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Fractionationation of purified terpenoids from red algae *Hypnea musciformis* (Wulfen) J.V. Lamouroux. and *Kappaphycus alvarezii* (Doty) Doty Ex P.C. Silva. By Gc: Ms analysis

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Abstract
Terpenoids represent the earliest biomolecules, having been reported from earth sediments from 2.5 billion years ago. Most common phytochemical in the plants that shows high diversity. This feature in turn reflects their unique biological activities and there by forms the resource for traditional human exploitation. In this study the terpenes composition from the red algae *Hypnea musciformis* and *Kappaphycus alvarezii* were identified and estimated by using Gas Chromatography- Mass Spectrum. The crude methanolic extract of *Hypnea musciformis* and *Kappaphycus alvarezii* were purified by Column Chromatography. The analysis of the purified fraction of *Hypnea musciformis* and *Kappaphycus alvarezii* revealed the presence of 8 major peaks and 12 major peaks of terpenoids respectively. Significant differences were found in the terpenes composition in both algae. The total number of terpenoid composition was found to be highest in *Kappaphycus alvarezii.* The main terpene component was the Hexadecanoic acid, methyl ester in *Hypnea musciformis* where as in β-amyrin in *Kappaphycus alvarezii* as per the concentration percentage.

Keywords: terpenoids, red algae, methanol extract, gas chromatography- mass spectrum, column chromatography

1. Introduction
Algal biomass and algae-derived phytochemicals have been attributed to a wide range of potential applications for human nutrition and health products. Among marine organisms, seaweed is a promising alternate for novel drug production because it is relatively renewable by aqua culture. These sea weeds were rich source of bioactive compounds which were structurally different with valuable pharmaceutical potentials [1]. Natural products were derived from various plant groups such as terrestrial plants, microorganisms, vertebrates, invertebrates, marine organisms, lower plant groups and marine algae. Bioproducts are an important source of new raw drugs and chemical entities for further development of lead molecules [2]. Marine algae contain more than 60 trace elements in a concentration much higher than terrestrial plants. Marine macroalgae produce a diverse array of secondary metabolites such as terpenes, sterols, polyphenols, acetogenins and others which were characterized by a broad spectrum of biological activities [3]. Many industrial products such as agar, algin and carrageenan were extracted from seaweeds and also, they were consumed in Asian countries like China, Japan, Korea as food. Currently, there is an increased interest in phyto products with valuable medicinal properties, such as terpenoids. Terpenes are a large class of naturally-occurring organic compounds; they are also known as isoprenes, as their structure is based on repeating isoprene units. They constitute one of the largest group of phytochemicals. Many pharmacological studies on algae have reported that the chemical compounds derived from marine algae have biological potentialities in terms of anti-inflammatory, anticancer, anti-HIV, antimutagenic and free radicals scavengers [4, 5].
Nowadays, there is an increased demand in natural resources such as terpenoids in terms of their valuable medicinal properties [6]. Interestingly, volatile constituents, terpenoids and steroids are found in considerable amount in red algal seaweeds. These detected compounds were valuable antitumor agents [7]. Some of the terpenes such as sesterterpenes and triterpenes from marine sponges were reported to have pharmacological significance as they were used as most potent drugs against various life-threatening diseases [8]. Recently, it was reported that diterpenoids and sesquiterpenoids from marine algae have cytotoxic, antiviral and algicidal activities [9]. There were also reports showing the complementary cardioprotective effects of flavonoid metabolites and terpenoid constituents of *Ginkgo biloba* extract [10]. Algal terpenes

constitute a wide and well-documented group of marine natural products with structures differing from their terrestrial plant biosynthetic analogues. Biologically and ecologically relevant terpenoids were isolated from brown seaweeds such as Sargassaceae and Cystoseiraceae families. The members of Phaeophyceae such as Dictyotales produce a large array of bioactive secondary metabolites having a broad defensive action. The single genus *Dictyota* has reported with a wealth of terpenes. [11] reported the presence of 19 terpenes in *Padina pavonica* and 20 terpenes in *Hormophysa triquetra*, in addition to 5 sterols recorded from both the species. Triterpenic acids exhibit various biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, cytotoxic, and cardiovascular effects [12].

