

## 4. EXTRACTION, ISOLATION AND CHARACTERIZATION OF PHYTOCHEMICALS FROM *G.ACEROSA*

### 4.1 Introduction

It is now well known that marine organisms possess several valuable, potential compounds with the ability to treat different diseases. The aim of this chapter of the thesis was to prepare a powder from *G.acerosa* and to extract, isolate and characterize the phytochemicals using different techniques. Further, the study also investigated the polyphenol and flavonoid content of the algae and its isolated compounds.

### 4.2 Methods

As described in chapter 3, *G.acerosa* was processed and the dried powder of the algae which was subjected to extraction using solvents of varying polarity. The extracts obtained were screened for their phytoconstituents qualitatively, followed by quantification of polyphenol and flavonoid contents and separated by HPLC. The functional groups present in the extracts were analyzed by FT-IR and the antioxidant activities of the extracts were determined. The ethyl acetate extract was separated by open column chromatography and the isolated compounds were characterized by GC-MS and NMR techniques.

### 4.3 Results

#### 4.3.1 Qualitative screening of Phytochemicals in *G.acerosa*

Table 4.1 shows the phytochemicals identified in the red alga *G.acerosa* by sequential extraction using solvents including hexane, dichloromethane (DCM), ethyl acetate, ethanol, methanol and water. The data showed that hexane was a good solvent to isolate alkaloids, glycosides followed by oils, fats and flavonoids. The solvent DCM was beneficial in isolating phytosterols, glycosides, coumarins followed by alkaloids, tannins, flavonoids, oils, fats and protein. The other solvent ethyl acetate proved to be better than the previous two solvents to isolate tannins, flavonoids, terpenoids, phytosterol, glycoside, oils, fats, carbohydrate, coumarins

along with alkaloids and protein. The solvent ethanol was good to isolate flavonoids, phytosterol, glycoside and also tannins, terpenoids, oils, fats, carbohydrate, coumarins and protein. Similarly methanol was efficient in isolating tannins, terpenoids, phytosterol, glycosides, protein, carbohydrates followed by flavonoids, oils, fats, resins and coumarins. Water was a good solvent to extract carbohydrates followed by flavonoids, protein, glycoside and coumarins.

Table 4.1 Phytochemical screening of *G.acerosa* extracts obtained by sequential extraction.

Name of the phytochemical compound	Hexane	DCM	Ethyl acetate	Ethanol	Methanol	Water
Tannins	-	+	+++	++	+++	-
Alkaloids	+++	++	++	-	-	-
Flavonoids	+	+	+++	+++	++	++
Terpenoids	-	-	+++	++	+++	-
Saponins	-	-	-	-	-	-
Phytosterol	-	+++	+++	+++	+++	-
Glycoside	+++	+++	+++	+++	+++	+
Oils & Fats	++	++	+++	++	++	-
Protein	-	+	+	+	+++	++
Carbohydrates	-	-	+++	++	+++	+++
Resins	-	-	-	-	++	-
Coumarin	-	+++	+++	++	++	+

### 4.3.2 Quantitative screening of phytochemicals

In another set of experiment, the polyphenol concentration of the algal extracts was quantified.

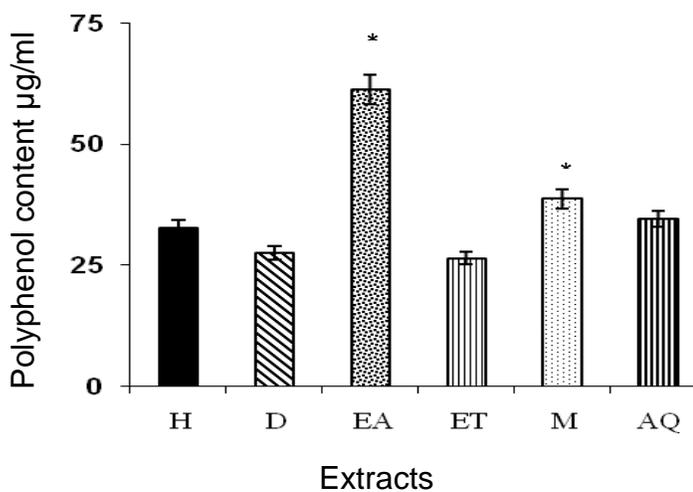


Figure 4.1 Bar chart showing the amount of algal polyphenol extracted from the red algae *G.acerosa* using hexane (H), DCM (D), ethyl acetate (EA), ethanol (ET), methanol (M) and water (AQ).

Data are expressed as mean  $\pm$  S.D, n=3, \*p<0.05. Note that ethyl acetate extract was found to have more concentration of polyphenol than the other extracts.

Following this, the flavonoid concentration of the algal extracts was determined.

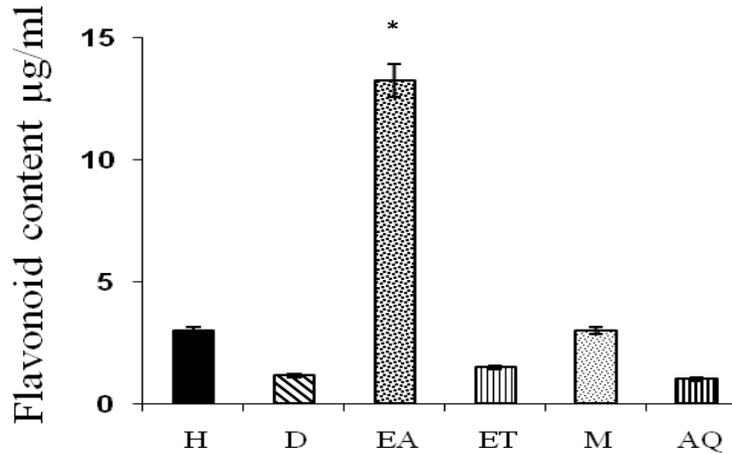


Figure 4.2 Bar charts showing the quantification of flavonoids extracted from the red algae *G.acerosa* using hexane (H), DCM (D), ethyl acetate (EA), ethanol (ET), methanol (M) and water (AQ).

Data are expressed as mean  $\pm$  S.D, n=3, \*p<0.05. Note that ethyl acetate extract was more effective in extracting the flavonoids when compared to the other solvents.

#### 4.3.3 HPLC analysis of algal extracts

The extracts of *G.acerosa* were separated by HPLC.

