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Research Article

GC-MS Based Metabolomics Analysis and Characterization of Rosmarinic Acid from Tropical Seagrass *Halodule pinifolia*

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ABSTRACT

Study investigated the metabolomics analysis of seagrass for acquiring diverse group of metabolites which offers various prospects for novel, financially significant constituents conveyed to the spotlight. GC-MS analysis revealed the presence of thirty five compounds includes metabolites, flavonoids, sugars, aminoacids and plant hormones. Rosmarinic acid (RA) is a pharmacologically active phytoconstituent that has so far been reported on the family of Boraginaceae and Lamiaceae. Present finding records the presence of RA from tropical seagrass *Halodule pinifolia* of Cymodoceaceae family collected from vellar estuary and is the first report on a tropical seagrass distributed in India. The existence of RA was identified by co-TLC, HPLC-PDA-MS and further it was confirmed by isolation of RA through prep-TLC and structural characterisation via NMR analysis. Study has explored a newer marine source, *H. pinifolia* for RA, which has an emerging potential preclinical chemical entity and its biosynthesis can be enriched by plant tissue culture.

Keywords: Seagrass; Cymodoceaceae; *Halodule pinifolia;* Metabolomics; Rosmarinic acid

INTRODUCTION

Seagrass, a characteristic asset of beach front biological community offers various prospects for novel, financially significant constituents and are less investigated with respect to phytochemical constituents. Investigation on antimicrobial, antioxidants, metabolite synthesis and nutritive reviews bought seagrasses to the spotlight, as a wellspring of novel bioactive compounds [1,2,3]. The bioactive compounds can be either primary or secondary metabolites which are derived from the metabolic process in plants. Profiling of primary metabolites are crucial for the growth and development of plants, while secondary metabolites is not essential but are important for the plants to overcome the stress by maintaining the subtle balance in the environments. In recent years, metabolomics has become a well-accepted approach for the holistic measure of all plant metabolites quantitatively or qualitatively, so that a clear metabolic picture of specific plant species can be obtained. Seagrasses cover two families Hydrocharitaceae and Cymodaceaceae with 14 species reported all over India and are vanishing owing to the environmental and geopolitical instabilities, and therefore it is hard to acquire important secondary metabolites, thereby losing many potential compounds which are undiscovered. Many of the bioactive compounds have been reported as pharmacologically active constituents and rosmarinic acid (RA), a naturally occurring phenolic compound commonly found in the medicinal plants of family Lamiaceae and Boraginaceae [4]. Being a potential compound extracted from *R. officinalis*, its action towards human health was brought to the limelight for researchers to investigate more on different source of pharmaceutical applications. Earlier findings document antioxidant [5], anti-inflammatory [6], anti-bacterial [7], anti-mutagenic [8], astringent [9], anticholinesterase [10], hepatoprotective [11], cardioprotective [12] and anti-viral activities [13] of RA. Due to its valuable bioactive properties, numerous studies have been recently focussing for identifying a rich source for RA.). Though, there has been a report on the occurrence of RA from temperate seagrass family Zosteraceae, no studies have been published so far in respect to metabolomics analysis of tropical seagrasses. Hence, there has been a considerable interest on metabolomics analysis; to purify and characterize RA from tropical seagrass *Halodule pinifolia* of Cymodoceaceae family thereby biosynthesis of RA can be enhanced by the micropropagation of seagrasses under *in-vitro* conditions.

Materials and Methods

Plant material

Halodule pinifolia collected from the Vellar Estuary were washed with native water to remove the

epiphytes and sand particles. The collected material was authenticated by Kannan and Thangaradjou [14] and was frozen immediately using liquid nitrogen in order to avoid the metabolite changes triggered by the enzymatic reactions. Prior to metabolite extraction, the presence of epiphytes or endophytes in *Halodule pinifolia* was confirmed by microscopic observation [15] and *in-vitro* culture of seagrass [16].

