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Faculdade de Ciências e Tecnologia (FCT)



Master Thesis Report

**Screening for biological activities with application in
aquaculture in halophytes from the Algarve coastline**



Thesis Co-supervisors

Ph. Dr. João Varela Ph. Dr. Luísa Custodio

Marine Biotechnology Group

João Pedro Palmeira (nº 27882)
Faculdade de Ciências e Tecnologia
Universidade do Algarve, Campus de Gambelas

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List of abbreviations and acronyms

BHT	Butylated hydroxytoluene
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HBSS	Hank's buffered salt solution
IC ₅₀	Half maximal inhibitory concentration
PMA	Phorbol myristate acetate
RPMI	Cell culture media developed at Roswell Park Memorial Institute
RT	Room temperature
SOD	Superoxide dismutase

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Resumo

A produção de organismos aquáticos em sistemas de aquacultura é frequentemente afectada pelo surgimento de doenças infecciosas, sendo a causa de perdas económicas, problemas ambientais e colocando em risco a segurança do consumidor. A forma convencional de controlo de agentes patogénicos era por meio de antibióticos e compostos sintéticos. Este procedimento teve no entanto consequências sob a forma de agentes patogénicos mais resistentes, assim como a contaminação química dos produtos de aquacultura e dos ecossistemas locais. Para evitar os efeitos nefastos do uso de antibióticos, estes foram grandemente substituídos pela vacinação em aquaculturas de países ocidentais. Existe no entanto ainda um uso massivo de antibióticos em sistemas de produção do terceiro mundo, pelo que são necessárias alternativas terapêuticas de baixo custo. Uma forma alternativa de conseguir o controlo de doenças em aquacultura, é pelo uso de compostos orgânicos bioactivos com propriedades antibióticas, antioxidantes e imuno-estimulantes. Estas biomoléculas existem naturalmente em bactérias, algas, fungos, plantas superiores e outros organismos. Ácidos gordos, nucleótidos, monossacarídeos, polissacarídeos, peptídeos, polifenóis e terpenóides são exemplos destas substâncias. Uma fonte promissora de compostos bioactivos são as halófitas ou plantas tolerantes ao sal. Em comparação com outras traqueófitas, as halófitas têm mais defesas moleculares, de forma a lidarem com o stress oxidativo do seu habitat. Muitas halófitas foram tradicionalmente usadas como um recurso alimentar e medicinal por culturas africanas e asiáticas. Este trabalho científico avaliou as

propriedades antibióticas, antioxidantes, imuno-estimulantes e quelantes de metais de: *Atriplex halimus* L., *Arthrocnemum macrostachyum* Moric., *Carpobrotus edulis* L., *Juncus acutus* L. e *Plantago coronopus* L., provenientes da costa Algarvia. As propriedades antibióticas foram testadas contra: *Listonella anguillarum*, *Photobacterium damselaepiscicida* e *Vibrio fischeri*. As propriedades imuno-estimulantes foram testadas com ensaios de cytochrome *c* e Griess em fagócitos provenientes de *Sparus aurata*. O extrato de éter de *J. acutus* inibiu o crescimento de *P. damselaepiscicida*. *A. halimus*, *A. macrostachyum*, *C. edulis*, *J. acutus* e *P. coronopus* exibiram propriedades antioxidantes e quelantes para cobre e ferro. Estas halófitas mostram potencial como fontes de compostos bioactivos com aplicação em aquacultura e outras áreas.

Palavras chave: Halófitas, Patógenos em aquacultura, Extrato de plantas, Imuno-estimulantes, Vibrio.

Abstract

Infectious diseases often hamper the production of aquatic organisms in aquaculture systems, causing economical losses, environmental problems and consumer safety issues. The conventional way aquaculture producers had to control pathogens was by means of synthetic antibiotics and chemicals. This procedure had consequences in the emergence of more resilient pathogens, drug contamination of seafood products and local ecosystems. To avoid the repercussions of antibiotic use, vaccination has greatly replaced human drugs in western fish farms. However there is still massive unregulated antibiotic use in third world fish farms, so less expensive therapeutic alternatives for drugs are desperately needed. An alternative way to achieve disease control in aquaculture is by using natural bioactive organic compounds with antibiotic, antioxidant and/or immunostimulant properties. Such diverse biomolecules occur in bacteria, algae, fungi, higher plants and other organisms. Fatty acids, nucleotides, monosaccharides, polysaccharides, peptides, polyphenols and terpenoids, are examples of these substances. One promising source of bioactive compounds are salt tolerant plants. Halophytes have more molecular resources and defence mechanisms, when compared with other tracheophytes, to deal with the oxidative stresses of their habitat. Many

halophytes have been used as a traditional food and medical supply, especially by African and Asian cultures. This scientific work evaluated the antibiotic, antioxidant, immunostimulant and metal chelating properties of *Atriplex halimus* L., *Arthrocnemum macrostachyum* Moric., *Carpobrotus edulis* L., *Juncus acutus* L. and *Plantago coronopus* L., from the Algarve coast. The antibiotic properties were tested against *Listonella anguillarum*, *Photobacterium damsela piscicida* and *Vibrio fischeri*. The immunostimulant properties were tested with cytochrome *c* and Griess assays on *Sparus aurata* head-kidney phagocytes. *J. acutus* ether extract inhibited the growth of *P. damsela piscicida*. *A. macrostachyum*, *A. halimus*, *C. edulis*, *Juncus acutus* and *P. coronopus* displayed antioxidant, copper chelating and iron chelating properties. These plants show potential as sources of bioactive compounds with application in aquaculture and in other fields.

Keywords: Halophyte, Pathogens in aquaculture, Plant extract, Immunostimulants, Vibrio.

1. Introduction

1.1 Aquaculture and infectious diseases

Aquaculture has greatly increased its relevance as colocar resumo em portuges a source of nourishment to satisfy the ever-increasing demand of the world food markets, displaying a fourfold growth in the last twenty years (Naylor *et al.*, 2000). Despite the fact that it reduces pressure on wild stocks and provides aquatic products year round, aquaculture causes environmental problems, which have yet to be solved in a great extent. Examples of ecosystem damage caused by fish farming ranges from nutrient excess in surrounding waters (Boyd & Massaut, 1999) to genetic contamination of wild populations (Crozier, 1993; McGinnity *et al.*, 2003). The need to exploit low commercial value fisheries to use as feed in aquaculture of top predatory species has also attracted criticism (Tidwell & Geoff, 2001).

A major issue facing aquaculture nowadays, present as well in industrial farming, is animal disease control and prevention. The high densities of stock kept in modern aquaculture operations and resulting waste products create a favourable medium for pathogen proliferation and transmission. Because of the stressful conditions to which they are exposed, the immune system of farmed animals is often compromised, further exposing them to infection. High stocking densities, handling, transport and low water quality, are major immunosuppressants (Shoemaker, Evans & Klesius, 2000; Saurabh & Sahoo, 2008). This is particularly relevant in fish and crustaceans. However, opportunistic pathogens can affect cultivated sessile molluscs as well (Ford *et al.*, 2002).

Infectious diseases are the largest single cause of economic loss in all kinds of aquaculture. Gram-negative bacteria of the genera *Aeromonas*, *Listonella*, *Photobacterium* and *Vibrio* are the most common pathogens, causing epizootic outbreaks in the large majority of cultured species. Pseudo-fungal diseases, caused by *Saprolegnia* spp. and *Fusarium* spp., are the second most common infections, with salmonids and crustaceans (Decapoda) being particularly prone to them (Marking, Rach & Schreier, 1994; Subasinghe, Bondad-Reantaso & McGladdery, 2000). Gram-positive bacterial infections (*Mycobacterium marinum*, *Streptococcus* spp.) can cause the loss of livestock, especially in freshwater aquaculture species like tilapia (*Oreochromis* spp.) (Shoemaker, Evans & Klesius, 2000; Ghittino *et al.*, 2003). Fungal, parasitic and viral

infections are widely spread in all forms of marine and freshwater aquaculture as well. This is a matter of great concern for the industry of aquaculture because of the associated economical losses, the environmental impacts on local ecosystems, and human consumption safety issues.

Several of these pathogens such as *Aeromonas* spp. (Gracey, Burke & Robinson, 1982), *Mycobacterium marinum* (Ghittino *et al.*, 2003), *Streptococcus* spp. (Appelbaum, 1992) and *Vibrio* spp. (Blake *et al.* 1979) can cause potential zoonosis, leading to serious public health questions over the safety of aquaculture products. Diseased stock in an aquaculture facility can have dire consequences for local marine ecosystems as well. Water borne pathogens can be transmitted to wild populations over a wide range of species, and not only to species directly related to the farmed stock. For example, the bacterium *Aeromonas salmonicida* largely affects aquaculture Salmonids, but it is capable of infecting many other fish groups (Bullock & Stuckey, 1975; Austin, 1993). In addition of captive animals and local wild populations sharing the same waters (except in facilities that are totally isolated like those inland) the problem of disease transmission is increased by escaped livestock. Disease-carrying feral fish have already caused serious declines or even the extinction of isolated wild populations that had little defence against pathogenic strains to which they were never exposed (Heggberget *et al.*, 1993). Under some circumstances, organisms are bred or raised not only for human consumption but also as head-start to boost wild populations. In this case, disease prevention is very important, as testing for the presence of pathogens in apparently and otherwise healthy stock is not completely flawless (Thorburn, 1996).

One of the ways aquaculture producers have to avoid and fight pathogens is by means of antibiotics and chemicals. Massive use of antibiotics was common practice in all forms of fish aquaculture both as a prophylactic and to treat infection. Their use was viewed as a more economic alternative than reducing stock densities and applying additional hygienic measures (Grave, Markestad & Bangen, 1996). However, the unregulated drug use had many harmful effects. Antibiotics, and other compounds used to treat disease, evade the aquaculture confinement via uneaten food, animal waste and lost stock. Commonly used antibiotics like quinolones are not easily biodegradable, remaining active for long periods of time in the sediment or being transported by currents to distant locations (Hansen, Lunestad & Samuelsen; 1992; Coyne, Hiney & Smith, 1997). Also it has been shown that residual amounts of antibiotics, metals and other compounds used to treat infectious diseases in livestock, remain in the tissues of

commercialised fish and invertebrates (Angulo, Nargund & Chiller, 2004; Sapkota *et al.*, 2008).

The selective pressure of antibiotics in fish tissue and in the surroundings of a fish farm has resulted in more virulent strains of bacteria, able to resist most antibiotics. Ever increasing doses and new drugs are needed to keep pathogens in check. Some of the metabolic weaponry acquired by these bacteria has the potential of being transmitted by horizontal gene transfer to terrestrial ecosystems, hypothetically reaching human pathogens (Kruse & Sørum, 1994; Rhodes *et al.*, 2000; Tendencia & De La Peña, 2001; Chelossi *et al.*, 2003). Also as aforementioned, several common pathogenic agents in aquaculture are zoonosis, creating a more direct route for human infection by antibiotic resistant bacteria (Ghittino *et al.*, 2003). Human groups especially at risk from the environmental contaminants and antibiotic resistant pathogen carriers include aquaculture workers, populations living near fish farms and consumers of aquaculture products (Sapkota *et al.*, 2008).

