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Bio-prospecting of macro-algae for potential industrial dyes

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Synthetic dyes have been used for different purposes in various fields, but their continued usage has presented both environmental and health challenges. Therefore, alternative safer dye sources are inevitable. Algae have different pigments with potential for exploitation and usage in different socio-economic sectors. The aim of this study was to bio prospect dyes from macro-algal species of the Indian Ocean marine ecosystem. Six algal samples were collected from Coastal beaches along the Kenyan Indian Ocean during the wet and dry seasons. The samples were processed and used for pigment extraction and screening. Pigments were extracted using ethanol, acetone, diethyl ether and hexane in sequential with distilled water. Subsequently, the crude extracts were analyzed for pigment component using spectrophotometry and qualitatively for presence of active components. From the six species, *Ulva reticulata* was the best dye producer in ethanol and distilled water. All extracts were coloured green except those from *Galaxaura subverticillata* which gave a dark red extract in 80% hexane and a brown extract in distilled water. The crude extracts also contained different active components, with phenols being the most common component in ethanol and acetone extracts. This study demonstrates that macro-algae species from the Indian Ocean ecosystem contain useful pigments for biotechnological exploitation. Future studies should focus on increasing the pigment content through genetic manipulation of macroalgae and analysis of the pigments using modern methods such as the gas chromatography-mass spectrometry (GC-MS).

Key words: Marine biodiversity, marine bio-resources, macro-algae, pigments.

INTRODUCTION

Algae are a wide group of pigmented eukaryotic organisms naturally inhabiting both fresh aquatic and marine ecosystems. They are divided into six wide groups (phyla) based on the dominating pigment which gives the algae its colour (Abayomi et al., 2009). Members of the *Chlorophyta* are green in colour due to the presence of abundant chlorophyll pigment. They contain chlorophyll *a*, *b,* carotenes and xanthophylls.

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Members of *Phaeophyta* comprise brown algae, which contain xanthophylls and chlorophyll *a*. The *Rhodophyta* are made up of red algae and contain chlorophyll *a*, phycoerythrin, phycocyanin, carotenes and xanthophylls. Members of class *Chrysophyta* are made up of diatoms and golden-brown algae, which contain chlorophyll *a* and carotenes. The *Pyrrophyta* consists of dinoflagellates and contain chlorophyll *a,* xanthophylls and carotenes. Members of the *Euglenophyta* are made up of euglena, which contain chlorophyll *a, b*, carotenes and xanthophylls (Bibeau, 2009).

Synthetic dyes are widely used in textile industry, leather tanning, paper production, food technology (Slampova et al., 2001), agricultural research, in light-harvesting arrays, photo electrochemical cells (Wrobel et al., 2001), and hair coloring among other sectors as enumerated by Forgacs et al. (2004). These dyes have an accumulated effect on long-term usage. Almost 89% of hydrogen peroxide hair dyes have been shown to be mutagenic, with half of the components showing various degrees of mutagenicity (Tai et al., 2016). Waste effluent from textile industry mainly consists of excess dyes washed off during the dyeing process and has been reported to cause the highest toxicity to marine life as compared to effluent from pulp and paper industry (Flohr et al., 2012). Allura red, a dye mostly used in baking and beverage industries, has been reported to cause adverse health effects in humans including allergies, cardiac disease and in some cases, multiple sclerosis upon long term use (Rovina et al., 2016). In a study carried out by Sener et al. (2011), Indigotin and methylene blue dyes were reported to cause acute generalized exanthematous pustulosis in some individuals. Thus, it is important to bio prospect for alternative safer dyes.

Due to health and environmental problems caused by the wide usage of synthetic dyes, most industries are considering the adoption of natural dyes and colorants. Recently, green microalgae have gained importance in industries to produce commercially important products such as food ingredients, fertilizers, bio plastics, pharmaceuticals, chemical feed stock and biofuels (Skjånes et al., 2013). Algal pigments have also been quantified using high throughput techniques such as high-performance liquid chromatography and spectrophotometry as an integral part of water quality monitoring and general experimental research on phytoplanktons (Thrane et al., 2015). Of great importance are the chlorophylls, xanthophylls and carotenoids which have been commercially exploited in the pharmaceutical, cosmetic and food industries as demonstrated by Wang et al. *(*2015). Additionally, a recent study by Moldovan et al. (2017) demonstrated the potential usage of phycoerythrin, a red pigment obtained from the macro-algal *Glacilaria* sp. in the textile industry to dye cotton fabrics. Algal pigments are easy to extract as they are either water or organic solvent soluble (Gupta et al., 2013). Chlorophyll and carotenoidss are fat soluble and can be extracted from thylakoid membranes with organic solvents such as acetone, methanol or dimethylsulfoxide. The phycobillins and peridinin are water soluble and can be extracted from algal tissues after the organic extraction of chlorophyll. Large scale extraction of algal pigments involves disintegration of the biomass, followed by treatment with an organic solvent mixture. The pigment can then be extracted from the supernatant of the solvent mixture by various methods including chromatographic methods.

