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RESEARCH ARTICLE

PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF THE THREE DIFFERENT SEAGRASS EXTRACTS

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Abstract

Antibacterial activities of the three sea grass Cymodocea serrulata, Halophila ovalis and Halodule pinifolia were tested against ocular pathogens E.Coli, Enterococcus faecalis, Corynebacterium, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Methicillin Sensitive Staphylococcusaureus, Methicillin Sensitive Staphylococcus saphrophyticus and Methicillin Sensitive Staphylococcus epidermidis using different solvents hexane, ethyl acetate, chloroform and ethanol namely were investigated. The chloroform and ethylacetate extract showed maximum activity. In the case of phytochemical analysis the ethylacetate, ethanol and chloroform extracts showed positive activity with phytoconstituents such as phenols, steroids, terpenoids, flavonoids, alkaloids, glycosides, saponins, and tannins but sugars and quinine showed negative activity. Further experiments are underway to isolate active compounds in controlling the growth of pathogens.

Introduction:-

The eye is a unique organ which gets infected from external sources or through internal sources were the micro-organisms carried in person. The infections spread to adjacent tissue, from the conjunctiva to the cornea, the inner eye, the orbit and to the brain. (Willimson and Sorsby, 1950). The common eye infections are conjunctivitis, blepharitis, internal and external hordeolum, microbial scleritis, canaliculitis, keratitis, dacryocystitis, preseptal cellulitis, orbital cellulitis, endophthalmitis and panophthalmitis (Modarrres et al., 1998). Ocular medications such as eye drops or an ointment are used to treat and prevent eye diseases. Many traditional herbal eye drops are prepared from many medicinal plants combination which cures ophthalmic disorders.

The marine species from the sea offers a huge numbers of novel compounds (de Vries and Beart 1995), and it consists of numerous natural molecules to be evaluated for drug activity (Gerwick and Bernart, 1993). Many marine natural products were isolated from sponges, coelenterates tunicates, opisthisbranch mollusks, echinoderms, Sea grass, Seaweeds, bryozoans. (Prakash Williams et al., 2007).

Most studies have focused on coral reefs and mangroves, while seagrasses have generally ignored (Durate, 2000). Marine and estuarine submersed aquatic angiosperms, or seagrasses, produce antimicrobial compounds that may act to reduce or control microbial growth. Numerous medicines and chemicals are also prepared from sea grass and their associates. Only few reports are available regarding antibacterial, antialgal, antifungal and antiviral activities of the seagrass against pathogens (Harrison and Chan 1980; Bernard and Pesando 1989; Devi et al., 1997; Bhosale et al., 2002; Harrison 1982; Ballesteros et al., 1992; Jensen et al., 1998; Premnathan et al., 1992). The present study was planned to investigate the antibacterial activity and phytochemical analysis of the seagrasses against ocular pathogens.

Material and Methods:-

Sample Collection:-

Fresh seagrass samples of Cymodocea serrulata, Halophila ovalis and Halodule pinifolia were collected from the intertidal region of the Mandapam coast (Lat. 09˚ 17.417'N; Long. 079˚ 08.558'E) during the year 2014. They were brought to the laboratory washed in seawater to remove the macroscopic epiphytes and extraneous matter, and then rinsed in distilled water, and shade dried for 10 days. The samples were powdered and stored in a refrigerator.

Preparation of Crude Extract:-

Sea grass powder were soaked in 2L organic solvents with the increasing order of polarity viz., hexane, ethyl acetate, chloroform and ethanol (1:4 w/v), and kept for 10 days in a shaker. The extraction was repeated thrice, pooled and filtered through Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary flash evaporator. The dry aqueous extracts were lyophilized and stored in a refrigerator until further analysis.

Antibacterial Assay:-

Eye infected pathogens (E.Coli, Enterococcus faecalis, Corynebacterium, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Methicillin Sensitive Staphylococcusaureus, Methicillin Sensitive Staphylococcus saphrophyticus and Methicillin Sensitive Staphylococcus epidermidis) used in this study were collected from Dr. Agarwal Eye Hospital, Tamilnadu, India.

