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ISOLATION AND CHARACTERIZATION OF STIGMASTEROL FROM THE SEA GRASS *HALOPHILA OVALIS*

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ABSTRACT
The present investigation was focused to isolate bioactive compounds from *Halophila ovalis* and check the activity against ocular pathogenic bacteria. **Methods:** Fresh seagrass samples of *Halophila ovalis* were collected from Mandapam coast, Ramanathapuram district, Tamilnadu. Seagrasses were extracted by using different solvents. For this study four ocular pathogenic bacteria were choosed. Antibacterial activity and MIC was calculated. The potential seagrasses were analyzed by qualitative phytochemical studies and TLC. The seagrass crude extract were subjected to PTLC. The best fractions were analyzed by TLC then it is subjected to FTIR, LCMS and NMR analysis. **Results:** Based on antimicrobial activity and MIC, it shows that chloroform extract showed maximum activity and it is further subjected to compound isolation. **Conclusion:** From this present study, it can be concluded that Stigmasterol was isolated from chloroform extract of *Halophila ovalis.*

KEYWORDS: Seagrass, TLC, LCMS, FTIR, NMR.

INTRODUCTION
Marine algae are the potential and diverse source of bioactive compound therefore it plays an important role in chemotherapy (Rangaiah *et al.,* 2010). Seagrasses are marine angiosperms which act as a primary producer and are highly productivity in origin. Though seagrasses comprises few species, their importance to estuarine and coastal marine environments and to pharmaceuticals is significant. They supply as the basic energy source for more or less complicated food web.

During recent year microbiologists and pharmacologists showed increased attention towards marine algae and seagrasses because of their potential bioactive substances. It is believed that these bioactive substances have medical importance so it can be used as potential drugs. There are many evidence regarding marine algae used as treatment for human in extensive. There have been of number of works on antimicrobial activity from marine sea grass (Sreenathkumar *et al.,* 2010 and Raja Kannan *et al.,* 2010). Hence the present investigation was focused to isolate bioactive compounds from seagrass and check the activity against ocular pathogenic bacteria.

MATERIALS AND METHODS
Fresh and healthy seagrass samples of *Halophilaovalis* was collected from valbay village, Mandapam coast, Ramanathapuram district, Tamilnadu. To remove extraneous matter the samples were washed with seawater and then with clean water. The samples were dried at room temperature and ground into a fine powder and it was extracted with various organic solvents such as hexane, ethanol, ethyl acetate and chloroform using cold percolation method (Handa *et al.,* 2008).

Antibacterial activity of crude extracts (hexane, ethanol, ethyl acetate and chloroform) of seagrasses was tested against selected ocular pathogens *Corynebacterium xerosis, Bacillus subtilius, Escherichia coli, Enterococcus faecalis,* by disc diffusion assay. Discs loaded with 5% DMSO served as negative control and the antibiotics gentamycin used as positive control (Bauer et al., 1966).

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

The methanol and chloroform solvents were used as stationary phase and it is transferred into the container about 1cm in depth from the bottom. The extracts to be analyzed were diluted with respective solvents and then spotted with help of capillary tube and placed inside the chamber in the ascendant position. After the

development 1cm from the top of plate it is taken out and vaporized. The plates were viewed under UV and the $R_f$value is calculated (Striegel and Hill, 1996).

Preparative Thin Chromatography of the chloroform extract was carried out on the silica gel plate. 2000µl of the extract was then applied as a single band of 160mm length on the activated TLC plate using a capillary tube. The plates were then developed with the 100ml of standardized solvent system, Hexane: EthylAcetate: (90:10) in the twin trough chromatographic chamber. After the successful development, the plate was examined under the UV Chamber at 254nm. The developed plate was then scaled marked, scratched out along with the silica gel, with a sharp blade and collected in the eppendorf tube. The solid was further confirmed by below mentioned test and it is subjected to ^{1}H-NMR, FT-IR and LCMS to ascertain the chemical structure.