Because of the danger that their use represents to aquaculture, western world fish farms have largely replaced antibiotics by vaccination (Alderman & Hastings, 1998). Any synthetic compound to be used in the treatment of fish destined for human consumption must be on an approved substance list. Also quinolones and several other human prescription drugs have been outlawed for their use in aquaculture or are only available by veterinary order (Grave, Markestad. & Bangen, 1996). However in third world countries, which contribute to over 90% of the global production, chemicals like chlorine are widely used in all forms of mainstream aquaculture. These compounds are frequently used both as a way to treat infections and as prophylactics (Bakken, 2004; Bondad-Reantaso *et al.*, 2005). It is thus crucial to find alternative ways to control disease in fish farms that do not employ the use of artificial drugs and chemicals. Possible solutions must also be readily available at a low cost; otherwise it is unlikely they will be embraced in developing nations. Natural therapies and prophylactics are a new field in aquaculture, but have already shown promising results. One of such therapies is the use of probiotics, which have helped prevent *Vibrio* spp. infection on shrimp farms in Southeast Asia (Moriarty, 1997, 1998; Irianto & Austin, 2002).

1.2 Use of Bioactive compounds in aquaculture

Another recent pathway by which the health of aquaculture livestock can be maintained without man-made drugs is by using biologically active organic compounds, normally present in bacteria, algae, fungi, higher plants and other organisms. Fatty acids, nucleotides, monosaccharides, polysaccharides, peptides, polyphenols and terpenoids, are among the groups of biomolecules with interest for aquaculture. These substances may exhibit antibiotic, antioxidant and immunostimulant properties, and are easily included in specifically prepared diets or bioencapsulated in live food. Beneficial effects of incorporating these compounds in animal feed include enhanced growth, immunity, osmoregulation, stress tolerance in addition to larvae survival and quality. Livestock fed diets supplemented with bioactive substances also display, enhanced resistance towards bacterial, fungal, pseudo-fungal, parasitic and viral infections (Waagbo *et al.* 1993; Roberts, Davies & Pulsford, 1995; Burrells *et al.*, 2001; Esteban *et al.* 2001; Chotigeat *et al.*, 2004; Skjermo & Bergh, 2004; Bricknell & Dalmo, 2005; Kumar *et al.*, 2005; Cheng *et al.*, 2007; Dubber & Harder, 2008; Harikrishnan *et al.*, 2009; Zhang *et al.*, 2009).

Besides avoiding man made chemicals, the use of biologically active compounds in animal diets could have additional health benefits for the final consumer of aquaculture products. A major example is cold-water fish enriched with marine microalgal omega-3 fatty acids, which are highly beneficial to human health (Kris-Etherton, Harris & Appel, 2003, Ward & Singh, 2005).

Immunostimulating compounds positively modulate innate and adaptive immune responses in aquaculture fish. These substances trigger humoral and cellular defence mechanisms by acting on receptors and intercellular gene activation. Desirable effects on innate fish immunity include enhanced cytotoxic, phagocytic, respiratory burst, lysozyme and natural haemolytic complement, activities. These innate immune responses serve as first line of defence against pathogens and keep them in check until an efficient acquired immune response has been developed, leading to an optimised capacity to fight infectious diseases (Esteban *et al.* 2001; Dügenci, Arda & Candan, 2003; Skjermo & Bergh, 2004; Bricknell & Dalmo, 2005; Cheng *et al.*, 2007; Saurabh & Sahoo, 2008; Zhang *et al.*, 2009). Some compounds appear to significantly enhance the efficacy of vaccination (Burrells *et al.*, 2001; Saurabh & Sahoo, 2008).

Immunostimulating compounds can play an important role in reinforcing the immune systems of farmed animals in industrial aquacultures as prophylactics during high stress periods such as transport, vaccination, sea transfer and reproduction (Bricknell & Dalmo, 2005).

It has been suggested that natural antioxidant compounds protect living tissues and act as a buffer against oxidative stresses (environmental toxins, pollutants, stress by handling and overcrowding). Some organic compounds like L-ascorbic acid have dual properties, functioning both as a natural antioxidant and immunostimulant (Waagbo *et al.* 1993; Roberts, Davies & Pulsford, 1995; Chotigeat *et al.*, 2004; Rau *et al.*, 2004).

Related to antioxidant activity, some biomolecules exhibit desirable metal-chelating properties. These compounds bind to harmful metals in living tissues and, allow them to be excreted or, diminish their negative impact on organisms. In practice metal-chelating compounds could be used to detoxify fish (Cuajungco & Lees, 1998, Ebrahimzadeh, Pourmorad & Bekhradnia, 2008). Further research will most likely lead to new bioactive compounds with desirable properties.

The search for bioactive substances with interest for aquaculture does not have to be restricted to marine organisms. Many species of terrestrial vascular plants (Tracheophytes) produce compounds that can act as natural antioxidants, antibiotics and immunoestimulants for human and animal use. A great number of these plants have been used during millennia to treat human ailments (Kapil & Sharma, 1997; Nascimento *et al.*, 2000; Choudhary *et al.*, 2007). Terrestrial plant compounds have already showed anti-fungal and immunostimulant properties in aquaculture fish (Harikrishnan *et al.*, 2009; Zhang *et al.*, 2009).

Salt tolerant and salt requiring plants show signs of being a good source of new compounds with applications in aquaculture and other fields. Halophytes are a polyphyletic functional group that displays biochemical, physiological and morphological adaptations, allowing them to grow and reproduce in saline environments (O'Leary & Glenn, 1994; Manousaki & Kalogerakis, 2011). Being present in about half the flowering plant families, halophytes represent 2% of angiosperm species, approximately 6000 species (Le Houerou, 1993; Glenn, Brown & Blumwald, 1999).

Halophytes are exposed to several kinds of oxidative stress in their habitat (normally arid, semi-arid, salt marsh and coastal environments), including intense solar radiation, ionic and osmotic stress, which cause adverse effects on plant growth at physiological

and biochemical levels (Munns, 2002; Munns, James & Lauchli, 2006). These conditions promote the development of unusually high levels of reactive oxygen species (ROS) during otherwise normal cellular activities such as photorespiration and β -oxidation of fatty acids (Parida & Das, 2005). ROS have damaging oxidative effects on macromolecules such as proteins and nucleic acids and may cause peroxidation of membrane lipids. To avoid the harmful effects of oxidative stress, halophytes rely on protective bio-active compounds and physiological adaptations (such as salt exclusion, excretion, succulence, transport and compartmentalization and osmotic adjustment). Organic compounds present in salt tolerant plants include natural antioxidants (ascorbic acid, carotenoids, glutathione, methylated metabolites, phenols, polyphenols, thiols and thioredoxin) and ROS scavenging enzymes (ascorbate peroxidase, catalase, glutathione peroxidase and superoxide dismutase). These biomolecules are present in larger amounts in halophytes when compared to most land plants and their capacity to detoxify radicals under conditions of salt stress is an important stress-tolerance adaptation (Tsugane *et al.* 1999; Ghanem *et al.*, 2010). Related with their origin in different higher plant families, halophytes differ in their degree of salt tolerance. Plants with an increased tolerance to salinity (such as *Salicornia bigelovii* Torr. (Chenopodiaceae), which is able to grow at twice seawater salinity) exhibit a higher ROS scavenging activity (Flowers, Troke & Yeo, 1977; Bowler, Van Montagu & Inze, 1992). Halophytes also possess osmotically active compounds (such as saccharides, polyhydroxyalcohols, organic acids, amino acids, betaines, and tertiary sulfonium substances) that decrease the osmotic potential, protect biomolecules against the damaging effects of salinity and maintaining homeostasis (Arabawa & Timasheff, 1985; Gagneul *et al.*, 2007; Ghanem *et al.*, 2010). Any of these biomolecules has potential for its use in aquaculture as natural antioxidants, antibiotics, immunostimulants and even for their nutritional value. They also could hold future interest for biomedical sciences.

1.3 Halophyte Species

The xero-halophytes *Arthrocnemum macrostachyum* Moric. (glaucous glasswort), *Atriplex halimus* L. (Mediterranean saltbush) and *Carpobrotus edulis* L. (sour fig or Hottentot's fig), and the halophytes *Juncus acutus* L. (spiny rush) and *Plantago coronopus* L. (buck's horn plantain), can be considered potential sources of novel bioactive molecules with applicability for human medicine and aquaculture fields (**Figs. 1. and 2.**).

A. macrostachyum and *A. halimus* (Amaranthaceae) are native Circum-Mediterranean species (Al-Saleh *et al.*, 1997, Andueza *et al.*, 2005). *C. edulis* (Aizoaceae) is a South African species, now established in several Mediterranean climate countries, namely coastal California, Western Australia and Mediterranean Europe countries, including the coast of Portugal where is considered invasive (Collins & Scott, 1982; Draper *et al.*, 2003; Moragues & Traveset, 2005). *J. acutus* (Juncaceae) is a cosmopolitan species, native to most Palearctic (Kirschner *et al.*, 1999). Originally a Eurasian, Middle East and North African species, *P. coronopus* (Plantaginaceae) is now cosmopolitan as well (Braza, Arroyo & Garcia, 2010). In Portugal these species are found mainly in coastal and salt marsh habitats.

In their native ranges *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus* are used for traditional phytomedicine purposes. Traditional African (*C. edulis*), Chinese (*J. acutus* and *P. coronopus*), Middle-East and Moroccan medicine (*A. macrostachyum*, *A. halimus*, *J. acutus* and *P. coronopus*) have adopted these halophytes to treat a wide range of medical conditions. These include the treatment of gastrointestinal, hepatic, oral, respiratory, tumoral, urinary tract and skin diseases (Van der Watt & Pretorius, 2001; Chiang *et al.*, 2002, 2003; Chen & Chen, 2004; Springfield *et al.*, 2003; Gálvez *et al.*, 2005; Khan *et al.*, 2006; Springfield & Weitz, 2006; Sassi *et al.*, 2007; Beghalia *et al.*, 2008; Daoudi *et al.*, 2008; Buwa & Afolayan, 2009; Rahman *et al.*, 2011; Metíner, Özkan & Ak, 2012). Besides *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus*, other closely related species of halophytes in the genera *Arthrocnemum* (90 species in Salicornioideae, the phylogenetics of *A. macrostachyum* are not fully resolved), *Atriplex* (250 species), *Carpobrotus* (12 species), *Juncus* (300 species) and *Plantago* (265 species) are used for their medicinal



Fig. 1. General view of featured halophytes and their reproductive morphology. (A) Glaucous glasswort *Arthrocnemum macrostachyum*. (B) *A. macrostachyum* inflorescence. (C) Mediterranean saltbush *Atriplex halimus*. (D) *A. halimus* inflorescence. (E) Sour fig *Carpobrotus edulis*. (F) *C. edulis* flower.

properties (**Table 1.** to **Table 4.**) (Willis, 1980; McArthur & Sanderson, 1984; Kirschner *et al.*, 1999; Springfield & Weitz, 2006; Saleh, 2011). *Plantago major* L. has been the subject of most studies as traditional Chinese medicine herb (Mouhajir *et al.*, 2001).