Therefore, since there is dire need for alternative and safer natural dyes, the research team surveyed and documented macro-algal species from the Indian Ocean marine ecosystem while focusing on: (i) determination of solvent suitability for dye extraction among the selected macro-algal species, and (ii) evaluation of dye properties for potential industrial and medicinal applications.

MATERIALS AND METHODS

Study sites and sampling

The algal samples were collected from both public and private beaches along the Kenyan Indian Ocean during the dry season in January 2015 and during the wet season in August 2015. The sample sites included Pyrates, Nyali, Mama Ngina, Kibarani, Malindi, Watamu and Vasco da Gama beaches of the North coast as well as Diani beach in the south coast. Six macro-algal species were targeted as identified using morphological characteristics with comparison to the algae database (http://www.algaebase.org/) and a dichotomous key based on morphological characteristics matching those already documented in the algae encyclopedia. The species were identified as: Ulva *reticulata* (Nyunja et al., 2009), *Rhizoclonium grande* (Gupta, 2012), *Galaxaura subverticillata*, *Ulva pertusa*, *Chaetomorpha viellardi* and *Enteromorpha muscoides* (Silva et al., 1996). Wet algal samples weighing about 150 g were hand-picked, cleaned in sea water and preserved in sterile plastic bags inside cool boxes for transportation to the laboratory at Technical University of Mombasa, Kenya.

Sample preparation and dye extraction

Algal samples were rinsed using distilled water to remove mud and debris. Samples were then dried in a hot air oven maintained at 110°C for a period of 2 h to ensure complete drying. The dry algae were ground separately using a blender then refined to a powder using pestle and mortar. A sequential extraction with organic solvents and distilled water was carried out. First, 10 g of powder from each algae species were weighed and mixed with 100 ml of 80% organic solvent. Two polar solvents (ethanol and acetone) and two non-polar solvents (diethyl ether and hexane) were used. The setup was replicated three times and left in the dark for 24 h to maximize the extraction. Next, the liquid extract was strained using a muslin cloth then the extraction repeated for another 24 h using 100 ml of distilled water to extract the water-soluble pigments. The extracts from overnight incubation were vacuum filtered to obtain debris free dye extracts, then concentrated in a rotor vacuum evaporator (ROVA-2L/3L) to remove excess organic solvents. This was followed by centrifugation at 1800 rpm for 30 min to efficiently separate the organic solvent phase and the dye phase. The liquid dye extract was carefully pipetted from the organic solvent phase and used in subsequent analysis.

Determination of properties of dye extracts

The crude extracts were qualitatively tested for presence/absence of active compounds (glycosides, flavonoids, phenols, alkaloids, saponins and tannins), with prior removal of chlorophyll by saponification method in the presence of 1 M sodium hydroxide as described by Li et al. (2016). To 50 µl extract, 200 µl of 1% sodium hydroxide was added and the mixture was left in the dark at room temperature. The mixture was then centrifuged at 15000 rpm for 5 minutes and the clear layer of the extract pipetted into an Eppendorf tube for analysis. To test for the presence/absence of flavonoids, Shinodas test was used. For phenols, gelatin test was used. For alkaloids, Mayer's test was used. For tannins, phelic chloride test was used. For saponnins, bicarbonate test was used and for glycosides, Keller-kiliani test was used (Senguttuvan et al., 2014; Sheela, 2013).

The unsaponified extracts were tested for pigments composition using calorimetric spectrophotometry. This was done by making a 10x dilution of the concentrated extracts using the solvent used in extraction. The diluted sample was subjected to wavelength scanning using an absorbance spectrophotometer (T70 UV/VIS). Peaks of the absorbance wavelengths were recorded during the progress of the scanning.

