Seaweeds *Calliblepharis jubata* and *Fucus vesiculosus* Pigments: Antidermatophytic Activity

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Article

Seaweeds *Calliblepharis jubata* and *Fucus vesiculosus* Pigments: Antidermatophytic Activity

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Abstract: Seaweeds have been the target of much attention due to the known bioactivities of some of their compounds. Although many studies about the application of seaweeds in various areas are available, these data are mostly about the raw extracts and not about specific compounds, such as seaweeds pigments. Moreover, the fungal infections of the skin, nails and hair caused by dermatophytes, are the most common fungal infections in humans. These pathologies require long periods of topical and/or systemic treatment that are associated with adverse effects and contraindications. This study had two objectives, the first was to evaluate the possibility of isolating and characterizing the pigments of *Calliblepharis jubata* and *Fucus vesiculosus*; the second was to assess their antifungal activity. The extraction of pigments was performed using a method of extraction by exhaustion; the purification was carried out by column chromatography. Three techniques were used to characterize the pigments, thin layer chromatography (TLC), UV-visible spectrophotometry and Fourier-transform infrared spectroscopy (FTIR). The antifungal activity was evaluated using the microdilution method, following the EUCAST international standards, on three dermatophytes, *Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum canis*. It was possible to observe that the extract obtained from the seaweed *C. jubata* and *F. vesiculosus*, corresponding to the purified pigment phycobiliprotein, and the crude extract, an enriched extract, obtained from the seaweed *C. jubata* showed antifungal activity against the three fungal agents of human skin infection.

Keywords: seaweeds; pigments; natural extracts; antifungal activity; UV spectrophotometry; TLC; FTIR

1. Introduction

Seaweed extracts are a desirable substitute to traditional therapies due to several factors. One of these advantages is their degradability into less harmful substances for the environment [1]. The interest in seaweed has been growing in the past few years due to the wide range of biological actions, including anticancer, anti-inflammatory, antioxidant, anti-obesity [2–4] .

The seaweeds can be divided into three phyla and the principal characteristic responsible to that division are the different pigments that they possess (Table 1). Chlorophyll (Chl), carotenoids and phycobiliproteins (PBPs) are the three main categories of pigments [5–9].

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Table 1. Pigments detected in Seaweeds.

Phylum	Chl	PBPs	Carotenoids	References
Chlorophyta	*a*, *b*		β-Carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin	[5–8,10]
Ochrophyta	*a*, *c*		β-Carotene, fucoxanthin, and violaxanthin	
Rhodophyta	*a*	Phycocyanin Phycoerythrin	β-Carotene, lutein, and zeaxanthin	

Calliblepharis jubata is a benthic seaweed, which belongs to phylum Rhodophyta, class Florideophyceae, genus *Calliblepharis* Kützing, species *Calliblepharis jubata* (Goodenough & Woodward) Kützing [11].

C. jubata is a brownish red seaweed with a thallus made up of holdfast with branches that grows into an upright frond and then spreads out into a dichotomous or unevenly split blade. The frond's shape might vary although it frequently has a cylindrical or hardly compressed stipe. It has blades that are 30 cm long, 6 mm wide, and have slender branching. The branches have a lengthy, tendril-like appearance. The blade surface and branch edges produce long branchlets, or proliferations [11].

As a gametangial, *C. jubata* have both male and female reproductive organs. Around April, the female reproductive structures (cystocarps), which have a diameter of 1-2 mm, start to emerge as noticeable globose formations on the branchlets. Tetrasporangia, which are known to occur in April, June, and July, are the sexual reproductive structures. Although they occasionally appear sparsely on the blades, they are found on the chanceless. The tetrasporangia grow in the cortex with zonately organized tetraspores [11].

The studies on this seaweed have focused mostly on its polysaccharide composition. *C. jubata* has hybrid iota or iota-kappa carrageenan with extremely low content of kappa-carrageenan [12].

F. vesiculosus belongs to the phylum Ochrophyta, class Phaeophyceae, genus *Fucus* Linnaeus, species *Fucus vesiculosus* Linnaeus [11]. This seaweed is normally approximately 40 cm long (although fronds can grow longer) and have a color range of olive green to olive brown to reddish brown to almost black. It uses a tiny disc-shaped holdfast to attach to rocky superficies. There are some studies reporting some activities and, or some application of this seaweed. *F. vesiculosus* can be used in agriculture as a fertilizer, in bioremediation as a biosorbent of heavy metals because of his high capacity to capture environmental pollution [12]. Despite of having good quantities of proteins, minerals, and fatty acids it's not advisable to ingest this seaweed due to the capacity to absorb heavy metals. In pharmacology, this seaweed has reports in helping with problems related to obesity and cellulite, it has anti-cancer potential, helps reducing blood pressure pathologies [13,14].

Natural pigments exist in a wide range of hues and have been widely used throughout history in daily life. A few examples worth mentioning are food production, textile and paper industries, water science and tecnhology, as well as agricultural research and practise. Pigments exhibit advantageous biological activities such as antioxidants, antidiabetic, antiangiogenic, anti-inflammatory and anticancer. These qualities make them suitable for use in these various industrial contexts [2–4] As a result, they have a great chance of meeting current market needs, which increasingly focus on the health and biotechnology sectors in search of natural substances and products with established positive impacts on human health [15,16].

Seaweeds inhabit aqueous habitats with a rich diversity of microorganisms. Seaweeds consequently acquired some antibacterial qualities. They can be viewed as suitable alternatives to the resistance to traditional antimicrobial. In several publications, seaweeds have been suggested as potential sources of compounds with antimicrobial proprieties. However, knowledge about the antimicrobial activities of certain components of such pigments remain scarce, although seaweeds produce a wide range of pigments. Studies about these pigments have mostly focused on their

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antioxidant potential [6,17–20]. A few studies highlight the antibacterial properties of carotenoids particularly the action of fucoxanthin against Gram-positive and Gram-negative bacteria [6,21–23]. It has also been shown that lutein has antiviral properties against the hepatitis B virus (HBV) [24,25].

Antifungal resistance has been identified as a significant public health problem in the twenty-first century by the World Health Organization (WHO). This resistance mechanism may be due to clinical factors such as insufficient dosage, inaccurate diagnosis, inappropriate or irresponsible treatment, microbiological factors like genetic alterations or both aspects [26].

Opportunistic fungal infections have significantly increased in recent years [27,28]. Although fungi do not generate pandemics or epidemics, the frequency of severe systemic fungal infections has dramatically increased, mostly due to the sharp rise in the number of immunosuppressed patients [29,30]. The dermatophytes infections have considerably grown, even in immunocompetent hosts [29]. Natural compounds with antifungal activities have also received considerable attention during the past few decades, namely from terrestrial plants and seaweeds [29,31]. Due to the increasing number of fungal infections, the emerging resistance to the traditional antifungals, the limited number of antifungals available, and because superficial mycosis and onychomycoses require long periods of topical and systemic treatment that are associated to adverse effects, contraindications, and sometimes interaction with other drugs, it is essential to screen for novel antifungal compounds. The objective of the present work was to isolate and characterize *C. jubata and F. vesiculosus* pigments and extracts, and to evaluate the pigments' antifungal effect against fungi agents of skin infections. Also was secondary, to observe if a crude and enriched extract can be key to a better welfare future.

2. Results

Due to the lack of studies on the seaweed pigments it were used several techniques to be able to do the most rigorous possible identification.

2.1. Pigment Purification

The purification of the pigments from crude extracts was achieved by column chromatography (CC). Throughout the experiments several mobile phases were studied to observe wish one was more efficient in the separation of the pigments. It was tested four mobile phases: *n*-hexane: acetone (7:3, v/v), Petroleum ether: acetone (7:3, v/v), Methanol: acetone (7:3) and n-hexane: methanol (7:3, v/v). Of all mobile phases studied the only one that showed to be able to separate the pigments was *n*-hexane: acetone (7:3, v/v); with this the pigment separation occurred more efficiently (Figure 1A). In fact, it is possible to see the separation of pigments with different "colors". This technique is based on the observation at naked eye, because is possible to observe the different fragment thought the column. In the selected fragment, it is possible to see the start and the end of the yellow, aqua-blue, and green pigment.