In some cases the ancient knowledge on these and other medicinal plants, has been lost or limited to certain ethnicities (Batanouny, 2001). The therapeutic ethnobotanical use of these species has attracted the interest of biochemistry researchers, for the prospect of developing new drugs and therapies

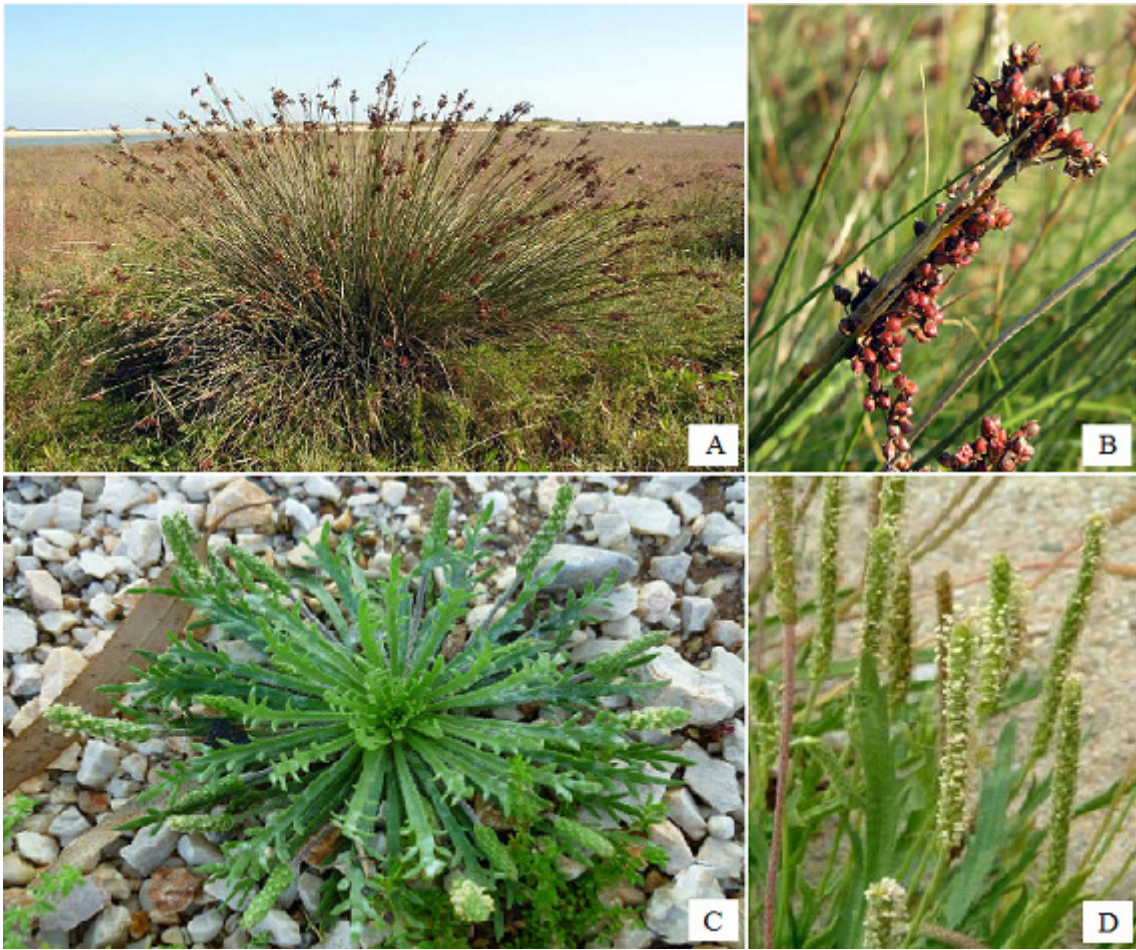


Fig. 2. General view of featured halophytes and their reproductive morphology (part two). (A) Spiny rush *Juncus acutus*. (B) *J. acutus* inflorescence. (C) buck's horn plantain *Plantago coronopus*. (D) *P. coronopus* inflorescence.

Table 1. Traditional uses, *in vitro* properties and bioactive compounds of medicinal glassworts (*Arthrocnemum* spp.).

Halophytes	Traditional uses	<i>In vitro</i> Properties	Bioactive Compounds
<p><i>A. glaucum</i> (Moric.) K.Koch</p> <p><i>A. indicum</i> Willd.</p> <p><i>A. macrostachyum</i></p>	<p>Alexipharmic (<i>A.indicum</i>) (Agoramoorthy <i>et al.</i>, 2008)</p> <p>Antibiotic (<i>A macrostachyum</i>) (Rahman <i>et al.</i>, 2011)</p> <p>Seed oil production (<i>A. glaucum</i>) (Khan <i>et al.</i>, 2006)</p>	<p>Antibiotic activity: <i>Bacillus cereus</i>, <i>Clostridium</i> <i>perfringens</i>, <i>Listeria ivanovii</i>, <i>Klebsiella pneumonia</i>, <i>K. oxytoca</i>, <i>Staphylococcus</i> <i>aureus</i>, <i>S. epidermis</i> and <i>Yersinia enterocolitica</i> (<i>A macrostachyum</i>) (Rahman <i>et al.</i>, 2011)</p> <p>Antifungal activity (<i>A. glaucum</i>) (Radwan & Hamdy, 2004)</p> <p>Hypoglycaemic activity (<i>A macrostachyum</i>) (Shabana <i>et al.</i>, 1990)</p>	<p>Alkaloids, anthraquinones, coumarins, flavonoids, saponins, sterols, tannins and triterpenes (<i>A. glaucum</i> and <i>A macrostachyum</i>) (Al-Saleh <i>et al.</i>, 1997) (Radwan & Hamdy, 2004)</p>

Table 2. Traditional uses, *in vitro* properties and bioactive compounds of medicinal salt bushes (*Atriplex* spp.).

Halophytes	Traditional uses	<i>In vitro</i> Properties	Bioactive Compounds
<p><i>A. confertifolia</i> (Torr. and Frem.)</p> <p><i>A. halimus</i></p> <p><i>A. hortensis</i> L.</p> <p><i>A. semibaccata</i> R. Br.</p> <p><i>A. stocksii</i> Boiss.</p> <p><i>A. vestita</i> Thunb.</p>	<p>Antibiotic and anti-inflammatory (<i>A. hortensis</i>, <i>A. semibaccata</i> and <i>A. stocksii</i>) (Sassi <i>et al.</i>, 2007)</p> <p>Arthritis and gastrointestinal conditions (<i>A. halimus</i>) (Beghalia <i>et al.</i>, 2008)</p> <p>Human nourishment (edible leaves) (<i>A. hortensis</i>) (Khan <i>et al.</i>, 2006)</p>	<p>Antibiotic activity: <i>B. cereus</i>, <i>B. subtilis</i>, <i>L. ivanovii</i>, <i>K. pneumonia</i>, <i>K. oxytoca</i>, <i>S. aureus</i> and <i>S. epidermis</i> (<i>A. halimus</i> and <i>A. vestita</i>) (Rahman <i>et al.</i>, 2011) (Abaas & Isa, 2011)</p> <p>Antioxidant activity (<i>A. hortensis</i>) (Bylka, Stobiecki & Frański, 2001)</p> <p>Anticarcinogenic Activity (<i>A. confertifolia</i>) (Capua <i>et al.</i>, 2010)</p> <p>Antifungal activity (<i>A. halimus</i>) (Abd-Ellatif, Rahman & Deraz, 2011)</p> <p>Hypoglycaemic activity (<i>A. halimus</i>) (Aharonson, Mishkinisky & Sulman, 1969)</p>	<p>Flavonoids, glycosides, proteins and triterpenes (<i>A. halimus</i>, <i>A. hortensis</i> and <i>A. stocksii</i>) (Siddiqui <i>et al.</i>, 1994) (Bylka, Stobiecki & Frański, 2001) (Rahman <i>et al.</i>, 2011)</p>

Table 3. Traditional uses, *in vitro* properties and bioactive compounds of medicinal ice plants (*Carpobrotus* spp.) and spiny rushes (*Juncus* spp.).

Halophytes	Traditional uses	<i>In vitro</i> Properties	Bioactive Compounds
<p><i>C. edulis</i></p> <p><i>C. quadrifidus</i> (L.) L. Bolus</p> <p><i>C. mellei</i>(L.) L. Bolus</p> <p><i>C. muiirii</i>(L.) L. Bolus</p>	<p>Antibiotic and antiinflammatory</p> <p>Treatment of gastrointestinal, oral, respiratory (tuberculosis) and skin conditions</p> <p>Human nourishment (edible fruit)</p> <p>(<i>C. edulis</i>, <i>C. quadrifidus</i>, <i>C. mellei</i> and <i>C. muiirii</i>) (Van der Watt & Pretorius, 2001) (Springfield <i>et al.</i>, 2003) (Springfield & Weitz, 2006) (Buwa & Afolayan, 2009)</p>	<p>Antibiotic activity: <i>B. cereus</i>, <i>B. subtilis</i>, <i>Moraxella catarrhalis</i>, <i>Mycobacterium aurum</i>, <i>M. tuberculosis</i>, <i>M. smegmatis</i>, <i>Pseudomonas aeruginosa</i>, <i>S. aureus</i>, <i>S. epidermis</i> and <i>Streptococcus pneumoniae</i> (<i>C. edulis</i>, <i>C. quadrifidus</i>, <i>C. mellei</i> and <i>C. muiirii</i>) (Van der Watt & Pretorius, 2001) (Springfield <i>et al.</i>, 2003) (Martins <i>et al.</i>, 2005) (Springfield & Weitz, 2006) (Buwa & Afolayan, 2009)</p>	<p>Flavonoids, lipids, phenols, tannins, triterpenes, citric acid and malic acid (<i>C. edulis</i>, <i>C. quadrifidus</i> and <i>C. muiirii</i>) (Van der Watt & Pretorius, 2001) (Springfield <i>et al.</i>, 2003) (Martins <i>et al.</i>, 2011)</p>
<p><i>J. acutus</i></p> <p><i>J. rigidus</i> Desf.</p>	<p>Antiinflammatory, diuretic (treatment of kidney and urinary tract conditions) and sedative (<i>J. acutus</i> and <i>J. rigidus</i>) (Mouhajir <i>et al.</i>, 2001) (Chen & Chen, 2004) (Khan <i>et al.</i>, 2006) (Daoudi <i>et al.</i>, 2008)</p>	<p>Anti-eczematic activity (<i>J. acutus</i>) (Awaad, 2006)</p> <p>Immunostimulatory activity (<i>J. acutus</i> and <i>J. rigidus</i>) (Daoudi <i>et al.</i>, 2008)</p> <p>Algicide activity (<i>J. acutus</i>) (Dellagreca <i>et al.</i>, 2002) (Dellagreca <i>et al.</i>, 2004)</p>	<p>Dihydrodibenzoxepins, 9,10-dihydrophenanthrenes, phenanthrenes, phenolic glycosides and pyrenes (<i>J. acutus</i>) (Dellagreca <i>et al.</i>, 2002) (Dellagreca <i>et al.</i>, 2004) (Dellagreca, Previterra & Zarrelli, 2005) (Awaad, 2006)</p>

Table 4. Traditional uses, *in vitro* properties and bioactive compounds of medicinal plantains (*Plantago* spp.).