2. Materials and methods

The marine red algae seaweeds such as *Hypnea musciformis*, *Kappaphycus alvarezii*, were collected on June 2017, from the Mandapam coast (latitude 9˚ 17' N, longitude79˚ 22' E), Gulf of Mannar. Identity was confirmed by referring algal flora and substantiated by matching with samples of CMFRI, Kochi. The samples were then ground and the crude extract was prepared by Soxhlet extraction method. About 20 g of powdered material was uniformly packed into a thimble and extracted with 250 ml of methanol. The process of extraction has to be continued for 24 h. Further, the extract was poured into a beaker and kept on a hot plate and heated at 30-40ºC till all the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC till future use. Fractionation of the crude methanolic algal extract was done by silica gel Column chromatography using petroleum ether: ethyl acetate as solvent combinations. The eluted fractions were then subjected to TLC and further analysed by GC-MS. For thin-layer chromatography (TLC), the samples were loaded on a pre-coated TLC aluminium sheets Si60 F254 (20 × 10 cm) and eluted using petroleum ether and ethyl acetate as solvents and adapted in a TLC development chamber. TLC identification was performed by treating with a solution of sulfuric acid (1 M) followed by gradual heating on a heating plate.

2.1 GC-MS analysis

For GC-MS analysis, the sample was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 ìM film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Helium was used as carrier gas with a flow rate of 1 mL/min; the injector was operated at 200 °C and column oven temperature was programmed as 50 - 250 °C at a rate of 10 °C / min injection mode. The following MS conditions were maintained i.e., ionization voltage of 70 eV; ion source temperature of 250 °C; interface temperature of 250 °C; mass range of 50 - 600 mass units. A chromatogram was obtained and the mass spectrum of the unknown components were compared with the spectrum of the known components available in the NIST library.

3. Results and Discussion

The crude methanolic extract of *Hypnea musciformis* and *Kappaphycus alvarezii* were purified by column chromatography. Each fraction was eluted using petroleum ether and ethyl acetatae as solvent combinations. The fraction eluted using 95:5 solvent combination of *H. musciformis* and 50:50 solvent combinations of *K. alvarezii* showed significant amount of terpenoids as detected by using GC-MS spectra technique. Parallelly, the fractions eluted by column chromatography were subjected to thin layer chromatography for confirming the presence of terpenoids. Retention time and the relative abundance of each compound were recognized. The analysis of the 95:5 purified fraction of *H. musciformis* revealed the presence of 8 major peaks of terpenoids (Fig:1; Table 1) compatible with their fragmentation patterns. The 50:50 purified fraction of *K. alvarezii* showed the presence of 12 major peaks. (Fig: 2; Table 1). Table 1 summarized the terpene components, retention time (Rt), molecular weight (M.W.), molecular formula (M.f) and concentration percentage of *H. musciformis* and *K. alvarezii.*