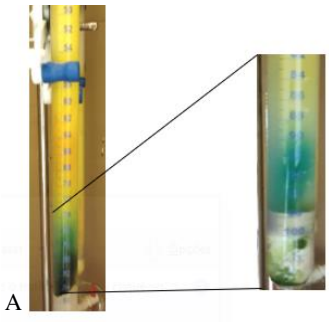

Figure 1. A- Representation of separated pigments in the bottom of the column at two stages of the elution using *n*-hexane: acetone using *n*-hexane: acetone (7:3, v/v).

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2.2. Pigment Characterization

2.2.1. Pigment Characterization by Thin Layer Chromatography (TLC)

This method was used to evaluate the yield of pigments present in each fraction collected in the column chromatography and to identify them based on the Retention factor (Rf) value assigned in literature. This enables to decide which pigments to use for further studies. The ones chosen to proceed in the subsequent phases of the work are presented in Tables 2 and 3. The extracts chosen were the crude extracts and the ones that appeared to be isolated or with a maximum of two pigments. Tables 2 and 3 also present the retention factors calculated for the pigment of each extract used.

Table 2. *F. vesiculosus* pigments characterization through the Rf value obtained in TLC.

	Nomenclature of the pigments	Retention Factor (Rf)	Pigments	References
Ethanol extracts	A	0,54	Chlorophyll *b*	[32–38]
		0,85	β-carotene	
	B	0,48	Fucoxanthin	
	C	0,48	Fucoxanthin	
	D Crude Extract	0,7	*NA	
		0,13	Neoxanthin	
		0,19	*NA	
		0,65	*NA	
		0,9	β-carotene	
		0,95	β-carotene	
Acetone Extracts	G	0,38	*NA	
	H	0,06	Neoxanthin	
	I Crude Extract	0,11	Neoxanthin	
		0,38	*NA	
		0,45	Lutein	
		0,02	Chlorophyll *c*	
		0,59	Fucoxanthin	
		0,85	*NA	

*NA – Not identified.

Table 3. *C. jubata* pigments characterization through the Rf value obtained in TLC.

	Nomenclature of the pigments	Retention Factor (Rf)	Pigments	References
Acetone Extracts	J	0,82	*NA	[32–36]
	L	0,53	Chlorophyll *b*	
	M (Crude Extract)	0,04	Chlorophyll c	
		0,42	Lutein	
		0,62	*NA	
		0,68	Chlorophyll *a*	
		0,74	Zeaxanthin	
		0,81	*NA	
		0,84	*NA	
		0,86	*NA	

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		0,91	β-carotene
Ethanol extracts	O	0,53	Chlorophyll *b*
		0,7	Violaxanthin
	R (Crude Extract)	0,75	*NA
		0,86	*NA
		0,91	β-carotene

*NA – Not identified.

2.2.2. Ultraviolet and Visible (UV-Vis) Spectrophotometry

The UV-Vis spectrophotometry (Figure 2) was only performed for the pigments that appear to be isolated by TLC. The analysis of the spectrums and the identifications of the pigments was done based on the absorbance wavelength found in the literature and is presented in Table 4. Through analysis of the spectrums is possible to understand that the Chl *a* is present in several fractions of the extracts. The extracts D and I are the crude extracts of *F. vesiculosus* and based on the spectrophotometric data, these extracts only have the characteristic peaks of Chl *a*. In the case of *C. jubata* the crude extracts M and R already show several peaks, showing to be more enriched extracts by spectrophotometry.

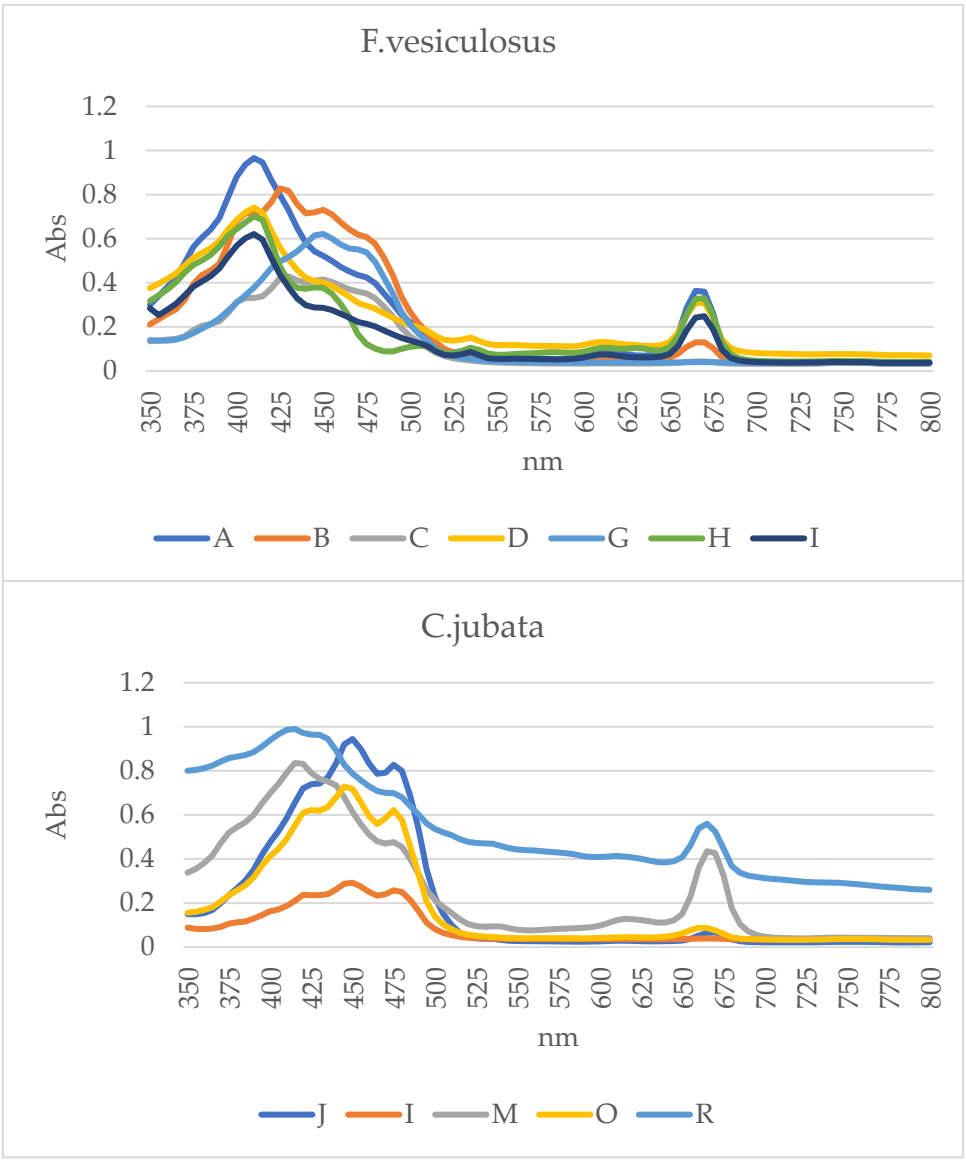

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Figure 2. Visible spectra from the seaweed pigments fractions.

Table 4. Identification of the pigment though the absorbance peaks.