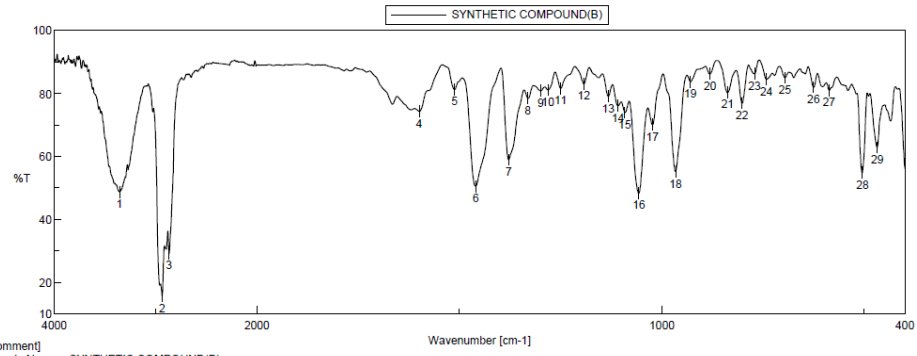
RESULTS

The chloroform extract of *Halophila ovalis* showed significant activity against *Corynebacterium xerosis* and *Escherichia coli.* The ethyl acetate extract of *Halophila ovalis* showed moderate activity against *B.subtilis.* The significant activity was observed against *C. xerosis, E.coli and E.fecalis.*

The phytochemical analysis of *Halophila ovalis* shows the presence of steroid in hexane hexane, ethanol and aqueous extract. Alkanoids present only in the chloroform extract. Flavonoids, terpenoids, tannins and phenols were present in ethylacetate extract of *Halophila ovalis.* Steroids, glycosides, saponins and phenol were present in ethanol extract of *Halophila ovalis.* Steroids, glycosides, saponins, terpenoids and phenol were present in hexane extract of *Halophila ovalis*.

TLC analysis of active extract revealed steroid compounds. The R_f value of steroid compound are 0.90, 0.85, 0.75, 0.58 and 0.41 noted respectively. Preparative thin layer chromatography revealed 42 mg of solid. The resulted solid sample showed a single spot in TLC. Further the solid is confirmed as steroid compound by Salkowski reaction and Liebermann burchard reaction.

The FT IR spectrum of steroid compound showed totally 29 peaks. In that a major peaks occurred at strong band of -OH group at 3354 cm^{-1}, aliphatic -CH at 2865 cm^{-1}, C=C group at 1598, 1511 cm^{-1} and C-O at 1298, 1281 cm^{-1}. The stretching and bending vibrations of methyl part were noticed by the intense band 2935 cm-1 and medium intensity band at 1459cm^{-1}. Peaks at 1249 and 696 cm^{-1} indicate OH bond vibrations. C-H vibrations of the unsaturated part were observed at 881 cm^{-1}. C-C vibrations were found at 1626 cm^{-1}. Based on FTIR analysis the functional groups were determined as stigmasterol.