Metabolite extraction and GC-MS based metabolomics analysis of *H. pinifolia*

Halodule pinifolia (1 kg, freeze dried) were macerated separately with three different solvents such as chloroform (Chl), ethylacetate (EtAc) and methanol (MeOH). Prior to the metabolomics analysis, proteins and other crude cellular components were removed via filtration with 10kDa cutoff filter. Filtered extracts were concentrated by vacuum evaporation at 40 ° C and the dry weight of each extract was found to be 4.53 g/kg (Chl), 2.39 g/kg (EtAc) and16.53 g/kg (MeOH) which was further, resuspended using respective solvents for qualitative and quantitative detection. Methoxamine (MO)-derivatization was performed by using an aliquot of different solvent extracts by adding 80 ml of methoxamine hydrochloride (10mg/ml) in pyridine and shaking at 30 ° C for 1 hour. Timethysilyl etherification was performed by adding MSTFA (80 mL) at 37 ℃. GC-MS was performed using Agilent 7890A liquid chromatograph coupled to TOF mass spectrometer. Derivatized sample (1mL) was separated on 30^{-m} x 0.25^{-mm} fused silica capillary column coated with 0.25^{-mm} CP-SIL 8 CB low bleed. The injector temperature was 23 ℃ and the split ratio was set at 1:25. The scanned mass range was 85-600 *m/z* and the detector voltage was set at 1500 V.

Preparative High performance Liquid Chromatography analysis

Rosmarinic acid in the extracts was eluted with the help of standard using an Agilent 1260 series preparative HPLC (Agilent Technologies Inc., Chemetrix) equipped with binary pump. It was fitted with C18 column (Phenomenex; 25 cm x 10 mm x 5 μm particle size) and 10 μL of 400 mg/ mL sample volume was repeatedly injected for the isolation under optimized condition. The mobile phase consisted of aqueous phosphoric acid (0.3% v/v; pH - 1.76; solvent A) and aqueous acetonitrile (10% v/v; solvent B) at a flow rate of 1mL/ min. Chromatography was performed with linear gradient elution from 0 to 70% solvent B in 20 min. Individual constituents were monitored using HPLC-diode array detector and the fractions were collected following the targeted peak picking method. The eluted compound were structurally elucidated

Spectrophotometric determination of rosmarinic acid

Rosmarinic acid standard at a concentration of 1 x 10^{-3} g/mL was prepared by dissolving 0.0901 g in 250 mL of methanol was used as stock solution. Solutions of different concentrations (5, 10, 15, 20, 25, 30 μM) were made by diluting the stock solution in methanol. Determination of rosmarinic acid was carried out based on the complex reaction of rosmarinic acid with Fe^{2+} ions following the procedure described by Ondrejovic et al. [17]