Extract	Peaks (nm)	Pigment	Reference
A	415, 665	Chl *a*	[36–42]
B	410,430,455	Fucoxanthin	
C	410,430,455, 670	Fucoxanthin enriched with Chl *a*	
D	415, 670	Chl *a*	
G	455, 475	β-carotene	
H	415, 450, 500, 535, 670	Enriched Chl *a*	
I	415, 670	Chl *a*	
J	425, 450, 475 ,670	Enriched Extract	
L	420 ,450, 480	Enriched Extract	
M	420, 475, 675	Enriched Extract	
O	425, 450, 475, 665	Violaxanthin enriched with Chl *a*	
R	415, 435, 480, 665	Enriched Extract	
S	495, 630	Mixture of PBS	

2.2.3. FTIR-ATR Analysis

The analysis of the extracts by FTIR-ATR allows identifying the types of chemical bonds the compounds have (Table 5 and Figures 3–10). In this case, lyophilized samples that had visible substrate were analyzed because it was not possible to collect a sample for analysis. The analysis that will follow was carried out considering the information found in literature, although the information on pigments and this type of analysis is still very limited.

All the pigment extracts showed a "noise", which was associated with the humidity present in the air and carbon dioxide. This disturbance was visible in grater predominance between 400-700 cm^{-1} (chemical bounds related C-O bounds of various compounds) and 1800-2300 cm^{-1} (carbon dioxide area).

Pigments A and B present the spectra peaks substantially in the same areas. However, analyzing the main peaks present in the FTIR-ATR spectra and the respective connections allow us to reach the conclusion that it can be either a chlorophyll or a carotenoid.

By observing the graphs and the peaks present, it is possible to affirm that the extract pigments C and H have only one same peak. According to the literature, this peak is a C-N bond that is associated with chlorophylls. Most of all peaks, despite not being the same, represent the same chemical bonds, thus allowing the conclusion that both extracts are carotenoids, and C has a CO-C-O bond, indicating the presence of fucoxanthin.

The extract D and I are the crude extracts of *F. vesiculosus* and despite of the spectra are not very similar, but both have bounds related strongly with the presence of chlorophylls and carotenoids.

The extracts R and M refer to the crude extract of ethanol from *C. jubata*. With the detected peaks of FTIR-ATR, it was possible to identify chemical bound related with the presence of carotenoids, phycoerythrin and chlorophylls.

Taking into account the information found and the peaks detected for extract S, its spectra indicate an extract-enriched in phycoerythrin with traces of chlorophyll.

In Table 5 it is possible to observe the identified peaks of the pigment's extracts

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Table 5. FTIR-ATR bands identification and characterization of the pigments from the extracts.

Wave number (cm^{-1})	Bond	Possible compound	A	B	C	D	H	I	M	R	S	References
1034	C-N	Phycoerythrin/ Chlorophyll	-	-	-	-	-	-	+	-	-	
1035	C-N	Phycoerythrin/ Chlorophyll	-	-	-	-	-	-	-	+	-	
1037	C-N	Phycoerythrin/ Chlorophyll	-	-	-	-	-	-	-	-	+	
1039	C-N	Phycoerythrin/ Chlorophyll	-	-	+	-	+	-	-	-	-	
1057	C-O	Common bond present in the compounds	+	-	-	-	-	-	-	-	-	
1076	(>P=O) stretching	Phosphated compound	-	-	-	+	-	-	-	-	-	
1149	C-O stretch	Common bond present in the compounds	-	-	-	-	-	+	+	-	-	
1152	C-O stretch	Common bond present in the compounds	-	-	-	-	-	-	-	+	-	
1159	C-O stretch	Common bond present in the compounds	+	-	-	-	-	-	-	-	-	[40,43–49]
1176	CO-C-O	Fucoxanthin	-	-	+	-	-	-	-	-	-	
1180	CO-C-O	Fucoxanthin	-	-	-	-	+	-	-	-	-	
1372	C-N	Phycoerythrin/ Chlorophyll	-	-	-	+	-	-	-	-	-	
1377	C-N	Phycoerythrin/ Chlorophyll	+	-	-	-	-	+	+	-	-	
1403	-COO-	Chlorophyll/ Phycoerythrin	-	-	+	-	-	-	-	+	+	
1455	CH_2 scissoring	Carotenoid	-	-	-	-	-	+	+	-	-	
1457	CH_2 scissoring	Carotenoid	+	-	-	-	-	-	-	-	-	
1458	CH_2 scissoring	Carotenoid	-	-	-	+	-	-	-	-	-	
1615	C=C aromatic	Chlorophyll	-	-	-	-	-	+	-	-	-	

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1617	C=C aromatic	Chlorophyll	-	-	-	+	-	-	-	-	-
1618	C=C aromatic	Chlorophyll	-	-	-	-	+	-	-	-	-
1634	C=O	Phycoerythrin	-	-	-	-	-	-	-	-	+
1636	C=O	Carotenoid	-	-	+	-	-	-	-	-	-
1651	C=O	Phycoerythrin	-	-	-	-	-	-	-	+	-
1733	- C=O	Caro/chl	-	+	-	-	-	-	-	-	-
1738	- C=O	Caro/chl	-	-	-	-	-	-	+	+	-
1739	- C=O	Caro/chl	-	-	-	-	-	+	-	-	-
1742	- C=O	Caro/chl	+	-	-	-	-	-	-	-	-
2852	O-CH_3	Carotenoids	-	+	-	-	-	+	+	+	-
2853	O-CH_3	Carotenoids	+	-	-	-	+	-	-	-	-
2921	CH_2 stretch	Carotenoids	-	-	-	-	-	-	+	-	-
2922	CH_2 stretch	Carotenoids	+	+	-	-	+	+	-	+	-
2926	CH_2 stretch	Carotenoids	-	-	+	-	-	-	-	-	-
2936	CH_3 stretch	Carotenoids / Phycoerythrin	-	-	-	-	-	-	-	-	+
2937	CH_3 stretch	Carotenoids / Phycoerythrin	+	-	-	-	-	-	-	-	-
3010		Chlorophyll	-	-	-	-	-	+	-	-	-
3011		Chlorophyll	-	-	-	-	+	-	+	+	-

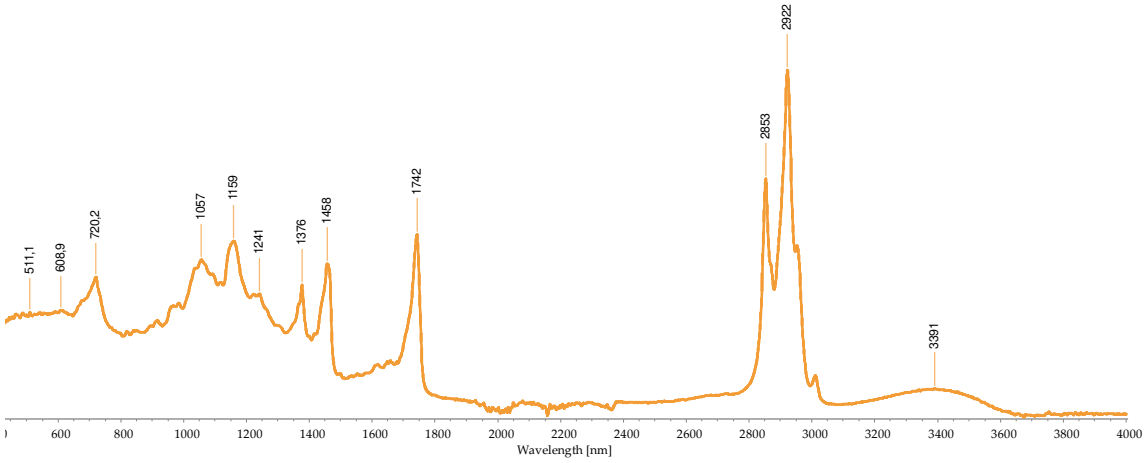

Figure 3. FTIR-ATR spectra of the extract A.

