International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Identification of Substances from the Extracts of Sea Weeds Grateloupia Filicina and Gracelaria Corticata from Visakhapatnam Coast Using Chromatography Techniques

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Abstract

Sea weeds belonging to the Rhodophyceae (red algae) are an important source of bio active substances of medicinal value. Many of these substances are used in the drug development to treat diseases caused by bacterial and fungal infections, inflammation, pain, arthritis, as well as cancerous diseases. The paper chromatographic studies reveals that Grateloupiafilicinacontained photosynthetic pigment carotene and Gracelariacorticatacontained the amino acid leucine. The thin layer chromatographic studies indicates that Grateloupiafilicinacontained amino acid phenylalanine and Gracelariacorticatacontains Leucine. All the three substances having pharmaceutical importance

Key words: Grateloupiafilicina, Gracelariacorticata, Chromatography, Retention time, Carotene, Luciene, Phenylalanine.

Introduction:

Marine thalloid algae are rich in bioactive substances that could be used as functional ingredients in the development of drugs to treat diseases of humans as well as animals. The people of Asian countries use seaweeds as food and also as medicinal herbs since 5,000 years carotenoids and catechins, phlorotannins. The medicinal use of macroalgae goes back at least 5,000 years to ancient china. The people of china considered the seaweeds as a source of bioactive substances as they are able to produce different types of secondary metabolites participating in various biological activities of the consumers (Liu et al., 2012). As the researchers revealed the various biological activities exhibited by the marine algal originated substances, the global Pharmaceutical industry is now giving extensive efforts to extract various substances of medicinal value from seaweeds. Phaeophyceae, Rhodophyceae contain maximum number of species having medicinal uses.

Many species of seaweeds possess bioactive substances which inhibit the growth of some of the bacterial pathogens, (Rajasulochana et al., 2009). Seaweeds provide nutritious food of low calorific

value and rich in vitamins, minerals, proteins, polysaccharides, steroids and dietary fibre (Govindaswamy et al., 2012).

Many Marine thalloid algal species produced different types of substances as secondary metabolites which are antimicrobial, anthelmintic and cytotoxic (Newman et al., 2003). The above bioactive substances included polysaccharide, cyclic peptides, lipids, glycerols, alkaloids, diterpenoids, phlorotannins, quinines, polyketides and sterols (Cabrita et al., 2010, kayalvizhi et al., 2012).

Since past two decades seaweeds were considered as commercially important marine renewable resource for extracting substances in the manufacturing of noval drugs to treat microbial infections, inflammations and even fatal diseases such as cancer (Elena et al., 2003). Seaweeds are capable of synthesizing bioactive substances as secondary metabolites, (Del et al., 2001). There are many compounds derived from seaweeds which functions as antibacterial, antiviral, antifungal (Nair et al., 2007) and anticoagulant (Athukorala et al., 2006). They also contain antioxidant molecules such as ascorbate and glutathione which are highly reactive in fresh condition. Carotenoids, catechins and phlorotannins are secreted as secondary metabolites (Yuan et al., 2005). The polyphenol and phlorotannin from brown seaweeds function as antioxidants and antibacterial compounds (Kuda et al., 2007).

This study has been undrertaken to isolate and evaluate prospective bioactive substances from marine thalloid algaeGrateloupiafilicinaand Gracilariacorticata from the rocky shore of Visakhapatnam. An attempt has been made to identify the substance present in the above two species of seaweeds using chromatographic techniques for further synthesis of novel drugs.

Materials and methods

Collection of samples and Identification

Grateloupiafilicinaand Gracilariacorticatasamples were collected from rocky shore behind VUDA park of the Visakhapatnam on 21.02.2020 during low tide time at 1:44 pm. The collection site and the thallus of the two genera of algae were shown in the plate 1.

Preparation of samples for extraction

The collected seaweeds samples were sorted out to remove other types of algal fronds from the collection. The sorted samples were cleaned and the necrotic parts were removed. To remove sand, unwanted matter and any associated debris, the seaweeds were washed in running tap water. Then the sea weed is kept in 4% hydrogen peroxide in a 1 L. capacity glass finger bowl for 20 min to remove all epiphytes and bacteria attached to the blades of the thallus. The thallus, then removed from the finger bowl, with a clean forceps, transferred into a 5 L. Borosil glass tray, washed in running tap water gently, 3 times, each time 5 minutes to remove the traces of hydrogen peroxide. Finally the thallus is washed in distilled water. Then the sea weed was cut into pieces and dried in direct sunlight for 3 hours in a day in open air (Mean temperature 31˚C) until the fragments of the sea weed thalli become dried and easily made into small pieces when smashed by hand. After completely drying, the two species of dried seaweeds were ground to a fine powder in a mortar and pestle manually.

