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Molecular Identification, Antimicrobial and Antioxidant Activities of the Tropical Seagrass *Halophila stipulacea* Grown in El-Bardawil Lake, Egypt

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Abstract: *Halophila stipulacea* a tropical seagrass entered the Mediterranean Sea from the Red Sea after the opening of the Suez Canal in 1869. Up to date, no genetic studies of *Halophila stipulacea* from El-Bardawil Lake are available. In order to verify the molecular identity of El-Bardawil lake isolates of *Halophila stipulacea*, employing Random Amplified Polymorphic analysis (RAPD) which carried out using six decamer primers, 26 amplified bands were produced, the highest number of amplified fragments (11 fragments) was produced by primer OP C04 and the OP B07 whereas OPC03 was the lowest primer (7 fragments). In this study, aqueous extract from *Halophila stipulacea* was assayed against some microorganisms using zone inhibition technique. The extract showed moderate activity against Gram-positive bacteria and high activity against filamentous *Aspergillus niger* fungus. In addition the antioxidant activity of the methanolic extract was studied and showed a moderate activity of the *Halophila stipulacea* seagrass reached 40%. The total phenolic content in the seagrass extract was 0.523 mg tannic acid equivalent /g.

Key words: *Halophila stipulacea*- El-Bardawil Lake - Random Amplified Polymorphic DNA analysis (RAPD) – antimicrobial activity - antioxidant activity.

INTRODUCTION

El-Bardawil Lake is an important fishing area for many species that use the seagrass beds as nursery grounds. Information concerning seagrasses of the Mediterranean Sinai coast is very scarce. Ruggiero and Procaccini, 2004 reported that *Halophila stipulacea* is a tropical seagrass entered the Mediterranean Sea from the Red Sea after the opening of the Suez Canal in 1869. Furthermore, the seagrasses of El-Bardawil Lake have not been studied since 1977, when Lipkin surveyed the seagrasses vegetation of Sinai. He reported that *Ruppia cirrhosa* occupied almost the whole western third of the lagoon, but may disappear completely during severe winters (Lipkin, 1977). Geneid and El-Hady, (2006) reported that two seagrass species *Ruppia cirrhosa* and *Cymodocea nodosa* were recorded in El-Bardawil Lake from spring 2003 to winter 2004.

The ecological importance of the marine flowering plants, seagrasses, is not only due to their extraordinarily high rate of primary production, but also to their ability to serve as nurseries, providing a habitat and protection from predators for many diverse benthic organisms. Consequently, several ecological studies on these species have been extensively carried out to determine the environmental "health" of coastal and estuary ecosystems (Dawes, 1998). Despite this, analysis and chemical elucidation of the secondary metabolite products from seagrasses has only recently been undertaken, highlighting antifouling, antibacterial, antiviral, anti-inflammatory, antiprotozoal, antidiabetic activities and cytotoxic bioactivities (Rowley *et al*., 2002; Hua *et al*., 2006 and Orhan *et al* ., 2006; Kumar *et al.*, 2008 and Kong *et al.*, 2008; Qi *et al*., 2008).

Seagrasses belonging to the genus *Halophila* are widely distributed along the western coasts of the Indian Ocean, Red Sea and South-eastern Florida coasts (Den Hartog, 1970). This genus has been the subject of many ecological studies whereas few phytochemical investigations have been conducted to date. The opening of the Suez Canal in 1869 facilitated the expansion of *H. stipulacea* into the Mediterranean Sea. In 2002 *H. stipulacea* became only the second seagrass to make a transoceanic migration with the discovery of a 300 m^2 mono-culture of *H. stipulacea* in a single bay on the Caribbean coast of Grenada, West Indies (Ruiz and Ballantine, 2004). It is one of the nine macrophyte species that are considered as invasive playing a significant role in the recipient ecosystems, taking the place of keystone species and being economically harmful (Boudouresque and Verlaque, 2002). The success of *H. stipulacea* as an invasive in the Mediterranean Sea can be attributed to its rapid vegetative expansion (Marba´ and Duarte, 1998), habitat flexibility (Pereg *et al*., 1994), tolerance of a wide salinity range (Por, 1971), adaptation to high irradiance (Schwarz and Hellblom, 2002), and ability to grow at depths from the intertidal zone to greater than 50 m (Beer and Waisel, 1981). The rapid growth and pervasiveness of *H. stipulacea* is similar to another aggressive invasive macrophyte, *Caulerpa taxifolia* (Boudouresque and Verlaque, 2002 and Anderson, 2005). The genetic and morphological variability was