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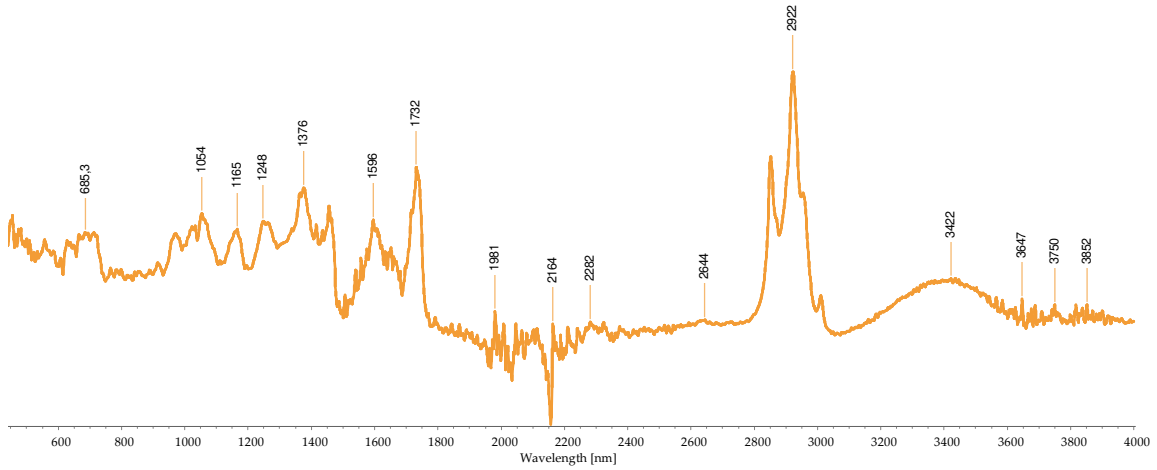

Figure 4. FTIR-ATR spectra of the extract B.

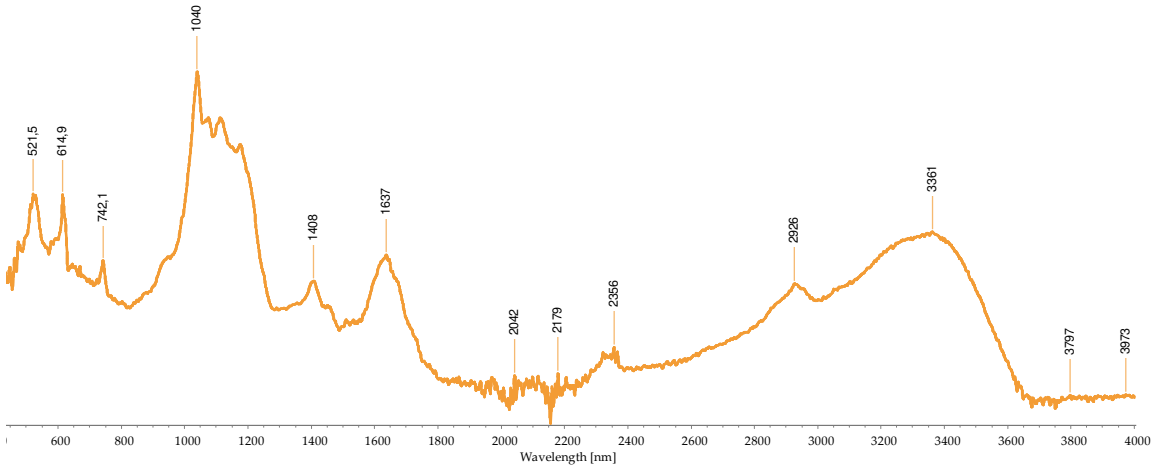

Figure 3. FTIR-ATR spectra of the extract C.

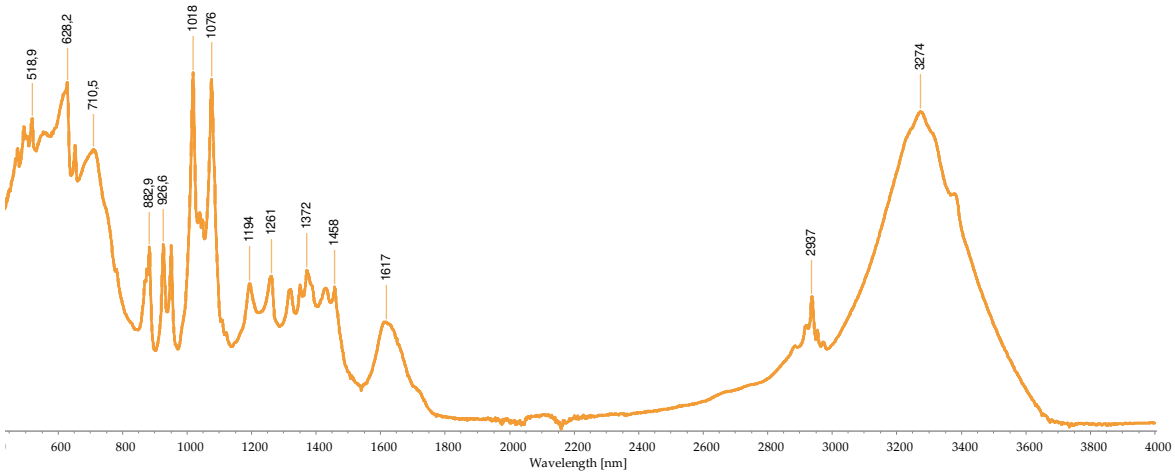

Figure 5. FTIR-ATR spectra of the extract D.

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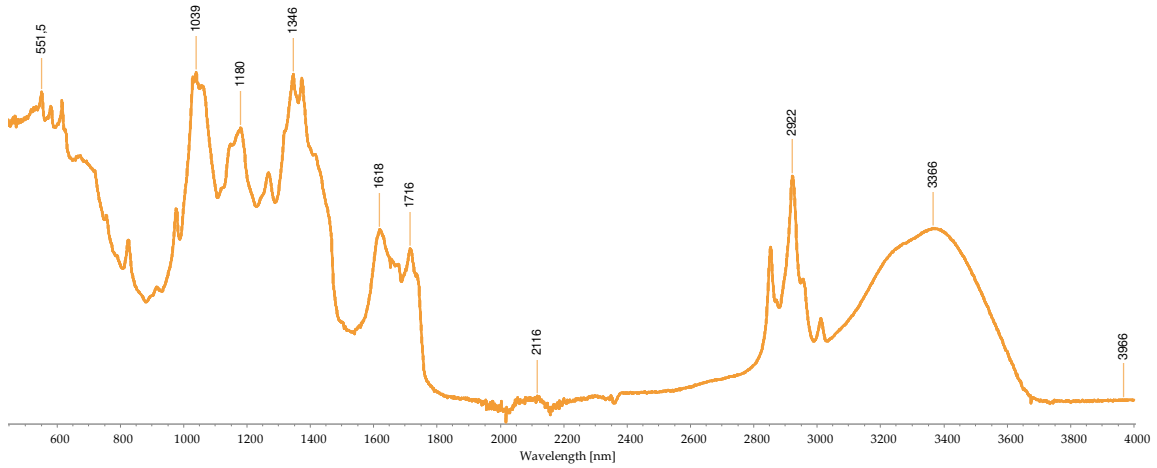

Figure 6. FTIR-ATR spectra of the extract H.