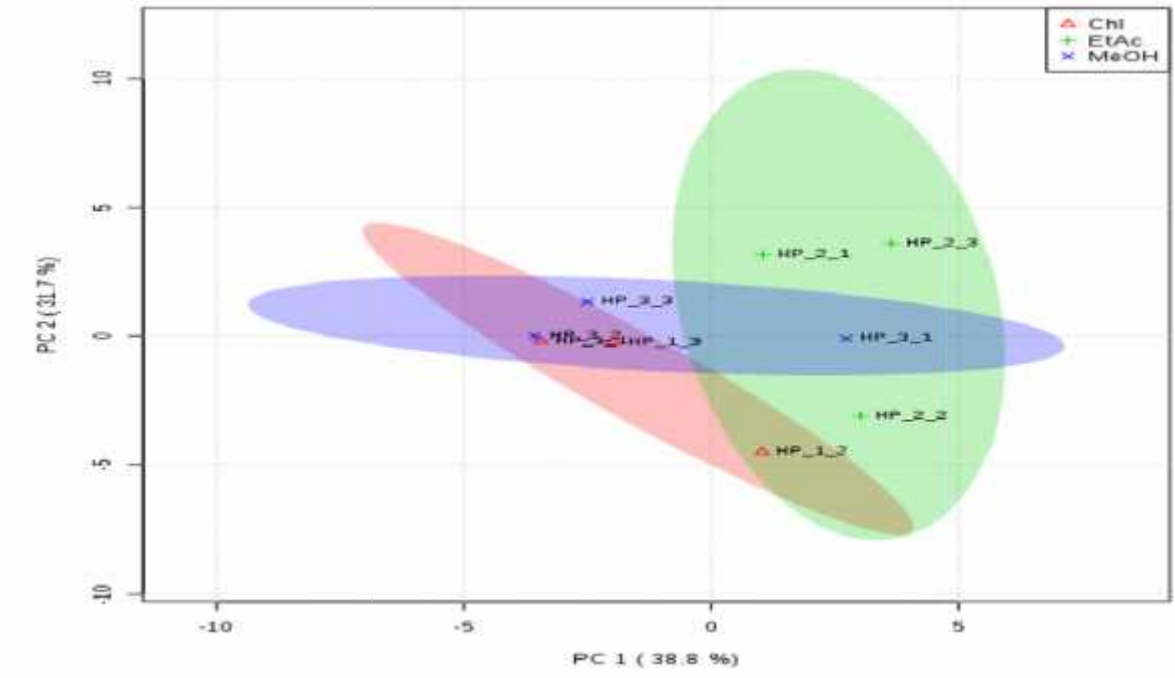

Figure 1. Score plot of Principal component 1 and 2 of PLS-DA results obtained from LC-TOFMS data for three different solvent extracts of *H. pinifolia* (Chl (HP_1), EtAc (HP_2);MeOH (HP_3) each group with triplicates).

Table 1. GC-TOFMS derived metabolites from *H. pinifolia*

S. No	Compounds	Chemical formula	m/z	Monoisotopic Mass (g/mol)
Chloroform extracts				
1.	Gallic acid	$C_7H_6O_5$	170.12	170.021
2.	Vanillic acid	$C_8H_8O_4$	168.14	168.0422
3.	Catechol	$C_6H_6O_2$	110.11	110.036
4.	2,3-Dihydroxy-1-4-(4-hydroxy-3-methoxyphenyl)-propane-one	$C_{10}H_{12}O_5$	212.20	212.068
5.	Catechin	$C_{15}H_{14}O_6$	290.268	290.079
6.	Gallocatechin	$C_{15}H_{14}O_7$	306.27	306.073
Ethyl acetate extracts				
7.	p-coumaric acid	$C_9H_8O_3$	164.158	164.0473
8.	Vanillin	$C_8H_8O_3$	152.1473	152.0473
9.	Isobutyric acid	$C_4H_8O_2$	88.1051	88.0524
10.	Isoleucine	$C_6H_{13}NO_2$	131.1729	131.0946
11.	Isovaleric acid	$C_5H_{10}O_2$	102.1317	102.0680
12.	Nicotinic acid	$C_6H_5NO_2$	123.1094	123.0320
13.	Phenylacetic acid	$C_8H_8O_2$	136.1479	136.052
14.	Fumaric acid	$C_4H_4O_4$	116.0722	116.0109