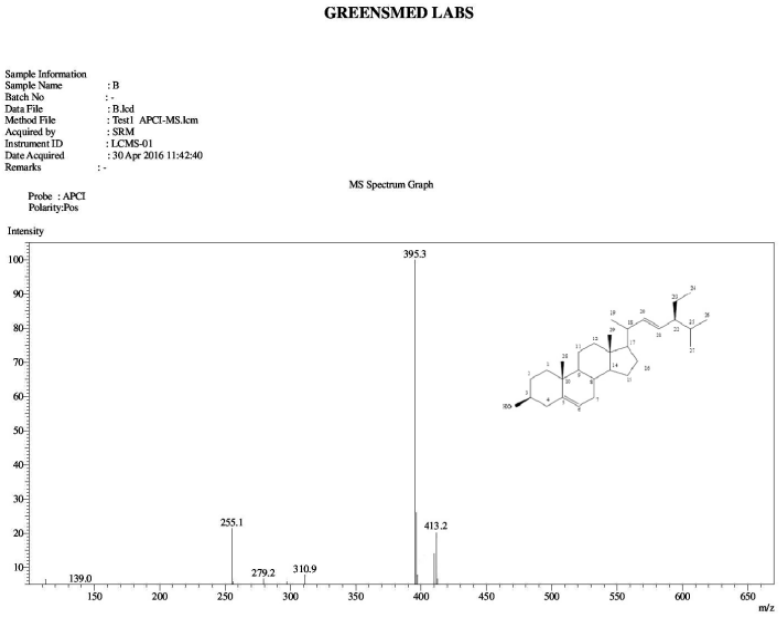
[Comment]
Sample Name SYNTHETIC COMPOUND(B)
Comment KBr Pellet
User GOUTHAMI
Division QC
Company GREENSMED LABS

[Data Information]
Creation Date 4/30/2016 11:45 AM
Data array type Linear data array
Horizontal Wavenumber [cm-1]
Vertical %T
Start 399.193 cm-1
End 4000.6 cm-1
Data pitch 0.964233 cm-1
Data points 3736

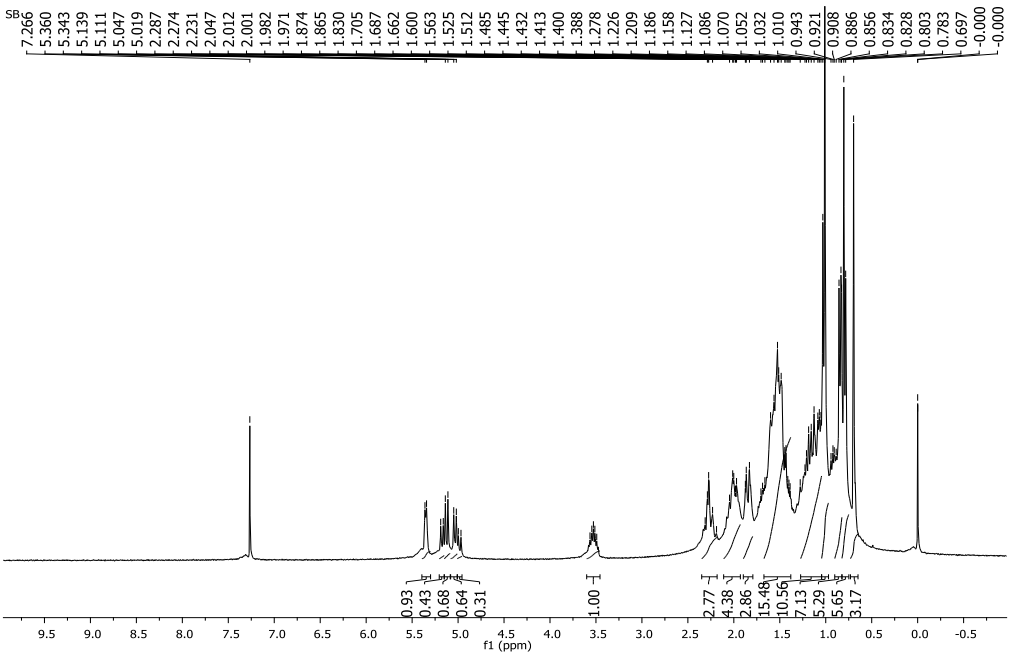
Result of Peak Picking

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3354.6	48.587	2	2935.1	15.615	3	2865.7	29.151	4	1598.7	74.193	5	1511.9	81.133
6	1459.8	50.467	7	1377.9	58.822	8	1330.6	78.293	9	1298.8	80.723	10	1281.5	80.977
11	1249.6	81.470	12	1192.8	82.942	13	1132.0	78.943	14	1107.9	75.915	15	1091.5	73.832
16	1056.8	48.210	17	1023.1	69.966	18	966.2	55.059	19	929.5	83.585	20	881.3	86.074
21	837.9	80.151	22	802.2	76.772	23	771.4	86.113	24	741.5	84.341	25	696.2	84.857
26	625.8	81.855	27	587.2	80.874	28	505.3	54.672	29	468.6	62.720			