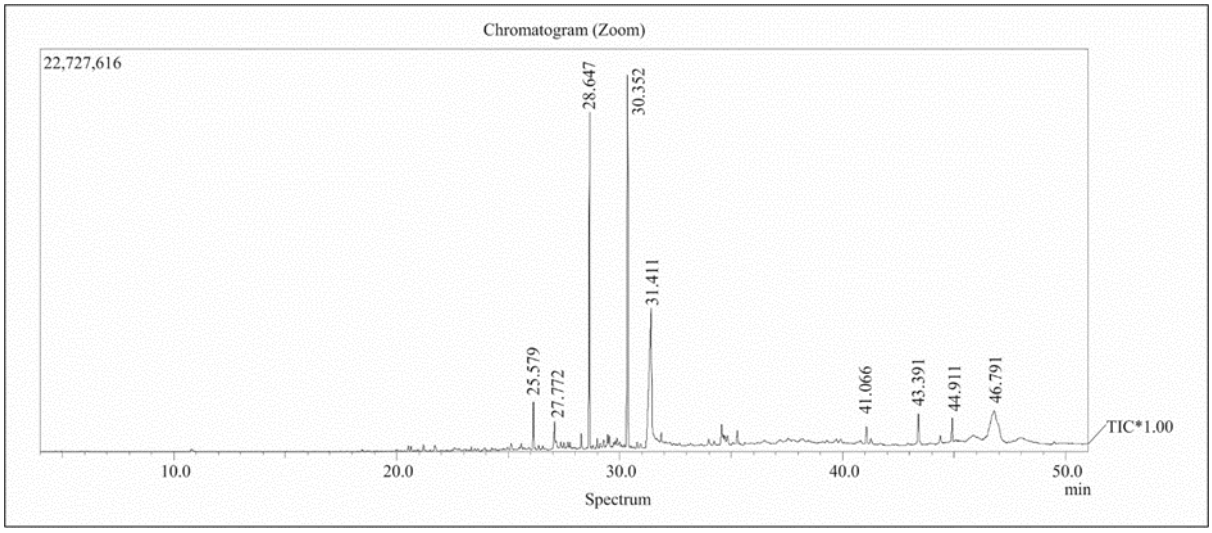

Fig 1: GC- MS spectra showing terpenes composition of *Hypnea musciformis*

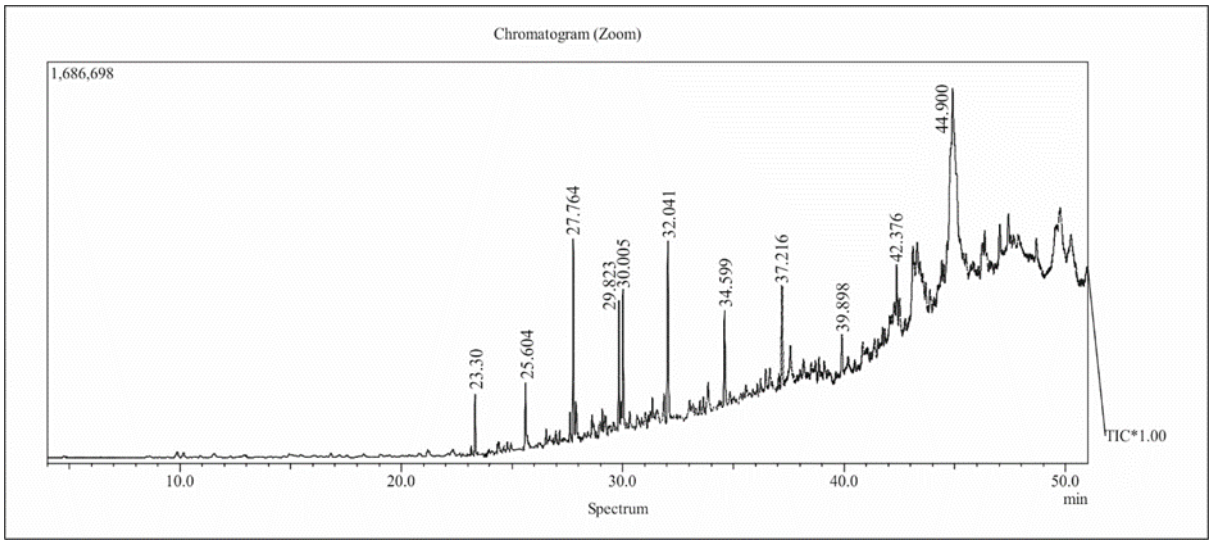

Fig 2: GC-MS spectra showing terpenes composition of *Kappaphycus alvarezii*

Table 1: Terpene components identified in *Hypnea musciformis* and *Kappaphycus alvarezii* by GC-MS.