Halophytes	Traditional uses	<i>In vitro</i> Properties	Bioactive Compounds
<i>P. asiatica</i> L. <i>P. coronopus</i> <i>P. lanceolata</i> L. <i>P. major</i> L. <i>P. media</i> L.	Antibiotic, antitumoral, antifungic, antiinflammatory, antiviral, diuretic and hepatoprotective Human nourishment (edible leaves and seeds) (<i>P. asiatica</i> , <i>P. coronopus</i> , <i>P. lanceolata</i> , <i>P. major</i> and <i>P. media</i>) (Chiang <i>et al.</i> , 2002) (Chiang <i>et al.</i> , 2003) (Gálvez <i>et al.</i> , 2005) (Çelik & Aslantürk, 2006) (Khan <i>et al.</i> , 2006) (Türel <i>et al.</i> , 2009) (Metíner, Özkan & Ak, 2012)	Antibiotic activity: <i>Escherichia coli</i> , <i>P. aeruginosa</i> and <i>S. aureus</i> (<i>P. coronopus</i> and <i>P. major</i>) (Freitas <i>et al.</i> , 2002) (Çitoğlu & Altanlar, 2003) Anticarcinogenic activity (<i>P. asiatica</i> , <i>P. Coronopus</i> , <i>P. Lanceolata</i> , <i>P. major</i> and <i>P. Media</i>) (Gálvez <i>et al.</i> , 2003) (Chiang <i>et al.</i> , 2003) Antifungic, antigenotoxic and antiviral activity (<i>P. major</i>) (Chiang <i>et al.</i> , 2003) (Çitoğlu & Altanlar, 2003) (Çelik & Aslantürk, 2006) Antinematode activity (<i>P. major</i>) (Aballay, Parraguez & Insunza, 2005) Antioxidant activity (<i>P. coronopus</i> and <i>P. lanceolata</i>) (Gálvez <i>et al.</i> , 2005) Immunostimulatory activity (<i>P. Asiatica</i> and <i>P. Major</i>) (Chiang <i>et al.</i> , 2003)	Flavonoids, phenols, phenylpropanoids, monoterpenoids, triterpenoids and iridoid glycosides (<i>P. asiatica</i> , <i>P. coronopus</i> , <i>P. lanceolata</i> , <i>P. major</i> and <i>P. media</i>) (Taskova <i>et al.</i> , 2002) (Gálvez <i>et al.</i> , 2005) (Al-Mamun <i>et al.</i> , 2007)

1.4 Objectives

This work evaluated the potential of biomolecules of *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus*, from the Algarve coast, to be used in aquaculture.

Antibiotic, antioxidant (free radical scavenging and metal chelation) and immunostimulant properties were screened. The antibiotic properties were tested *in vitro* against 3 Gram-negative bacteria species: *Listonella anguillarum*, *Vibrio fischeri* (Vibrionaceae) and *Photobacterium damsela piscicida* (pasteurellosis). These pathogens were selected for their relevance as causative agents of morbidity and mortality in fish farms. The immunostimulant properties were tested *in vitro*, using head-kidney phagocytes from a major aquaculture species, the gilthead seabream *Sparus aurata* L.

2. Materials and methods

2.1 Sample Collection

Aerial parts of *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus* were collected on the Algarve coast (Faro Beach and Ludo, in May 2010), oven dried at 40 °C, milled and stored at - 20°C.

2.2 Preparation of the extracts

2.2.1 Organic extracts

Organic extracts were obtained by means of a sequential extraction procedure using solvents of increasing relative polarities. Aliquots (1 g) of milled samples were homogenized and sequentially extracted with hexane, ether, chloroform, methanol and water. The extracts were centrifuged (400 g, 15 min, RT), and the supernatant carefully removed. The forementioned procedure took place until a clear supernatant layer was

achieved in each individual sample. The extracts were evaporated (temp. < 40°C), the dried extract was weighed and dissolved in dimethyl sulfoxide (DMSO), to obtain solutions in the final concentration of 500, 260, 100, 70 and 50 mg/mL.

2.2.2 *Water based plant extracts*

Milled samples (3 g) in Falcon tubes were homogenized and extracted with Hank's buffered salt solution (HBSS). The extracts were centrifuged (400 g, 15 min, RT), and the supernatant carefully removed. The forementioned procedure took place until a clear supernatant layer was achieved in each individual sample. The extracts were freeze-dried and dissolved in HBSS. (to obtain solutions with the final concentration of 50, 10, 5 and 1 mg/mL.

2.3 *Testing of antibiotic activity*

2.3.1 *Pathogen collection, culture and growth curve determination*

Samples of *L. anguillarum*, *P. damsela piscicida* and *V. fischeri* were obtained from Dr. Paulo Pedro (LAQ-University of Algarve and Dr. Teresa Baptista (Escola Superior de Turismo e Tecnologia do Mar de Peniche). The bacterial strains were inoculated on 4 mm thick culture medium (Marine Agar, Conda Laboratories) and salt enriched Tryptic Soy Agar (Himedia). In the Microbiology Laboratory of CIMA (Center for Marine and Environmental Research), Petri dishes were inoculated to produce isolated colonies, using sterile swabs in a Biohazard laminar flow hood (Steril VBH C2 Biohazard Cabinet). The Petri dishes were incubated at room temperature for *L. anguillarum* and *P. damsela piscicida* and at 12 °C for *V. fischeri*. Samples from individual colonies were then inoculated in 200 mL Erlenmeyer flasks containing liquid growth medium Marine Broth (Conda Laboratories) or Salt enriched Tryptic Soy Broth (Himedia) at the above mentioned temperatures. Cell Growth was monitored through spectrophotometric (Jenway Genova 6305 UV/Vis) readings of optic density at 650 nm in order to

determine the period of exponential growth. New sub-cultures in fresh medium were done every 48 hours.

2.3.2 Antibigram Testing

Antibiotic activity testing was made adapting the Kirby-Bauer disk diffusion method (Drew *et al.*, 1972). The bacterial inoculum for each of the diffusion method tests was obtained from exponentially growing cultures of the selected bacteria, grown to a density of colony forming units (CFU) of approximately $1-2 \times 10^8$ CFU/mL. The corresponding optical density was established by previous monitoring of the same cultures both by optical density at 600nm and quantification of CFU from appropriate dilutions. Petri dishes, containing 25mL (4 mm thick) of the chosen agar medium, were inoculated using a sterile swab. Sterile paper disks (6 mm of diameter), were impregnated with 10 μ L of plant extract, and dried for 15 minutes at room temperature. The disks were then put on the Petri dishes (**Fig. 3**) and the set was let to pre-diffuse for 15 minutes before incubation. All procedure took place in a laminar flow chamber. The Petri dishes with *Listonella anguillarum* and *Photobacterium damsela piscicida* were incubated at room temperature. *Vibrio fischeri* was incubated at 12 °C. After the incubation period, all Petri dishes were examined and the antibacterial activity evaluated on the basis of the presence and diameter (in millimetres) of an inhibition area surrounding the disks. Tested extracts included water based and organic fraction extracts (50 mg/mL). When low antibiotic activities were detected, disks containing higher extract concentration (70, 100, 260 and 500 mg/mL) were used. Paper disks containing or not 10 μ L of HBSS, were used as negative controls. Disks impregnated with ampiciline and tetracycline (10 μ g of antibiotic per disk), were used as positive controls.

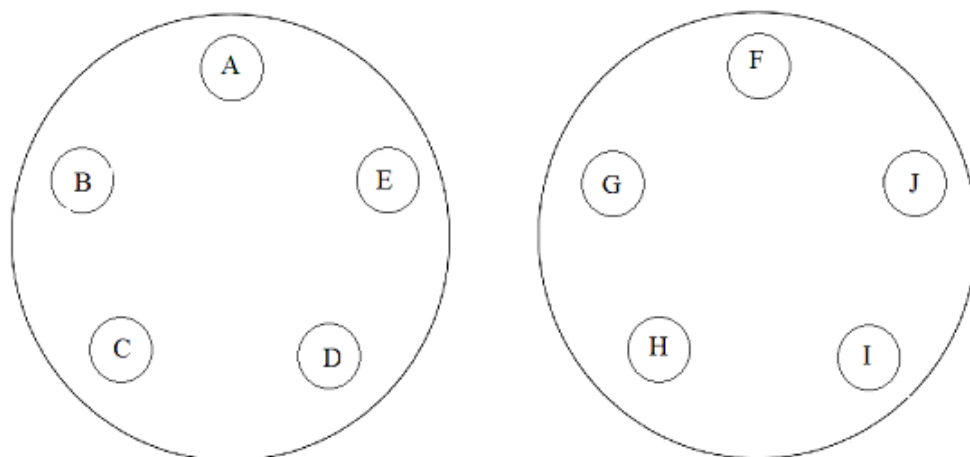


Fig. 3. Disposition of paper disks with halophyte extracts, antibiotics, paper and solvent controls, inside the marine agar Petri dishes. (A) *A. macrostachyum*, (B) *A. halimus*, (C) *C. edulis*, (D) *J. acutus*, (E) *P. coronopus*, (F) ampiciline, (G) tetracycline, (H) DMSO, (I) HBSS and (J) paper.

2.4 Testing of antioxidant activity

Antioxidant and metal-chelating activities of the water based extracts (10, 5 and 1 mg/mL) were tested in 6 replicates. Also for IC₅₀ (half maximal inhibitory concentration) determination 4 replicates of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/mL were tested.

2.4.1 Antioxidant activity screening by DPPH free radical method

Testing for antioxidant activity was made using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method (Mensor *et al.* 2001). In a 96-well plate (**Fig 4.**), 200 μ L of DPPH (0,047 mg/mL) were added to 22 μ L of extract. The same amount of DPPH was also added to 22 μ L of HBSS (Hank's Balanced Salt Solution) and BHT (butylhydroxytoluene at 1 mg/mL), respectively as negative and positive controls. Wells containing 200 μ L of HBSS and 22 μ L of plant extract were used as colour controls.

After the procedure, the plate was left in the dark for 30 minutes. Absorbance was read at 517 nm (Biotek plate reader Synergy 4).

