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Report on the identification of alkaloids from *Sargassum tenerrimum*

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ABSTRACT

Sargassum tenerrimum was collected from the shores of Vainguinim in Goa. Preliminary phytochemical investigations of this seaweed revealed the presence of alkaloids which was further confirmed by their chromatographic analysis. Alkaloids were extracted in methanol and re-precipitated in Conc. Ammonium hydroxide. The precipitate was dissolved in Chloroform and it was observed that alkaloids were retained and resolved as a single band even after treatment with charcoal for removal of pigments. Sequential fractionation (water, chloroform) of the chloroform extract resulted in identification of four alkaloids Ephedrine, Cuscohygrine, Pyrvinium and Doxapram by LC-MS.

Keywords: Alkaloids, Ephedrine, Cuscohygrine, Pyrvinium, Doxapram.

Introduction

Seaweeds, a sustainable resource in the marine environment, serves as raw material in many industries for the production of agar, alginates and carrageenans and are also widely utilized as food in Asian countries (Mishra *et al.*, 1993). Recent studies reported that seaweed and their extracts possess bioactivities of medicinal value (Mhadhebi *et al.*, 2014). The compounds isolated from seaweeds have provided important leads for development of new improved drugs against cancer, microbial infections and inflammation (Elena *et al.*, 2001). In recent years, seaweeds have also gained attention as potential natural antioxidants (Subbiah and Bhuvaneswari, 2016). One such seaweed is *Sargassum* which is reported to be a rich source of bioactive secondary metabolite compounds like terpenoids, flavonoids, sterols, alkaloids and sulphated polysaccharides (Wijesinghe and Jeon, 2012). These isolated compounds show diverse biological properties like analgesic, anti-inflammatory, anti-oxidant and anti-microbial. Hence, *Sargassum spp.* are reported to have great potential use in pharmaceutical and nutraceutical areas (Guven *et al.*, 2010).

One of the interesting groups of secondary metabolites which provide the potent drug leads are the alkaloids. These are crystalline compounds which react with acids to form salts. In plants, they exist as salts or *N* –oxides. Colored alkaloids are very rare, for example, berberine is yellow and the salts of Sanguinarine are copper-red in color (Hesse, 2002).

Alkaloids help plants by protecting them from predators and environmental stress (Hesse, 2002). At present, approximately 600 alkaloids have been examined for their bio-medicinal properties and many have gained importance as drugs in the pharmaceutical industries [E.g. Taxol isolated from *Taxus brevifolia*; Vincristine and Vinblastine isolated from *Catharanthus raseus* (Madagascar) showed anti-cancer property] (Hadi, 2002). Alkaloids (lombine, cononaridine & mataranine A and B) isolated from plant *Vocanga foetida* have been reported to have strong antimicrobial activity against *Staphylococcus aureus.* Though these reports on alkaloids are mostly pertaining to terrestrial plants, the report on seaweed alkaloids is not extensive. Hence, the main aim of this study was to detect, extract, isolate and identify the alkaloids from *Sargassum tenerrimum* that may possess therapeutic potential

Materials and Methods

Collection of Seaweed sample

Sargassum tenerrimum J.Agardh 1848 (Phaeophy ceae: Fucales: Sargassaceae) growing abundantly was collected from the rocky shores of Vainguinim (Lat.15°45'N; Long.73°81'E) beach (Goa) during the postmonsoon month of October 2017. In the lab, seaweeds were thoroughly washed with tap water followed by distilled water. Part of the washed seaweed was stored separately in a Petri plate with some distilled water and sent for identification. Rest of the washed seaweeds were dabbed dry and then spread out onto more blotting paper and left to dry in shade at room temperature. Shade dried seaweeds were ground into a fine powder using a blender. The powdered samples were then stored at 4°C until further use.

Preliminary phytochemical screening

Preliminary alkaloid detection tests were carried out in methanolic extract of seaweeds as described by Harborne (1998). Coffee was used a positive control and methanol was used as a negative control.

a) Wagner's test: Few drops of Wagner's reagent were added along the sides of test tube containing few mL of filtrate. Formation of reddish-brown precipitate was taken as positive.

Wagner's Reagent : Iodine (1.27 g) +potassium iodide (2 g) dissolved in 100 mL distilled water.

b) Hager's test: Few mL of Hager's reagent was added to a few mL of filtrate and the occurrence of a prominent yellow color confirmed the test as positive.