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investigated in two western Mediterranean populations of *H. stipulacea* using RAPD-PCR markers (Procaccini *et al*. 1999). High genetic variability within and between the two populations was found.

Antioxidants in biological systems have multiple functions, including depending against oxidative damage and in the major signaling pathways of cells. The natural antioxidants (Phenolic compounds) play a key role in antioxidative defense mechanisms in biological systems and they act as free radical scavengers. (Gokce and Haznedaroglu 2008). McMillan *et al*., 1980 reported the presence of unidentified sulphated phenolic compounds from nine different species of *Halophila* including *Halophila stipulaceae*, unidentified sulphated and nonsulphated flavones from *Halophila ovalis* and *H. minor* complex (McMillan, 1986), flavones and flavone glycosides from *Halophila johnsonii* (Meng *et al*., 2008). Antibacterial activity against a series of microorganisms has been described for methanolic and ethyl acetate extracts of *H. ovalis* from the South Indian Sea (Kumar *et al*., 2008). Furthermore, chemoecological implications of the introduction of both exotic species, the mollusc *Syphonota geographica* and the seagrass *H. stipulacea*, in the Mediterranean Sea were discussed by Mollo *et al*. (2008).

The aim of this study is to detect the genetic signatures of *H. stipulacea* collected from the eastern part of El- Bardawil Lake and some of it's biological activities.

Method:

Study Area:

El-Bardawil lake is a shallow hypersaline lagoon located along the northern shore of the Sinai Peninsula between longitudes 320 40’ and 330 30’ E and latitudes 310 03’ and 310 14’ N. The lagoon, which is a natural depression, is separated from the Mediterranean Sea by a long, 300-1000 m wide, arrow-shaped sand bar. It has been described as a wetland of major international importance since it is a major bottleneck for migrant water birds passing through the Eastern Mediterranean region where wetlands are scarce (Meininger and Atta, 1990). Human impact on Bardawil lagoon is minimal due to the unexploited surrounding area, thus it is considered one of the cleanest water mass in the region (Varty *et al*., 1990). The lagoon extends for about 90 km and has a maximal width of 22 km. The lake is very shallow (from 0.5 to 2 m deep), and thus warms up very quickly during summers and cools easily during winters. Three openings (Boughaz) connect the lagoon to the sea. Two of these are man-made (the western Boughaz I and the Middle Eastern Boughaz II), while the third one is natural (eastern Boughaz III at the Zaranik protectorate). The main water supply of the lagoon comes from the Mediterranean Sea, and flows constantly through these three openings (Ibrahim *et al*., 1987).

Sample Collection:

Halophila stipulacea was collected from El Bardawil Lake in 2008. The seagrass covered a surface of the eastern part of lake. *H. stipulacea* formed a homogeneous population, in which no other large-sized macrophytes were observed in this part (Fig. 1).

Varela-Álvarez *et al*., (2011) showed that *H. stipulacea* consisted of thin creeping rhizomes, 0.25-0.30 cm wide, from which pairs of leaves were issued at regular intervals on the dorsal side. Pairs of leaves were attached to the rhizomes by petioles. The leaves were 3-5 cm long, 0.7- 0.8 cm wide, 1 or 2 at each shoot node, linear-oblong, thin and hairy. The margin of the leaves was spinulose and their surface was crossed by 4 to 14 pairs of veins. The rhizomes were irregularly branched and were fixed to the sandy substratum by roots issued at each node of the rhizomes.