Preparation of seaweed extracts

7 grams of each species of seaweed powder was soaked separately in 150 ml of 10% TCA (Trichloroacetic acid) solvent for 18 hours in 250 ml glass beakers, followed by homogenization at interval of 2 hours a day using homogenizer. After complete homogenization the slurry was transferred in to 10 ml polythene centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes at 4^0C temperature.

The supernatant thus obtained was separated and subsequently used for conducting paper chromatography and thin layer chromatography for both species (Keiser, 2013). The chromatograms thus obtained were depicted in the results section (plates 2 and 3). The R_f values were calculated for paper and thin layer chromatograms obtained for Grateloupiafilicina(sample-A) and Gracilariacorticata (sample - B) were compared with standard chromatograms (Keiser, 2013 and Schweitzer, 2014).

Paper chromatography

Preparation of solvent system and experimental procedure

A rectangular piece of Whatman No.1 chromatography paper is cut with the help of scissor (Whatman No.1, 18 cm long and 8 cm breadth) without touching the paper to avoid unwanted color development on the paper. With the help of micro pipette known volume (1.0µl) of sample is placed on the chromatography paper each spot at a distance of 1.5 cm, on the base line drawn about 0.8 cm from the lower end of the chromatography paper. Care was taken to confine the extract to a spot of 0.5 cm in diameter. Two spots, one with the extract of Grateloupiafilicina(Sample – A) and another spot with the extract of Gracilariacorticata (Sample – B) were deposited on the chromatography paper by applying 1.0 µl portions. After drying, the paper was hanged from steel rod present on the top of the rectangular glass chromatography chamber. The free end of the chromatography paper was allowed to sit in 70:30 ratio 96% ethanol and water as a solvent system present in the steel trough near the bottom of the chromatography chamber. Care was taken that the solvent do not touch the base line where spots of the extract present. It would take approximately 4-8 hours run time for the mobilization of molecules on the chromatography paper about 3/4 distance of the paper from the base line (Keiser, 2013).

In this study with ethanol and water as a solvent system, it was observed that within 2 hours the solvent travelled to a distance of 10 cm which is maximum. The strip of paper was then removed. A line will be drawn on the paper indicating the distance that the solvent travelled through the paper. The paper was dried at room temperature and Ninhydrin was sprayed uniformly using a sprayer on the paper. Then the chromatography paper was again air dried and was just warmed up for 5 minutes. The obtained chromatogram paper was depicted in the figure1.

Analysis of paper chromatogram:

The colored spots formed on the chromatography paper were first identified. The maximum distance that the molecules travelled towards the solvent front from the base line and formed a colored spot was measured with a graduated scale. The Retention Factor (R_f) value is calculated for sample A and sample B adopting the following formula.

Retention factor (R_f) =Distance between the colored spot and the extract spot near the base line ÷ Distance travelled by solvent front.

The results were given in the results section.

Thin layer chromatography

Preparation of solvent system and experimental procedure

In the present study, n-butanol, acetic acid, water were taken in 3:1:1 ratio top prepare 200 ml of solvent by volume (Schweitzer, 2014) to use as solvent system.This solvent system was transferred to 1000 ml beaker and it is covered with aluminum foil for saturation for 1 hour. Readymade (Merck Sciences Pvt.Ltd) silica gel plates are taken and cut into square shape (12 cm length × 12 cm breadth) with the help of scissor without touching with fingers. A base line was drawn at a distance of 1.5 cm above the lower edge of the plate. A known volume (0.2 µl) of sample is released in 2 places on the silica gel plates where the base line was drawn at intervals of 1.5 cm distance with the help of a micro pipette. Care was taken to confine the sample to spot of minimum areas possible (about 0.5cm in diameter). After air drying, the silica gel plates were placed in the beaker containing solvent in such a way, about 3/4 distance of the plate from the base line was submerged in the solvent.
In the Thin Layer Chromatography the usual maximum run time would be four hours.
The plates are then removed from the solvent and the maximum limit until which the molecules in the extract mobilized on the silica gel plate from the baseline was marked. After air drying the silica gel plate, Ninhydrin was sprayed on the plates with the help of sprayer. Again the silica gel plates were air dried and kept in hot air oven for 10 minutes at 100^0C. The coloured spots developed were thus marked, the distance travelled by the solvent and the distance of the solvent front were measured with a graduated scale. Retention Factor (R_f) was calculated using the following formula. The obtained results were given the results section.