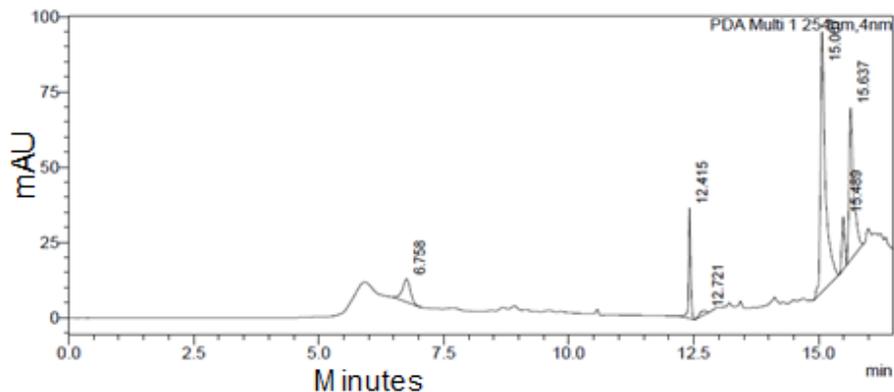


Figure 4.3 The original HPLC chromatogram of hexane extract from *G. acerosa*. The chromatogram shows the presence of 4 different compounds in the extract.

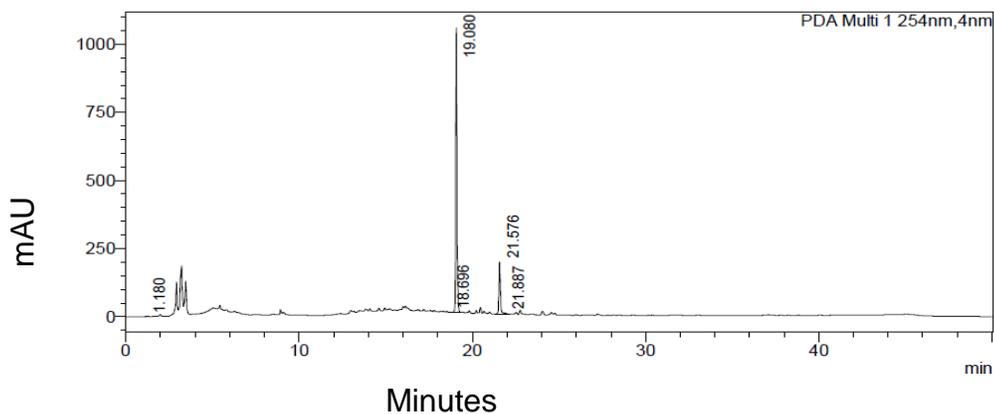


Figure 4.4 The HPLC chromatogram of DCM extract from *G. acerosa*. The chromatogram shows the presence of 4 different compounds in the extract

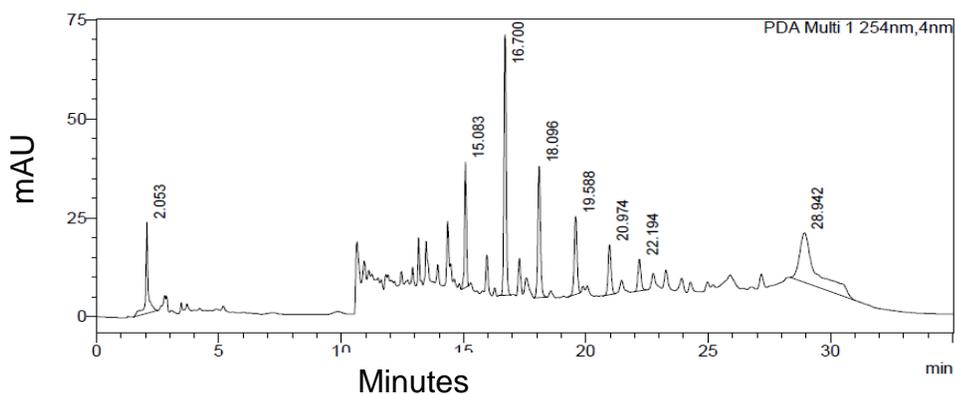


Figure 4.5 The HPLC chromatogram of ethyl acetate extract from *G. acerosa*. The chromatogram shows the presence of 8 different compounds in the extract.

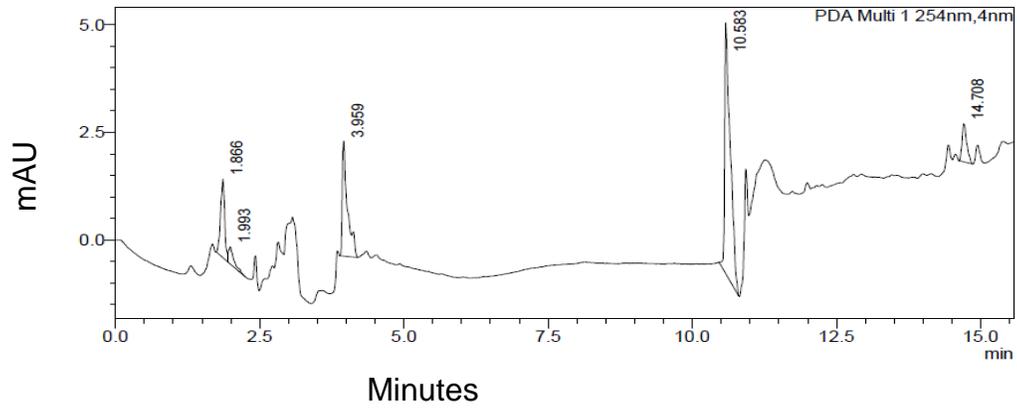


Figure 4.6 The HPLC chromatogram of ethanol extract from *G. acerosa*. The chromatogram shows the presence of 5 different compounds in the extract.

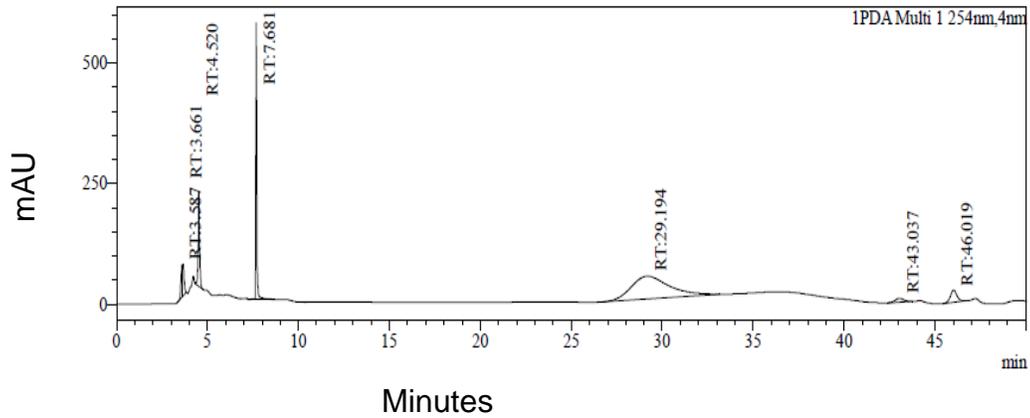


Figure 4.7 The HPLC chromatogram of methanol extract from *G. acerosa*. The chromatogram shows the presence of 6 different compounds in the extract.