Antibacterial activity was determined using the disc diffusion method (Bauer et al., 1966). Based on this method the sterile filter paper discs 6 mm in diameters (Whatman No.1), were loaded with 100µg, 250µg, 500µg and 1000µg/ml of crude extracts of the seagrasses and air-dried. Discs were loaded with 5% DMSO (Dimethyl sulphoxide) was used as negative control and amikacin was used as Standard. The discs were placed on Muller Hinton agar (HiMedia, India) plates which was inoculated by the test organisms and incubated for 24 h at 37^0 C. Zone of inhibition was recorded in millimeters and mean values were reported and it was compared with Standard Chart.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration was determined using broth microdilution method (NCCLS 2003; Mazzanti et al., 2000; Devienne and Raddi, 2002). Serial double dilutions are prepared with a solution of maximum active seagrass extracts: Dimethylsulfoxide 95:5 in a 96-well microtiter plate over the range of 7-3,125 µg/mL.

Overnight broth cultures of each strain were prepared and the final concentration of the microbe in each well was adjusted to 2×10^3 cfu/mL and 5 µL of culture was inoculated in each well. Plates were incubated at 37°C for 24 h. The MIC defined as the lowest concentration of the seagrass extract at which the microorganism does not demonstrate visible growth and the absorbance of each well were determined using an automatic ELISA tray reader adjusted at 630 nm (SLT Spectra). The samples were analysed in duplicate and the assay was repeated twice. The antibiotic amikacin was employed as positive control. The wells showing complete absence of growth are identified and 10 µL of each well is transferring to Mueller–Hinton agar plates for bacterium and incubated at 37ºC. Values are expressed as mean ± standard error and they are calculated.

Phytochemical analysis:-

The Crude extract of the seagrasses were subjected to Phytochemical screening to detect the presence of phenols, steroids, terpenoid, flavonoids, alkaloids, glycosides, saponins, sugars, quinine and tannins by the standard method using different solvents such as hexane, ethyl acetate, chloroform and ethanol (Kokate, 2006).

Results:-

Table 1 shows the antibacterial activity of the seagrass Cymodocea serrulata extracts against nine ocular pathogens. Control disks (DMSO) showed no inhibition. The antibiotic amikacin inhibited all the pathogens. The extracts obtained using chloroform, Ethyl acetate and Ethanol showed maximum activity against E.Coli (8.66mm, 9mm and 7.33mm), Corynebacterium sps (7mm, 8mm). There is no zone of inhibition were recorded in remaining six pathogens.

Table: 1:- Antibacterial activity of Cymodocea serrulata against Ocular Pathogens.

S.NO	Bacterial pathogens	Chloroform	Ethyl acetate	Ethanol	Hexane
		Zone of inhibition (mm in diameter)			
1	**B. subtilis**	-	-	-	-
2	**Corynebacterium**	7	8	-	-
3	**E. femelis**	-	-	-	-
4	**E. coli**	8.66	9	7.33	-
5	**P. aeruginosa**	-	-	-	-
6	**K. pneumonia**	-	-	-	-
7	**MSSA**	-	-	-	-
8	**MSSE**	-	-	-	-
9	**MSSS**	-	-	-	-

- No Zone of inhibition

Table 2 shows the antibacterial activity of the seagrass Halodule pinifolia extracts against nine ocular pathogens. Control disks (DMSO) showed no inhibition. The antibiotic amikacin inhibited all the pathogens. The extracts obtained using chloroform, Ethyl acetate and Ethanol showed maximum activity against E.Coli (8.33mm, 9mm and 8mm), Corynebacterium sps (13.66mm, 11mm, 14.33mm in hexane). There is no zone of inhibition were recorded in remaining six pathogens.

Table: 2:- Antibacterial activity of Halodule pinifolia against Ocular Pathogens.

S.NO	Bacterial pathogens	Chloroform	Ethyl acetate	Ethanol	Hexane
		Zone of inhibition (mm in diameter)			
1	**B. subtilis**	-	-	-	-
2	**Corynebacterium**	13.66	11	-	14.33
3	**E. femelis**	-	-	-	-
4	**E. coli**	8.33	9	8	-
5	**P. aeruginosa**	-	-	-	-
6	**K. pneumonia**	-	-	-	-
7	**MSSA**	-	-	-	-
8	**MSSE**	-	-	-	-
9	**MSSS**	-	-	-	-

- No Zone of inhibition

The antibacterial activity of the seagrass Halophila ovalis extracts against nine ocular pathogens were shown in Table 3. Control disks (DMSO) showed no inhibition. The antibiotic amikacin inhibited all the pathogens. The extracts obtained using chloroform, Ethyl acetate and Hexane showed maximum activity against E.Coli (7.6mm, 7.3mm and 7mm), Corynebacterium sps (11.66mm, 13mm and 14.66mm). The extracts obtained using Ethyl acetate against Enterococcus femelis is 7.66mm. There is no zone of inhibition were recorded in remaining five pathogens.