Hager's Reagent: Saturated aqueous solution of picric acid.

c) Mayer's test: Few drops of Mayer's reagent were added from the sides of the tube containing few mL of filtrate. Formation of a creamish yellow precipitate indicated positive result.

Mayer's Reagent: Mercuric chloride (2.72 g) dissolved in distilled water (20 mL). Potassium iodide (10 g) was dissolved in distilled water (40 mL) separately. Both solutions were mixed and distilled water was added to make a final volume of 200 mL).

Preparation of Extracts

Two different extracts were prepared: (i) Dissolved Chloroform Extract (DCE) and (ii) Chloroform Extract (CE)

Preparation of Dissolved Chloroform Extract (DCE)

About 40g of sample was weighed and added into 400 mL of methanol (containing 10% acetic acid). This mixture was kept for 4 hours on a shaker under dark conditions at room temperature. The suspension was then centrifuged (10,000 rpm, 10 min). The supernatant was then concentrated by rotary evaporation to one fourth of the original volume and was precipitated by adding a few drops of Conc. Ammonium hydroxide. The obtained precipitate was washed in 1% NH_4OH and centrifuged further at 3000 rpm for 10 min. This pellet that precipitated was dissolved in chloroform (120 mL), which was further concentrated to about 2-4 mL under vacuum by rotary evaporation (Hultin and Torssell, 1965).

Preparation of Chloroform Extract (CE)

40g of sample was weighed and extracted in 120 mL of chloroform. The volume was further concentrated and reduced to 50 mL using rotary evaporator. The extract obtained was further fractionated thrice with water (50mL) in a separating funnel. The funnel was agitated 4-5 times for proper mixing of the liquids and allowed to stand. The lower layer of chloroform (Alkaloid Containing) was collected and retained.

CE was further clarified to get rid of all the pigments present in the extract by passing it through charcoal placed over the filter paper. TLC was run using CE fraction to check the presence of alkaloids. Having tried multiple solvent systems reported by Wagner and Bladt (1996), a modified solvent system of Petroleum ether: Ethyl acetate: Diethyl amine (8:1:1) was found to be effective and used to run the silica plates.

TLC analysis for Detection of Alkaloids in DCE and CE

Both extracts obtained from *S. tenerrimum* were spotted on silica gel plates (Merck 105550, Aluminum oxide 60 F254, neutral, 20cm X 20cm X 200µm) to separate the constituent bioactive compounds. 10 µl aliquots of DCE and CE, were used. The plates were run in the solvent system of Petroleum ether: Ethyl acetate: Diethyl amine (8:1:1), till the solvent front reached 2/3rd of the plate. The plates were then dried and observed under UV trans-illuminator for any visible separation of bands, followed by spraying with Dragondroff's and Marquis spraying reagents to detect color spots (Harborne, 1998).

Separation and Identification of Compound by LC-MS

LC-MS enables efficient separation of alkaloids which are otherwise difficult to separate when in a mixed consortia of the extract. Rapid analytical screening and high sensitivity makes it an ideal technique for identification. After resolving the ideal molecule by TLC, preparative TLC was done to obtain a higher yield of the desired alkaloid compounds which was then solubilised in Chloroform, and 3µl of this was injected into the LC-MS system (MS Q-TOF, Agilent Technologies) by modifying the protocol of Ptak *et al.* (2009) as per requirement of the present study. Gas temperature was kept at 250°C. The ion source was Dual AJS ESI (IIT, Bombay). Flow rate was maintained at 0.3mL/min. Pressure was maintained within 0-1200 bar and the stop time was set to 30 minutes.

Results

At the site of sample collection, *Sargassum* was found attached to the rocky shores in the sub-tidal region and floating in the water. The collected sample of *Sargassum* was identified as *S. tenerrimum* with the help of a Taxonomist and Botanist, Prof. Vijay Kerkar, Department of Botany, Goa University.

Alkaloids were detected through qualitative phyto chemical analysis on the methanolic extract of *S. tenerrimum* (Table-1).

