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ORIGINAL ARTICLE

Alpha-amylase Inhibition and Antioxidant Activity of Marine Green Algae and its Possible Role in Diabetes Management

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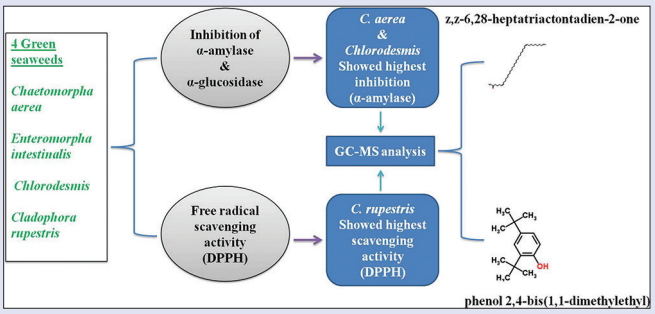
ABSTRACT

Aim: In the continuing search for safe and efficient antidiabetic drug, marine algae become important source which provide several compounds of immense therapeutic potential. Alpha-amylase, alpha-glucosidase inhibitors, and antioxidant compounds are known to manage diabetes and have received much attention recently. In the present study, four green algae (*Chaetomorpha aerea*, *Enteromorpha intestinalis*, *Chlorodesmis*, and *Cladophora rupestris*) were chosen to evaluate alpha-amylase, alpha-glucosidase inhibitory, and antioxidant activity *in vitro*. **Materials and Methods:** The phytochemical constituents of all the extracts were qualitatively determined. Antidiabetic activity was evaluated by inhibitory potential of extracts against alpha-amylase and alpha-glucosidase by spectrophotometric assays. Antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl, hydrogen peroxide (H_2O_2), and nitric oxide scavenging assay. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out to determine the major compound responsible for its antidiabetic action. **Results:** Among the various extracts screened, chloroform extract of *C. aerea* (IC_{50} – 408.9 µg/ml) and methanol extract of *Chlorodesmis* (IC_{50} – 147.6 µg/ml) showed effective inhibition against alpha-amylase. The extracts were also evaluated for alpha-glucosidase inhibition, and no observed activity was found. Methanol extract of *C. rupestris* showed notable free radical scavenging activity (IC_{50} – 666.3 µg/ml), followed by H_2O_2 (34%) and nitric oxide (49%). Further, chemical profiling by GC-MS revealed the presence of major bioactive compounds. Phenol, 2,4-bis (1,1-dimethylethyl) and z, z-6,28-heptatriactontadien-2-one were predominantly found in the methanol extract of *C. rupestris* and chloroform extract of *C. aerea*. **Conclusion:** Our results demonstrate that the selected algae exhibit notable alpha-amylase inhibition and antioxidant activity. Therefore, characterization of active compounds and its *in vivo* assays will be noteworthy.

Key words: 2,2-diphenyl-1-picrylhydrazyl, alpha-amylase, alpha-glucosidase, *Chaetomorpha aerea, Chlorodesmis, Cladophora rupestris*

SUMMARY

- Four green algae were chosen to evaluate alpha-amylase, alpha-glucosidase inhibitory, and antioxidant activity *in vitro*
- *C. aerea* and *Chlorodesmis* showed significant inhibition against alpha-amylase, and *C. rupestris* showed notable free radical scavenging activity
- No observed activity was found against alpha-glucosidase
- GC-MS analysis of the active extracts reveals the presence of major compounds which gives an insight on the antidiabetic and antioxidant activity of these algae.



Abbreviations used: DPPH: 2,2-diphenyl-1-picrylhydrazyl, BHT: Butylated hydroxytoluene, GC-MS: Gas chromatography-mass spectrometry.