Analysis for further clarification that the stigmasterol molecular weight was analysed using Liquid Chromatography Mass Spectrometry (LCMS). The structure of stigmasterol, theoretically generated using software chemoffice so that the amount of molecular weight (MW) is observed on the peak of 413.2 (M+ $C_{29}H_{48}O$), 395 (100%); 255.1 (22%).



The structure of the stigmasterol compound was analysed using ^{1}H Nuclear Magnetic Resonance (NMR). Results of ^{1}H NMR were analyzed and then combined to determine the bonds to construct a structure of the stigmasterol. The ^{1}H-NMR report the peaks at δ ppm: 0.78 (3H, s, -CH_3); 0.85 (6H, d, -CH_3); 0.88 (6H, d, -CH_3); 0.90 (3H, d, -CH_3); 0.92-1.08 (8H, m, -CH_2); 1.22-1.70 (10H, m, -CH_2); 1.83-1.87 (2H, m, -CH); 1.97-2.01 (3H,m, -CH); 2.23-2.28 (2H, m, -CH); 3.52 (1H, m, -CH); 5.01-5.04 (1H, m, =CH); 5.11-5.13 (1H, m, =CH); 5.34-5.36 (1H, m, =CH).



DISCUSSION

In this study the chloroform extract of *Halophila ovalis* showed significant activity against *Corynebacterium xerosis* and *Escherichia coli.* The ethyl acetate extract of *Halophila ovalis* showed moderate activity against *B.subtilis.* The significant activity was observed against *C. xerosis, E.coli and E.fecalis.* 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). 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.*

The phytochemical analysis of *Halophila ovalis* shows the presence of steroid in hexane hexane, ethanol and aqueous extract. Alkanoids present only in the chloroform extract. Flavonoids, terpenoids, tannins and phenols were present in ethylacetate extract of *Halophila ovalis.* Steroids, glycosides, saponins and phenol were present in ethanol extract of *Halophila ovalis.* Steroids, glycosides, saponins, terpenoids and phenol were present in hexane extract of *Halophila ovalis.* Athiperumalsami *et al.,* 2008 screened four seagrasses such as *Halophila ovalis*, *S. isoetifolium*, *C. serrulata* and *H. pinifolia* and reported 15 phytochemicals from benzene and petroleum ether extract of *S. isoetifolium*.

In present study, TLC analysis of chloroform extract of *Halophila ovalis* revealed, steroid compounds. The R_f values of steroid compound were 0.90, 0.85, 0.75, 0.58 and 0.41. Based on this R_f values it is subjected to preparative thin layer chromatography to obtain a large compounds. From the results, 42 mg of colourless crystalline powder was yielded from from chloroform extract of *Halophila ovalis*. The resulted solid sample showed a single spot in TLC, using different solvent systems, including Hexane: ethyl acetate (95:5); chloroform: ethanol (9.5:0.5), ethyl acetate: Hexane (50: 50) after the extraction and evaporation of crystals and its melting point is 164 – 166^o C. This result was supported with earlier literatures Kamboj and Ajay kumar saluja, (2011). He eluted stigmasterol, showed single spot in TLC using several solvent systems including chloroform: ethanol (9.8:0.2), ethyl acetate: ethanol (9.8: 0.2), chloroform: ethyl acetate (4:1) and it is a white crystalline powder (100mg) with melting point (144-146^{o}C).