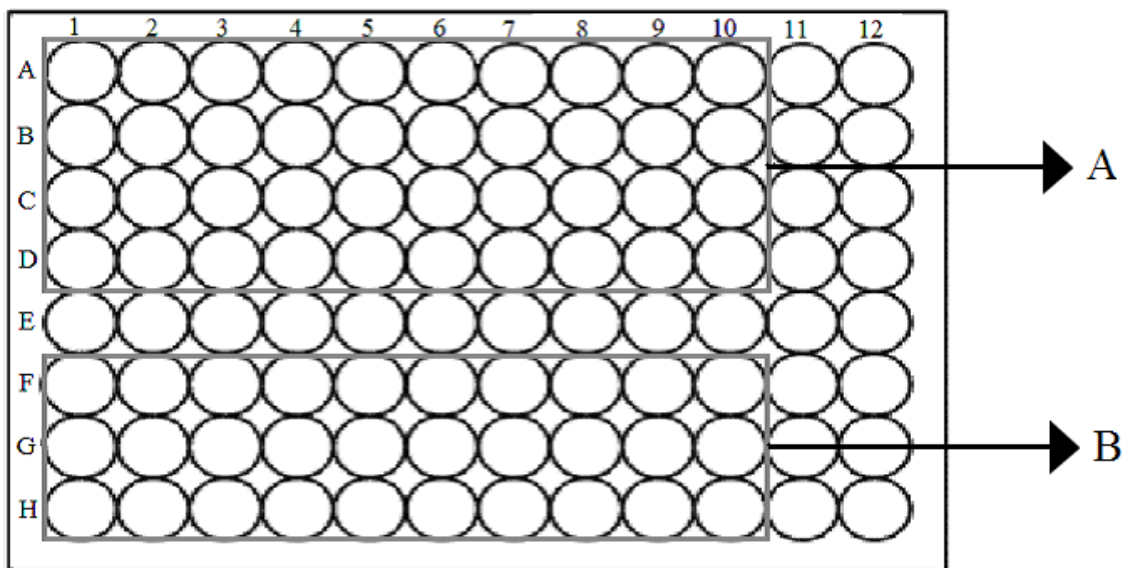


Fig. 4. Scheme of sample 96-well plate used for the antioxidant and metal chelating IC_{50} determination of water based halophyte extracts. **A** represents trial wells. **B** represents colour controls.

2.4.2 Metal chelating activity for copper

Testing for metal chelating activity of Cu^{2+} was made according to the method described by Saiga, Tanabe & Nishimura, 2003. In a 96-well plate, 200 μ L of sodium acetate buffer (50 mM, pH 6), 100 μ L of copper sulphate (50 μ g/mL) and 6 μ L of pyrocatechol violet (40 mM in sodium acetate) were added to 30 μ L of extract. In the negative and positive controls the extracts were replaced by 30 μ L of, respectively, HBSS and EDTA (ethylenediaminetetraacetic acid at 1 mg/mL). For color controls, wells containing 30 μ L of extract and 306 μ L of sodium acetate buffer, were used. Absorbance was read at 632 nm.

2.4.3 Metal chelating activity for Iron

Testing for metal chelating activity of Fe^{2+} was made according to the method described by Ebrahimzadeh, Pourmorad & Bekhradnia, (2008). In a 96-well plate, 200 μL of distilled water and 30 μL of FeCl_2 (0.1 mg/mL), were added to 30 μL of extract. In the negative and positive controls the extracts were replaced by 30 μL of, respectively, HBSS and EDTA. For colour controls, wells containing 30 μL of extract and 230 μL of distilled water were used. After 30 minutes, 12.5 μL of ferrozine were added to the set, except for the colour controls. Absorbance was read at 562 nm.

2.5 Testing of immunostimulant activity

The testing of immunostimulant activity was made using the water based extracts (10 mg/mL). Each extract was tested in triplicate using the following methods.

2.5.1 Fish collection

Gilthead seabream (*Sparus aurata*) with similar weights were collected from a local facility (Ramalhete Biological Station) and acclimatized to laboratory conditions for two weeks before the start of the experiment (the specimens were kindly provided by Dr. Jorge Dias, and acclimatized by Vera Rodrigues and Helena Teixeira at the aquaculture laboratory in Gambelas, University of Algarve. Live specimens were slightly anaesthetized in a bucket of tank water containing 2-fenoxietanol (60 $\mu\text{g/L}$), and transported as stress-free as possible, to the local where head-kidney extraction took place. Specimens were killed by an overdose of 2-fenoxietanol before kidney extraction.

2.5.2 Isolation of head kidney phagocytes

Isolation of head kidney was done accordingly to the procedure described by Sørensen *et al.* (1997). The head kidney was removed aseptically and passed through a 100 mm nylon mesh. Leibovitz culture medium (L-15, Sigma-Aldrich) supplemented with penicillin (100 IU/mL), streptomycin (100 $\mu\text{g/mL}$), 20 U/mL heparin and 2% FBS (fetal bovine serum) was used on the phagocytes. The resulting cell suspension was

centrifuged (400g, 15 min, 15 °C). The culture medium was changed by L15 containing 0.1% FBS and the volume reduced to 1-2 mL. The cell suspension was again centrifuged (400 g, 5 min, 15 °C). The cells were re-suspended on L15 medium plus 5% FBS, pipetted to a 96-well plate (100 µL per well) and kept in the dark, at room temperature. The viable cell concentration was determined by the try-pan blue exclusion assay (Strober, 2001). After 4 hours the medium was replaced with fresh medium of equal composition and the cells verified for attachment. The cells were incubated for 24h after the extraction. The cells of different fish were kept separated or were combined, according to different authors (Tafalla & Novoa, 2000; Díaz-Rosales *et al.*, 2007).

2.5.3 *Determination of respiratory burst activity by Cytochrome c*

The culture medium was removed and the cells were washed with HBSS. 50 µL of cytochrome *c* were added to the cells. Trial wells received 50 µL of water-based extracts. 50 µl of PMA (phorbol myristate acetate at 10 µg/ml) and PMA plus SOD (superoxide dismutase 300 U/ml), replaced the extracts respectively in the positive and negative controls. As cytochrome *c* exhibits photosensitivity, the whole procedure took place in under low light conditions. The absorbance was measured after 30 minutes at 550 nm, and the measurement was repeated every 30 minutes for 3 hours. A total of 9 fish were used, of which 4 had their head-kidney cells combined in 2 trials (two fish represented in each cell pool). The remaining fish had their cells treated separately in 5 different trials.

2.5.4 *Determination of respiratory burst activity by Griess assays*

The cell attachment medium was replaced by 100 µl of RPMI and 20 µl of extract. For negative controls, 100 µl of HBSS was used. After 30 minutes, 50 µl of each well was removed and added to 100 µl of Griess solution in a new 96-well plate. The set was let incubate for 20 minutes at room temperature and sheltered from the light. Absorbance was measured at 540 nm. A total of 9 fish were used, of witch 4 had their head-kidney cells combined in 2 trials (two fish in each cell pool). The remaining fish had their cells treated separatly in 5 different trials.

2.6 Data presentation and statistic treatment

The results are presented as mean±standard error of mean (SEM). Data was submitted to a statistical analysis, using Duncan test by the means of SPSS Statistics 17.0 software and assuming $p < 0.05$ (level of significance of 95%).

3. Results and Discussion

3.1 Antibiotic activity

To test the antibiotic activity of halophyte extracts, *L. anguillarum*, *P. d. piscicida* and *V. fischeri* were selected based upon their relevance as a source of infection in aquaculture systems, and their standardized culture guidelines. In particular *V. fischeri* is the most widely used aquatic test organism in toxicology studies (Kaiser, 1998; Kaplan & Greenberg, 1985; Gonzalez, Osorio & Santos, 2003; Thompson, Austin & Swings, 2006). *L. anguillarum*, the causative agent of haemorrhagic septicaemia, is the most common cause of economic loss among the selected pathogens, having a cosmopolitan distribution and being reported to cause infection in more than 48 warm and cold water fish species (Actis *et al.*, 1999; Austin & Austin, 1999). *P. d. piscicida* is also common, particularly in Mediterranean countries where it is the cause of pseudotuberculosis in mainstream aquaculture species, including gilthead seabream, seabass (*Dicentrarchus labrax* L.) and sole (*Solea senegalensis* Kaup. and *Solea solea* L.) (Zorrilla *et al.*, 1999; Magariños *et al.*, 2001, 2003; Díaz-Rosales, 2007).

The freeze dried strain of *V. fischeri* was inoculated on liquid medium (Marine Broth, Conda Laboratories) and successfully recovered. However no growth was obtained once re-inoculated in solid Marine Agar medium. Dormancy in *V. fischeri* (Thompson, Austin & Swings, 2006) as an explanation for growth absence on Marine Agar plates was ruled out, since the used inoculum came from a liquid culture. Probably the unnoticed growth was the result of unexpected long duplication time or unappropriate incubation conditions. In fact a generation time for *V. fischeri* of 16 hours would require an incubation for at least 2 weeks for the detection of visible CFU. Thus finally the antibiotic properties were exclusively tested on *L. anguillarum* and *P. d. piscicida*.

Table 5. Inhibition halos of *L. anguillarum* caused by antibiotics (positive controls), solvent and paper controls (negative controls).

Controls	Inhibition halos (mm)
Ampiciline	0
Tetracycline	12 ± 1
DMSO	0
HBSS	0
Paper	0

The growth of *L. anguillarum* was not inhibited by the water or organic halophyte extracts. Ampiciline, the solvent (DMSO and HBSS) and paper controls did not inhibit *L. anguillarum*. However it showed sensibility to the tetracycline impregnated paper disks, displaying a mean of 12 mm growth inhibited area surrounding the disk (**Table 5.** and **Fig. 5**).

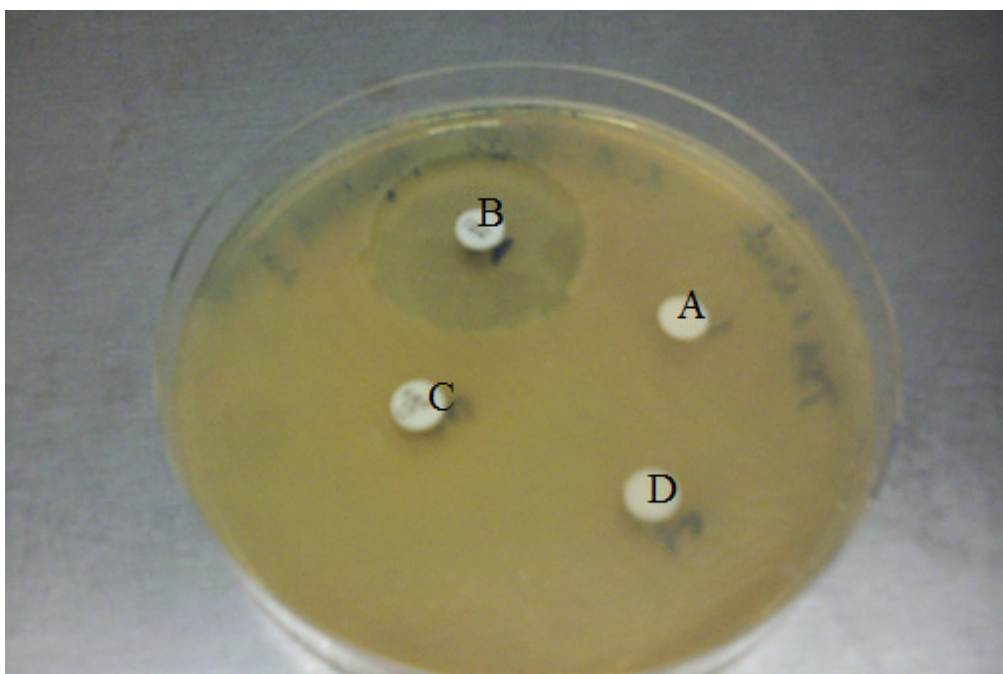


Fig. 5. Marine agar Petri dish with *L. anguillarum*, control plate after incubation. A) ampiciline, B) tetracycline, C) HBSS, D) filter paper.