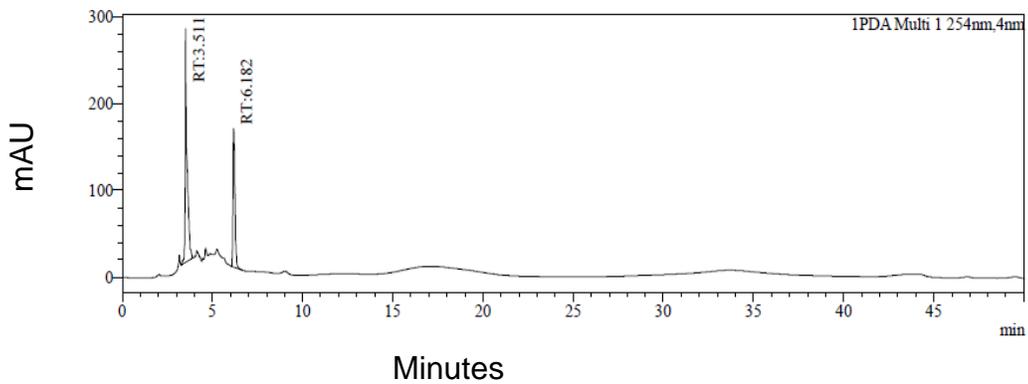


Figure 4.8 The HPLC chromatogram of water extract from *G. acerosa*. The chromatogram shows the presence of 2 different compounds in the extract. These chromatograms are typical of 3 such different experiments.

### 4.3.4 FT-IR analysis of algal extracts

The identification of the functional groups in *G.acerosa* were identified by FT-IR analysis.

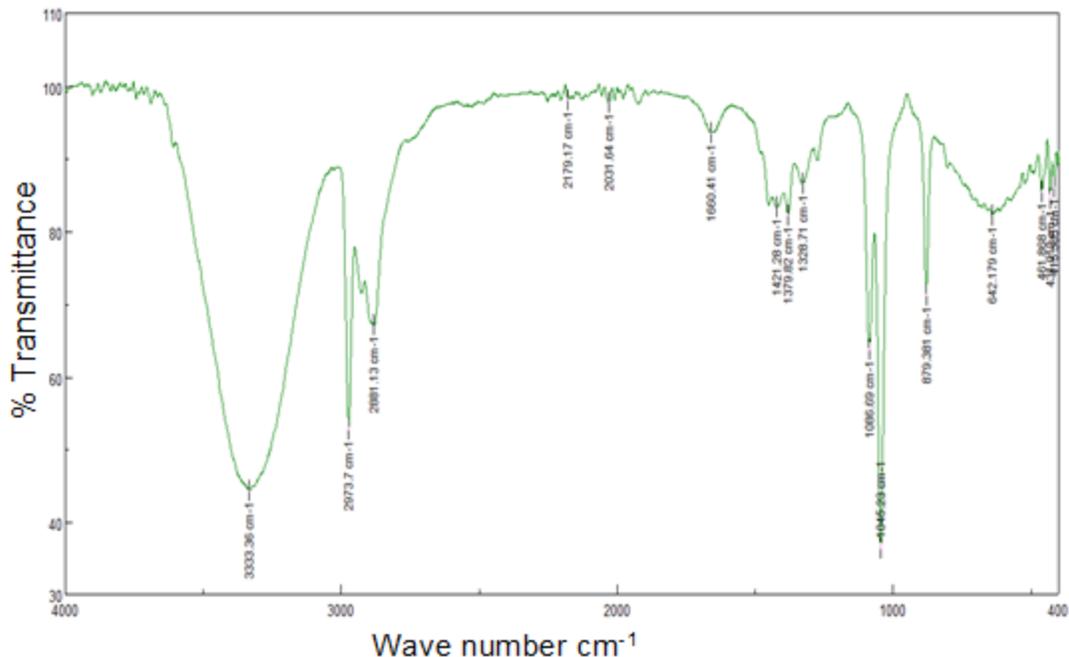


Figure 4.9 FT-IR spectra of hexane extract from *G. acerosa*.

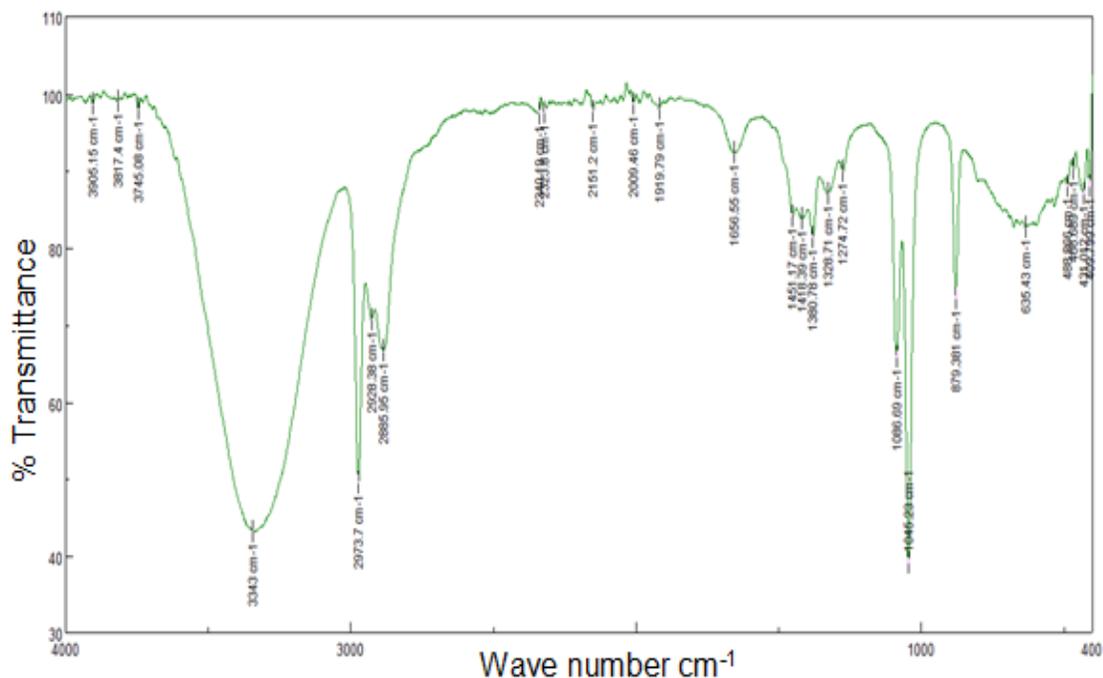


Figure 4.10 FT-IR spectra of DCM extract from *G. acerosa*.