RESULTS

Distribution of the macro-algal species

The adoption of conventional taxonomic principles revealed a varying distribution of the six different macro-algal species (*U. reticulata, U. pertusa, R. grande, E. muscoides, G. subverticillata* and *C. viellardi*) in the studied sites. Mama Ngina had four of the species used in the study while Vasco da gamma had three. The rest of the sampling sites were dominated by single species except, Diani beach which did not have any of the algae species. *U. pertusa* was present in four sites sampled: Mama Ngina, Pirates, Nyali and Vasco da Gamma beaches. *U. reticulata* and *C. viellardi* were present in Mama Ngina and Vasco da gamma sites. *R. grande* was exclusively found in Kibarani site as the dominating algal species. *G. subverticillata* was found in Mama Ngina and Malindi sites, while *E. muscoides* only occurred at Watamu site.

Solvent suitability for dye extraction

A comparison of all the extracts from the different solvents showed that, ethanol and distilled water are the best polar solvents for extraction of algal pigments while hexane is the best non-polar solvent for extraction. *U. reticulata* is the best dye producer, followed by *R. grande*, *U. pertusa* and finally *C. viellardi* (Figure 1).

A principle component analysis (Figure 2) showed that the concentration of extracts from *U. pertusa* and *R. grande* using acetone were not significantly different from each other but were significantly different from water extract of *G. subverticillata*. Likewise, *U. reticulata* and *E. muscoides* extracts of hexane and diethyl ether were not significantly different from each other but were significantly different from ethanol extract of *C. viellardi*.

Dye absorbance characteristics

A spectrophotometric analysis determined the minimum and maximum absorbance wavelength for each dye extract, suggesting the possible pigment components. A peak at 444 and 630 nm was present in all species extracts, suggesting a chlorophyll component which is characteristic of most algae (Table 1). Two peaks at 450 and 453 nm were observed in distilled water extracts of *G. subverticillata* and *R. grande.* Acetone extracts of *G. subverticillata* and *E. muscoides* had two similar peaks at 450 and 540 nm. *U. pertusa* acetone extracts had a clear absorbance peak at 480 nm.

Active components of algal dyes

Extracts from the different macro-algal species had different active compounds depending on the solvent used. Tannins were detected in ethanol and acetone extracts of *U. pertusa* and *U. reticulata.* Flavanoids were detected in hexane and diethyl ether extracts of *U. reticulata* and in diethyl ether extract of *R. grande.* Phenols were detected in all extracts of ethanol and acetone. Saponins were present in ethanol and acetone extracts of *U. reticulata, U. pertusa* and *R. grande.* Additionally, saponins were detected in distilled water and acetone extracts of *G. subverticillata.* Notably, alkaloids were only detected in acetone extracts of *U. reticulata* and *U. pertusa,* while Glycosides were present in distilled water extracts of *U. pertusa, R. grande* and *E. muscoides.*

DISCUSSION

The results of this study have shown that the Coastal Indian Ocean of Kenya harbor a diverse macro-algal species that are distributed in different locations. The availability of four of the algae species isolated at Mama Ngina could be attributed to the fact that the site is a shipment area. The ships could possibly harbor different algae species on their surfaces, which find their way into the site. Likewise, the relatively high algae diversity at Vasco da Gama could be attributed to the nature of the site. It is an estuary of the Sabaki River, thus could receive a variety of algae species harbored by the draining waters. The rest of the sites do not experience a mix up of water from different environments, hence the trend in dominance of one species in each site. On the other hand, a lot of human activities at the Diani beach could be responsible for the lack of algae species at the site. The availability of macro-algae species at different sampling sites in all the sampling seasons suggests that