Results

Identification of algal species

The collected species of thalloid algae were identified as Grateloupiafelicina and Gracilariacorticata (Umamaheswararao and Sriramulu, 1970). The characters matched to the description of Umamaheswararao and Sriramulu (1970) to confirm the algal species as above were briefly given below.
Grateloupiafilicina(plate 1a)belonging to the order Halymeniales was identified by the red color of its dark blackish crust from which several erect axes up to 2-4(-10) cm high arise. The axes were compressed. Erect thalli were four times dichotomously branched.

Gracilariacorticata (Plate 1b) belonging to the order Gracilariales was identified by the red color of its thallus, erect thallus,12cm in length, arising singly from a discoid holdfast. Stipe very short, terete and 5 mm long. Blades were linear, 8.5 cm long, 4mm wide; apices obtuse, acute in finer branches. Blade surface and margins were smoother.

Identification of the separated components was based on comparison of obtained R_f values with the R_f value of standards using the same mobile phase. The R_f values obtained were in agreement with those obtained by Schweitzer (2014), with the same solvent system.

Paper chromatography (plate 3)

Two visible bands appeared with solvent system 70:30 ratio 96% ethanol and water. The distance travelled by the unknown molecules present in the 10% Trichloroacetic acid extract of Grateloupiafilicinawas measured as 8.9 cm on the chromatography paper, the solvent front is 10 cm.
R_f value of sample (A) i.e., Grateloupiafilicinais 0.89.

The R_f values obtained for sample A (0.89) is compared with the standard chromatograms, it shows the presence of photosynthetic pigment which is identified as carotene.
When the R_f values obtained for sample B i.e., Gracilariacorticata (0.73) is compared with the standard chromatograms, it shows the presence of an amino acid which is identified as Leucine.
(band B, R_f0.73)

Thin layer chromatography

In the present study, with n-butanol, acetic acid, water in 3:1:1 ratio by volume (Schweitzer, 2014) as solvent system, it was observed that within 2 hours the solvent front was formed at a maximum distance near the opposite end of the silica gel plate.

The calculated R_f values in TLC for sample A i.e., Grateloupiafilicinais 0.68 (fig.2b). It shows the presence of amino acid which is identified as phenylalanine, when compared with the standard given by Schweitzer 2014 with the same solvent system.
The calculated R_f value for sample B i.e., Gracilariacorticata is 0.73 (fig.2b). It shows the presence of amino acid which is identified as leucine, when compared with the standard given by Schweitzer 2014, with the same solvent system.

Discussion

Macro thalloid algae are important livestock that can be harvested from marine environment. They can be cultured in the open sea farms for large scale industrial production of substances such as enzymes, carotenoids, xanthophylls, amino acids in addition to chlorophylls. Some of these substances having pharmaceutical importance. Marine thalloid algae can be cultivated in large quantities for the industrial extraction of the above substances of pharmaceutical importance. Other potential economic importance of the marine thalloid algae are antibacterial substances.

Antibacterial substances such as hydroquinones, terpenoids, phenols, brominated phenols and poly phenols were also extracted from species of chlorophyceae, phaeophyceae and rhodophyceae (Faulkner, 2002). The secondary metabolites namely saponins, flavonoids, steroids and alkaloid phenols were rich in seaweeds. These substances are extensively in the preparation of drugs. Studies on the

phytochemistry and antibacterial activity of selected red sea weeds from Manapadcoastal area, Thoothukudi, India indicated that those species of red seaweeds can be used to extract natural food colorants and substances of antibacterial activity (Adikalaraj et al., 2011).
In the present paper chromatography study, carotene and leucine were identified in Grateloupiafilicinaand Gracilariacorticata respectively.