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INTRODUCTION

Diabetes mellitus is a major endocrine disorder characterized by chronic hyperglycemia. It mainly affects the humans due to defects in insulin secretion or resistance.[1] Pancreatic alpha-amylase and alpha-glucosidase are key enzymes in the small intestine. These enzymes play a major role in the digestion of starch yielding glucose and maltose, leads to increased postprandial glucose levels.[2] Hence, reducing the starch digestion rate by inhibition of enzymes such as alpha-amylase and alpha-glucosidase is the best way for the management of diabetes.[3] Oxidative stress induced by free radical is also one of the causative factors for diabetes. Antioxidant compounds play a major role in scavenging free radicals and control of oxidative stress related diseases such as diabetes.[4]

Recently, marine algae and their bioproducts are getting greater attention toward the treatment of diabetes.[5] There is a great interest in screening marine algae for therapeutic drugs, as their ability to produce secondary metabolites has been extensively documented. Marine algae contain considerable amounts of polyphenols, which are effective antioxidants and have particular biological activities.[5] However, only a few studies have investigated the biological potential of Indian marine algae. In this study, four seaweeds (*Chaetomorpha aerea*, *Enteromorpha intestinalis*, *Chlorodesmis*, and *Cladophora rupestris*) belongs to the class of *Chlorophyta* were sequentially extracted using various solvents and screened for *in vitro* alpha-amylase, alpha-glucosidase, and various free radical scavenging assays (2,2-diphenyl-1-picrylhydrazyl [DPPH],

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hydrogen peroxide (H_2O_2), and nitric oxide). Preliminary phytochemical and gas chromatography-mass spectrometry (GC-MS) analyses were carried out to determine the probable inhibitory constituent.

MATERIALS AND METHODS

Algal samples

Fresh algae were collected from Parangipettai (Latitude 11°30'14"N; Longitude 79°45'8" E), Cuddalore (*C. aerea, E. Intestinalis,* and *Chlorodesmis*) and Kovalam (*C. rupestris*) (Latitude 12°79'25"N; Longitude 80°25'30"E) Chennai, Tamil Nadu, India during September 2012. The collected algae were cleaned, and holdfasts were removed, and they were shade dried, powdered, and stored under −20°C until extraction. The algal samples were identified and authenticated by Dr. P. Kaladharan, Principle Scientist and Scientist In Charge, Calicut Regional Centre of Central Marine Fisheries Research Institute.

Preparation of algal extracts

The dried algal samples (25 g) were milled and extracted using 250 ml of solvents such as chloroform, ethanol, and methanol for 24 h by using Soxhlet apparatus. Each filtrate was concentrated to dryness under reduced pressure using rotary evaporator (Super Fit, Rotavap, model, PBU-6, India). The samples were lyophilized by using freeze dryer (Lark, Penguin Classic Plus, India) and stored in a refrigerator at 2–8°C for use in subsequent experiments.

Preliminary phytochemical screening

Preliminary phytochemical screenings were carried out, as per the standard protocols of Harborne.[6]

In vitro alpha-amylase inhibition study

The alpha-amylase inhibitory activity was determined as described by Jayasri *et al.*[7] with minor modifications. Briefly, 250 µl of algal extracts with varying concentrations (125–500 µg/ml) and 250 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing alpha-amylase (porcine pancreatic alpha-amylase Sigma, St. Louis, USA) solution (0.5 mg/ml) were incubated for 10 min at 25°C. After preincubation, 250 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5 s intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 500 µl dinitrosalicylic acid (Sigma, St. Louis, USA) color reagent. The tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 5 ml of distilled water, and absorbance was measured at 540 nm in the ultraviolet (UV)-visible spectrophotometer (Systronics AU-2700, India). The experiments were performed in triplicates, and the alpha-amylase inhibitory activity was calculated as percentage inhibition, using the formula.

$$\% \text{ Inhibition} = \left(\left[\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}} \right] / \text{Abs}_{\text{control}} \right) \times 100$$

In vitro alpha-glucosidase inhibition study

The alpha-glucosidase inhibitory activity was determined as described by Jayasri *et al.*[7] with slight modifications. Briefly, 50 µl of algal extracts with varying concentrations (250–1000 µg/ml) and 100 µl of 0.1 M phosphate buffer (pH-6.9) containing alpha-glucosidase (SRL, India) solution (1.0 U/ml) were incubated in 96 well plates at 25°C for 10 min. After preincubation, 50 µl of 5 mm p-nitrophenyl alpha-D-glucopyranoside (Sigma, St. Louis, USA) in 0.1 M phosphate buffer (pH-6.9) was added to each well at 5 s intervals. The reaction mixture was then incubated at 25°C for 5 min. After incubation, absorbance readings were recorded at 405 nm using a plate reader (Bio-TEK, USA) and compared to the control which contained 50 µl of buffer solution in place of algal extract. The experiments were performed in triplicates, and alpha-glucosidase inhibitory activity was calculated as percentage inhibition. The percentage of enzyme inhibition was calculated as follows.