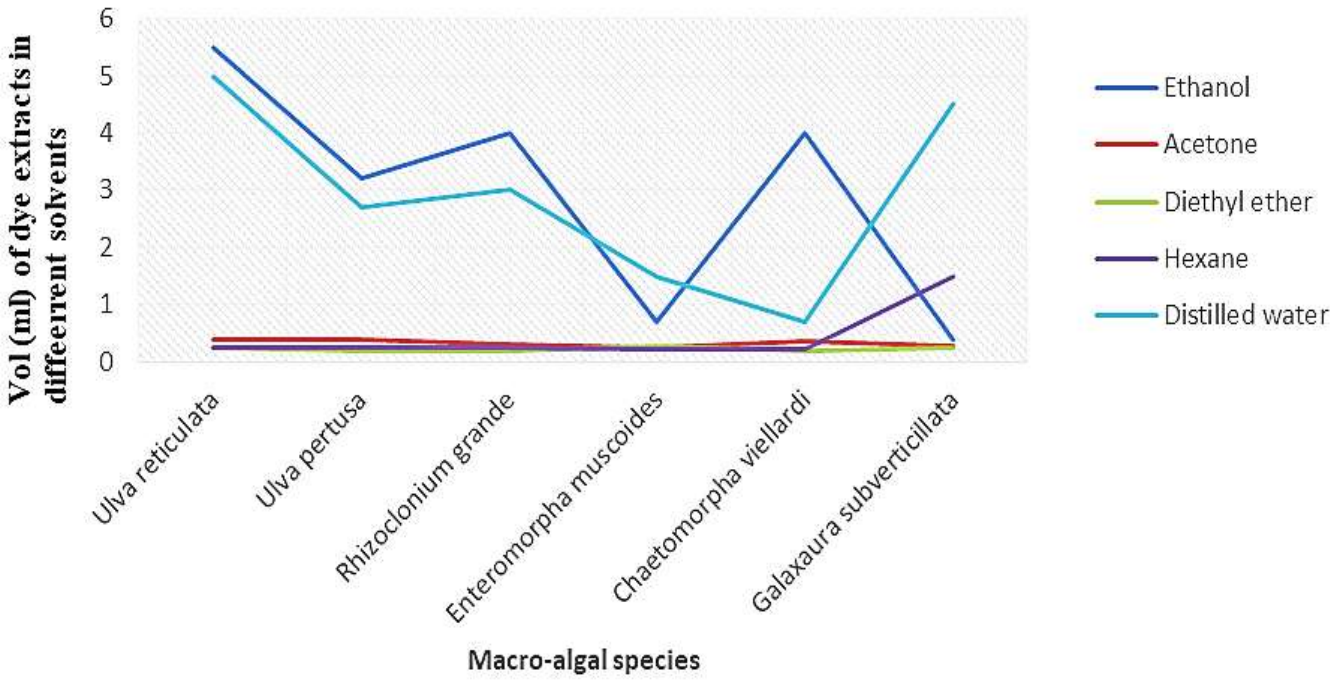

Figure 1. A line graph showing comparison of mean-volume of the dye extracts.