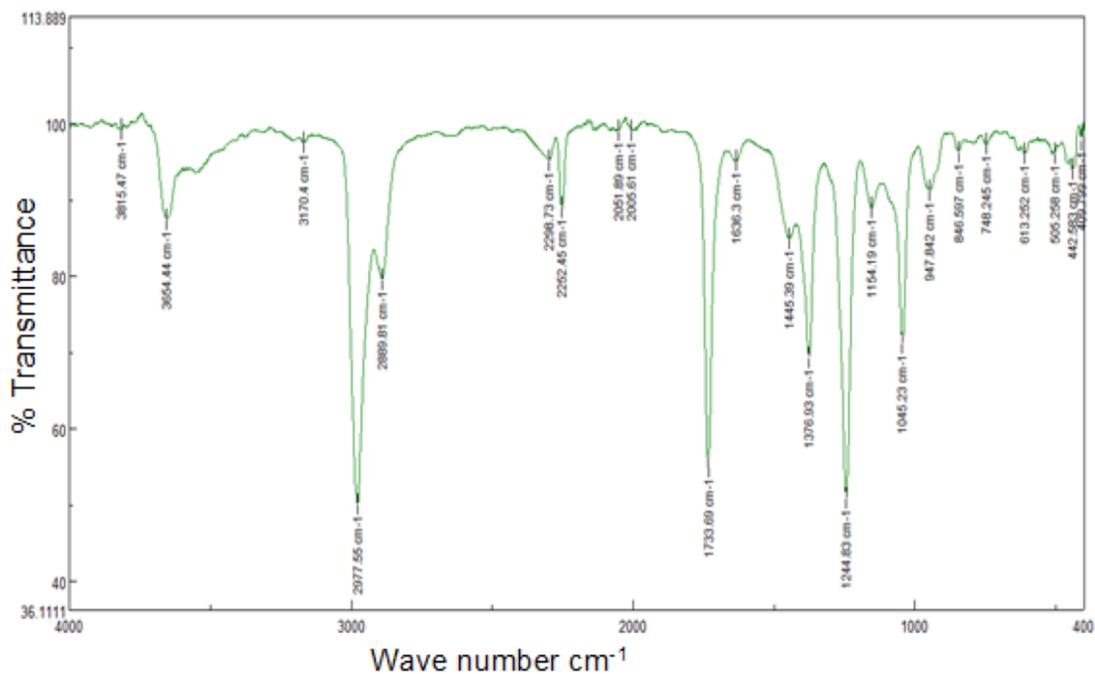


Figure 4.11 FT-IR spectra of Ethyl acetate extract from *G. acerosa*.

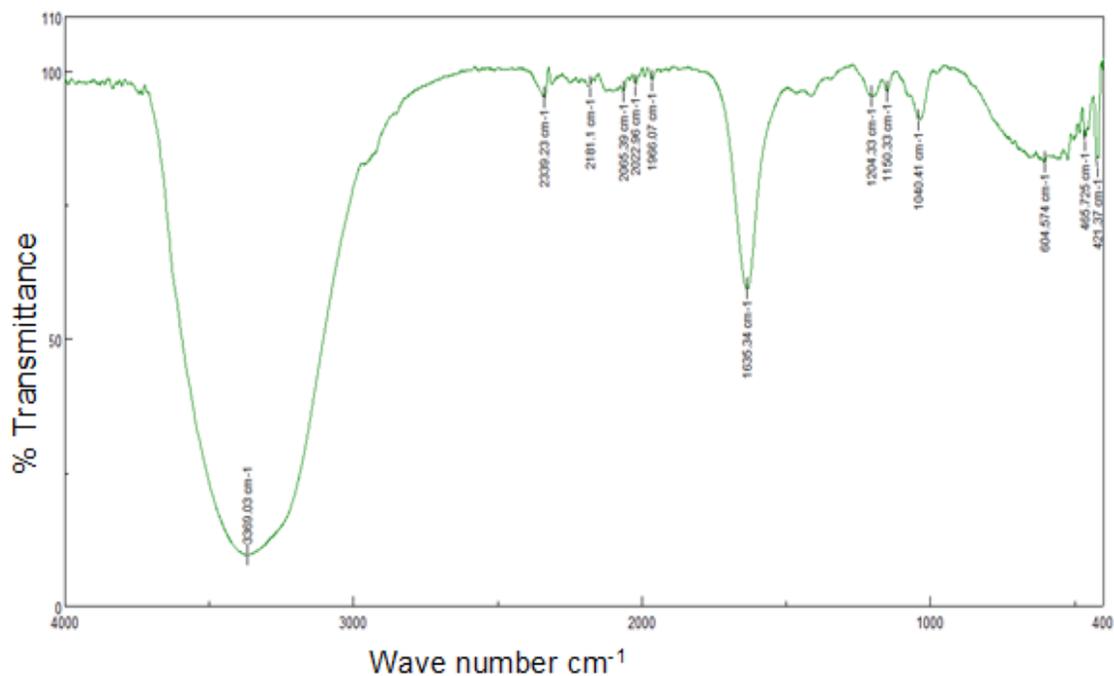


Figure 4.12 FT-IR spectra of ethanol extract from *G. acerosa*.

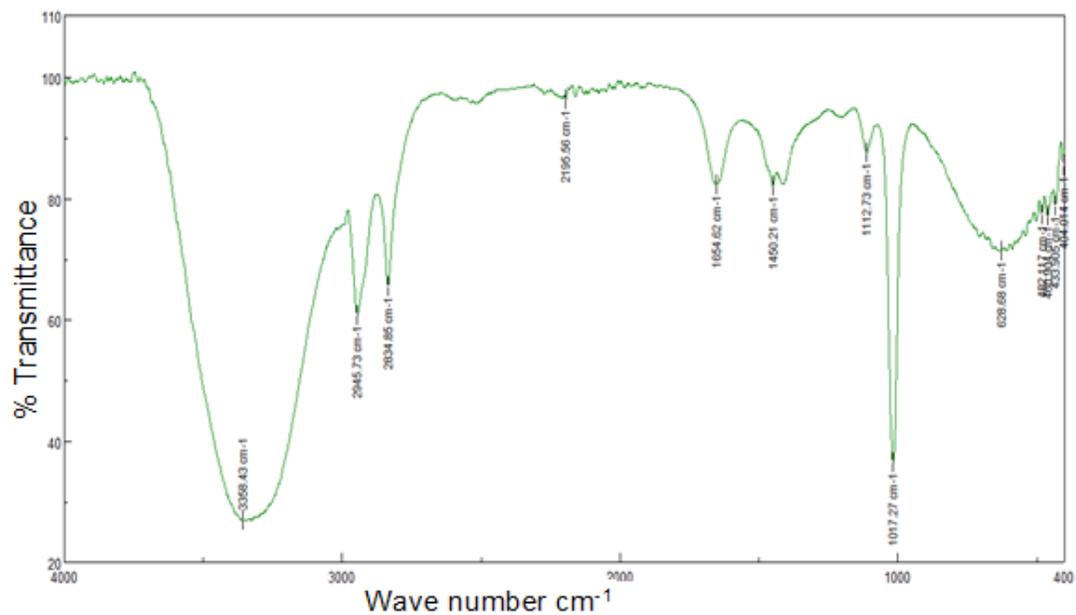


Figure 4.13 FT-IR spectra of methanol extract from *G. acerosa*.