$$\% \text{ Inhibition} = \left(\left[\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}} \right] / \text{Abs}_{\text{control}} \right) \times 100$$

Free radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl)

Free radical scavenging activity was determined according to the method of Mensor *et al.*[8] Briefly, 500 µl of 0.3 mM alcoholic solution of DPPH (Himedia, India) was added to 2.5 ml of test samples at varying concentrations (250–1000 µg/ml). The samples were incubated in dark for 30 min, and absorbance was measured at 518 nm using UV-visible spectrophotometer (Systronics AU-2700, India). Synthetic antioxidant butylated hydroxytoluene (BHT) were used as positive control. The experiments were performed in triplicates, and scavenging activity was expressed as percentage inhibition, using the following formula.

$$\% \text{ Scavenging} = \left(\left[\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}} \right] / \text{Abs}_{\text{control}} \right) \times 100$$

Hydrogen peroxide radical scavenging activity

H_2O_2 radical scavenging activity was determined according to the method of Ruch *et al.*[9] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (50 mM, pH 7.4). Briefly, 1 ml of test samples of varying concentrations (250–1000 µg/ml) were added to the H_2O_2 solution and incubated for 10 min. Absorbance was measured at 230 nm against blank solution containing phosphate buffer without H_2O_2. Synthetic antioxidant ascorbic acid was used as positive control. The experiments were performed in triplicates, and scavenging activity was expressed as percentage scavenging, using the following formula.

$$\% \text{ Scavenging} = \left(\left[\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}} \right] / \text{Abs}_{\text{control}} \right) \times 100$$

Nitric oxide scavenging activity

The nitric oxide scavenging activity was determined according to the method of Marcocci *et al.*[10] Briefly, 2 ml of the test extracts with varying concentrations (250–1000 µg/ml) were incubated with 0.5 ml of sodium nitroprusside (5 mM) for 2 h at 27°C. Aliquot 1 ml of the incubated solution and mixed with 0.6 ml of Griess reagent (1.0 mL sulfanilic acid reagent [0.33%] in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthyl ethylenediamine dichloride [0.1%]). The absorbance was measured immediately at 550 nm, and synthetic antioxidant BHT was used as positive control. The experiments were performed in triplicates, and scavenging activity was expressed as percentage scavenging, using the following formula.

$$\% \text{ Scavenging} = \left(\left[\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}} \right] / \text{Abs}_{\text{control}} \right) \times 100$$

Gas chromatography-mass spectrometry analysis

GC-MS (Perkin Elmer, Clarus 680 GC coupled to a Clarus 600 MS) analysis was performed for the detection of major compounds present in the methanol extracts of *C. rupestris* and chloroform extracts of *C. aerea*. Column used in GC-MS was Elite-5MS (30.0 m, 0.25 mm ID, 250 µm df). Carrier gas used was helium at a constant flow rate of 1 ml/min. Electron Ionization mode with electron energy set at 70 eV was used. About 1 µl of extract diluted with methanol was injected to GC-MS, and the compounds were identified based on the molecular structure, molecular mass, and calculated fragment ratio of resolved spectra with that of mass spectra available from the library. Spectral data were interpreted using the database of National Institute Standard and Technology (NIST).

Statistical analysis

All the data reported were expressed as mean ± standard error of the mean. Statistical analysis was performed using two-way ANOVA. The values were considered to be significantly different when the $P < 0.0001$ compared to the baseline values. Graph-Pad Prism, version 5 (GraphPad software, Inc.) was used for statistical analysis.

