peak Area%	
Octanoic acid	4.98	144	0.02	Fungicide,Pesticide,Candidicide*
Nonanoic acid	6.47	158	0.32	Herbicide[34]
2H-Pyran-2-one,tetrahydro-4-hydroxy-6-pentyl	8.22	186	0.34	Antimicrobial &anticancerous[35]
Undecylenic acid	9.09	184	0.46	Not reported
12-Hydroxydodecanoic acid	9.63	216	0.84	Antimicrobial,antioxidant,antiallergic,antiaging ,antitumor,anti-inflammatory[36]
2(4H)-Benzofuran,5,6,7,7a-tetrahydro-4,4,7a-trimethyl	10.43	180	1.50	Antimicrobial[37]
Dodecanoic acid	10.71	200	2.16	Not reported
Dihydrojasmone	11.21	166	0.99	Antimicrobial & antifungal[38]
Azelaic acid	11.81	188	1.43	Antimicrobial[39]
Tetradecanoic acid	13.13	228	16.46	Antimicrobial & anti-inflammatory[40]
9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	13.30	196	1.91	Anticancerous[41]
2-Pentadecanone,6,10,14-trimethyl	14.07	268	2.26	Repellent to arthropod[42]
Pentadecanoic acid	14.32	242	2.55	Antioxidant, antibacterial[43]
2H-Pyran-2-one,tetrahydro-6-nonyl	14.83	226	0.23	Not reported
n-Hexadecanoic acid	15.82	256	29.02	Antiinflammatory[44]
11,13-Dimethyl-12-tetradecen-1-ol acetate	16.61	282	0.82	Antioxidant &antitumour activity[45]
Trans-13-Octadecenoic acid	18.10	282	2.70	Antimicrobial[46]
Octadecenoic acid	18.50	284	11.17	Free radical scavenging activity[47]
Erucic acid	19.07	338	1.23	Not reported
z)-3,7,11-Trimethyldodec-2-enoic acid,methyl ester	19.93	254	0.34	Not reported
7-Methyl-Z-tetradecen-1-ol acetate	21.44	268	1.03	Anticancer,anti-inflammatory,hepatoprotective[48]
Cis-11-Eicosenoic acid	21.85	310	0.26	Not reported
Tricyclo[20.8.0.0(7,16)]triacontane,1(22),7(16)-diepoxy	22.74	444	0.37	Antidiabetic type II[49]
Bis(2-ethylhexyl)phthalate	23.88	390	21.60	Antibacterial & antifungal[33]

*Dr.Duke'sphytochemicals&ethanobotanical databases

Table 6:- GC MS analysis of Spirogyra Ethyl acetate extracts

Name of the compound	RT	Mol.Wt	Peak Area%	
Nonanoic acid	6.93	158	0.10	Herbicide[34]
Diethyl adipate	8.23	202	0.34	Antimicrobial anti inflammatory[50]
Ethyl 5-methylhexonate	8.51	158	0.00	
Diethyl pimelate	9.65	216	0.62	Antioxidant & antimicrobial[51]
Nonanoic acid,9-oxo-,ethyl ester	9.94	200	0.55	Antioxidant,anti inflammatory, anticancerous[52]
Ethyl hydrogen suberate	10.63	202	0.16	Antioxidant anti inflammatory[53]
Diethyl suberate	10.96	230	0.89	Antifungal ,antibacterial[54]
3-Cyclopentylpropionic acid,ethyl ester	11.12	170	0.18	Antibacterial[55]

Nonanedioicacid,monomethyl ester	11.87	202	0.66	Antitumour [56].
Nonanedioicacid,dimethyl ester	12.16	216	1.98	Antimicrobial,anti inflammatory[57]
12-Oxododecanoic acid,ethyl ester	12.43	242	0.05	Antiseptic ,antimicrobial,anti oxidant[58]
Thiazolo[3,2-a]pyrimidin-5-one,7-methyl-2,3-dihydro-	12.57	168	1.60	Antibacterial ,antibiofilm[59]
Tetradecanoic acid	13.17	228	4.87	No report
Decanedioic acid	13.34	258	0.21	No report
Tetradecanoicacid,ethyl ester	13.45	256	1.92	Anticncerous[60]
2-Pentadecanone,6,10,14-trimethyl-	14.08	268	0.93	Not reported
Pentadecanoic acid	14.23	242	0.27	Antimicrobial[61]
Ethyl 13 -methyl-tetradeconate	14.33	270	0.36	Antibacterial[62]
Dodecanedioicacid,dimethyl ester	14.59	258	0.26	Antibacterial,antimicrobial,antidiabetic[63]
Hexadecanoicacid,methyl ester	15.20	270	0.13	Antimicrobial anti inflammatory[64]
n- Hexadecanoic acid	15.88	256	10.81	Antiinflammatory[44]
Hexadecanoicacid,ethyl ester	16.11	284	1.15	Antimicrobial,anti oxidant [58]
Octadecanoic acid	18.53	284	1.79	Free radical scavenging activity[47]
Octadecanoic acid,17-methyl-,methyl ester	18.91	312	0.33	Antioxidant,antimicrobial[65]
3-Buten-2-one,4-(3-hydroxy-6,6-dimethyl-2-methylenecyclohexyl)-	19.24	208	0.34	No report
Z)-3,7,11-Trimethyldodec-2-enoic acid,methylester	19.83	254	0.21	Insecticidal[66]
Cholest-22-ene-21-ol,3,5-dehydro-6-methoxy-,pivalate	20.76	498	0.68	Anticancerous, antiviral
Octadecanoic acid,10-oxo-,methyl ester	21.53	312	0.38	No report
(E)-9-Octadecenoic acid ethyl ester	21.83	310	1.04	No report
i-Propyl 11,12-methylene – octadecanoate	22.23	338	0.16	Antimicrobial[68]
Ethyl Olelate	22.57	310	0.36	Flavour[69].
Bis(2-ethylhexyl)phthalate	23.97	390	65.86	Antibacterial & antifungal[33]
Docosanoic acid ,ethyl ester	24.82	368	0.16	No report
Eicosanoic acid,2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl ster	25.03	470	0.08	Antibacterial[70]antioxidants [71]
Eicosanoicacid,ethyl ester	27.61	340	0.56	No report
Cholestan-3-ol,2-methylene-,(3β,5α)-	28.46	400	0.01	Antioxidant[72]

RT: Retention time, Molwt: Molecular weight

Conclusion:-

Aeromonashydrophila 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. Spirogyra 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, antioxidant&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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