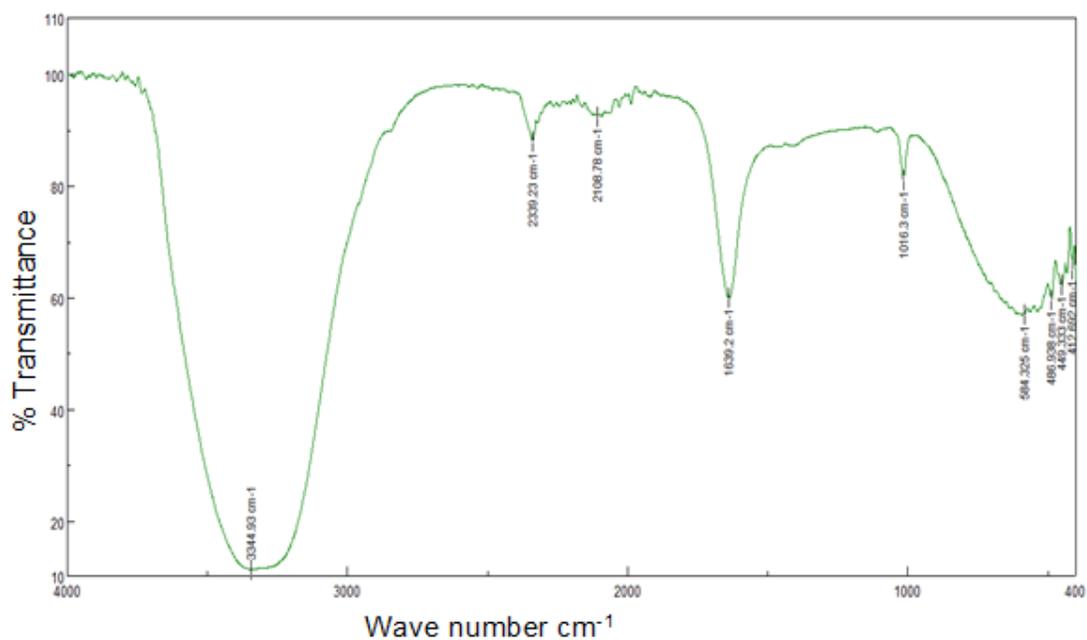


Figure 4.14 FT-IR spectra of water extract from *G. acerosa*.

These spectra are typical of 3 such different experiments.

Table 4.2 Identification of functional groups in the algal extracts by FT- IR analysis

	Component peaks	Hexane extract	DCM extract	Ethyl acetate extract	Ethanol extract	Methanol extract	Aqueous extract
1° and 2° Amines	N-H stretch	3333.36	3343		3358.43	3369.03	3344.93
Alkanes	C-H stretch	2973.7	2973.7	2977.55	2945.73		
Aldehydes	C-H stretch of C=O	2881.13	2885.95	2889.81	2834.85		
Aldehydes/ Ketones/ Proteins	C=O stretch	1660.41	1656.55		1654.62	1635.34	1639.2
Carboxylic acids/ glycosides	C=O stretch			1733.69			
Aliphatic amines (or) Esters and Ethers	C-N stretch (or) C-O stretch	1086.69	1086.69	1045.23	-	1040.41	1016.3
Terpenes	C=C stretch		1656.55			1654.62	

#### 4.3.5 Open column chromatography of ethyl acetate extract from *G.acerosa*

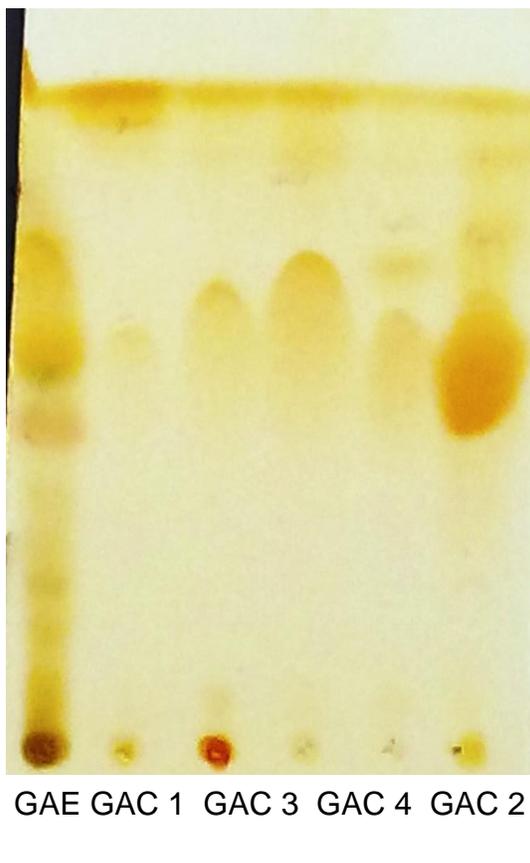


Figure 4.15 Separation of the ethyl acetate extract from *G.acerosa* (GAE) by column chromatography using petroleum ether and ethyl acetate. The compounds isolated were separated by thin layer chromatography using ethyl acetate and hexane. The chromatographic separation yielded four compounds. The compounds were labelled as GAC 1, GAC 2, GAC 3 and GAC 4.

Among these four compounds, GAC 1 and GAC 2 were single compounds and hence their structure was analyzed by NMR and GC-MS techniques.