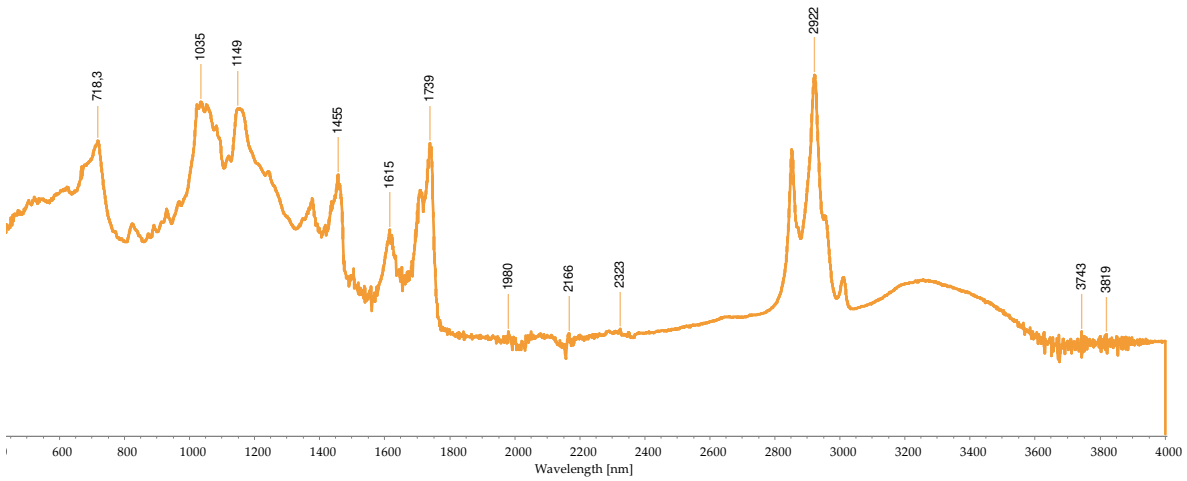

Figure 7. FTIR-ATR spectra of the extract I.

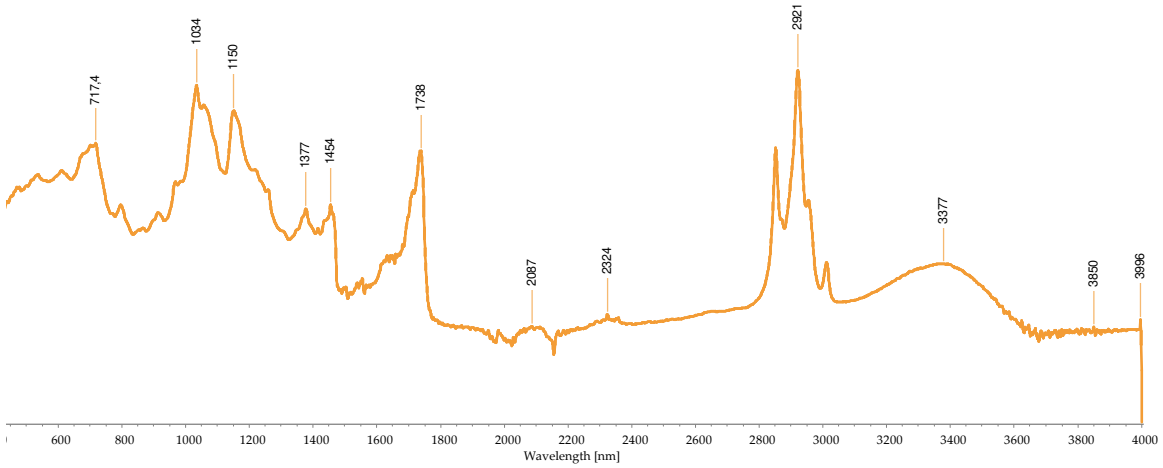

Figure 8. FTIR-ATR spectra of the extract M.

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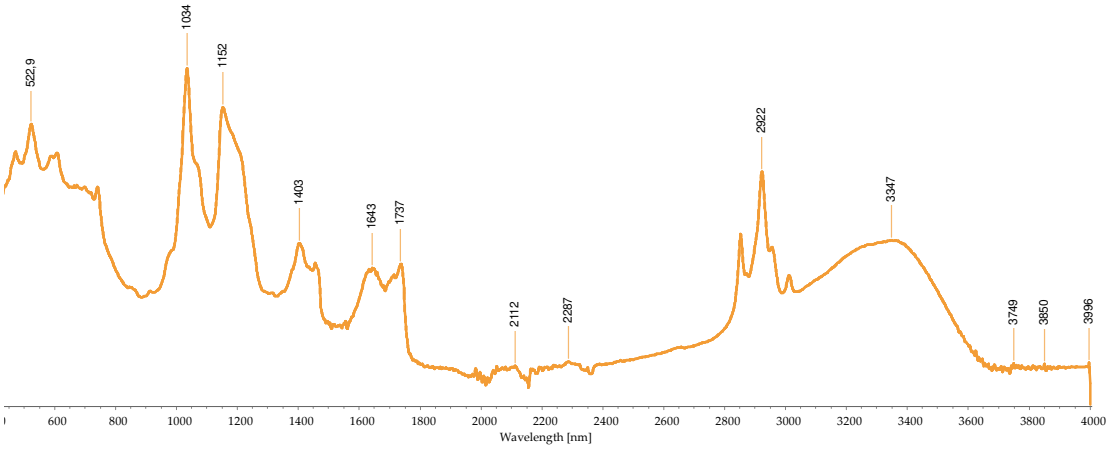

Figure 9. FTIR-ATR spectra of the extract R.

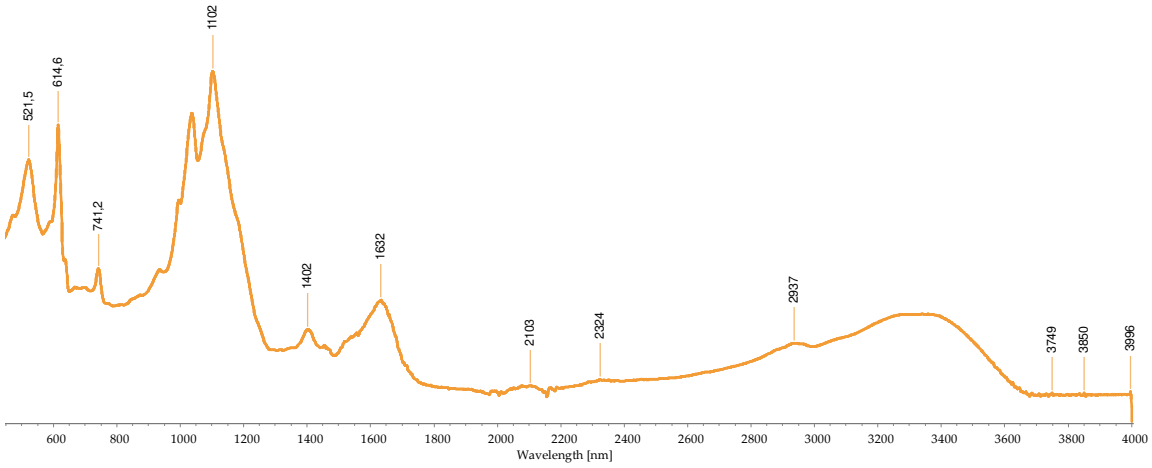

Figure 10. FTIR-ATR spectra of the extract S.

2.3. Concentration of the Isolated Pigments

The concentration of the purified fractions was determined from the dry weight of the extract (Table 6). The crude extract (D,I,M,R) has, as expected, the highest concentration.

Table 6. Pigment concentration from *F. vesiculosus* and *C. jubata.*

F. vesiculosus Pigments	Concentration (µg/mL)	*C. jubata* Pigments	Concentration (µg/mL)
A	266.66	**J**	133.33
B	233.33	**L**	100
C	166.66	**M**	766.67
D	1666.7	**O**	100
G	166.67	**R**	6900
H	200	**S**	11133
I	1833.3		

2.4. Antifungal Activity

After the confirmation of the extract's sterility, their anti-dermatophitic effect was evaluated on *Trichophyton mentagrophytes, T. rubrum* and *Microsporum canis* by microdilution assays following the

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standard methods according to the E.DEF 9.3 EUCAST. The extracts J, L, M and O did not shown inhibition of the growth of the fungi tested.

None of pigments extract of *F. vesiculosus* showed fungal growth inhibition, not even the crude extracts.

In Table 7 are represented the MIC values for pigment extracts R and S of *C. jubata*. Only these pigment extracts that showed antifungal activity against the fungi tested, *T. mentagrophytes*, *T. rubrum* and *M. canis*. These pigments were tested for three weeks.

Table 7. Minimum inhibitory concentration (MIC) of extract R and S and itraconazole for *T. mentagrophytes*, *T. rubrum*, *M. canis*, over three weeks (W).