RESULTS AND DISCUSSION

Seaweeds and their organic extracts pose wide array of bioactive compounds with potential health benefits.[11] Marine seaweeds that can reduce postprandial hyperglycemia by inhibiting enzymes such as alpha-amylase and alpha-glucosidase is found to be an effective strategy for the management of diabetes.[12] In this study, four seaweeds (*C. aerea*, *E. intestinalis*, *Chlorodesmis,* and *C. rupestris*) collected from the southern coast of India were screened for alpha-amylase, alpha-glucosidase inhibitory activity, and antioxidant potential *in vitro*. Among the four seaweeds, *C. aerea*, *E. Intestinalis,* and *C. rupestris* were previously reported for its antibacterial and antioxidant activity.[13,14] However, none of the seaweeds have been screened for alpha-amylase and alpha-glucosidase inhibitory activity to elucidate the hypoglycemic activity *in vitro*. Therefore, the present study validates alpha-amylase and alpha-glucosidase inhibitory activity *in vitro*.

Initially, the extracts were screened for the presence of various phytocompounds [Table 1]. The phytochemical analysis indicates the presence of phenols, flavonoids, alkaloids, lipids, glycosides, and tannins. The extracts were then assayed for alpha-amylase inhibitory activity. The results obtained were showed in Figures 1 and 2. Among the four seaweeds, *Chlorodesmis* methanol extract (72%) and *C. aerea* chloroform extract (86%) exhibited highest inhibition against alpha-amylase at a concentration of 500 µg/ml. The IC_{50} values were found to be 147.6 and 408.9 µg/ml, respectively. Moreover, methanol extract of *E. Intestinalis* (59%) and *C. rupestris* (14%) showed moderate to low alpha-amylase inhibitory activity. Previously, these green seaweeds (*C. aerea* and *C. rupestris*) were reported for its antibacterial activity[14,15] and, to the best of our knowledge, this is the first time report on alpha-amylase inhibitory activity of these four seaweeds. Our results also demonstrate that these extracts did not influence the activity of alpha-glucosidase enzyme. This study confers that the phytocompounds present in these green algal extracts may not be responsible for significant alpha-glucosidase inhibition.

Further, seaweeds were assayed for free radical scavenging activity and/or antioxidant potential of the selected extracts. The extracts were screened for three different free radical scavenging activity (DPPH, H_2O_2, and nitric oxide) [Figures 3, 4a and b]. The results indicate that methanol extract of *C. rupestris* showed the highest scavenging activity against DPPH (72%), H_2O_2 (34%), and nitric oxide (49%). Among them, *C. rupestris* showed highest scavenging activity against DPPH with an IC_{50} value of 666.3 µg/ml. Methanol extract of *C. aerea* also showed moderate to low activity against DPPH (32%), H_2O_2 (16%), and nitric oxide (24%). Methanol extracts of *Chlorodesmis* (20%) and *E. Intestinalis* (5%) showed very less scavenging ability against DPPH and no activity was found against H_2O_2 and nitric oxide at a concentration of 1000 µg/ml. Earlier studies report that *Cladophora glomerata*, and *E. Intestinalis*, collected from Konya, Turkey possess notable free radical scavenging activity. Due to high phenolic content, *C. glomerata* methanolic and aqueous extracts exhibit highest scavenging activity as compared to *E. Intestinalis*.[13] In accordance with previous studies, our result confers that *Cladophora* species exhibit potent antioxidant and/or free radical scavenging activity *in vitro*.

Chemical profiling by GC-MS, based on the retention time (RT) and molecular mass, revealed the presence major phytoconstituents.

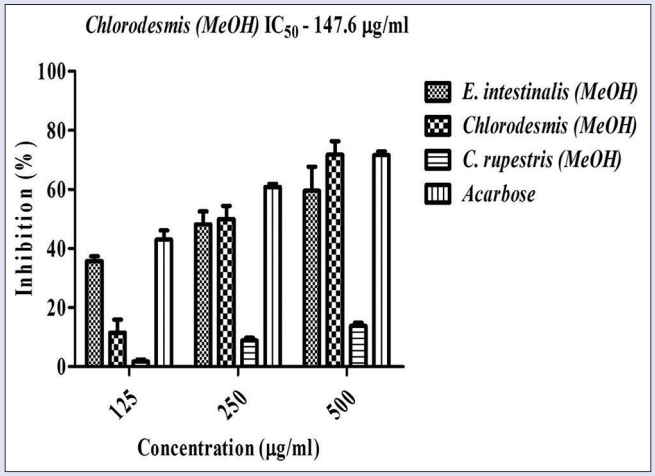

Figure 1: Effect of various seaweed extracts on alpha-amylase inhibition *in vitro*. Acarbose is used as positive control and absorbance was measured at 540 nm. Values are means ± standard deviation ($n = 3$), ***$P < 0.0001$ considered as significant