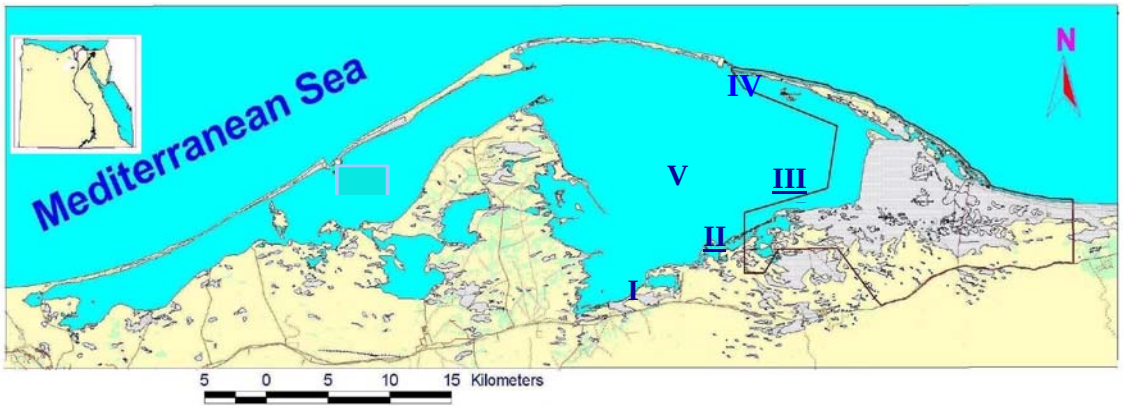

Fig. 1: Map showing *H. stipulacea* location in El-Bardawil Lake, North Sinai, Egypt.

Stations	Name	Latitude	Longitude
I	El-Telol	$31^0 04\backslash 37\backslash\backslash$	$33^0 13\backslash 36\backslash\backslash$
II	El-Rodh	$31^0 05\backslash 58\backslash\backslash$	$33^0 15\backslash 03\backslash\backslash$
III	El- Zaranik	$31^0 07\backslash 03\backslash\backslash$	$33^0 16\backslash 51\backslash\backslash$
IV	Boughaz II	$31^0 12\backslash 15\backslash\backslash$	$33^0 15\backslash 41\backslash\backslash$
V	M. El-Telol	$31^0 08\backslash 35\backslash\backslash$	$33^0 15\backslash 40\backslash\backslash$

***Random Amplified Polymorphic DNA Technique (RAPD*)**:

The seagrass *H. stipulacea* was collected from each chosen location, held at -4°C for subsequent DNA extraction. DNA extraction and purification were performed using a bead-beating method (Abd El- Razik *et al*., 2007). Six random decamer primers (Operon technologies, Inc.; Alameda, California, EUA) were initially screened for consistently reproducible and scorable amplified bands. PCR mixture was prepared according to the instructions provided with Taq polymerase; catalog# M8301) purchased from Sigma Chemical Co., St. Louis. Cycling conditions included an initial 4-min melt at 93°C followed by 44 cycles of 92°C for 1 min, 37°C for 1 min, and 72°C for 2 min. The final cycle was 92°C for 1 min, 37°C for 1 min, and 72°C for 8 min. PCR products were separated in 1.8% agarose gels with DNA Marker (100 bp DNA ladder, Invitrogen, USA) and stained with ethedium bromide, run in 1X TBE buffer at a constant voltage of 80 v. All tested primers produced strong, reproducible PCR products (bands) were selected for further study. The reproducibility of the RAPD markers was tested by performing PCR reactions with different concentrations (20 to 200 ng) of DNA template, with at least three independent DNA extractions from the same sample (Besnard *et al*., 2001).