Table 6. Inhibition halos of *P. d. piscicida* caused by antibiotics (positive controls), solvent and paper controls (negative controls).

Controls	Inhibition halos (mm)
Ampiciline	16± 2
Tetracycline	10± 1
DMSO	0
HBSS	0
Paper	0

Of the tested halophyte extracts, *P. d. piscicida* displayed a 3 mm inhibition halo surrounding the ether fraction of *J. acutus*. Increasing the concentration of *J. acutus* ether extract (50, 70, 100, 260 and 500 mg/ml were tested) did not result in a higher inhibition. *P. d. piscicida* revealed sensitivity to both antibiotic controls, causing growth inhibition halos of 16 mm (ampiciline) and 10 mm (tetracycline). *P. d. piscicida* growth was not inhibited by the solvent and paper controls (**Table 6.** and **Fig. 6.**).

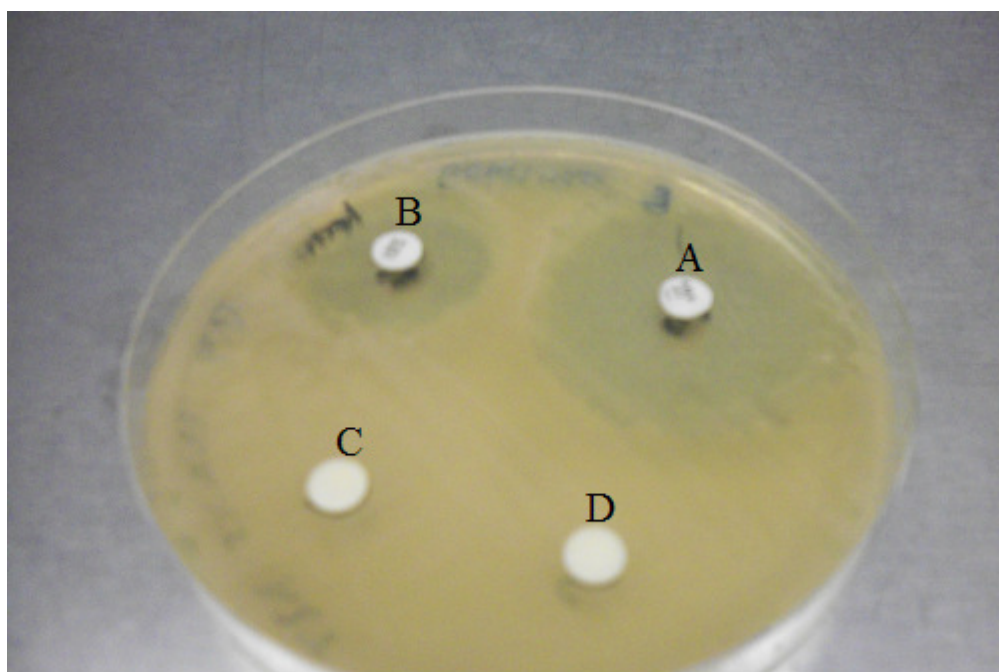


Fig. 6. Marine agar Petri dish with *P. d. piscicida*, control plate after incubation. A) ampiciline, B) tetracycline, C) HBSS, D) filter paper.

The results suggest the presence of organic compounds in *J. acutus* which inhibit the growth of *P. d. piscicida*. Research done by several authors gives evidence about the nature of these biomolecules. Five phenolic glycosides were isolated from *J. acutus*: oxyresveratrol 2-*O*- β -D-glucopyranoside; resveratrol 3',4'-*O*, *O'*-di- β -D-glucopyranoside; markhamioside F; canthoside B; and caffeic acid glucorhamnoside. These compounds displayed a significant anti-inflammatory and anti-eczematic activity (Awaad, 2006). Dellagrecia *et al.*, 2002, isolated nine 9,10-dihydrophenanthrenes, three phenanthrenes and a pyrene, with algicide properties from *J. acutus*. These included the novel compounds: 5-(1-Ethoxy-ethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene, 5-(1-phytoxy-ethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene, 2,7-dihydroxy-1-methyl-5-vinylphenanthrene, 2,7-dimethoxy-1,6-dimethyl-5-vinylphenanthrene and 2,7-dihydroxy-1,6-dimethylpyrene (Dellagrecia *et al.*, 2002).

Dellagrecia *et al.*, 2004 isolated twenty-five 9,10-dihydrophenanthrenes, four phenanthrenes, a dihydrodibenzoxepin, and a pyrene, from *J. acutus*. Two novel phytotoxic dimeric phenanthrenoids have also been isolated from the rhizome methanolic extract of *J. acutus* (Dellagrecia, Previtiera & Zarrelli, 2005).

For *A. macrostachyum*, *A. halimus*, *C. edulis* and *P. coronopus*, the antibiotic properties described by several authors suggests that further antibiotic screening and testing is needed for these halophytes. One potential explanation for absence of antibiotic activity resides on variation in phytochemical profiles in the tissues of halophytes according to location, genetics and season, among other factors. Chokoe *et al.*, 2008 reported differences on antimicrobial testing on *C. edulis* extracts based on seasonal changes of phytochemicals. Organic fraction extracts (obtained by increasingly high polarity solvents: hexane, dichloromethane, ethyl acetate, acetone and methanol) from *C. edulis* leaf samples collected during the spring and autumn, were compared for their antibiotic activity against *E. coli*, *Enterococcus faecalis*, *P. aeruginosa* and *S. aureus*. The spring extracts were more potent against all test organisms, having lower MIC (minimum inhibitory concentration) values than the autumn extracts. When taking the total activity of each extract into account, the autumn extracts showed higher efficacy when compared with the spring samples. The compounds are identified as flavonoids, tannins, malic and citric acids. Other group displaying variation of antimicrobial molecules are the plantains. Among the compounds present in *Plantago*, one group of interest for therapeutic properties are the iridoid glycosides (**Fig. 7**).

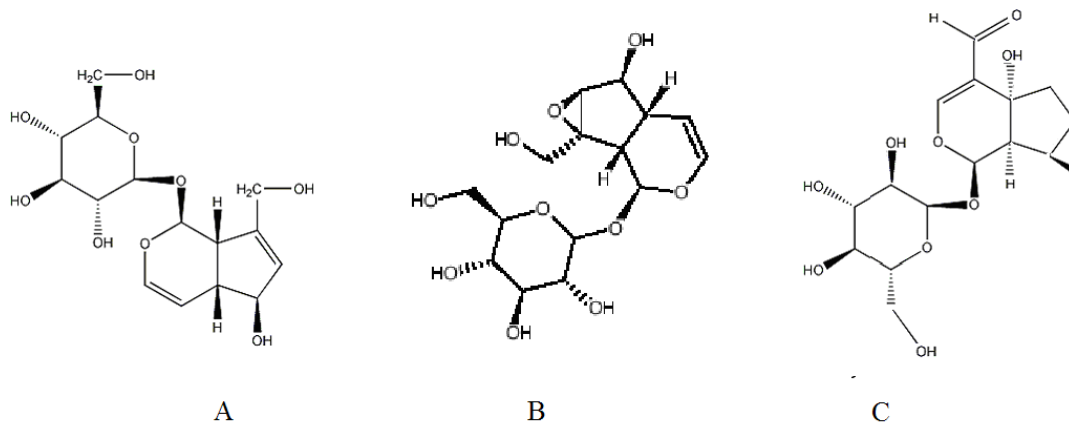


Fig. 7. Iridoid glycosides present in *Plantago spp.* (A) aucubin, (B) catalpol and (C) plantarenaloside.

These secondary metabolites function as a natural deterrent against herbivory and plant pathogens (Marak, Biere & Van Damme, 2000; Biere, Marak & Van Damme, 2004). These compounds have antibiotic, antifungal, anti-inflammatory, anti-viral and hepatoprotective activities (Davini *et al.*, 1986, Suh *et al.*, 1991; Shim *et al.*, 2007). Genetic, ontogenic, phenotic, populational and seasonal variation of iridoid glycosides is described for *P. lanceolata* and *P. major* (Darrow & Bowers, 1997; Tamura, 2002; Tamura & Nishibe, 2002; Fuchs & Bowers, 2004; Zubair, 2010; Quintero & Bowers, 2012; Zubair *et al.*, 2012). (Bowers, 1996). For *P. lanceolata*, based upon phytochemical sampling in different seasons, targeting the concentrations of catalpol, aucubin and acteoside present in the aerial parts, it is possible to make an assessment of the most desirable period to harvest leaves for their therapeutic properties. The levels of aucubin steadily increased from spring to midfall from 2,1 to 4,8% of dry matter. The levels of acteoside increased from 3,4 to 7,1% over the same period. The data suggested that midfall was the appropriate period to harvest leaves for medicinal uses (Tamura, 2002; Tamura & Nishibe, 2002).

3.2 Testing of antioxidant activity

3.2.1 Antioxidant activity screening by DPPH free radical

Reactive oxygen species are continuously produced in vertebrate tissues as natural byproducts of aerobic metabolism. These reduced forms of oxygen include: hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot), nitric oxide (NO), singlet oxygen ($^1\text{O}_2$), superoxide anion ($\text{O}_2^\cdot-$) and the non radical peroxynitrite (ONOO^-). ROS are damaging to most cellular constituents (lipids, proteins and nucleic acids) and involved in the progression of several diseases. Oxy radical-induced damage is normally maintained at low levels due to the presence of cellular antioxidant defences. Vertebrate antioxidant defences include ROS scavenging enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase. Hydrophobic and hydrophilic antioxidant biocompounds such as ascorbic acid, tocopherol, carotenoids, fatty acids, and glutathione, which are synthesized or obtained from external sources, are also used to prevent ROS induced tissue damage. Oxidative stress is experienced when these endogenous antioxidant defences become overwhelmed by prooxidant forces (Babich, Palace & Stern, 1993; Krapfenbauer *et al.*, 2003). In fish, oxidative stress has been associated with exposure to heavy metals (Rau *et al.*, 2004; Wang, Chung & Tung, 2004), high stock densities (Caipang *et al.*, 2009; Aksakal *et al.*, 2011), infectious diseases (Shoemaker, Evans & Klesius, 2000; Tanaka *et al.*, 2002; Saurabh & Sahoo, 2008; Ali, Hashem & Al-Salahy, 2011), insecticides (Pena-Llopis, Ferrando & Pena, 2003; Parvez & Raisuddin, 2005; Monteiro *et al.*, 2006), nanomaterials (Oberdörster, 2004; Zhu *et al.*, 2008a, 2008b), nitrogen compounds (Yang *et al.*, 2010) and high temperatures (Parihar & Dubey, 1995). Nutritional deficiencies also make fish prone to oxidative stress, especially in the case of non synthesized micro-nutrients such as ascorbic acid, retinol and tocopherol (Montero *et al.*, 2001). Effects of oxidative stress in fish include a compromised immune response (Montero *et al.*, 2001), growth inhibition and peroxidation of polyunsaturated fatty acids (Zhu *et al.*, 2008a).