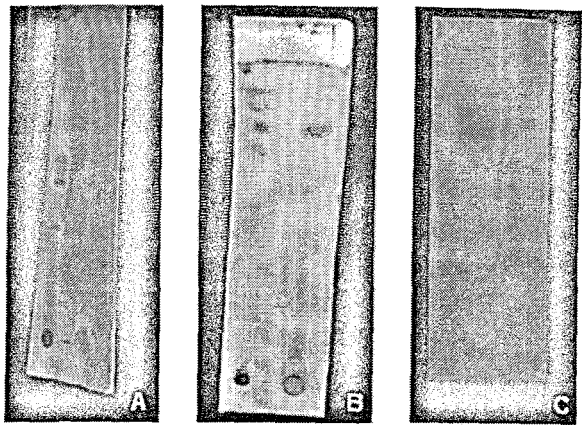
Table-1. *Detection of Phytochemical*

	Tests For Alkaloids		
	Mayer's test (Colour Reaction)	Wagner's test (Colour Reaction)	Hager's test (Colour Reaction)
T+R (Methanol)	Pale green to creamish yellow	Pale green to reddish-brown	Pale green to prominent yellow
Positive control (Coffee+R)	Pale green to creamish yellow	Pale green to reddish-brown	Pale green to prominent yellow
Negative control (Methanol+R)	Pale green (No colour change)	Pale green (No colour change)	Pale green (No colour change)

T=Test Extract, R=Reagent, Coffee+R= Positive control, Methanol+R= Negative control

The TLC resulted in visual separation of four bands in DCE and one band for CE. These results were validated by repeating the TLC thrice. When the plates were observed under UV for fluorescence, similar separation was observed with four bright, pinkish-orange colored fluorescence bands for DCE and one for CE (Fig. 1A).

For better resolution, plates were sprayed with Marquis Reagent and Dragendorff's Reagent. Marquis Reagent reacted to give brownish and green colored spots in CE (Fig. 1B), whereas no color developed with Dragendorff's Reagent in either of the extracts (Fig. 1C). The visualization of brownish spots suggests that the resolved constituted molecule could be an alkaloid. As seen from the Fig.1B, the spots in the left lane on the TLC plate were of DCE, while the right lane on the plate was of CE (Figs.1A, 1B and 1C).



(A)TLC plate under UV; (B) TLC plate sprayed with Marquis reagent ; (C) Plate sprayed with Dragendorff's reagent

Fig.1. *TLC Profile of Alkaloids*

The de-pigmentation of CE by passing through activated charcoal was successful and resulted in improved resolution of the resolved molecules. The chromatogram showed a single crisp and clear band (Fig. 2B), in comparison to the three bands that were observed in the pigmented CE chromatogram in Fig. 2A. The R_f value of the band was calculated to be 0.46. Since CE processing resulted in a single band, it was further subjected to LC-MS for identification.

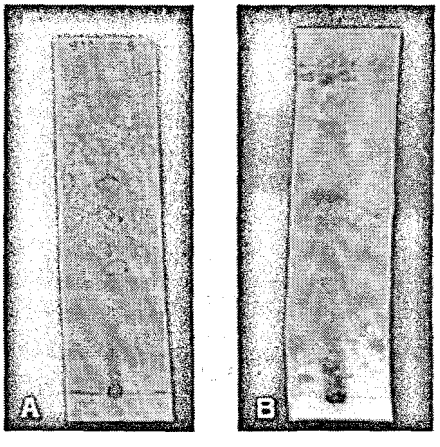
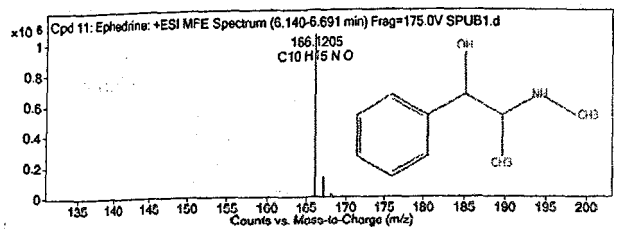
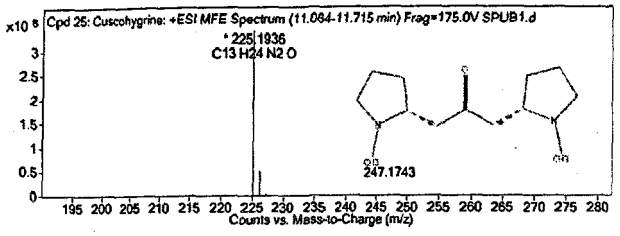