### 4.3.6 Structural analysis of GAC 1

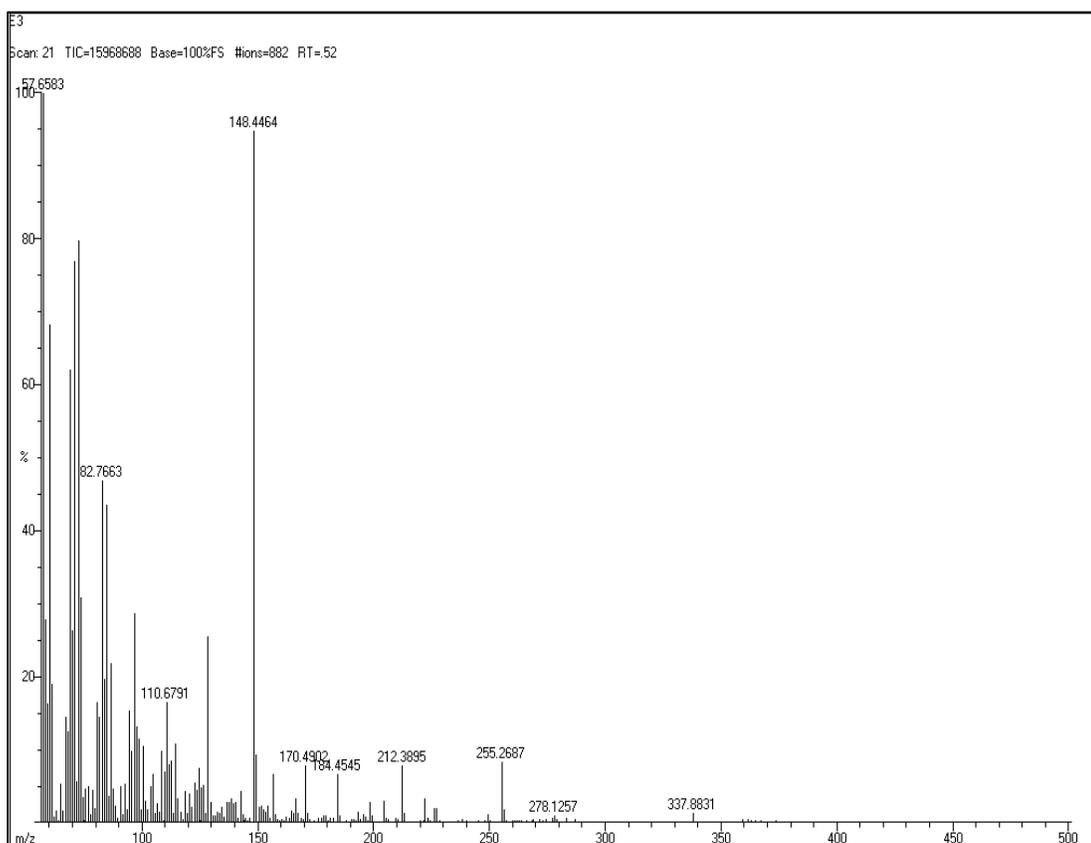


Figure 4.16 NMR spectra of GAC 1 isolated from *G.acerosa*.

The results revealed that GAC 1 as a White solid;  $^1\text{H}$  NMR:  $\delta$ 0.88-1.68 (m, 29H), 2.25 (m, 2H), 7.52 (m, 1H), 7.70 (m, 1H);  $^{13}\text{C}$  NMR:  $\delta$ 14.1, 22.6, 29.3, 29.6, 29.7, 31.9, 39.2, 174.02; IR (nujol) 2955, 2924, 2853, 1738, 1463, 1456. HRMS Result Obtained: 255.2687, theoretical 255.2562.

Based on the NMR data the structure of GAC 1 was determined.

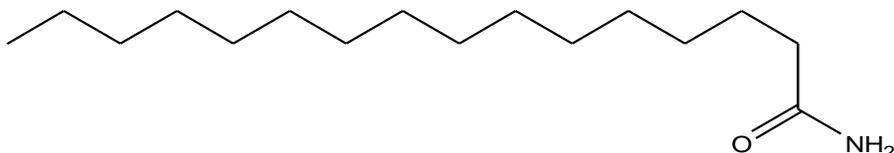


Figure 4.17 Structure of GAC 1 isolated from the ethyl acetate extract of *G.acerosa*.

Based on the structural analysis GAC 1 was identified as Palmitamide, IUPAC name: Hexadecanamide, Molecular Formula: C<sub>16</sub>H<sub>33</sub>NO, Molecular mass: 255.43.