Methanol Extracts				
15.	Caftaric acid	$C_{13}H_{12}O_9$	312.229	312.048
16.	Coutaric acid	$C_{18}H_{27}N_3O_4$	349.4247	349.200
17.	Ferulic acid	$C_{10}H_{10}O_4$	194.184	194.0579
18.	Resveratrol	$C_{14}H_{12}O_3$	228.247	228.0786
19.	*trans*-Cinnamic acid	$C_9H_8O_2$	148.1586	148.0524
20.	Naringenic acid	$C_{15}H_{12}O_5$	272.2528	272.0684
21.	Sinapic acid	$C_{11}H_{12}O_5$	224.21	224.0684
22.	Glucoside aglycone	$C_{16}H_{32}O_6$	320.426	320.22
23.	2,4-trans abscisic acid	$C_{15}H_{20}O_4$	264.321	264.136
24.	Oxydisuccinic acid	$C_4H_6O_4$	118.088	118.027
25.	Gibberilin A4	$C_{19}H_{24}O_5$	332.396	332.162
26.	Indole acetic acid	$C_{10}H_9NO_2$	175.187	175.063
27.	Caffeic acid	$C_9H_8O_4$	180.1574	180.0422
28.	Rosmarinic acid	$C_{18}H_{16}O_8$	360.3148	360.084
29.	Quercetin	$C_{15}H_{10}O_7$	302.2357	302.0426
30.	Proanthocyanidin	$C_{30}H_{24}O_{12}$	576.5044	576.1267
31.	L-Glutamic acid	$C_5H_9NO_4$	147.1293	147.053
32.	feruryl tartaric acid	$C_4H_6O_6$	150.0868	150.0164
33.	Procyanidin B3	$C_{30}H_{26}O_{12}$	578.526	578.142
34.	Hydroxy benzoic acid	$C_7H_6O_3$	138.122	138.0316
35.	Hypoxanthine	$C_5H_4N_4O$	136.1115	136.0385