Physically, carotene is a yellow orange pigment belongs to carotenoid family. It is a precursor for the synthesis of vitamin A. All micro algae consists of carotene. α- carotene is present in Cryptophyta and Cholorphyceae. The more common type of carotene in marine environment is β-carotene. The two types of carotenes differ in the position of double bond in the hydrocarbon ring. With reference to their chemical structure, Carotenes are naturally occurring polyunsaturated hydrocarbons. They consists of 40 carbon atoms in a single molecule. The number of hydrogen atoms are variable with no elements. Some carotenes consists of hydrocarbon rings at their terminal portions of the molecule either at one end or on both sides of the molecule (Morancais, Mouget and Dumay, 2018).

Carotene functions as photoreceptors in the thallaoid algae, absorbs light and helps in the electron transport. They are also effective antioxidants, substrates for formation of lipoxygenase and contending with the Arachidonic Acid decrease leukotriene formation in the cells (Nowicki and Murray, 2020).
The long carbon chain, conjugated double bonds allow the carotene molecule easily isomerize, absorb light and oxygen, subject to auto oxidation. Therefore when added to food carotenes are unstable (Dini, 2019)

In the present study the yellow-orange band and the $R_f$value obtained suggested the presence of carotene in the extract of Grateloupiafilicina. This study reports the presence of carotene for the first time in Grateloupiafilicina. Further work shall be undertaken to study the type of carotene and the quantity per unit weight present in the above species of macro algae.
As carotenes has more health benefits, Grateloupiafilicinacan be used as a natural source of this substance for industrial production. Grateloupiafilicinacan be cultivated in the open sea farms for large scale biomass production.

Leucine is essential for protein synthesis it can be obtained only from diet. The dietary sources of leucine are meat, dietary products, all types of beans and legumes. A minor metabolite of leucine known as β hydroxyl β methyl butyric acid, along with leucine promote protein synthesis in humans (Wilkinson et al. 2013).

The suggested daily intake of leucine for men is 56 g per day and women 46 g per day. leucine helps repairing tissues, healing wounds, build muscles, repair muscle and prevent muscle degeneration. Further leucine controls blood glucose levels, helps in secretion of growth hormone in children and muscle building, controls obesity by appetite regulation and acts as an anti-aging agent (Momaya, 2022)

Leucine was obtained in the extract of Gracilariacorticata in the Paper chromatography and also in the Thin Layer Chromatography. So this study revealed that Gracilariacorticata is good natural source of leucine. G.Coriticata is a popularly cultured seaweed in Tamilnadu and Kerala coast of India which is

used as a natural food colouring agent. This study emphasizes that, this species of seaweed can also be used for extraction of leucine for pharmaceutical use.

One of the essential amino acid namely phenylalanine (Phe.) can be supply to the body of organisms only through food, animals required this amino acid for synthesis of proteins, melanin and catecholamine- a neurotransmitter. Phe. is the precursor for the synthesis of L- tyrosine. The oxidation of Phe. in the body of organisms requires metal ions such as magnesium and iron. In addition thiamin, biopterin, riboflavin, ascorbate, pantothenate, vitamin B6, niacin, lipoate, ubiquinone for its oxidation (Kohlmeier, 2003). The daily adult requirement for Phe. and tyrosine is 39 mg / Kg body weight (Young and Borgonha, 2000).

The structure of phenylalanine resembles epinephrine, dopamine and tyrosine. Phenyl alanine is metabolized in to tyrosine, which further metabolized to catecholamine- a neurotransmitter, thus used as antidepressant along with phenylethylamine (PEA). Administration of phenylalanine is a good results in adult and pediatric patients (Kapalka, 2010). Reviews of research studies revealed that the use of phenylalanine as antidepressant is very useful (Sabelli, 2002).
In the present study on Grateloupiafilicina, phenylalanine was identified for the first time in the Thin Layer Chromatography with R_f value 0.68 (Schweitzer 2014). Keeping in view the various antidepressant properties of phenylalanine, further studies are necessary to quantify per kg yield of phenylalnine from the dry/wet biomass of G.filicina.

Since the obtained substances are having pharmaceutical importance, quantitative study on the carotenes, phenylalanine and leucine from the above thalloid algal species will be conducted in future for the industrial level extraction of these substances.