Compound Name	Molecular weight	Molecular formula	*H.musciformis* Area. %	RT	*K.alvarezii* Area. %	RT
Hexadecane	$C_{16}H_{34}$	226	-	-	1.64	23.330
Eicosane	$C_{20}H_{42}$	282	0.54	25.579	5.91	32.041
Hepta decane	$C_{16}H_{34}$	226	-	-	2.43	25.643
Octadecane	$C_{18}H_{38}$	254	_	_	5.30	27.764,
Heneicosane	$C_{21}H_{44}$	296	0.27	27.772	3.07, 4.48, 1.85,	34.599, 37.216, 39.898,
Tricosane	$C_{23}H_{48}$	324	_	_	1.35, 2.32	27.880, 42.376
2- Pentadecanone	$C_{14}H_{28}O_2$	228	18.49	28.647	_	_
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	21.82	30.352	3.27, 3.72	29.823, 30.005
n-Hexadedcanoic acid	$C_{16}H_{32}O_2$	256	21.30	31.411	_	_
Hexadecanoic acid,ethyl ester	$C_{18}H_{36}O_2$	284	0.50	43.391	_	_
Beta amyrin	$C_{30}H_{50}O$	426	_	_	47.17	44.900
Heptadecanoic acid,methyl ester	$C_{18}H_{36}O_2$	284	0.13	44.911	_	-
11- octadecanoic acid,metyl ester	$C_{18}H_{36}O_2$	284	0.33	46.791	_	_

Highest number of terpenoids were observed in *K. alvarezii* as compared to *H. musciformis.* There was a significant difference in terpenes content between the studied species. It was noticed that some of the terpene compounds in the studied seaweeds showed marginal differences in their retention time. This may be attributed by the sequence of increasing polarity of the separated compounds that was detected in GC-mass detector of each species. Some compounds such as Eicosane, Heneicosane, Hexadecanoic acid, methyl ester was common among both species and they also showed difference in their retention time. The terpene components in *H. musciformis* were separated at retention time interval from 25.57 to 46.79 min as shown in Figure 1. Terpenes of *Kappaphycus alvarezii* detected at the retention time interval ranging from 23.30 to 44.9 min (Fig.2). The main terpene component was the Hexadecanoic acid, methyl ester in *H. musciformis* followed by n-Hexadedcanoic acid, 2-Pentadecanone where as in *Kappaphycus alvarezii* Beta amyrin, a triterpenoid followed by Tricosane and Heneicosane as per the concentration percentage recorded.

The identified compounds possess many biological properties. Three bioactive components such as alpha-amyrin, beta-amyrin and lupeol from *Dandelion* root extract have been reported as anticancerous compounds [13]. The mechanism of anti-inflammatory action of β-amyrin isolated from the leaves of *Costus igneus* was reported [14]. Alpha-and beta-amyrins were two promising bioactive natural products (pentacyclic triterpenes) that have been shown to exhibit various pharmacological actitivies such as anti-inflammatory, antihyperglycemic, antioxidant, gastroprotective, hepatoprotective, and hypolipidemic effects at nontoxic concentrations. n-hexadecanoic acid - palmitic acid (R/T 17.25) was reported to possess antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha is a reductase inhibitor. Nonadecane, Eicosane, Heneicosane and Tricosane belong to the higher alkane group that were useful in the production of biopesticides, candles, aviation fuels, lubricating oil and serve as anti- corrosive agents. Eicosane, one of the compounds in paraffin wax, was used in making candles while Tricosane was long have been used as bio-pesticides, aviation of fuel and lubricating oil [15]. It was reported that GC-MS analysis of hexane extract of *Oedogonium* strain (KU680468 (SSU/18S rDNA); KU680573 Rbcl) revealed the existence of Eicosane, 1-Nonadecene, Tricosane, Heneicosane and Nona hexacontanoic acid [16]. Hexadecanoic acid was reported earlier as a component in the alcohol extract of the leaves from *Kigelia pinnata* and *Melissa officinalis* for their medicinal potentialities [17]. The GC–MS profile of *Phormidium fragile* showed the presence of 27 compounds,