Concentration		**µg/mL**						
Extracts		**R**			**S**			**Itra.**
Weeks		**W=1**	**W=2**	**W=3**	**W=1**	**W=2**	**W=3**	**W=1,2,3**
T. mentagrophytes	MIC	3450	>3450	>3450	2783.25	5566.5	5566.5	1
	MIC_{50}	1725	>3450	>3450	>5566.5	2783.25	2783.25	0.5
	MFC	3450	>3450	>3450	2783.25	5566.5	>5566.5	>1
T. rubrum	MIC	3450	3450	3450	2783.25	2783.25	5566.5	1
	MIC_{50}	1725	1725	1725	Nd*	Nd*	2783.25	0.5
	MFC	3450	>3450	>3450	2783.25	2783.25	>5566.5	>1r
M. canis	MIC	3450	>3450	>3450	2783.25	2783.25	2783.25	1
	MIC_{50}	862,5	>3450	>3450	1391.625	1391.63	1391.63	0.5
	MFC	>3450	>3450	>3450	2783.25	5566.5	5566.5	>1

*Not determined.

During the first week after the preparation of the extracts, the MIC_{100} observed for the crude ethanol extract R was 3450 µg /mL for *T. mentagrophytes*. The concentration that inhibits 50% of the growth, the MIC_{50} of the extract R was 1725 µg/mL and the MFC was 3450 µg/mL. For *T. rubrum*, the MIC and MFC of the crude extract was 3450 µg/mL. For *M. canis*, the MIC and MFC of 3450 µg/mL were only observed in the first week of the assay. Throughout the 3-week period of assays, the pigments seemed to lose their effect.

The extract S was able to inhibit the growth of all the fungi tested. The isolated pigments showed a MIC_{100} and MFC of 2783.25 µg/mL in the tree fungus tested, during the first week after the preparation of the extract.

It was made a control with itraconazole. The results of the MIC and MIC_{50} of the itraconazole are together in the same column because they did not vary over the weeks, hence the representation W=1,2,3. The MIC and MIC_{50} of itraconazole against *T. mentagrophytes*, *T. rubrum* and *M. canis* were 0,5 µg/mL. This data is supported with bibliography where the minimum inhibition concentration in dermatophytes normally is not higher than 1 µg/mL [50–52].

3. Discussion

With the continuous rise of fungal infections and the increase in fungal resistance to traditional antifungals, there is a need to find new treatment alternatives. Seaweeds are potential sources of novel therapeutic agents since they produce a wide range of secondary metabolites that, when compared to the ones produce by terrestrial plants, have an extraordinary molecular structure and pharmacological effects, including anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, among others [53–55].

During the extraction protocols, it was observed that the extraction of pigments from seaweeds depends on several variables including the solvent type, extraction duration, and samples state

 doi:10.20944/preprints202310.0724.v1

(whether fresh, dried, frozen, or milled) in accordance with previous reports [56,57]. The entire extraction process was thought up to obtain the most efficient method of extracting pigments. Due to the distance from the beach to the laboratory, it wasn´t possible to use the fresh seaweed, so the fresh biomass was stored in the freezer until further used. After thawing, the seaweeds were washed, as described in the methods, and dried in an air forced oven at 40ºC. The temperature of drying is important because high temperatures can compromise the process of extraction influencing the concentration of pigments extracted. Despite of some studies reporting the use of fresh seaweed, in this study it was used dried milled seaweed because of the bigger accessibility of the solvents to all the structures of the seaweed [57]. If fresh seaweed was used the solvent would not reach all the seaweeds cellular structures and the extraction wouldn't be as efficient.

As previously mentioned, the efficiency of pigment extraction depends on several factors and one of them is the solvent. It is important to test the behavior of the solvent used for the extraction in the different seaweeds because their efficiency can vary with the pigments that are pretended. For example, most of the carotenoids are fat-soluble, so to monetize the extraction it must be used fat solvents like, acetone, alcohols, esters, and hexanes [58]. In this work two solvents were used, ethanol 96% and acetone 99%. The aim was to see which one of them was more efficient in the pigment extraction. At the naked eye, both seem to extract the pigment from biomass until the exhaustion but in the column chromatography the acetone extracts showed to be better separated through the column. The number of fractions is also higher than the ones obtained with in the ethanol. With this analysis it was conclude that the best solvent to extract pigment was acetone. This is in agreement with the literature, in which other studies conclude that acetone is the best solvent to extract pigments in general [56,58–60]. Another detail observed through the weeks of assays was that despite of being solubilized in water, the pigments extracted with acetone remain more stable than those who were extracted with ethanol. After several days, the ethanol extracts start to show some deposit in the bottom of the falcon. The explanation for this observation wasn´t found in literature, so more studies must be done to understand this finding.

In the course of this work, it was possible to perceive that the process of identification and characterization of pigments is complex, and it is not possible to use only a simple technique to accomplish it. To obtain a characterization more consistent three techniques were used to the identification of the pigments, UV-visible spectrophotometry, FTIR and TLC. Due to the lack of information about the characterization of seaweeds pigments, these techniques complement themselves and gave a result more trustworthy.

The PBPs have several applications in biotechnology, biomedicine, pharmacology, dye, cosmetics, and food colorants [61]. In the pharmacology, it has shown a strong antioxidant effect against reactive oxygen species (ROS). Some *in vitro* studies showed the PBPs anti-inflammatory, anti-viral and anti-tumor activities [62]. The extract S was identified as a phycobiliprotein and was the one with the highest bioactivity This extract showed antifungal activity against all the dermatophytes tested. There are already some reports of the antifungal and antimicrobial activities of the PBPs. Righini et al. [47] described the antifungal activity of PBPs against spore germination and mycelial growth of the phytopathogen fungi *Botrytis cinerea*. This class of pigment also showed antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and others [63,64]. Crude ethanol extract from *C. jubata* harbors more than one pigment, so, it can be considered an enriched extract. This presented antifungal and fungicidal activity against *T. mentagrophytes*, *T. rubrum* and *M. canis* at 3450 μg/mL. Consistently, the fraction isolated from this crude extract also exhibited antifungal and fungicidal activity against the three dermatophytes tested at a lower concentration (2783.25 μg/mL). Nevertheless, the bioactivity of the extracts R and S decrease during the 3-week period of assays, demonstrating that during this period of time the pigments are not stable. Further studies are needed to unravel how to maintain the stability of the pigments preventing degradation. The chlorophylls are the most widely distributed pigments in every seaweed, and they are highly sensitive to heat, light, oxygen, and enzymes leading to their easy degradation and color alteration [65,66]. The color change of the chlorophyll can occur due to replacement of the magnesium atom in chlorophyll´s structure to hydrogen ions, causes the structure of chlorophyll to shift to

 doi:10.20944/preprints202310.0724.v1

pheophytin becoming olive brown color [65,67]. The mainly reasons for the carotenoid's degradation could be oxidation and isomerization, which reduce the redness and yellowness of the extracts. Normally, carotenoids oxidize in the presence of oxygen, however other factors such light, heat, metal ions, and enzymes can also speed up the process [65,68]. Despite of the lack of information about the PBPs in the generality there are starting to appear their application as food colorants and for that is necessary to understand the stability of this pigments [69,70]. Studies have been showing that PBPs are stable under basic pH, and in this case, they remain stable from 0 to 50 °C. The color of the PBPs remains stable during 20 days after the extraction. So, from this information and from the results observed during the work the PBPs are the most stable pigments present in seaweeds [71].

The tendency of using natural sources of pigments has been growing due to environmental safety, conservation, and awareness. The natural pigments obtained from native flora and fauna are a desirable source for human usage because they are non-toxic, biodegradable, and non-carcinogenic [72,73]. Due to this characteristic and the properties of the pigments these are being studied to integrate certain products/materials.