Conclusion

All the three substances such as carotene, leucine and phenylalanine present in the two genera of red algae have high economic and pharmaceutical value. The study found that Grateloupiafilicinaand Gracilariacorticata are good sources of the above substances. The study recommends these two thalloid algal genera for industrial production of these compounds. The present study encourages further studies on the quantification of these substances in these two seaweeds.

Acknowledgement

The authors thank the Head, Department of Marine Living Resources, Andhra University, Visakhapatnam, India for permitting them to use the CSIR-NMITLI Laboratory facility to undertake this work. CSIR, New Delhi, India is also acknowledge for creating the facility in the department of Marine Living Resources.

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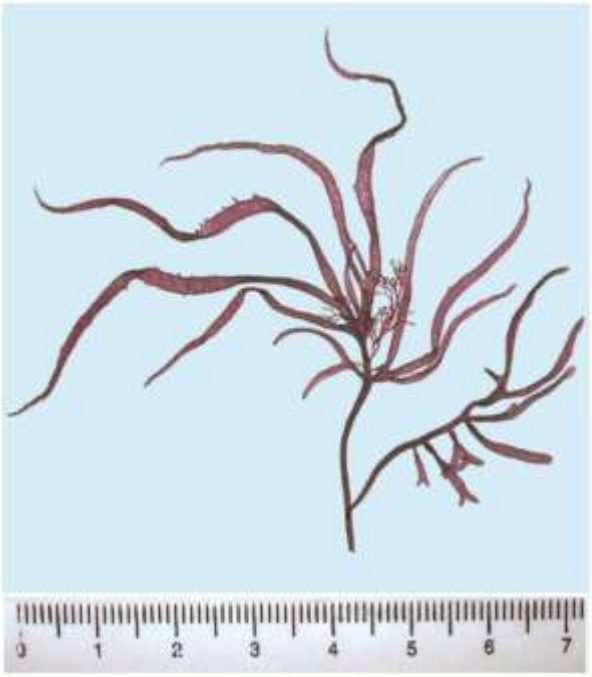

A. *Grateloupia filicina*

B. *Gracilaria corticata*

Plate : 1

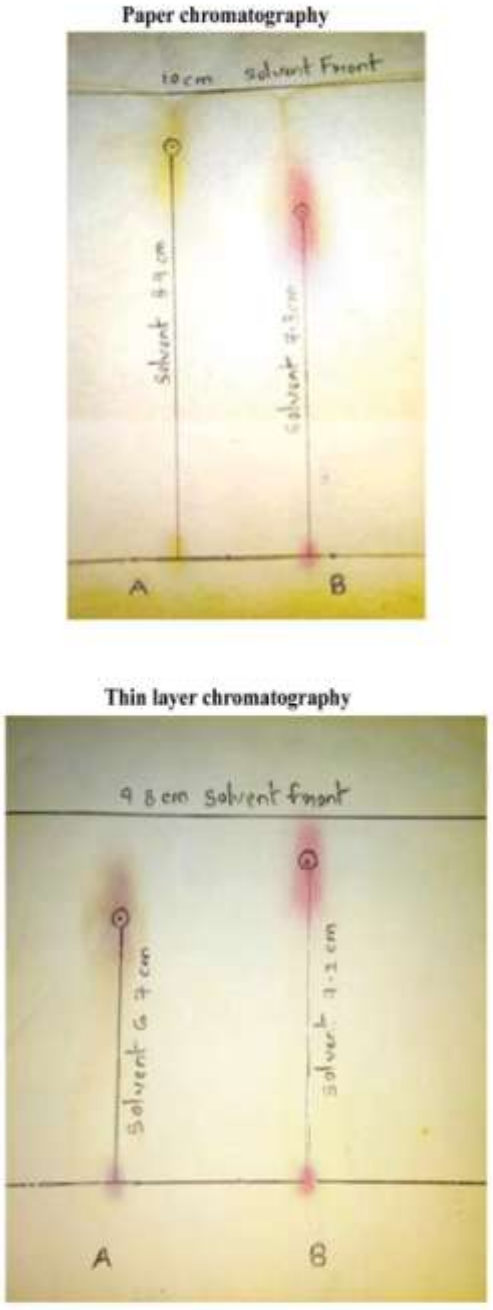

Figure : 1. Chromatograms obtained from extract of A. *Grateloupia filicina* and B. *Gracilaria corticata*