#### 4.3.7 Structural analysis of GAC 2

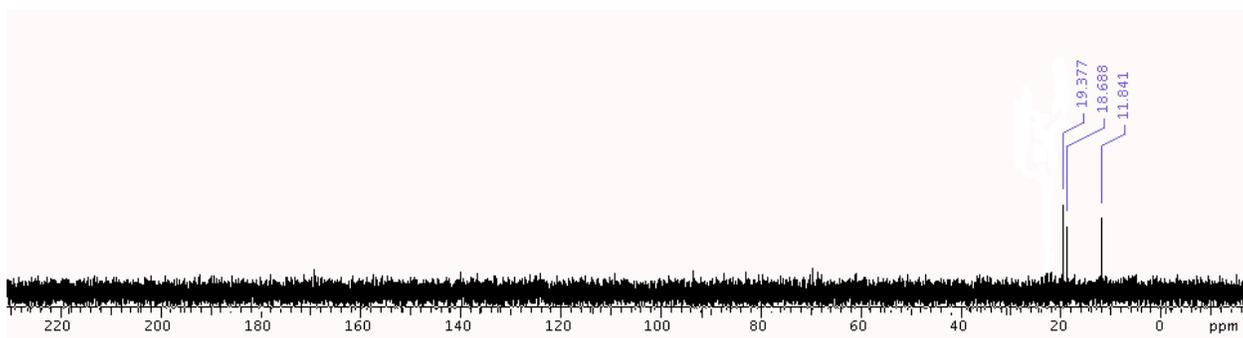


Figure 4.18 DEPT NMR OF CH<sub>3</sub> carbons in GAC 2

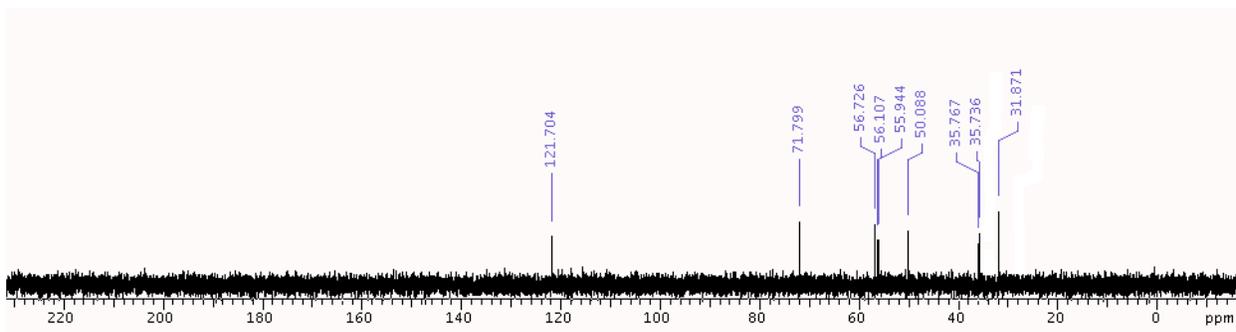


Figure 4.19 DEPT NMR of CH carbons in GAC 2

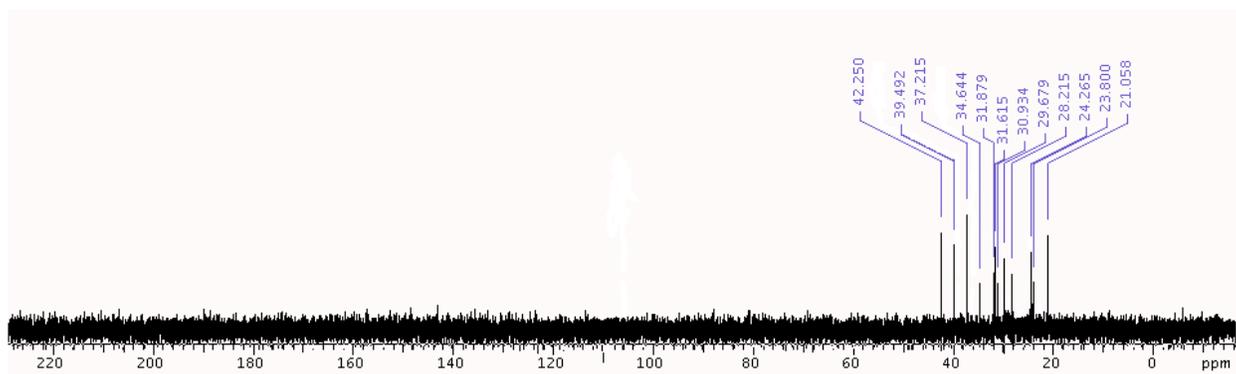


Figure 4.20 DEPT NMR of CH<sub>2</sub> carbons in GAC 2.

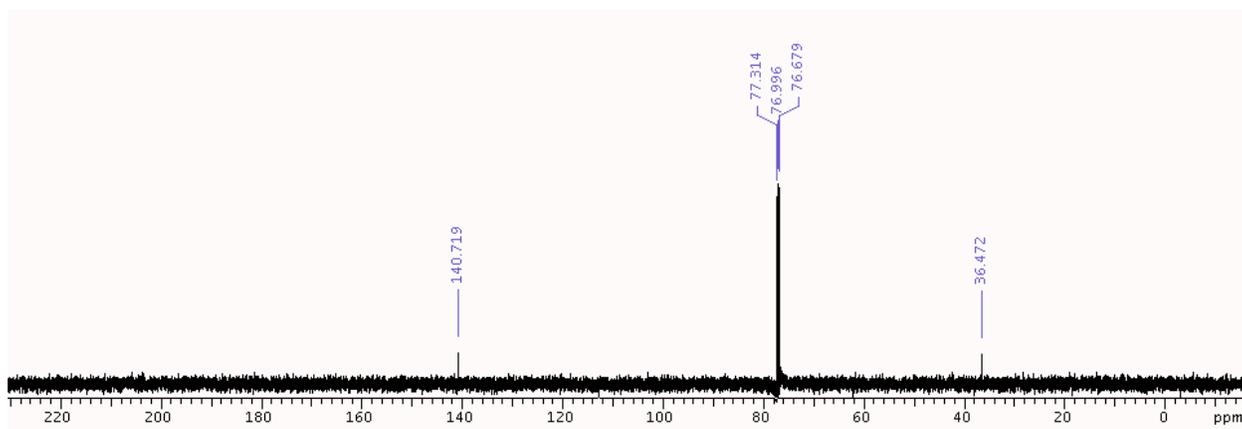


Figure 4.21 DEPT NMR of CH<sub>4</sub> carbons in GAC 2

Based on the NMR data, the structure of GAC 2 was determined. The compound was a White solid;  $^1\text{H}$  NMR:  $\delta$ 1.01 (m, 6H), 1.03-1.25 (m, 15H), 1.32-2.22 (m, 22H), 3.50-3.60 (m, 1H), 4.64-4.70 (s, 1H), 5.35 (m, 1H);  $^{13}\text{C}$  MR:  $\delta$ 11.8, 18.6, 19.3, 21.0, 23.8, 24.2, 28.2, 29.6, 30.9, 31.6, 31.8, 34.6, 35.7, 36.4, 37.2, 39.4, 42.2, 50.0, 55.9, 56.1, 56.7, 71.7, 121.7, 140.7.

Based on the structural analysis GAC 2 was identified as 2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14,15,16,17-tetradecahydro-10-methyl – 17- (7 methyloctan-2-yl)-1H-cyclopenta [ $\alpha$ ] phenanthren -3-ol of Molecular Formula  $\text{C}_{27}\text{H}_{46}\text{O}$  and Molecular mass 386.35.

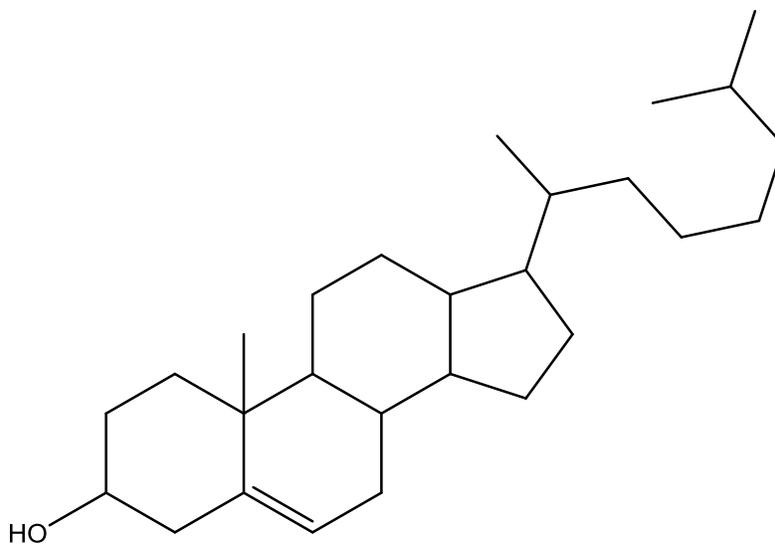


Figure 4.22 Structure of GAC 2 isolated from the ethyl acetate extract of *G.acerosa*.

## **4.4 Discussion**

### **4.4.1 Extraction of *G.acerosa***

Extraction is a major step in the analysis of plant samples because it enables the separation, characterization and identification of compounds from the sample. In the current study, *Gelidiella acerosa* was extracted sequentially at room temperature using solvents that differ in their polarity. This technique enabled the separation of different compounds in the alga based on their solubility without affecting their chemical, physical and biological properties. Previous studies have shown that sequential extraction is a favorable technique for isolation of plant ingredients in their native form [140]. The sequential extraction of the algae resulted in six different algal extracts which were qualitatively and quantitatively analyzed for the phytochemicals present in them.

### **4.4.2 Qualitative screening of phytochemicals in *G.acerosa***

Marine algae are the significant source of secondary metabolites which are chemically distinct and complex which enable the survival of the algae in the harsh conditions of the ocean. Phytochemical screening protocols are inexpensive methods that enable the identification of various phytochemicals in a mixture. The phytochemical analysis of the algal extracts identified different compounds in varying levels in each extract.