Table 1: Phytochemical screening of various extracts of the four seaweeds

Phytochemicals	Alkaloids	Phenols	Flavonoids	Proteins and amino acids	Lipids	Glycosides	Saponins	Tannins
Seaweed species: *Chaetomorpha aerea*								
Chloroform	−	+	+	−	+	−	−	−
Ethanol	+	+	−	+	−	−	−	−
Methanol	+	+	+	+	−	−	−	−
Seaweed species: *Enteromorpha intestinalis*								
Chloroform	+	+	−	+	+	−	−	−
Ethanol	−	+	+	+	−	+	−	−
Methanol	−	−	+	+	−	+	−	+
Seaweed species: *Chlorodesmis*								
Chloroform	+	+	+	−	+	−	−	−
Ethanol	−	−	+	+	+	−	−	−
Methanol	+	+		+		+	−	+
Seaweed species: *Cladophora rupestris*								
Chloroform	+	+	+	−	−	−	−	−
Ethanol	−	+	+	+	−	−	−	−
Methanol	+	+		+		−	−	+

+: Present; −: Absent

The methanol extract of *C. rupestris* and chloroform extract of *C. aerea* which showed the highest antioxidant and alpha-amylase inhibitory activity were analyzed by GC-MS and compared with the RT and mass spectra available in the data library of NIST. The chromatograms obtained were showed in Figure 5. Methanol extract of *C. rupestris* showed the presence of phenol 2,4-bis (1,1-dimethylethyl) and z,z-6,28-heptatriactontadien-2-one. This is early stated that the highest scavenging activity is proportional to its phenolic content.[16] Phenol 2,4-bis (1,1-dimethylethyl) present in the methanol extract of *C. rupestris* has previously isolated from a herb *Plumbago zeylanica* and reported for its antioxidant activity.[17] z,z-6,28-heptatriactontadien-2-one is another major compound identified in both methanol extract of *C. rupestris* and chloroform extract of *C. aerea*. However, no known antidiabetic compound was identified by GC-MS.

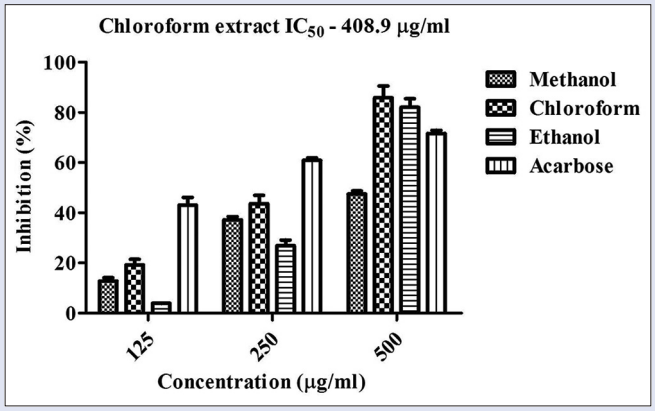

Figure 2: *In vitro* alpha-amylase inhibitory activity of *Chaetomorpha aerea*. Acarbose is used as positive control and absorbance was measured at 540 nm. Values are means ± standard deviation ($n = 3$), ***$P < 0.0001$ considered as significant