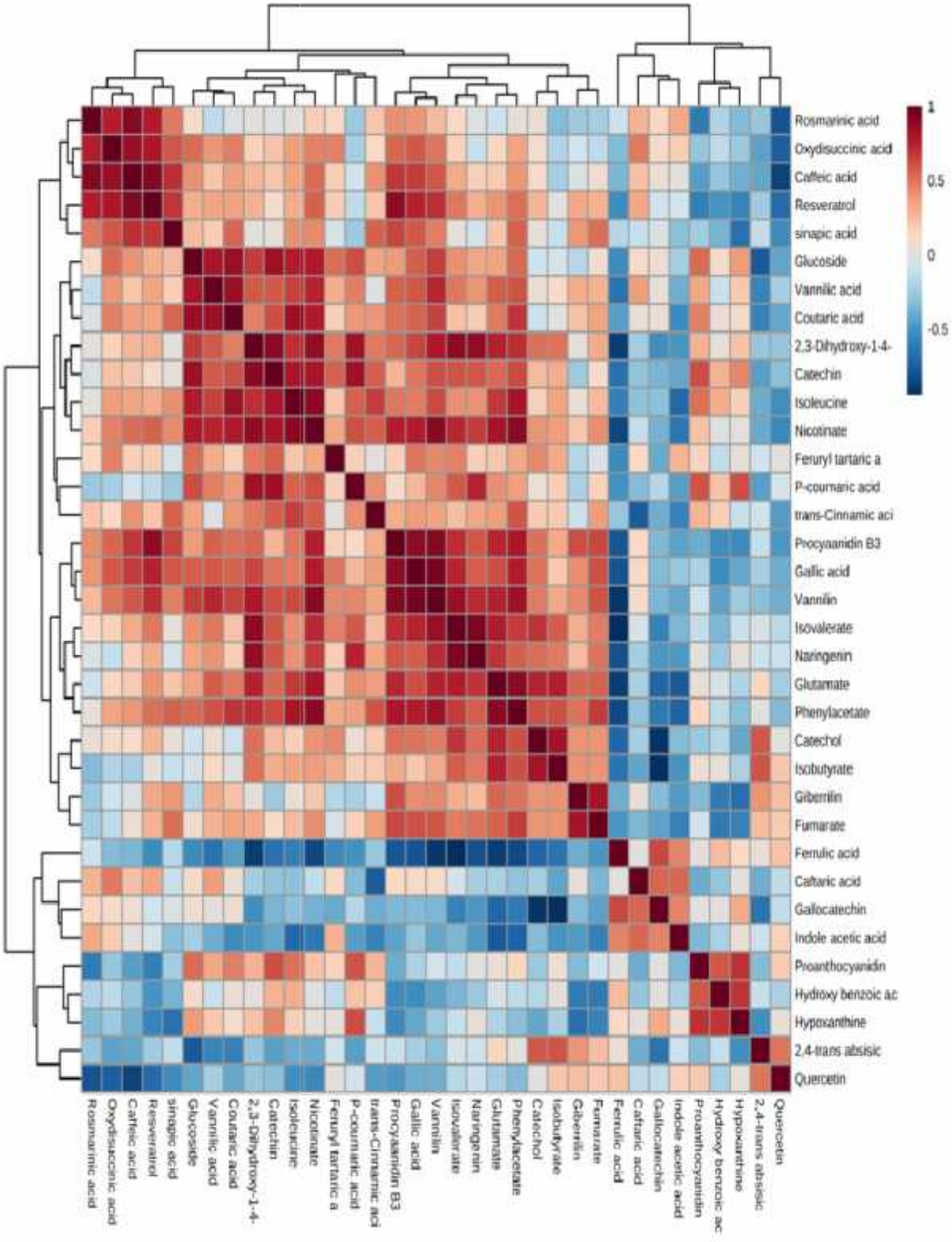

Figure 2. Correlation matrix of different metabolites derived from *H. pinifolia*