Often the colours of food are what catch the attention of consumers. Pigments have been studied for application in food industry because they are non-synthetic, safe food additives and because of their health properties [10]. Due to the antimicrobial properties shown by the pigments and because of the antifungal results presented above the pigments can be added to food products to increase their shelf life [21,54].

The textile industry is another sector where the use of the natural pigments has been growing most specifically in medical textiles. The hospital environment contains a huge amount of the microorganism and a great amount of textile materials (like uniforms, sheets, towels and so on) that accumulate microbes [7,23–25]. The emergence of studies on the antimicrobial properties of pigments makes their application in the textile industry more appealing, thus motivating the decrease in the use of heavy metals in this industry [7,72,74].

Due to the properties of the pigments, they can be integrated in the formulation of cosmetic products with various purpose being one of the them antimicrobial products, like antimicrobial creams [15,21,23,25,72,74]. The antifungal activity observed in our study indicates that the pigments extracted, characterize and selected might be used in topical applications to cure dermatophytosis.

4. Materials and Methods

4.1. Seaweed Harvesting Location

The seaweed specimens were harvested in the intertidal medio-littoral with predominance of rocky substrate with some intertidal pools, little tilted and exposed to the waves. *C. jubata* was harvest in Buarcos bay, located at 40°16′ North and 8°90′ West of Mondego Estuary and southern of Mondego Cape. *F. vesiculosus* was collected in Mondego Estuary, located at 40°08′ North, 8°50′ West. Both seaweeds were collected in the same day, on September 15th of 2021.

4.2. Biomass Treatment for the Pigment Extraction

After the harvesting, the samples were transported to the Marine Algae Laboratory hosted in the Coimbra University to be cleaned. The biomass was washed with the sea water, collected on the site of harvesting, to remove sands, stones, mollusks, and other specimens that may be mixed with the intended seaweed. In case the samples were not used immediately, they were put in plastic bags and stored in the freezer at -20 °C. For the biomass required to use immediately, after being washed with sea water, this is rinsed with sterile distilled water to eliminate salts [59,75].

Once eliminated the salts of the biomass, this was then dried. This step is important to remove all the water present in the samples. The seaweeds were then put in plastic trays, and taken to the forced air oven (Raypa) at 40 °C for 48 h [57].

 doi:10.20944/preprints202310.0724.v1

4.3. Preparation of the Sample for the Extraction of Pigments

The pigment extraction was adapted from various studies [76]. Several assays were performed to verify which method was more effective.

The first step for the extraction process, is the grinding of the biomass. The method used was by exhaustion, using two solvents. Samples were extracted with ethanol absolute (José Manuel Gomes dos Santos, Portugal), and pure acetone (Fisher chemical, Belgium). The purpose of using two solvents was to evaluate which one was more efficient and if the bioactivity of the pigments is affected by the solvents used for the extraction. To obtain the extracts, 5 g of milled and dried seaweed was covered with 100 mL of acetone and ethanol, respectively. This step was repeated three times with 1h between each extraction and all the extraction were in constant agitation. After the three extractions the extract was filtered with Gouch funnel (porosity: G3) and then concentrated in rotary vacuum evaporator (Witeg, Germany) until approximately 20 mL [10,40,59].

For the extraction of phycobiliproteins it was needed to prepare a solution of phosphate buffer of 0,1 M and pH 6.8. The extraction proceeded at the same way of the extraction of the other pigments, 5 g of seaweed was covered with 100 mL of phosphate buffer solution. After 1 h the sample was centrifuged for 20 min at 4000 rpm. The supernatant was collected, and the pellet was resuspended with another 100 mL of phosphate buffer solution. This step was repeated three times [77,78].

All the extractions were performed at room temperature (25 °C) and then store at 4 °C until further use.

4.4. Isolation and Characterization of Pigments

After the extraction, the pigments were isolated and characterized. For the isolation, it was preformed TLC and then Column Chromatography. TLC and Ultraviolet and Visible (UV-Vis) spectrophotometry were used to characterize the purified pigment. TLC method can be considered an isolation and characterization technique.

4.4.1. Chromatography Methods

Column Chromatography (CC) is the most common separation and purification technique, it can separate and purify liquid samples. With the aid of liquid mobile phase, a stationary solid phase adsorbs and separates the samples through it in column chromatography [79–81]. In this case, the pigments were separated according to their density. The different pigments have different polarities, and this characteristic allows them to adsorb in different regions of the column making possible to be separated. The elution solution used had different polarity to make the pigments desorbed the stationary phase and move throughout column.

For the separation and purification, a glass column of 50 mL was cleaned with acetone. After cleaning the column was added, to the bottom, a small piece of cotton and approximately 1 cm of sea sand. The stationary phase used was Silica (Silica gel, for chromatography, 0.060-0.200 mm, 60A, Acros Organics), 30 g were used and mixed with 40 mL of *n*-hexane solvent. The Silica doesn´t dissolve in the *n*-hexane, so it needs to be well mixed and poured almost immediately in the column. The solvent was collected in the bottom and then poured again into the top of the column to guarantee that all the Silica is removed from the sides of the column. After this, the column must be left undisturbed over night for the proper binding and prevent the existence of "holes" in the middle of the silica. Once the silica set, 5 mL of the crude extract were carefully introduced in the top of the column, so the pigments adsorb to the silica. After being bounded to the silica it was added the elution solvent *n*-hexane (Fisher chemical, Belgium): acetone (7:3, v/v). This eluent was poured frequently into the column for the separation of the pigments. As the extract runs along the column, it is possible to observe the separation of pigments due to the appearance of areas with different colors as can be seen in the Figure 7 After obtaining the fraction, there were stored in falcons at 4 °C until further use.

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4.4.2. Thin Layer Chromatography (TLC)

The affinity-based approach of TLC is used to separate the different fractions present in a sample. TLC is a flexible separation process that may be used to analyze both qualitative and quantitative samples [79,82,83]. In the development of this thesis, TLC was only used as a qualitative method. The TLC method was used for two reasons: 1) to evaluate if the fractions of pigments obtained from the column chromatography were in fact isolated; 2) to identify throw the retention factor (RF) the pigments present in which sample.

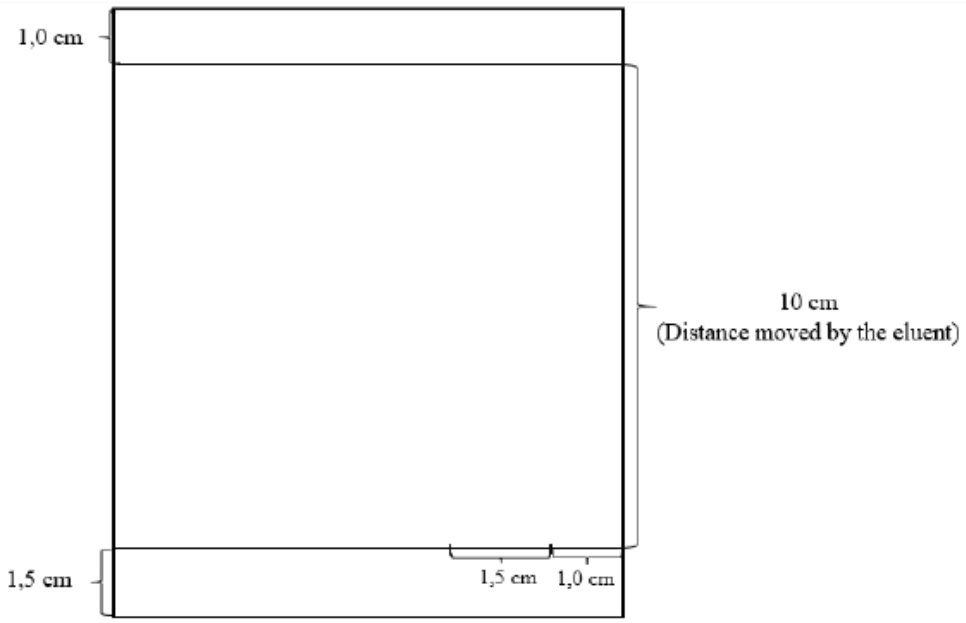

Figure 11. Representation of the measurements of a TLC plate.