Table: 3:- Antibacterial activity of Halophila ovalis against Ocular Pathogens.

S.NO	Bacterial pathogens	Chloroform	Ethyl acetate	Ethanol	Hexane
		Zone of inhibition (mm in diameter)			
1	**B. subtilis**	-	-	-	-
2	**Corynebacterium**	11.66	13	-	14.66
3	**E. femelis**	-	7.66	-	-
4	**E. coli**	7.60	7.33	7.00	7.00
5	**P. aeruginosa**	-	-	-	-
6	**K. pneumonia**	-	-	-	-
7	**MSSA**	-	-	-	-
8	**MSSE**	-	-	-	-
9	**MSSS**	-	-	-	-

- No Zone of inhibition

Minimum Inhibitory Concentrations of the Chloroform, Ethanol and Ethyl acetate extracts of the three seagrasses which showed maximum activity against Corynebacterium sps Escherichia coli, Bacillus subtilis, Enterococcus femelis pathogens are depicted in Table 4. Of the three seagrasses studied, minimum inhibitory concentrations of Halodule pinifolia extracts were effective in controlling the growth of Corynebacterium sps, Cymodocea serrulata extracts in Escherichia coli and Halophila ovalis extracts were effective in controlling the growth of Corynebacterium sps.

Table: 4:- MIC of maximum active seagrass extracts

Pathogens	Seagrass extracts	MIC (µg/ml)
Corynebacterium sps	Chloroform extract of Cymodocea serrulata	850
	Ethyl acetate extract of Cymodocea serrulata	875
	Chloroform extract of Halodule pinifolia	55
	Ethyl acetate extract of Halodule pinifolia	35
	Hexane extract of Halodule pinifolia	50
	Chloroform extract of Halophila ovalis	65
	Ethyl acetate extract of Halophila ovalis	65
	Hexane extract of Halophila ovalis	50
Escherichia coli	Chloroform extract of Cymodocea serrulata	90
	Ethyl acetate extract of Cymodocea serrulata	75
	Ethanol extract of Cymodocea serrulata	90
	Chloroform extract of Halodule pinifolia	90
	Ethyl acetate extract of Halodule pinifolia	70
	Ethanol extract of Halodule pinifolia	80
	Chloroform extract of Halophila ovalis	225
	Ethyl acetate extract of Halophila ovalis	90
	Hexane extract of Halophila ovalis	435
	Ethanol extract of Halophila ovalis	90
Bacillus subtilis	Chloroform extract of Halodule pinifolia	1875
	Ethyl acetate extract of Halophila ovalis	1650
Enterococcus femelis	Ethyl acetate extract of Halophila ovalis	85

Phytochemical analysis:-

Phytochemical analysis of Hexane, Chloroform, Ethyl acetate, Ethanol and Aqueous extracts of three seagrasses revealed the presence of phenols, steroids, terpenoids, flavonoids, alkaloids, glycosides, saponins, and tannins. Sugars and quinine were absent in all the three seagrasses (Table 5).

Table: 5:- Phytochemical analysis of the seagrasses using different extracts

Seagrass	Cymodocea serrulata					Halodule pinifolia					Halophila ovalis				
Compounds	**H**	**C**	**EA**	**E**	**AQ**	**H**	**C**	**EA**	**E**	**AQ**	**H**	**C**	**EA**	**E**	**AQ**
Steroids	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+
Alkaloids	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
Flavonoids	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-
Quinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
Saponins	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
Terpenoids	-	-	+	-	-	-	-	+	-	+	-	+	+	-	+
Tannins	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
phenols	-	-	+	+	-	-	-	+	+	-	-	+	+	+	+
Sugars	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

H- Hexane; C- Chloroform; EA- Ethyl Acetate; E- Ethanol; AQ- Aqueous

\+ indicates Present

\- indicates Absent

Discussion:-

For the past quarter century research on bioactive compounds from marine species has provided a broad and better support of marine natural products. Marine organisms collected from the coastal area of India have been shown to

possess a number of biological activities (Ely et al., 2004). There are number of reports which demonstrate the antimicrobial activity of mangroves, seaweeds, and other marine species and only limited reports were available for the sea grasses in the world and even very little information available from India. The study was planned to investigate and compare the ability of the different sea grass extracts to produce novel compounds which can be used for of remedial purpose. Antimicrobial activities found in sea grasses were considered to be an indication of synthesis of bioactive secondary metabolites Rengasamy et al., (2010).