Antimicrobial Activity of H. stipulacea:

In vitro antimicrobial susceptibility tests were performed using a panel which included both clinical pathogens and laboratory control strains, *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria), *Bacillus subtilis*, (Gram-postive bacteria), filamentous fungus as *Aspergillus niger* and yeast as *Candida albicans*, all of them belonging microbiology Lab. National Research Centre, Cairo. Antimicrobial activity of aqueous extract was tested by the paper disc diffusion method (Bauer *et al*., 1966). Antimicrobial activities were evaluated by measuring the diameter of inhibition zone (millimeters). Each test was carried out in duplicate.

Radical Scavenging Method For Antioxidant Activity:

The antioxidant was carried out by measuring the decolorizing capacity (bleaching) of each fraction against the stable 1,1-diphenyl -2- picryl- hydrazyl radical (DPPH). The color change due to radical scavenging can be measured using spectrophotometer at 517 nm (Deby and Margotteaux, 1970).

Determination of Total Phenolic Content:

The total phenolic content of the extracts was measured using the modified Folin-Ciocalteu method (Wolfe *et al*., 2003). An aliquot of the extract was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 g/ l) of sodium carbonate.

The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40^oC for colour development. Absorbance was then measured at 765 nm.

Samples from the extract were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content was expressed as mg/g Tannic acid equivalent.

Results:

All gels were analyzed using total lab (version 2.01). RAPD analysis (Fig. 2) was carried out using six decamer primers to observe the genetic signatures of the studied aquatic plant.

The RAPD primer codes and sequences are presented in Table (1) All examined samples followed the same PCR conditions to generate RAPD patterns. A total of 26 amplified bands were produced the number and size of fragments were amplified by these primers varied from 7-11 bands and 67.8-1804 bp. The highest number of amplified fragments (11 fragments) was produced by primers OP C04. Data were recorded as presence (1) or absence (0) of band products from the gel photographs (Table 2).

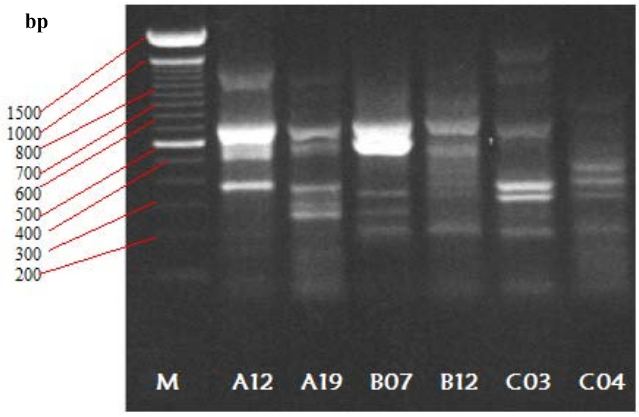

Fig. 2: Electrophoretic patterns of DNA amplified by A12, A19, B07, B12, C03 and C04 primers in studied macrophyte.

Table 1: Primer code, sequence, and total band generated.

Primer code	Primer sequence	Total BN
OPA12	3'-TCG GCG ATA G-5'	9
OP A19	3'- CAA ACG TCG G-5'	9
OP B07	3'- GGT GAC GCA G-5'	7
OP B12	3'- CCT TGA CGC A-5'	8
OPC03	3'- GGG GGT CTT T -5'	7
OPC04	3'- CCG CAT CTA C -5'	11

Table 2: The molecular weight and the presence or absence of band (0, 1) of population inferred from RAPD analysis.