In present findings the eluted compound was confirmed as steroid by Salkowski reaction and Liebermann burchard reaction. Pierre *et al.,* 2015 isolated sterol from *Odontonema Strictum* which showed single spot in TLC using several solvent systems including Ethyl acetate: Hexane (1:5), Ethyl acetate: Hexane: Chloroform (1.5: 2) and it yielded white crystalline powder (80mg) with melting point (134-136°C) and its R*f* value 0.55 gave a positive test to Liebermann Buchard and Salkowski reagents for steroidal nucleus (Kandati *et al.,* 2012; Volasoa *et al.,* 2014; Victor Njoku and Chidi Obi, 2009 and Elena *et al.,* 2011).

Stigmasterol isolated by preparative thin layer chromatography was further subjected to FT-IR, LC-MS and proton NMR to obtain a chemical structure. In our study, the IR spectrum showed totally 29 peaks. Of which major peaks occurred at strong band of -OH group at 3354 cm^{-1}, aliphatic -CH at 2865 cm^{-1}, C=C group at 1598, 1511 cm^{-1} and C-O at 1298, 1281 cm^{-1}. The stretching and bending vibrations of methyl part were observed at the intense band 2935 cm-1 and medium intensity band at 1459cm^{-1}. Peaks at 1249 and 696 cm^{-1} indicated OH bond vibrations. C-H vibrations of the unsaturated part were observed at 881 cm^{-1}. C-C vibrations were found at 1626 cm^{-1}. Based on FTIR analysis, the functional groups were determined as stigmasterol.

The stigmasterol obtained in our study was discussed in previous literatures such as, Pretsch *et al.,* 2000 obtained stigmasterol subjected to IR Spectroscopic analysis and observed absorption bands are 3547.41cm^{-1} of O-H stretching. Absorption at 3232.75 cm^{-1} is assumed as –HC= CH- structure, 3025 cm^{-1} =CH structure and 2857.75 cm^{-1} assigned C-H structure. Other absorption frequencies include 1638.83cm-1 as a result of C=C absorption and absorption frequency at 1071.28 cm-1 signifies cycloalkane. These absorption frequencies resemble the Stigmasterol (Grasselli, 1973).

In another study, the IR absorption spectrum showed absorption peaks at 3373.6cm-1(O-H stretching.); 2940.7 cm-1and 2867.9cm-1 (aliphatic C-H stretching); 1641.6cm-1 (C=C absorption peak); other absorption peaks includes 1457.3cm-1 (CH2); 1381.6cm-1 (OH def), 1038.7cm-1 (cycloalkane) and 881.6 cm-1 (Kamboj and Ajay kumar saluja, 2011). Manjula *et al.,* 2012 revealed the IR of stigmasterol showed peaks at 3345.5 cm-1 (br, OH), 2945.9 cm-1 (C-H str. in CH3 and CH2), 1649.8 cm-1 (C=C str.), 1452.6 cm-1 (C-H deformation in gem dimethyl), 1055.8 cm-1 (C-O str. of secondary alcohol).

In the present study, the molecular weight of stigmasterol was determined, by using liquid chromatography mss spectrum (LC-MS) technique. The molecular weight of stigmasterol was 412 which correspond to the molecular formula $C_{29}H_{48}O$. The peaks were observed at m/z 413 [M+], 395.3, 310, 279, 255 and 139. Chaturvedula and Indra Prakash, 2012 revealed the molecular weight by LC-MS and the peaks were observed at(m/z): 412 [M+], 394, 351, 314, 300, 271, 229, 213 and 55. In another literature FAB-MS spectroscopy used to obtain the molecular ion peaks of stigmasterol at 414 that correspond to molecular formula, $C_{29}H_{50}O$. Ion peaks were alsoobserved at m/z 367, 271, 255, 229,189, 175, 161, 133, 121, 105,107, 95, 81, 69, 55, 41 (Kamboj and Ajay kumar saluja, 2011).