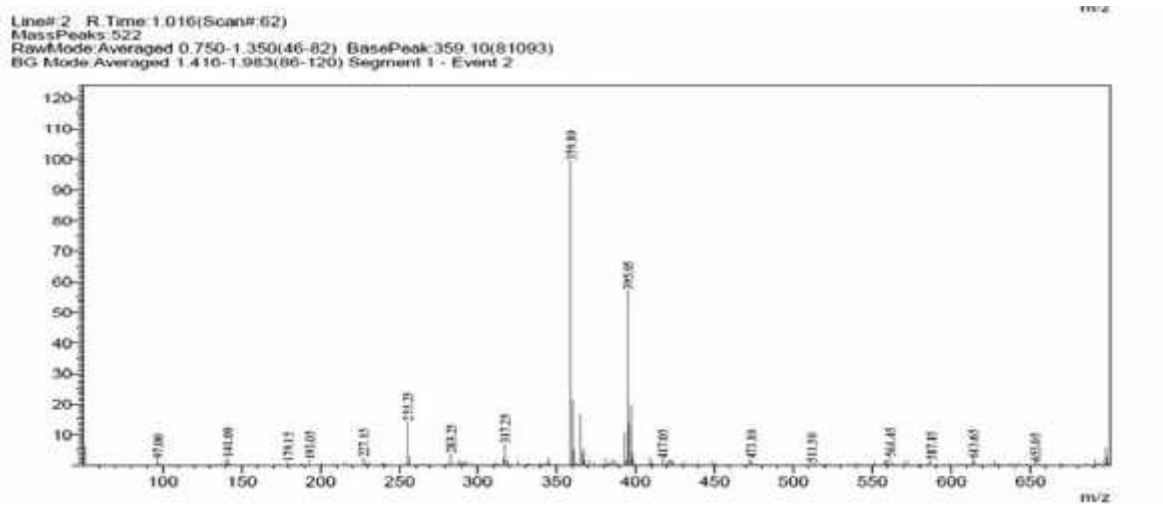

Figure 3. Mass spectra of Rosmarinic acid extracted from *H. pinifolia*

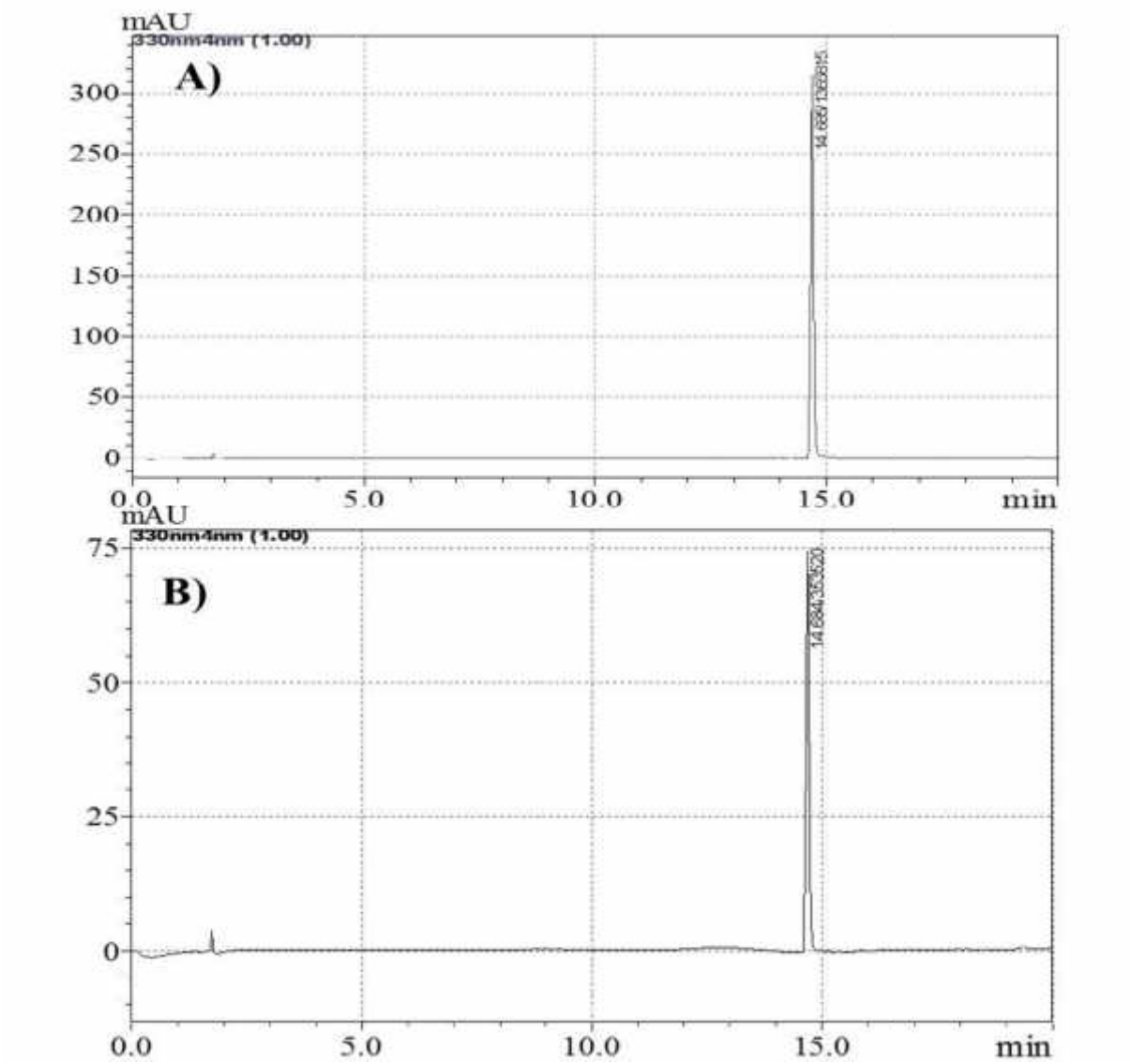

Figure 4. HPLC analysis of rosmarinic acid from *H. pinifolia* extracts at 330 nm. A) Standard Rosmarinic acid; B) Rosmarinic acid from *H. pinifolia*