M.W	(0,1)					
1804.348	0	0	0	0	1	0
1525	0	0	1	1	0	0
1261.46	0	1	0	0	0	0
1168.052	0	0	0	0	1	0
976.878	1	0	0	0	0	0
945.4	0	1	0	0	0	0
882.229	1	0	0	0	0	0
650	1	1	1	1	1	1
500	1	1	1	1	1	1
372.293	0	0	0	0	0	1
330.941	0	0	0	1	0	0
300	0	0	0	1	1	1
286.548	1	1	0	0	0	0
275.466	0	0	1	0	0	0
266.468	0	0	0	1	1	1
243.616	0	1	0	0	0	0
233.793	1	1	0	0	0	0
224.209	1	0	0	0	0	0
200	0	0	1	1	1	0
181.511	0	0	0	0	0	1
165.34	1	0	0	0	0	0
156.644	0	1	0	0	0	0
146.404	0	0	0	0	0	1
124.465	0	0	0	0	0	1
84.746	1	0	0	0	0	1
70	1	1	1	1	1	1

The antimicrobial activity of the aqueous extract of the seagrass was determined by measuring the diameter of zone of inhibition expressed in millimeters (mm), (Wilkinson , 2007). Aqueous extract of *H. stipulacea* was active against Gram positive bacteria (*Bacillus subtilis*), yeast (*Candida albicans*) and fungi (*Aspergillus niger*) with inhibition zone 15, 15 and 20 mm, respectively. Whereas the extract was not active against Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Table 3).

Table 3: The antimicrobial activity of *Halophila stipulaceae* extract.

Inhibition zone diameter (mm)	Tested microorganism				
	Bacteria			Fungi	Yeast
	Bacillus subtilis	*Pseudomonas aeruginosa*	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*
	15	-	-	20	15

Data shown that seagrass methanolic extract had a moderate antioxidant activity reached 40 %. The total phenolic content in the seagrass extract was 0.523 mg tannic acid equivalent /g.

Discussion:

The present study was carried out to determine the genetic identification of applied macrophyte using the Random Amplified Polymorphic DNA (RAPD) technique which offers a useful tool to investigate DNA polymorphisms. RAPD method are based on the assumption that bands of the same molecular weight, amplified with the same primer, represent homologous DNA sequences, several authors have demonstrated that the RAPD method is powerful tool in the assessment of genetic markers which are capable of discriminating between species or subspecies in a wide range of organisms, including fishes (Almeida and Sodre, 2002; Huang and Chen 2003; Hung *et al*., 2005; Qiubai *et al.,* 2006; Sofia *et al.,* 2008 and Khedkar *et al.,* 2009). These markers also represent an efficient and inexpensive way to generate molecular data and thus, they have been used successfully in various taxonomic and phylogenetic studies (Bektas and Belduz 2007). One of the major problems reported by users of RAPD is artifactual variation in banding patterns, though the magnitude of this problem varies greatly between laboratories (Penner *et al*., 1993). Nevertheless, the results presented here suggest that, with careful standardization of reagents and amplification conditions. The high representation of pseudo genes in the cloned fragments of the studied genome could be explained through a preferential PCR amplification due to the lower GC content of these sequences, which allows easier denaturation of the DNA fragment during the PCR cycling (Wagner *et al*. 1994).

In the present study, the analysis of RAPD data succeeded in screening the definition of the applied macrophyte populations. A total of 26 amplified bands were produced, the highest number of amplified fragments (11 fragments) was produced by primer OP C04 and the OP B07 whereas OPC03 was the lowest primers (7 fragments). Using the partial nuclear ribosomal DNA (rDNA ITS), Waycott *et al*. (2002) inferred relationships among species and biogeographic patterns in the 13 species of the seagrass genus *Halophila*. In fact, out of the 137 sequences of *Halophila* published in GenBank up to date, the rDNA ITS represent the vast majority, with only 5 sequences belonging to other molecular markers. Comparisons of the genetic polymorphism of this region between isolates from the Turkish coasts of the Aegean Sea and individuals from putative native (Red Sea) and introduced (Mediterranean) populations deposited previously in GenBank were performed. No intra-individual variability was found in the region considered among the isolates from Turkey (Varela-Àlvarez *et al*, 2011).