The results (Table 4.1) identified tannins, flavonoids, glycoside, alkaloids, oils, fats, protein, phytosterol carbohydrate, coumarins and terpenoids in the algal extracts. However, resins and saponins were not present in any of the extracts. These phytochemicals were present in varying levels in different extracts which may be due to the chemical nature of the phytochemicals and the solvent used for extraction. The ethyl acetate extract showed more phytochemicals which may be due to the midpolar nature of the solvent. The results of the current analysis

coincided with earlier observations in *G.acerosa* [13],[18] and thus confirming the algae as a rich source of bioactives.

#### **4.4.3 Quantitative screening of phytochemicals in *G.acerosa***

The major secondary metabolites that are targeted by the pharmacology, food and biomedical industries are the flavonoids and polyphenols. The quantitative analysis of the polyphenols in the extracts was carried out (Figures 4.2 and 4.3). The results revealed that the flavonoids and polyphenols were more concentrated in the ethyl acetate extract than the other extracts. The total polyphenol content was determined as 61.2 µg/100 mg and flavonoids as 13 µg/100 mg in the ethyl acetate extract. As these secondary metabolites are hydrophilic in nature, they get extracted in ethyl acetate. Flavonoids are the major phytochemicals widely distributed in plants. They have a characteristic flavone nucleus condensed with a benzene ring. They function as floral pigments, chemical messengers and inhibitors of the cell cycle. As flavonoids are phytochemicals, they cannot be synthesized in humans and animals in-situ.

Polyphenols are aromatic compounds ubiquitously found in plants and algae. Both polyphenols and flavonoids possess various biochemical properties including antioxidant [141], anti-inflammatory [142], antibacterial [143], anticancer [144] and hepatoprotective [145], activities. Similar studies have reported the occurrence of these phytochemicals in red algae [63],[64] but their characterization and applications are less explored.

#### **4.4.4 HPLC analysis of *G.acerosa* extracts**

High-performance liquid chromatography is an improved form of column chromatography used to separate a sample of its components under high pressure. The separation of the sample is based on the differences in the affinity of each component for the stationary and mobile phase [146]. The current study employed the reverse phase HPLC which utilizes a nonpolar stationary phase and a polar mobile phase for the separation of components in the crude extracts. The HPLC analysis revealed the presence of different phytochemicals in each extract. The

results are given in Figures 4.4- 4.8. The hexane, dichloromethane and water extracts revealed the presence of two to three compounds whereas the ethanol and methanol extracts exhibited five compounds. A maximum of eight compounds were present in the ethyl acetate extract. These results correlated with the preliminary phytochemical screening which showed that the phytochemicals were abundant in ethyl acetate extract.

#### **4.4.5 FT-IR analysis identified the functional groups in *G.acerosa***

FT-IR analysis generates an infrared spectrum that represents the absorption peaks which corresponds to the bonds between the atoms present in a sample. As each sample is unique, the spectrum generated is highly specific and enables the identification of the functional groups in a molecule/sample. The FT-IR spectra of the crude algal extracts (Figures 4.9 – 4.14) revealed the presence of various functional groups. The results are summarized in Table 4.2. The FT-IR analysis of hexane, dichloromethane, ethanol, methanol and aqueous extracts showed peaks at 3333.36, 3343, 3358.43, 3369.03 and 3344.93  $\text{cm}^{-1}$  which correspond to the N-H stretch indicating the presence of primary and secondary amines. The peaks at 2973.7, 2977.55 and 2945.73 in the hexane, dichloromethane, ethyl acetate and ethanol extracts correspond to the C-H stretch of alkanes. The IR peaks at 2881.13, 2885.95, 2889.81, 2834.85  $\text{cm}^{-1}$  in the hexane, dichloromethane, ethyl acetate and ethanol extracts correspond to the C-H stretch of C=O which indicated the presence of aldehydes groups.

Further, the peaks at 1660.41, 1656.55, 1654.62, 1635.34 and 1639.2  $\text{cm}^{-1}$  corresponded to the C=O stretch of ketones and proteins. The presence of C-N stretch of aliphatic amines and the C-O stretch of esters and ethers was identified by the IR peaks at 1086.69, 1045.23, 1040.41 and 1016  $\text{cm}^{-1}$  in hexane, dichloromethane, ethyl acetate, methanol and aqueous extracts. The ethyl acetate and ethanol extracts showed peaks at 1656.55 and 1654.62  $\text{cm}^{-1}$  which correspond to the C=C stretch of terpenes. The ethyl acetate extract showed a

sharp peak at 1733.69  $\text{cm}^{-1}$  that correspond to the C=O stretch of carboxylic acids, coumarins and glycosides. Further a single peak at 3654.44  $\text{cm}^{-1}$  reveals the presence of OH stretch of phenolics in the ethyl acetate extract. The outcomes of FT-IR analysis coincided with the phytochemical screening further confirming the presence of these active constituents in the algal extracts. The outcomes of the FT-IR analysis are in correlation with similar findings in marine algae [147], [148].

#### **4.4.6 Isolation and characterization of pure compounds from *G.acerosa***

##### **4.4.6.1 Open column chromatography of ethyl acetate extract (GAE)**

In the current study, the ethyl acetate extract demonstrated rich phenolic, flavonoid contents. Hence, the ethyl acetate extract was separated by column chromatography (Figure 4.15). As the crude extract is a mixture of various bioactives, the separation of these compounds was determined by the composition of the mobile phase used for separation. These compounds were concentrated in a rotary evaporator and the solvent traces was removed and analyzed further.

##### **4.4.6.2 NMR and GC-MS analysis of GACs**

The compounds isolated from GAE the GACs, were analyzed by GC-MS. Gas chromatogram-Mass spectrometer (GC-MS) is a hybrid analytical technique that utilizes the principles of both Gas Chromatography (GC) and Mass spectrometry (MS). The results of GC-MS revealed the mass of GAC 1 as 256.26 (Figures 4.16 and 4.17) and GAC 2 (Figures 4.18 – 4.22) as 386. The other two GACs were found to be mixtures. The algal compounds (GAC 1 and GAC 2) were subjected to *in silico* analysis.

## 4.5 Conclusion

The red alga *G. acerosa* was extracted and screened for various phytochemicals. The results of this chapter revealed that *G. acerosa* is a rich source of polyphenols, flavonoids. These compounds are good antioxidants and have anticancer capacity. Following this the ethyl acetate extract which showed high content of secondary metabolites were separated by column chromatography which yielded four compounds. These compounds were characterized and further subjected to *in silico*, *in vitro* and *in vivo* analysis for determining their therapeutic efficacy in cancer.