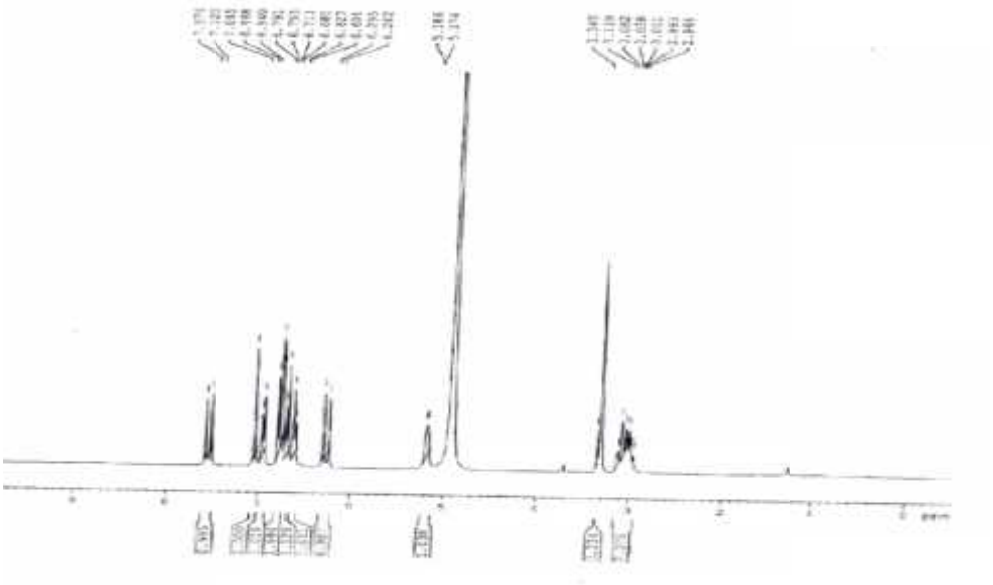

Figure 5. ^{1}H-NMR spectra of rosmarinic acid extracted from *H. pinifolia*

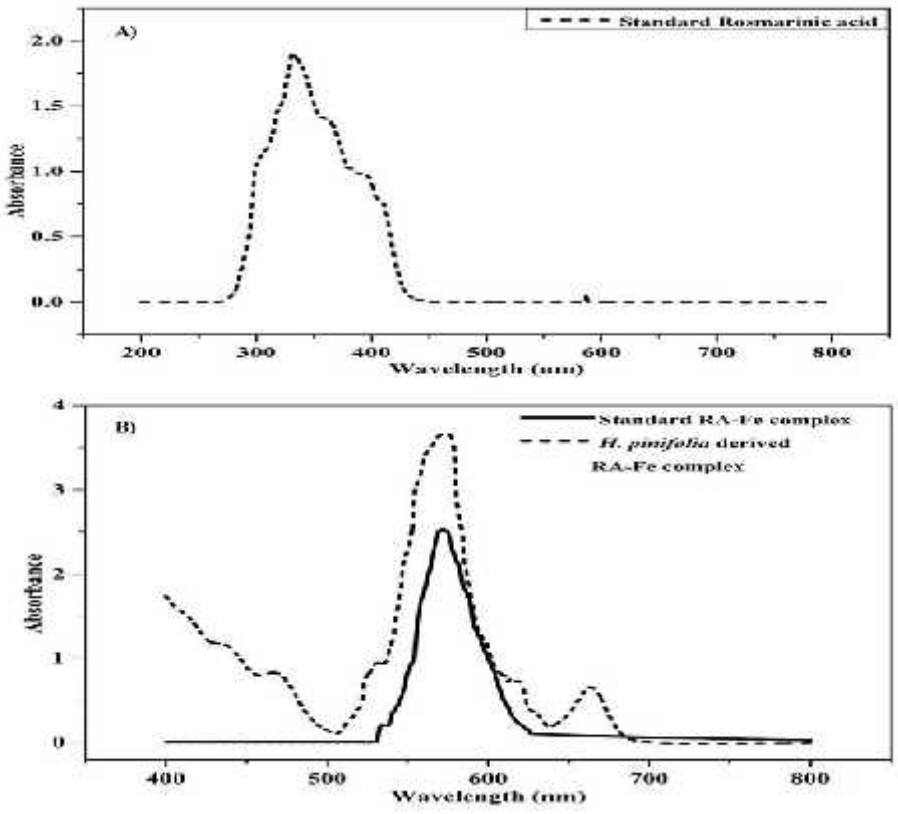

Figure 6. Absorption spectra of standard rosmarinic acid (A) and complexation of rosmarinic acid from *H. pinifolia* with Fe^{2+} ions (B)

Statistical analysis

Quantitative data obtained from the GC-MS was subjected to multivariate analysis using Metaboanalyst 3.0. To evaluate the relationships among groups of variable, normalized data were subjected to PLS-DA and the output consisted of score plot to show the contrast between the different

groups, while correlation matrix were used to identify the compound clusters.