which includes 8-octadecenoic acid methyl ester (31.30%) followed by 9-hexadecanoic acid methyl ester (Z) (14.83%), Hexadecanoic acid methyl ester (13.78%) and 24 compounds were distributed in different quantities [18, 19] reported the therapeutic values of *Euporbia longan* leaves mainly because of n- hexadecanooic acid and octadecanoic acid. Additionally minor proportions of 1, 2-benzene dicarboxylic acid-bis(2-methylpropyl) ester (1.93 %), hexadecanoic acid methyl ester (0.98 %), 9-octadecenoic acid (Z)-methyl ester (0.39 %) and eicosane (0.53 %) were also recognized in the bio-oil and this appears as principle acid in the biodiesel suggesting the transformation of bio-oil to biodiesel [20]. reported the main components of 9, 12 octadecadienoic acid, Octadec- 9enoic acid and 9,12-actadecadienoic acid from *Croton tiglium* seeds. These compounds were found to have potential antioxidant and anticancer activities. GC-MS analysis of ethyl acetate extract of *Goniothalamus umbrosus* revealed the presence of n-hexadecanoic acid. Some compounds such as octadecadienoic acid, Eicosane, Dotriacontane, Docosane, Octatriacontyl, Heptacosane and Tetracosane. have been already proposed to have a certain antimicrobial activity [21, 22]. confirmed several types of diterpenoids and sesquiterpenoids have been found to be the main secondary metabolites of the species belonging to Dictyotales. Heptadecane was observed to be in appreciable amount in *Hypnea cornuta* (26.38%) as mentioned by [23]. 2-pentadecanone was reported to exhibit insect repellence [24]. Terpenoids have multiple functions like inhibition of tumour proliferation via apoptosis triggered activity, and cation channel regulation. Similarly, terpenoids, forms intermediates in cholesterol biosynthesis i.e., regulate the marker enzyme 3-hydroxy-3- methyl glutaryl-coenzyme A reductase. Such dietary terpenoids are unique for the management of diseases like cancers or cardiovascular disorders [24]. Phytanates, a branched terpenoid fraction of fatty acid constituent of diet such as milk, butter, cheese, meat from cows, sheep, and some fish and fish oils. This may also be produced via the conversion of dietary phytol in the body. These molecules have been documented to activate PPARγ and the retinoid-X-receptor (RXR) i.e., the differentiation is induced in white and brown adipocytes [25]. Further, the molecules trigger PPARα to regulate metabolism of lipids in certain types of cell. Indeed, a phytol-enriched food will enhance the plasma and hepatic phytanic acid levels and induced the mRNA expression of PPARα target genes involved in peroxisomal and mitochondrial β- oxidation and metabolism of fatty acids. Thus, the present pool of terpenoids noticed in the red algae suggests their possibilities as future natural source of medicines.

4. Conclusion

The red algae *Hypnea musciformis* and *Kappaphycus alvarezii*were known for their nutraceutical potentials. Significant differences were found in the amounts and types of the terpenes among the two algae. The analysis by GC-MS revealed that *Hypnea musciformis* contain 8 terpene components whereas in *Kappaphycus alvarezii* 11 terpenes. The total concentration of terpenes in *K. alvarezii* recorded the highest percentage than those of *H. muscifromis*. The main terpene component was the Hexadecanoic acid, methyl ester in *H. musciformis* and in *K. alvarezii* was the betaamyrin. The pharmaceutical significance of terpenoids should be further analysed.

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