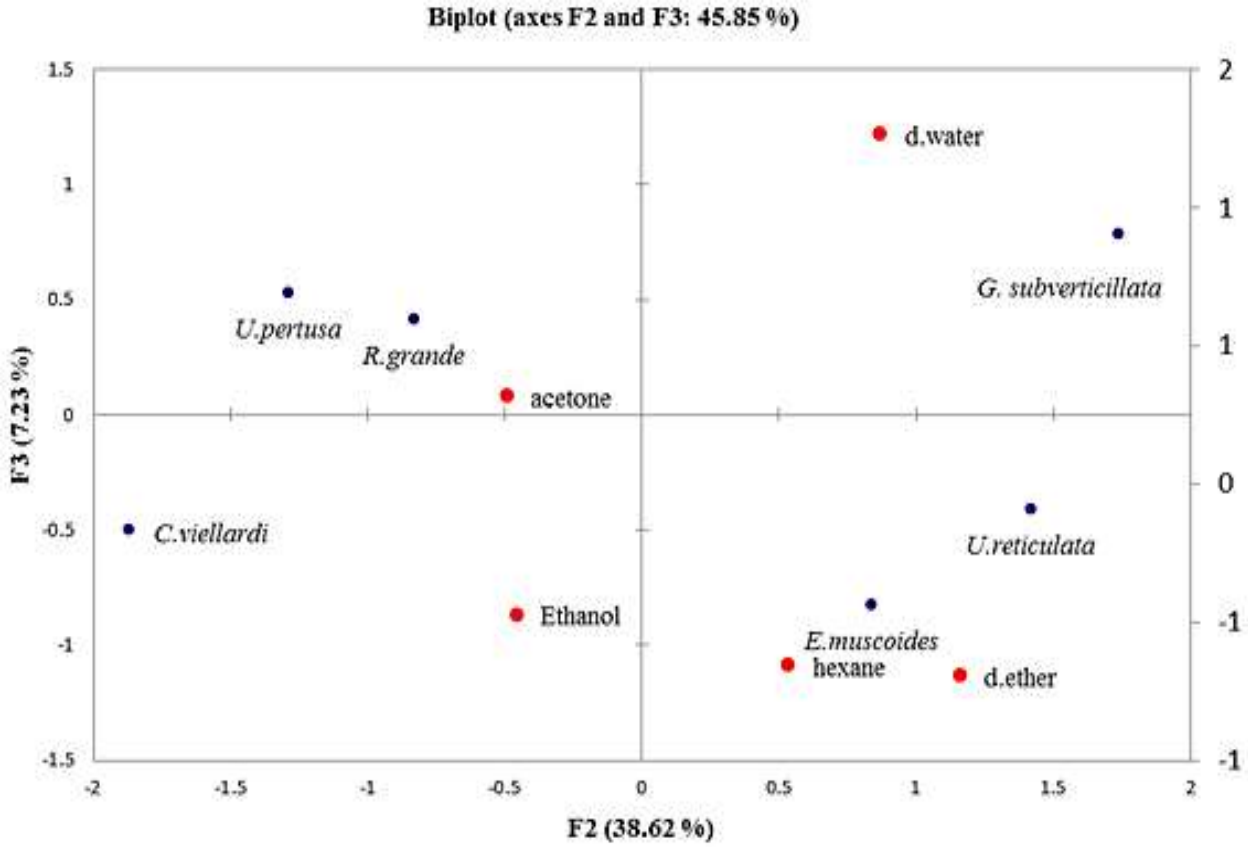

Figure 2. Principle component analysis of species versus extraction solvents. 'd.water' denotes distilled water; 'd.ether' denotes diethyl ether.

Table 1. Wavelengths and pigment identities of the extracts.

Species extract in solvent mixture	Wavelengths (nm) minimal and maximal	Pigment Identity
Ulva reticulata	444, 630	Chlorophyll *c*
	500, 550	Phycocyanin
Rhizoclonium grande	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	450, 453	Carotenoid
Chaetomorpha viellardi	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	500, 550	Phycocyanin
Galaxaura subverticillata	450, 540	Fucoxanthin
	450, 453	Carotenoid
Enteromorpha muscoides	444, 630	Chlorophyll *c*
	450, 540	Fucoxanthin
Ulva pertusa	430, 664	Chlorophyll *a*
	444, 630	Chlorophyll *c*
	480	Beta xanthin

although the algae could have found their way into the sites using different mechanisms, they have adapted to the conditions and as such become part of the ecosystem. It is also possible that the difference in macro-algae diversity at the sampled sites could be influenced by other environmental conditions including soil and water properties that are beyond the scope of this study.

Most industrial dyes are extracted using chemicals then dissolved in aqueous organic solvents or dimethyl sulfoxide (Valianou et al., 2009). In this study, the dyes were extracted using organic solvents in sequential with distilled water, therefore, by-passing the drying step during concentration. This has an advantage in that it is fast and efficient but has a drawback in that the amount of dyes can only be quantified based on the extent of pigment solubility in the extraction solvent, which could explain the diversity in volumes of pigment extracts observed in this study as shown in Figure 1. The pigment molecules dissolve completely in the solvent and cannot be evaporated to dryness without affecting the pigment structure. Anchoring the pigment extracts on ammonium sulphate is a potential way to capture them in a semi-solid state. The pigments form a dye cake that maintains their stability at room temperature

The principle component analysis in Figure 2, further emphasizes the variation in the solvents capability for pigment extraction. The non-polar solvents cluster together while the polar solvents vary greatly in their ability to extract pigments from different macro-algal species. All the three solvents have varying degree of polarity. It is no wonder each polar solvent cluster separately in the principle component analysis plot. The unmatched extraction efficiency observed with water and ethanol is attributed to the high polarity of these two solvents. Water, being highly polar, is efficient in extraction of lipophilic pigments (Warkoyo and Elfi, 2011). Similarly, ethanol has a high polarity as compared to other organic solvents due to presence of hydroxyl group in its structure, hence the efficiency in extraction as compared to the other solvents (Herrero et al., 2005). However, it should be noted that the high concentrates observed with ethanol and distilled water could be due to the good solvation ability of these two solvents and not necessarily high extraction capabilities. As such, more concentration of crude extracts with more sophisticated techniques like lyophilization is recommended to rule out this possibility.