In our study, The^{1}H-NMR (400MHz,$CDCl_3$) gives signal at δ ppm: 0.78 (3H, s, -CH_3); 0.85 (6H, d, -CH_3); 0.88 (6H, d, -CH_3); 0.90 (3H, d, -CH_3); 0.92-1.08 (8H, m, -CH_2); 1.22-1.70 (10H, m, -CH_2); 1.83-1.87 (2H, m, -CH); 1.97-2.01 (3H,m, -CH); 2.23-2.28 (2H, m, -CH); 3.52 (1H, m, -CH); 5.01-5.04 (1H, m, =CH); 5.11-5.13 (1H, m, =CH); 5.34-5.36 (1H, m, =CH).

Similar results were reported by Kamboj and Ajay kumar saluja, 2011 for proton NMR of stigmasterol at δ3.2(1H,

m, H-3), 5.26 (1H, m, H-6), 5.19(1H, m, H-23), 4.68(1H,m,H-22), 3.638 (1H, m, H-3), 2.38(1H, m, H-20), 1.8-2.0 (5H, m) ppm. Other peaks are observed at δ 0.76-0.89 (m, 9H), 0.91-1.05 (m, 5H), 1.35-1.42 (m, 4H), 0.69-0.73 (m, 3H), 1.8-2.00 (m, 5H), 1.07-1.13 (m, 3H), 1.35-1.6 (m, 9H) ppm.

In another study, Pierre *et al.*, 2015 reported the ^{1}H NMR spectrum of stigmasterol which varied between 0.736 to 5.378 ppm, This spectrum showed the presence of 6 high peaks which indicating the presence of six methyl groups at δ 0.736, 0.843, 0.967, 1.037, 1.200 and 1.534 ppm. The proton H-3 of a sterol moiety was appeared as a triplet of doublet at δ3.529 ppm. At δ 5.197 ppm and at δ 5.378 ppm showed peak in single in the region of the ethylene protons indicating the presence of three protons.

Pateh *et al.*, 2009 and Jamal *et al.*, 2009 also discussed theproton NMR of stigmasterol which showed the proton of H-3 as a multiplet at δ 3.2 and revealed the presence of signals for olefinic proton at δ 5.19 (m), 4.68(m), 4.6(m) and 2.38(m). Angular methyl proton at 0.69(s), 0.80(s) and 1.02(s) corresponds to C18 and C19 proton respectively.

Chaturvedula and Indra Prakash, 2012 reported ^{1}H NMR spectra of stigmasterol showed two methyl sinlgets at δ 0.71, and 1.03; three methyl doublets at δ 0.80, 0.82 and 0.91; and a methyl triplet at δ 0.83. It also showed protons at δ 4.98, 5.14, and 5.31 indicating the presence of three protons such as trisubsti-tuted and olefinic bond. Lieber-mann-Burchard reaction indicated stigmasterol is having a sterol skeleton (Kandati *et al*., 2012; Raju *et al*., 2012). In our study also, stigmasterol is confirmed by Lieber-mann-Burchard reaction ans salkowski test which indicates the presence of sterol.

Manjula *et al.*, 2012 also revealed the ^{1}H NMR of stigmasterol at δ 5.27-5.12 (d, m(1H, Vinylic proton), δ 4.9 d, (J=8 Hz) and 5.0 d (J=7 Hz) 2H, broad olefinic proton), δ 3.45 (m, 1H, CHOH), δ 1.14 to 2.21 (m, 18H, 9 X CH2 and 8H, CH proton), δ 0.62 to 1.09 (m, 18H, 6 XCH3) and the structures were confirmed by spectral analysis data reported in literature (Mohamed Khadeer Ahamed *et al*., 2007; Jain and Bari, 2010; Kamboj and Saluja, 2011).

CONCLUSION

From this present study, it can be concluded that Stigmasterol Stigmasterol was isolated from chloroform extract of *Halophila ovalis.*

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