The testing of halophyte antioxidants was made using the DPPH colorimetric method. The DPPH method was chosen for the antioxidant screening based on its simplicity, reaction rate and independence of sample polarity. The DPPH radical has a maximum

absorbance of 520 nm in its oxidized form, which decreases when the radical is reduced by antioxidant compounds (Koleva *et al.*, 2002). The antioxidant properties of the featured halophytes were compared with BHT, a commercial antioxidant (Molyneux, 2004). The antioxidant activity was calculated using IC₅₀ which represents the concentration of extract needed to inhibit 50% of oxidation *in vitro*.

Among the halophyte extracts, the lowest IC₅₀ values (and the most antioxidant activity, with statistically significant differences of $p < 0.05$ above 5 mg/mL) were displayed by *P. coronopus* and *A. macrostachyum*. The IC₅₀ value for *J. acutus* was lower than *C. edulis*. However at higher concentrations *C. edulis* exhibited more oxidant inhibition, with statistically significant differences of $p < 0.05$ above 5 mg/mL. For $p < 0.05$, *J. acutus* and *A. halimus* were significant above 10 mg/mL (**Table 7.** and **Fig. 8.**).

These results suggest differences in the amounts and/or types of compounds produced by these species. *P. coronopus* is a low rosette forming herb, being common in open rocky and gravel terrains. This species depends on antioxidant defenses, as well on morphological features (thick root system, numerous thin and hairy leaves, in contrast with most *Plantago spp.*) to grow in exposed locations. It is possible that under hydric stress, the protective role of antioxidant defenses becomes especially relevant in *P. coronopus*, also considering the lack of other adaptations such as leaf succulence (Golovko *et al.*, 2012). The antioxidant activity of *P. coronopus* is described by several authors (Chiang *et al.*, 2003; Gálvez *et al.*, 2005; Çelik & Aslantürk, 2006).

The second higher antioxidant activity was detected on *A. macrostachyum*. *A. macrostachyum* is an obligate halophyte, which is linked to more antioxidant and osmoprotectant activity (Redondo-Gómez, Mateos-Naranjo & Andrades-Moreno, 2010). This species is capable of growing in interstitial soil salinities up to 1000 mM (Khan & Bilquees Gul, 2002; Redondo-Gómez *et al.*, 2010).

C. edulis is a cover plant, occurring in dry rocky and dune habitats. *C. edulis* exhibits leaf succulence, and under conditions of sun induced stress it is capable of increasing the production of protective pigments. In comparison *J. acutus* thin erect leaves, expose a minimal area to solar radiation. Under conditions of sun or hydric stress, *J. acutus* leaves slowly dry starting from the tip, which protects the living tissue below. Besides salt marsh habitats, *J. acutus* also occurs in freshwater and inland environments (Sabovljevic & Sabovljevic, 2007; Weber, 2008).

The lowest antioxidant activity was detected on *A. halimus*. A possible explanation is that *A. halimus* depends more on morphological and physiological adaptations to

salinity and sun exposure, in comparison to antioxidant defenses. Young *A. halimus* leaves are coated with layers of vesiculated hairs (trichomes), which function in the regulation and secretion of salt. As leaves mature, these cells collapse, forming a light-reflective waxy layer of salt crystals, which protects tissues from solar radiation and water loss. In *A. halimus* stems, leaves also depend on mutual shading for photoprotection (Hassine *et al.*, 2009). It is suggested these adaptations make *A. halimus* less dependent on antioxidants for photoprotection (Streb, Tel-Or & Feierabend, 1997).

The results indicate that *A. macrostachyum* and *P. coronopus* are potential sources of antioxidant compounds. However it is possible that for all the featured species, a higher antioxidant production and yield, can be achieved by means of controlled growth conditions (such as exposing cultivated plants to oxidative stresses) and selective breeding ((Bowers, 1996; Pirie *et al.*, 2013). For plantains, the development of bioactive molecule rich cultivars has taken place for species such as *P. lanceolata* and *P. major* (Tamura, 2002; Tamura & Nishibe, 2002).

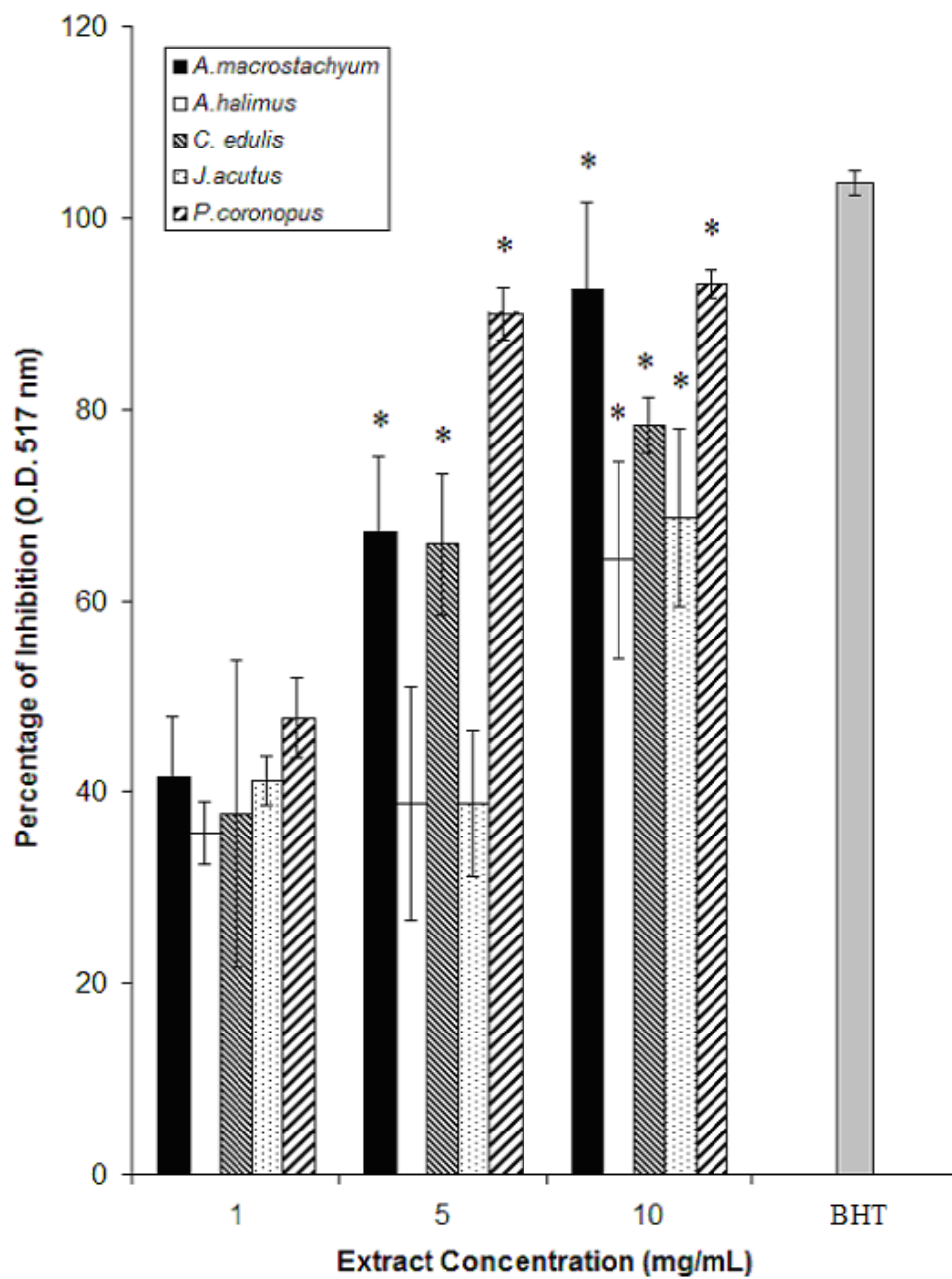


Fig. 8. Antioxidant activity of 1, 5 and 10 mg/mL water based halophyte extracts. The data represents means of 6 trial wells in a multichamber plate. The letter * denotes statistically significant differences ($p < 0.05$) when compared with negative control wells.

Table 7. Antioxidant activity IC₅₀ values of water based halophyte extracts.

	IC ₅₀ Values (mg/mL)
<i>A. macrostachyum</i>	2.04 ± 0.63
<i>A. halimus</i>	5.51 ± 0.88
<i>C. edulis</i>	3.11 ± 4.4
<i>J. acutus</i>	2.56 ± 1.11
<i>P. coronopus</i>	0.91 ± 0.23

3.2.2 Metal chelating activity for copper

Copper is a common heavy metal in the marine environment and a model prooxidant (Rau *et al.*, 2004). In teleost fish, copper is an essential element, being a part of key proteins and in cellular respiration (Watanabe, Kiron & Satoh, 1997). Excess copper is toxic, causing oxidative stress in tissues, disrupting sodium homeostasis and causing deposits in gills and intestinal tract (Woodward *et al.*, 1995). Copper can reach fish tissue by ingestion (in this case absorbed by the intestinal tract) or absorbed by the gill epithelium (in this case waterborne copper) (Woodward *et al.*, 1995). In fish, the liver is the main organ for copper accumulation and homeostasis (Lorentzen, Maage & Julshamn, 1998).

Metal chelators mobilize metal compounds held in tissues by forming soluble, stable complexes that can be excreted in feces and/ or urine (Ebrahimzadeh, Pourmorad & Bekhradnia, 2008). In plants, heavy metals induce oxidative and water stresses, not very different physiologically of the effects of salinity (Poschenrieder, Gunse & Barcelo, 1989; Nadjimi & Daoud, 2009). The same metabolic pathways that allow for salt tolerance in halophytes, also make these plants more resilient towards heavy metals, when compared with glycophytes. Of particular interest are the antioxidant defences of halophytes, which make these plants pre-adapted to cope with heavy metal oxidative stress (Zhu *et al.*, 2004). Independent research in a halophyte (*Mesembryanthemum*

crystallinum L.) and a glycophyte (*Lupinus luteus* L.), found evidence suggesting that copper and salt stress triggered similar responses in both plant species. It is possible that copper and salt stress responses in plants overlap to some extent, functioning in common integrated mechanical and chemical signals (Thomas *et al.*, 1998; Przymusiński, Rucińska & Gwoździński, 2004).