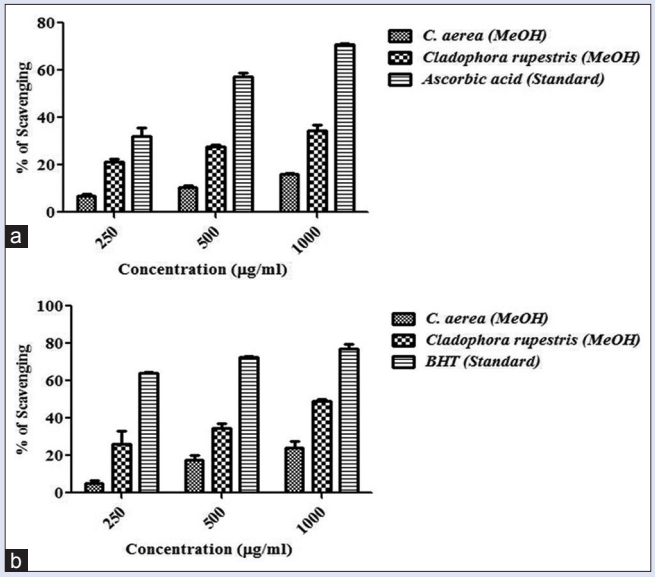

Figure 4: (a) Hydrogen peroxide radical scavenging activity of seaweed extracts. Ascorbic acid is used as standard and absorbance was measured at 230 nm. (b) Nitric oxide scavenging activity of seaweed extracts. Butylated hydroxytoluene is used as positive control and absorbance was measured at 550 nm. Values are means ± standard deviation ($n = 3$), ***$P < 0.0001$ considered as significant

CONCLUSION

Hence, it is anticipated that the tested algae produces a novel antidiabetic compound and further advancement of sophisticated spectroscopic techniques are required for the identification. The isolation and characterization of active compound will only give a conclusive data about the antidiabetic and antioxidant activity of these respective seaweeds and are underway.

Acknowledgment

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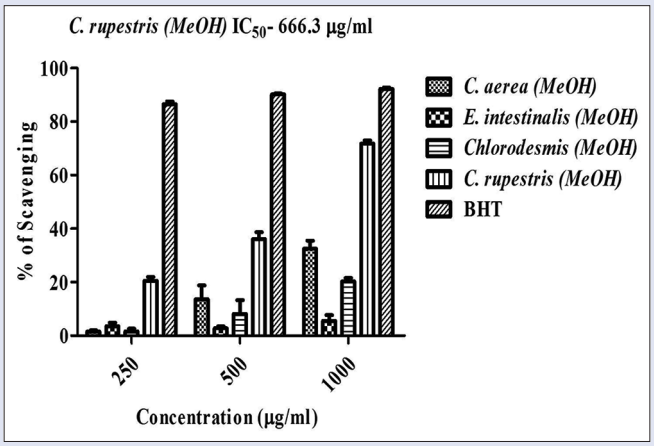

Figure 3: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of seaweed extracts. Butylated hydroxytoluene is used as positive control and absorbance was measured at 517 nm. Values are means ± standard deviation ($n = 3$), ***$P < 0.0001$ considered as significant

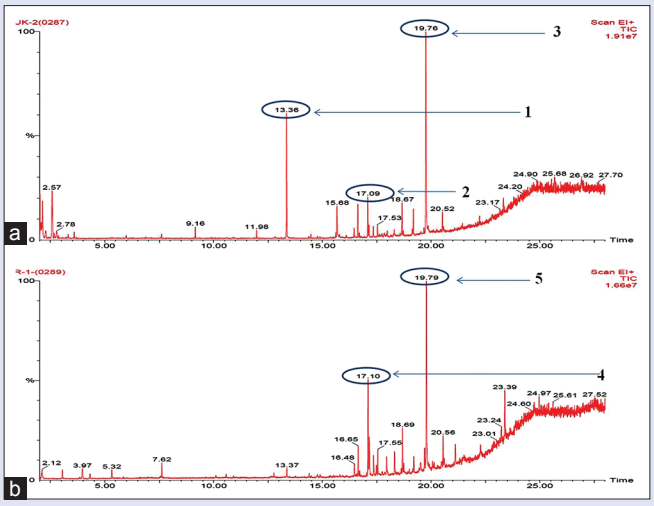

Figure 5: (a) Gas chromatogram of methanol extract of *Cladophora rupestris* (1) phenol, 2,4-bis (1,1-dimethylethyl), (2) z, z-6,28-heptatriactontadien-2-one, (3) z, z-6,28-heptatriactontadien-2-one. (b) Gas chromatogram of chloroform extract of *Chaetomorpha aerea*, (4) z, z-6,28-heptatriactontadien-2-one, (5) z, z-6,28-heptatriactontadien-2-one

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Conflicts of interest

There are no conflicts of interest.

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