The TLC method was adapted from Cotas et. al [40], with some modifications. For this method was used Silica gel plates (TLC Silica gel 60 F254, Supelco). The measures of the plates are represented in the Figure 7, and they change accordingly with the number of samples [40].

After isolating the samples, the next step was to activate the plates of silica gel (60 F254, Supelco) in the incubator at 120 °C for 5 min. Following the activation, it was applied 30 µL of each fraction obtained from the column chromatography. After the application of all the sample, the plate was introduced in a chromatography chamber with petroleum ether (Chem-lab NV, Belgium): acetone (7:3, v/v). The TLC was removed from the chamber when the eluent reached 1,0 cm from the top. After the evaporation of the eluent, at room temperature, the plate was observed at visible light. The pigments appear in different spots of the TLC due to their polarity and density. One way to identify the pigments present in the sample is by the calculation of the retention factor (Rf), which is defined as the ratio of the distance travelled by the spot of injection to the distanced moved by the eluent. The formula below is used to calculate the Rf values for each of the pigments saw [40,42].

$$RF = \frac{Distance\ moved\ by\ the\ pigment}{Distance\ moved\ by\ solvent}$$

4.4.3. Ultraviolet and Visible (UV-Vis) Spectrophotometry

Spectrophotometry is based on the interaction of eaves from the electromagnetic spectrum with molecules in the sample [84]. UV-vis is a sensitive molecular spectrophotometry technique that employs ultraviolet and visible light with wavelengths ranging from 200 to 780 nm. This spectroscopic approach relies on the extract absorption, scattering, diffraction, refraction, and reflection. UV and Vis light are only absorbed by chromophores, which are molecular functional groups in which electrons are stimulated at different frequencies [9,40,85].

 doi:10.20944/preprints202310.0724.v1

Pigments only absorb in particular wavelengths of visible light and reflect others. To be able to observe all the wavelengths and to have a clearer image the absorption spectrum of the pigments was recorded from 350 to 800 nm [76,86]. The spectrums were obtained from the spectrophotometer Spectra Plus (Molecular Devices)

4.4.4. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR is a low-cost method for examining the chemical bond of dried extracts. This method may be used to examine polysaccharides, pigments, and phenolic fractions, compound oxidation, and microplastics in seaweed. It is based on the vibration of the chemical bonds. When compared to chromatography, is less expensive and simpler to use, however it is less useful for biochemical quantification and quality analysis [79].

For the FTIR-ATR, the extracts were lyophilized (Alpha 1-2 LD plus, Christ) for 48 h. The FTIR-ATR spectra were recorded on Thermo Nicolet 380FTIR with smart Orbit diamond ATR accessory. All spectra are the average of two independent measurements from 400 to 4000 cm^{-1} with 64 scans, each at a resolution of 2 cm^{-1}.

4.5. Resuspension of the Pigment Fractions in Water

After the isolation and the selection of the pigments, they were resuspended in water to be used in the antifungal assays. This technique was based in the Cotas [76] and was realized in a rotary vacuum evaporator. Approximately 10 mL, 5 mL of distilled water was added to each faction of pigment. The water was added to the solvents for two reasons: 1) If the pigment was evaporated to exhaustion the pigment would attached to the walls of the balloon and only water would not be sufficient to resuspend them; 2) the point of ebullition of the water, *n*-hexane and acetone are different (100, 69 and 56 °C, respectively) so, as the solvents evaporate, the extract of pigments began to be in water since this is the last one to begin to evaporate.

4.6. Determination of the Concentration

For the determination of the pigment concentration and of the yield of the extraction process,1 mL of pigment was weighted after drying at 50 °C for 4 days.

4.7. Sterility Assays

To guarantee that the pigment extracts were sterile, they were inoculated in Potato Dextrose Agar (PDA) and Columbia agar plates and incubated for 7 days at 30 °C.

4.8. Antifungal Assays

4.8.1. Microorganism Culture and Growth Conditions

The fungal strains that were used in this study were *T. rubrum* IMF028, *T. mentagrophytes* IMF029, *M. canis* IMF035, *C. albicans* YP5314 and *C. parapsilosis* YP0515, fbelong to the Clinical Yeast Collection – University of Coimbra (CYC - UC) of the Institute of Microbiology of the Faculty of Medicine of the University of Coimbra.

T. mentagrophytes and *T. rubrum* were grown in (PDA, Difco) prepared following manufacturer´s instructions, and *M. canis* was grown in rice agar and incubated at 30 °C for 7 days.

4.8.2. Quantification of the Antifungal Activity

All the experiments were performed according the EUCAST E.DEF 9.3.1 standards. To obtain the fungal suspension, colonies were covered with 1 mL of sterile NaCl 0,85% solution. Then, the colonies were gently scraped and collected to a sterile falcon tube. This step allowed to obtain a suspension with spores and hyphal fragments and, to be able to count the number of spores present in the suspension it was needed to leave the hyphal fragments settle in the bottom of the falcon placed on ice. Once the hyphae settled, the supernatant was transferred to a different sterile falcon tube [87].

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The spore suspension was counted and diluted with NaCl 0,.5% to a concentration between $1x10^5$ CFU/mL and $2.5x10^5$ CFU/mL. Then, the suspension was diluted 1:10 with RPMI 1640 twice concentrated to obtain the final concentration of $2.5x10^5$ CFU/mL.

For the antifungal assays it was used sterile, disposable, 96 well microdilution plates with flat-bottom wells (Orange Scientific). 100 µL of inoculum was added to 100 µL of the extract. Different concentrations of extract were tested in a 2-fold dilution. The same process was made to the itraconazole (8 µg/mL). The multi-well plates inoculated with *Trichophyton* spp. were incubated at 35 °C and the multi-well plate containing *M. canis* were incubated at 30 °C. The tree plates were evaluated 4 days after the inoculation and the Minimum Inhibitory Concentration (MIC) was identified, as the lowest concentration at which an agent inhibits fungal growth. The EUCAST E.DEF 9.3.1 norm considers the MIC value as the concentration where no apparent growth is observed by the naked eye. For this study it was conducted three different assays with the same pigments

4.8.3. Minimum Fungicidal Concentration

The Minimum Fungicidal Concentration (MFC) is defined as the lowest concentration of an antifungal agent that induces 99 – 99.5% of fungi death [88]. To perform this assay, 30 µL of suspension of the well in which the inhibitory concentration was determined and of the wells with the two higher following concentrations were inoculated and spread in solid medium. The MFC´s for *T. mentagrophytes* and *T. rubrum* were determined in PDA medium while for *M. canis* this was done in rice medium. The plates were left for 4 days, at 35 °C in the case of the *T. mentagrophytes* and *T. rubrum* and the *M. canis* at 30 °C.

5. Conclusions

The present study reports for the first time the isolation and characterization by several tools (UV-visible spectroscopy, FTIR, TLC) of pigments of the seaweeds *F. vesiculosus* and *C. jubata*, as well as their antifungal activity against dermatophytes. This work allowed to understand that only an isolated pigment from *C. jubata* show antifungal activity against *T. rubrum*, *T. mentagrophytes* and *M. canis*, the extract S containing phycobiliproteins and the crude extract of ethanol R from which has several pigments.

These results demonstrate the possible application of seaweeds pigments as nutraceuticals, antimicrobials namely in antifungal treatments.

This work demonstrates that the purity rate of pigments is not a keystone for their antifungal activity. Were a crude extract and phycobiliproteins extract were the best extracts in the assays, above the others. Where the phycobiliproteins was a green extraction method and using FTIR-ATR and UV/V spectrophotometry characterization techniques accoupled with software program (which is already applied in the industrial) can be applied to the nutraceutical and natural pharmaceutical products.

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