One of the main objectives of this work is to evaluate the ability of *H. stipulacea* extracts to produce bioactive compounds of potential therapeutic interest. The production of antimicrobial activities was considered to be an indicator of this seagrass to synthesize bioactive secondary metabolites. It is believed that there are many producers of natural compounds unexplored in aquatic environment that could be potential sources to the reduction or control of bacterial diseases (Özbay and Alim, 2009). Recently, the antibacterial activity of some water plants has been described to motivate researches with other species (Morales *et al.*, 2006; Al-Bayati and Al-Mola, 2008 and Fareed *et al*., 2008). One advantage in studying aquatic plants is their high growth rate, which facilitates the large-scale production of extracts for the purpose of purification of active compounds (Martins *et al*., 2004; Wolff *et al*., 2008). The effect of aqueaus extract was active against tested Gram positive bacteria *(Bacillus subtilis)*, what indicates its potential as an antimicrobial compound producer. While it showed no activity against Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). This may be attributed to the fact that cell wall in Gram positive bacteria consist of a single layer, whereas Gram negative bacterial cell wall is a multi layered structure bounded by an outer cell membrane (Yoa and Moellering, 1995). These results can be related to volatile antibacterial compounds in the extracts (Tüney *et al*., 2006). This non response of Gram negative bacteria to extracts might be due to masking of antibacterial activity by the presence of some inhibitory compounds or factors in the extracts (Choudhury *et al*., 2005). The variation of antibacterial activity of the extracts might be due to distribution of antimicrobial substances, which varied from species to species. (Lustigman and Brown, 1991). Adomi (2006) reported that, the water and ethanol extracts of the stem bark of some medicinal plants were tested on Gram-positive and Gram-negative bacteria, where ethanol extract was inactive against any of the bacterial tested while the aqueous extract was active. On the other hand, Ergene *et al*. (2006) investigated both ethanol and aqueous extract of *Heracleum sphondylium* sub sp. As antimicrobial activities against Gram-positive and Gram-negative bacteria and reported that, both extracts showed antimicrobial activity against Gram-positive bacterium *Staphylococcus aureus*. The efficiency of secondary metabolites produced by the aquatic plants *Salvinia auriculata* and *Hydrocleys nymphoides* (collected from pond located in Brazil) in inhibiting the growth of bacteria of bovine origin (Rossi *et al*., 2011). In addition, the extract of *H. stipulacea* showed antifungal activity against *Aspergillus niger* and *Candida albicans* yeast, in this connection Abd El-Hady *et al*., (2007) reported that powdered substance of *Cymodocea nodosa* was exhibited the highest antifungal activity than that of *Ruppia cirrhosa* (the two seagrasses collected from Bardawil Lake), Haroon (2006) reported that the methanol extracts of some common and widely distributed macrophytes from Manzalah lake, showed inhibitory effects on *Aspergillus parasiticus* growth.

The total phenolic content in the seagrass extract was 0.523 mg tannic acid equivalent /g. Methanolic extract of *H. stipulacea* showed moderate antioxidant activity (40 %). Kannan *et al*., 2010 reported that seagrasses of Gulf of Mannar biosphere reserve, South India have antioxidant activities ranged from 25-75%. In general, phenolic compounds were commonly found in plants and have reported several biological activities including potential antioxidants and free radical scavengers apart from primary defense role . Reports revealed that seagrasses especially their polyphenol have the antioxidant activity (Gokce and Haznedaroglu, 2008).

Conclusion:

It was concluded that, by using the Random Amplified Polymorphic DNA (RAPD) technique we were able to confirm the molecular identity of *H. stipulacea* from El-Bardawil Lake. Aqueous extract of this aquatic plant exhibited good antifungal activity and its methanolic extract had moderate antioxidant activity, thus makes it interesting for investigation of its natural products components.

Recommendation:

Further studies for the isolation of active substances from *H. stipulacea* to control the pathogens and the study of their mode of action on the microbial cell should be in concern. Also to provide complete data of the antioxidant activity and characterization of the principle antioxidant agents, which can be used to treat various oxidative stress-related diseases in plants.

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