Fig. 2. *Shows the TLC of CE before (A) and after (B) de-pigmentation*

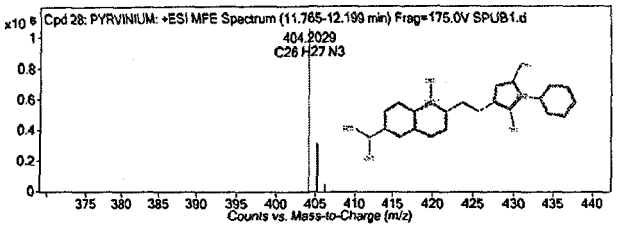
LC-MS profile indicated the presence of 100 different compounds. Each of the compounds was searched and tallied for on the EMBL-EBI, NCBI-Mesh and PubChem chemical databases to aid the identification. Literature pertaining to the identified compounds was reviewed for their reported source and occurrence. Only then, it was confirmed that, out of those separated, four were alkaloids namely, Ephedrine, Cuscohygrine, Pyrvinium and Doxapram (Fig. 3). These four alkaloids are well known molecules in the medical system and have often been isolated from terrestrial plants or synthesized artificially. This could very well be the first report suggesting the possibility of obtaining these molecules from seaweeds.



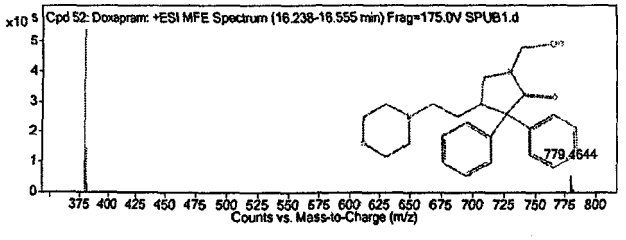
(a) Ephedrine



(b) Cuscohygrine



(c) Pyrvinium



(d) Doxapram

Fig. 3. *Zoomed molecular fragmentation spectra and structure of identified alkaloids by LC-MS*

Discussion

Natural products have long been used by many cultures and traditions in medical application and in recent times have been arduously explored for its bioactive pharmacopoeia by modern pharmaceutical companies. Natural products provide a skeletal framework of about 60% of the modern drugs that are now available (Cragg and Newman, 2013). About 80% antibacterial drugs and 90% of anti-malarial drugs are natural derivatives (Hagai and Deharo, 2011; Newman and Cragg, 2016). One interesting group of secondary metabolites that provides these potent drug leads are "Alkaloids".

Seaweeds are nutritious and a rich source of bioactive compounds such as vitamins, carotenoids, dietary fibers, proteins and minerals. One such seaweed is *Sargassum spp.* Many other biologically active compounds like terpenoids, flavonoids, sterols, sulphated polysaccharides *etc.* are isolated from different

Sargassum spp. with diverse biological activities like analgesic, anti-oxidant, anti-microbial, immunosuppressive, anti-inflammatory, anti-cholesterol, anti-hyperglycemia, anti-microbial and anti-tumor activity (Gurav *et al.*, 2014). Hence, *Sargassum spp.* has great potential to be used in pharmaceuticals and nutraceutical areas. In the present study, the methanolic extract indicated the presence of alkaloids in the *Sargassum tenerrimum* collected from Vainguinim Coast.

Even though several bioactive secondary metabolites are present in seaweeds that are capable of eliciting pharmacological effects in human beings, due to natural variability, their qualitative and quantitative composition in seaweeds may vary considerably (Thennarasan *et al.*, 2014). Therefore, to achieve maximal variety and quantity, ideal extraction methods need to be carefully chosen, to maintain sufficient purity and quantity of phytochemicals (i.e. Alkaloids in the present study). Recent trends in extraction techniques have largely focused on finding solutions to minimize the use of solvents, while enabling process intensification and a cost-effective production of high quality extracts (Chemat *et al.*, 2012). In this study, though both the approaches were tested, the extraction with direct chloroform was efficient in successfully extracting and retaining alkaloid even post-treatment. The extraction efficiency of the solvents used was evident as seen in the TLC results. The Ammonium hydroxide precipitation followed by re-dissolving it in chloroform had poor ability to retain the extracted alkaloids.