In this study the antibacterial activity of three different extracts of H. ovalis, C. serrulata and H. pinifolia against nine eye pathogens four were effective. Among them chloroform was more effective than the others. Hexane extract of two seagrass H. ovalis and H. pinifolia inhibited the growth against E.Coli and Corynebacterium. From this it exhibit that chloroform extract is suitable for deriving bioactive compounds from sea grasses. This investigation were supported the earlier reports thus, the methanolic extract of Enhalus acoroides were effective against S. aureus, K. pneumoniae and P. aeruginosa than the hexane extract (Alam et al., 1994). Among the seagrasses Halophila and Zostera were more effective than Cymodacea (Sreenath Kumar et al., 2008). Balasubramanian et al., (1974) reported that the lipid and water-soluble phenolic extracts of both leaf and root-rhizome fractions of H. pinifolia are the most promising of antibacterial activity. Rengasamy et al., (2010) reported that the methanol extract of H. pinifolia exhibited strong antibacterial activity against human pathogens. This result is in agreement with the present finding that H. pinifolia showed second maximum activity. Another study indicates that the six sea grasses were tested against clinically important UTI bacteria (Rengasamy et al., 2012). The result indicated that, C. serrulata and H. pinifolia exhibited predominant growth inhibitory activity against all the UTI bacteria.

Rengasamy et al., (2010) reported that the Hexane extract of the seagrasses doesn't show any activity against some human pathogens but in our present findings the hexane extracts of the seagrasses H. ovalis and H. pinifolia inhibited the growth against E.Coli and Corynebacterium.

Burkholder, (1966) showed in vitro properties against Gram positive bacteria, with minimum inhibitory concentration (MIC) ranging from 0.0063-0.2 mg ml^{-1}. For instance, the minimum inhibitory concentration of M. jodocodo against E.coli was 2.75 mg ml^{-1} while that of T. robustus against M. bourtardi was 15.75 mg ml^{-1} (Jonathan Gbolagade et al., 2007).

In our present findings minimum inhibitory concentrations of Halodule pinifolia extracts were effective in controlling the growth of Corynebacterium sps, Cymodocea serrulata extracts in Escherichia coli and Halophila ovalis extracts were effective in controlling the growth of Corynebacterium sps. Likewise, the minimum inhibitory concentration of the ethanolic plant extract ranged from 0.01 mg ml^{-1} to 100 mg ml^{-1} against pathogenic bacteria and fungi were reported by Liasu and Ayandele (2008).

In our study Phytochemical analysis of Hexane, Chloroform, Ethyl acetate, Ethanol and Aqueous extracts of three seagrasses revealed the presence of phenols, steroids, terpenoids, flavonoids, alkaloids, glycosides, saponins, and tannins. Sugars and quinine were absent in all the three seagrasses. This supports the earlier reports like Ergene et al., 2006 who revealed the presence of tannins, saponins, proteins, resins, reducing sugar, acidic compounds, alkaloids, cardiac glycosides and terpenoids in the phytochemical analysis of C. rotundata. The phytochemical compounds viz., glycoside, saponins, tannins, flavonoids, terpenoides and alkaloids have antimicrobial activity (Okeke et al., 2001). The preliminary phytochemical studies of the active fraction of root extracts of C. serrulata had variety of phytochemical constituents, namely alkaloids, carboxylicacid, coumarins, flavonoids, phenols, saponins, xanthoprotein, protein, steroids, tannins and sugar (Ravikumar et al., 1993).

Further work on the purification of individual groups of bioactive components may reveal the exact potential of the seagrass to inhibit ocular pathogenic microbes and encourage the development a novel broad spectrum herbal antimicrobial formulation in the future. Further purification of active compounds and structural elucidation can be used for drug discovery.

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