Different solvents can capture varying pigments based on their hydrophobicity with respect to the solvent. Non-polar solvents are efficient in the extraction of hydrophobic pigments present in high amounts in red algae which could be the reason why hexane only performed best in *G. subverticillata*, a red algae. Polar solvents on the other hand are efficient in extraction of hydrophilic pigments for instance phycoerythrin, phycocyanin and carotenoids as described by Warkoyo and Elfi (2011). This is clearly seen in Table 1, where chlorophylls are observed in all organic solvent extracts, while carotenoids are only observed in distilled water

extracts. Chlorophyll is insoluble in water but soluble in organic solvents. Four types of chlorophylls (*a, b, c1, c2* and *d*) have been documented. Chlorophyll *a* is a blue green pigment that has an absorbance minima and maxima at 430 and 664 nm, while chlorophyll c absorbs at 444 and 630 nm (Torres et al., 2015). They were the only types of chlorophyll identified in most of the extracts, suggesting that they are very essential to most algae species. This is consistent with the findings of Chakraborty and Santra (2008), who observed different levels of chlorophyll *a* and *c* in eight marine macro-algae species.

Chlorophyll *d* was not detected in any of the extracts, although Murakami et al. (2004) suggests that this pigment may sometimes be found in green algae. Besides chlorophyll, the extracts also contain accessory pigments which include fucoxanthin, phycocyanin, phycoerythrin, xanthophylls and carotenoids. Fucoxanthin was only found in distilled water extracts of the red algae, *G. subverticillata* and the green algae, *E. muscoides*. This is because it is a water-soluble pigment. Other studies have shown that red algae contain this accessory pigment in their chloroplasts (Holdt and Stefan, 2011). Phycocyanin is a blue pigment with absorbance wavelengths of 500 and 550 nm (Eriksen, 2008). In this study, the pigment was only present in ethanol extracts of two green algae; *U. reticulata* and *C. viellardi*, although Rahman et al. (2017) reported the presence of phycocyanin in *Cyanidioschyzon merolae*, a red algae. The presence of carotenoids in acetone extract of *R. grande* is consistent with the findings reported by Yoshii et al. (2004), who also extracted carotenoids from the genus, *Rhizoclonium.* Similar findings were also published by Warkoyo and Elfi (2011). With the analysis of crude extracts, background peaks are inevitable.

Mycosporine-like-amino acids have been reported to be the leading contaminants in most pigment extracts (Karsten et al., 2005; Sonntag and Sommaruga, 2007). As such purification with high throughput techniques prior to analysis is highly recommended in future studies to rule out any possibilities of altered wavelength due to mycosporines. Qualitative tests showed presence of a wide variety of phytochemicals. This is supported by Ibañez et al. (2012) and Sahayaraj et al. (2014) they also reported availability of a wide variety of phytochemicals in algae. Although, the amounts of phytochemicals were not quantified in this study, positive qualitative tests suggest that significant amounts could be present, which allows their detection with the qualitative tests. The presence of tannins and flavanoids in this study is consistent with the findings by Thomas et al. (2011), who carried out test for phytochemical compounds in petroleum ether extracts of *U. reticulata.* Phenols were present in all extracts of ethanol and acetone probably due to their polarity caused by the presence of a hydroxyl group in their structure. Similar findings have been reported by Cox et al. (2010) who determined phenolic content of six species of sea Weeds and Vijayavel and Martinez (2010) who studied antimicrobial activity of phenol extracts of *Ulva* species.

Conclusion

The Indian Ocean of the Kenyan Coast harbors macro-algal species which contain useful pigments that can be harnessed for biotechnological applications. From this study, three dyes; green, dark red and brown were obtained from green and red macro-algae species respectively. The dyes have a unique combination of pigments and active components. Future comprehensive studies should be conducted using modern methods such as the gas chromatography-mass spectrometry (GC-MS) to analyze the different pigments detected. This will help to elucidate the molecular structures of the bioactive compounds for further usage. Additionally, we recommend genetic manipulation studies be conducted on such macro-algae species in order to increase their pigment content for industrial applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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