An alkaloid is a compound that has nitrogen atom(s) in a cyclic ring. Numerous biological amines and halogenated cyclic nitrogen-containing substances are included in the term alkaloid. These halogenated alkaloids are specific to marine organisms and marine algae and could not be found in terrestrial plants (Perez *et al.*, 2016). Alkaloids in marine algae are classified in to three groups (Guven *et al.*, 2010); (i) Phenylethylamine alkaloids; (ii) Indole and Halogenated indole alkaloids and (iii) Other alkaloids, such as 2,7-naphthyridine derivatives. Alkaloids isolated from marine algae mostly belong to 2-phenylethylamine and indole groups (Perez *et al.*, 2016).

In the present study, the identification of the alkaloids namely, Ephedrine, Cuscohygrine, Pyrvinium and Doxapram from the total lot of 100 separated molecules is reported. The mass and the probable structural data obtained by LC-MS together with prevailing literature obtained from chemical databases was the key in identifying these molecules.

Ephedrine, a phenethylamine alkaloid, has been used as medication and a stimulant especially to prevent low blood pressure during spinal anesthesia. Ephedrine works by increasing the activity of the α and β adrenergic receptors in the vasculature by inducing vasoconstriction and to bronchodilation in the lungs, thus it has also been used for asthma, heart failure, rhinitis and urinary incontinence, and for its central nervous system stimulatory effects in treating narcolepsy and depression (Billington *et al.*, 2017). It is on the WHO Model List of Essential Medicines, 2015, as the most effective and safe medicines needed in a health system.

Pyrvinium, a relatively neutral molecule which can act as an anti-neoplastic agent and an anti-helminthic drug (against pinworms) is approved by the FDA (Ahmed *et al.*, 2016). Pyrvinium has the ability to inhibit Wnt(Wingless/Integrated) signaling through activation of casein kinase 1á (signal transduction regulators), thus playing a critical role in metazoan development, stem cell maintenance and human disease (Thorne *et al.*, 2010). Saraswati *et al.*, (2012) have reported it as a therapeutic molecule helping in mesenchymal stem cell (MSC) self-renewal, thus improving the clinical efficacy of MSC therapy.

Doxapram is a central respiratory stimulant having a brief duration of action (Lipman, 1993). Doxapram, independent of oxygen levels, is reported to directly stimulate the peripheral carotid chemoreceptors, possibly by inhibiting the potassium channels of type I cells within the carotid body, thereby stimulating catecholamines release. This results in the prevention or reversal of both narcotic - and CNS depressant-induced respiratory depression (Yost 2006; EMBL-EBI).

Cuscohygrine is a pyrrolidine alkaloid usually found in a variety of plants such as Datura, Hyoscymus, and Erythroxylum (Ionkova *et al.*, 1994; Glass, 1997). Decomposition of cuscohygrine often leads to formation of hygrine which is an established precursor of tropane alkaloids (hyoscyamine and scopolamine, both used in myriad pharmaceutical products). While hyoscyamine is used to treat bowel control problems, cramping pain caused by kidney and gall stones and Parkinson's disease, scopolamine helps in treating motion sickness, postoperative nausea and vomiting (Bahmanzadegana *et al.*, 2009; Kassel *et al.*, 2018). Information on cuscohygrine is very limited and is often reported in verification studies where cuscohygrine is considered to be a good marker to distinguish between chewing coca leaves and cocaine abuse (Rubio *et al.*, 2014).

On the basis of the available literature, the alkaloids identified in the present study have been reported either from terrestrial plants or are synthesized. Though Ephedrine and Cuscohygrine have natural origins as discussed, pyrvinium and doxapram seems to have been synthesized. In this context, this study could be the first record on the natural origin of these alkaloid molecules in seaweeds as there exist no previous reports. Hence, this study has no doubt raised the nutraceutical status of the brown seaweed *S. tenerrimum.*

Conclusion

Oceans possess large renewable resource of beneficial seaweeds. The chemically active biogenic compounds produced by seaweeds (alkaloids, terpenoids, steroids, lactones) have potentials as protective and human therapeutic agents. In the

present study, *Sargassum tenerrimum*, a well known brown seaweed, has been reported as a source of alkaloids (Ephedrine, Cuscohygrine, Pyrvinium, and Doxapram) of immense biomedical potential, strengthening its candidature for use in development of human therapeutics.

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Conflict of Interests

The authors declare that there are no conflicts of interests.

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