Results and Discussion

GC-MS analysis of metabolites from H. pinifolia

So far, metabolomics analysis of seagrasses has not yet been studied, which paved a way to study the diverse group of metabolites from seagrass *Halodule pinifolia*. Microscopic observation and *in-vitro* culture of seagrass *H. pinifolia* revealed the absence of epiphytes or endophytes during metabolite extraction. Hence, Metabolite accumulation pattern of *H. pinifolia* have been studied by GC-MS for different solvent extracts and the peak location were assisted by Chrome TOF software. In order to detect as many metabolites as possible, three solvents (chloroform, ethyl acetate and methanol) were used and some limitations had occurred while injecting the samples. In total 35 metabolites have been profiled from *H. pinifolia*, which includes phenolics, flavonoids, aminoacids, sugars and plant growth regulators (Table S1). Internal data structure obtained from GC-MS was normalized and was subjected to Partial Least Square-Discriminant analysis (PLS-DA) to determine the variance between the metabolites among different group i.e. different solvent extracts viz. Chl (HP_1), EtAc (HP_2) and MeOH (HP_3) each of with triplicates. Score plot of PLS-DA reveals the magnitude and direction of original variables with principal components. The first principal component resolved the metabolite profile of *H. pinifolia* accounted for 38.8% of variations, whereas the second principal component accounted for an additional variation of 31.7% (Figure 1). The relationship between two metabolite variables of *H. pinifolia* were determined by correlations matrix and the compounds of different solvent extract showed both positive and negative correlations. Among the thirty five compounds profiled, eight compounds such as quercetin, 2, 4-transabsisic acid, proanthocyanidin, Indole acetic acid, fumarate, ferulic acid, Isobutyrate and gibberellin showed the positive correlation and were clustered. Other compounds such as rosmarinic acid, Isovalerate, Gallic acid, etc., showed a strong negative correlation between the compounds of different extracts (Figure 2). Most of these compounds identified have not yet been reported on the seagrass family, but their occurrence has been reported on the terrestrial plants. Rosmarinic acid is one among the compounds of *H. pinifolia,* which has more pharmaceutical and nutraceutical effect and has not been reported so far in tropical seagrasses. Hence, further work was carried out with the purification and structural elucidation of rosmarinic acid using HPLC and NMR. The GC-MS fingerprint of the extract explored the presence of eight major secondary metabolites having molecular weight less than 500 daltons. The peak eluted at 14.68 min under gradient elution exhibited $[M-H]^+$ at m/z 359.10 in the negative mode confirmed rosmarinic acid, which has a molecular weight of 360 dalton (Figure 3). Further, the identification was confirmed by retention time match (14.68 min) and co-injection of extract mixed with standard RA under HPLC analysis (Figure 4 A&B). Karina and Bras [18] used methanol as eluent A and 0.1% phosphoric acid in water as eluent B. The study was similar to the present findings, but better resolution was observed when 0.05% formic acid was used. The purity of the isolated RA was found to be 98 % as determined by RP-HPLC method. The identity of the purified compound was established by proton NMR analysis (Figure 5). The chemical shift values were found to be matching with those of reported values [19]. RA in *H. pinifolia* was estimated by photometric method and is based on the complexation of RA with Fe^{2+}, which gives a dark blue colour causing a bathochromic shift (572 nm) and increasing the sample absorbance, while the standard RA showing maximum absorption at 332 nm. Ozturk et al. [20] reported a photometric method which uses zirconium (IV) ions to which RA make a complexation reaction giving a light-yellow colour which absorbs at 362 nm. Since, the use of Fe^{2+} ion is cheaper, eco-friendly and moreover, the interferences caused by the absorbance of non-complexed compounds in *H.pinifolia* extracts are found to be higher at 362 nm (Zr^{4+}) than 572 nm (Fe^{2+}), the present study attempted with the photometric method of using Fe^{2+} ions. Fig 4A illustrates the absorption spectrum of standard RA with the absorptivity coefficient found to be $\varepsilon_{332} = 1.91 \times 10^4\ L\ mol^{-1}\ cm^{-1}$. The comparison of Fe^{2+} complex with standard rosmarinic acid and RA from *H. pinifolia* extracts are illustrated in Fig. 4B. Standard RA absorbs at 332 nm and when it is mixed with the tris buffer containing $FeSo_4$, complexation occurs and the absorption undergoes red shift to 572 nm with the absorptivity coefficient of $\varepsilon_{572} = 2.54 \times 10^4\ L\ mol^{-1}\ cm^{-1}$. Reaction with *H. pinifolia* extract showed $\varepsilon_{572} = 3.662 \times 10^4\ L\ mol^{-1}\ cm^{-1}$. In addition to RA-$Fe^{2+}$ complex band, two more absorption bands at 654 nm and 664 nm were observed in *H. pinifolia* extract. In order to determine the concentration of RA in the *H. pinifolia* extract, standard curve was plotted using different concentration (5, 10, 15, 20, 25, 30 μM) of standard RA (Figure 6). The standard curve was generated with more than ten parallel measurements and the results obtained were found to be statistically significant ($P < 0.01$). The concentration of RA in the *H. pinifolia* extract was found to be 40 mg/g of DW extract. Hossain and Ismail [21] studied the HPTLC determination of RA in *Orthosiphon stamineus* and quantified the RA content to be ranging between 0.24 – 2.24 % for more than 20 samples in the same chromatographic run. In conclusion, the study has explored a newer marine source, *H. pinifolia* for RA, which is an emerging potential preclinical chemical entity. RA has been found to be the major secondary

metabolite of *H. pinifolia* and its biosynthesis can be enhanced by plant tissue culture techniques for bulk production.

Conclusion

Metabolomics analysis of *H. pinifolia* found to exhibit the diverse group of bioactive metabolites, which has not been reported so far and determined un-targeted metabolites by analytical and biological methods and the study has also explored a newer marine source, *H. pinifolia* for rosmarinic acid.

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