Osmoprotective compounds appear as well, to be significant for heavy metal tolerance mechanisms in halophytes. Proline, a synthesized osmoprotectant linked to ionic and osmotic stress, accumulates in halophytes in response to cadmium, copper, iron and other heavy metals. Under metal induced stress, proline acts in antioxidant defence, metal binding and signalling (Schat, Sharma & Vooijs, 1997; Shevyakova *et al.*, 2003; Sharma & Dietz, 2006; Lefeuvre *et al.*, 2009).

The copper chelating properties of the featured halophytes were compared with a commercial metal chelating compound, EDTA in a colorimetric test (Saiga, Tanabe & Nishimura, 2003).

The lowest IC₅₀ value (and the higher copper chelating activity, with statistically significant differences of $p < 0.05$ above 10 mg/mL) was displayed by *A. macrostachyum* (**Table 8.** and **Fig. 9**). *A. halimus* and *J. acutus* exhibited respectively the second and third lowest IC₅₀ values, with statistically significant differences of $p < 0.05$ above 10 mg/mL. *P. coronopus* displayed the highest IC₅₀ value, however for the 10 mg/mL concentration, it shows $p < 0.05$ significant differences and an chelating activity comparable to the forementioned species.

These results could be explained by the physiological adaptations and habitat of *A. macrostachyum* and *A. halimus*. *A. macrostachyum* is among the featured species, the most dependent and associated with marine environments, which suggests more contact with copper sources. *A. macrostachyum* and *A. halimus* resort to the uptake and accumulation of Cl⁻ and Na⁺ as part of their metabolism (in the shoot internodes for *A. macrostachyum* and in leaves for *A. halimus*) (Bajji, Kinet & Lutts, 1998). The glandular tissue responsible for the uptake of salt ions, is also capable of accumulating and secreting heavy metals such as copper. *A. macrostachyum* accumulates salt and heavy metals in tissues, while *A. halimus* secretes ionic compounds and metals in the leaf surface (Lutts *et al.*, 2004; Redondo-Gómez, Mateos-Naranjo & Andrades-Moreno, 2010). The accumulation of salt and metals in tissues, and the bioavailability of copper to *A. macrostachyum*, warrant more efficient chelating and osmoprotecting systems. These species are Chenopodiaceae, which is linked to specific osmoprotectants such as

glycinebetaine (Lefe´vre *et al.*, 2009). Salinity itself has been linked to the translocation of heavy metals from the roots to the aerial parts of plants (Fitzgerald *et al.*, 2003).

By comparison the least copper chelating activity was observed on *C. edulis*. A suggested explanation is that *C. edulis* normally has little contact with copper sources, and so biochemical chelators for this particular metal are less developed. The dune and rocky habitats that the sour fig inhabits are constituted by high drainage substrates. *C. edulis* also has a shallow root system.

Besides being potential sources of novel metal chelators, these species could be of special interest for decontamination of heavy metal polluted soils. *A. halimus* in particular, because of its adaptability and biomass production, has possible uses in phytoremediation research (Manousaki & Kalogerakis, 2011).

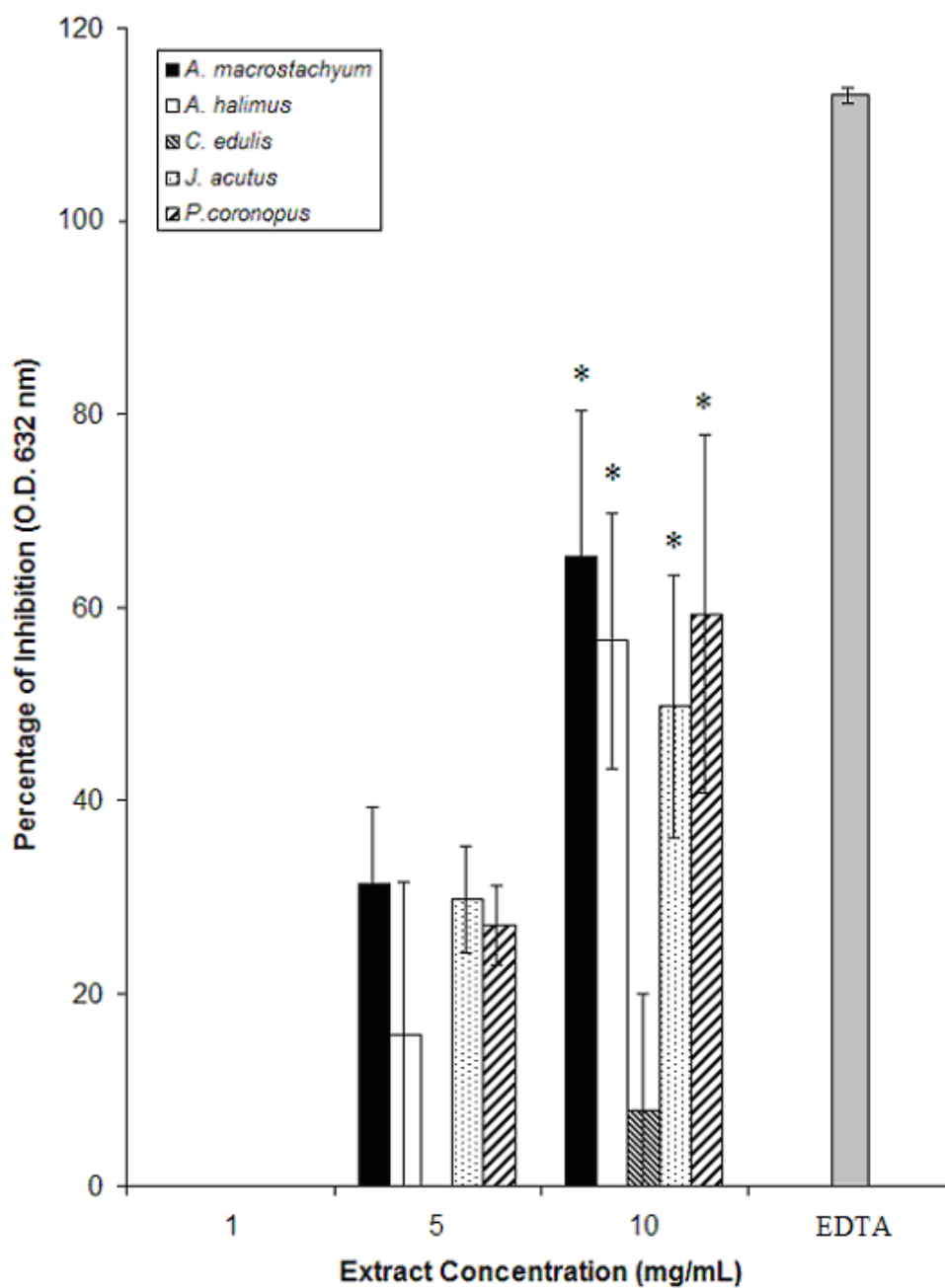


Fig. 9. Copper chelating activity of 1, 5 and 10 mg/mL water based halophyte extracts. The data represents means of 6 trial wells in a multichamber plate. The letter * denotes statistically significant differences ($p < 0.05$) when compared with negative control wells.

Table 8. Copper chelating activity IC₅₀ values of water based halophyte extracts.

Halophytes	IC ₅₀ Values (mg/mL)
<i>A. macrostachyum</i>	8.01 ± 4,2
<i>A. halimus</i>	9,26 ± 10.37
<i>C. edulis</i>	11.09±7,4
<i>J. acutus</i>	9,89 ± 13,83
<i>P. coronopus</i>	13,14 ± 20.76

3.2.3 Metal chelating activity for Iron

Iron is an essential micronutrient for fish, as a constituent of proteins involved in cellular respiration and oxygen transfer. Fish obtain iron via absorption mainly by the intestinal tract but also by the gill epithelium (Peuranen *et al.*, 1994). However excess iron is harmful as it causes the release of ROS, which damage cells and tissue. Also excessive waterborne iron can form deposits in gills, causing respiratory distress (Dalzell & MacFarlane, 1999).

The iron chelating properties of the featured halophytes were compared with a commercial metal chelating compound, EDTA in a colorimetric assay. The water based extracts of *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus* displayed iron chelating properties, with statistically relevant differences at concentrations of 1 mg/mL. The IC₅₀ values for all species are bellow 1 mg/mL (**Table 9.** and **Fig. 10.**).

Iron chelators originating from halophytes are indentified mainly as polyphenolic compounds. Species known for having iron chelators include *Anchusa azurea*, *Foeniculum vulgare*, *Limonium densiflorum*, *Melilotus spp.*, *Mesembryanthemum spp.*, *Pistacia lentiscus*, *Raphanus raphanistrum*, *Salicornia europaea*, *Salvia spp.*, *Sonchus oleraceus* and *Tamarix gallica* L. (Nehir & Sibel, 2004; Khan *et al.*, 2006;

Ebrahimzadeh, Pourmorad & Bekhradnia, 2008; Hanen *et al.*, 2009; Khaznadar, Vogiatzakis & Griffiths, 2009; Ksouri *et al.*, 2009; Medini *et al.*, 2011).

Iron chelating compounds could be of special use to freshwater aquaculture, as many river systems originate in ground water springs containing large amounts of naturally occurring waterborne iron, which limits aquaculture suitability in such areas (Vuori, 1995; Salami *et al.*, 2008).

Iron chelating phytochemicals have interest for human medicine. The treatment of a variety of medical conditions, such as chronic anemia, require the use of iron chelating and antioxidant therapy. Patients with thalassemia, and other chronic anemia disorders, require regular blood transfusions for quality of life and survival. However the iron released as a result of the breakdown of transfused red blood cells is not eliminated by human beings, and is deposited in the form of hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium (Rund & Rachmilewitz, 2005; Taher, Isma'eel & Cappellini, 2006). The resulting iron accumulation causes ROS mediated tissue damage, which can lead to complications such as endocrine abnormalities, endothelial dysfunction, hypothyroidism, heart and liver failure (Olivieri *et al.*, 1992; Taher, Isma'eel & Cappellini, 2006). The treatment of thalassemia includes both antioxidant and iron chelating therapies. This requirements could indicate potential in iron chelators present in halophytes, as research suggests that compounds such as phenols can serve a dual purpose as antioxidants and iron chelators. Current iron chelating therapy is limited by poor oral bioavailability, short plasma half-life and severe side effects of available compounds (Kukongviriyapan *et al.*, 2008).

Iron chelating compounds could also have potential for cancer therapy. Iron is an essential nutrient for cell growth, as it is necessary for nucleic acid synthesis and respiration. Due to their rapid proliferation, cancer cells have higher iron requirements when compared with normal cells in an organism. Iron chelators targeting cancer cells can deprive them of nutrients necessary for replication and halt their growth. Some research lines are focusing on lipophilic synthetic compounds as these enter cells more easily and target intracellular reserves of iron. Others are targeting natural compounds of plant origin (Whitnall *et al.*, 2006).

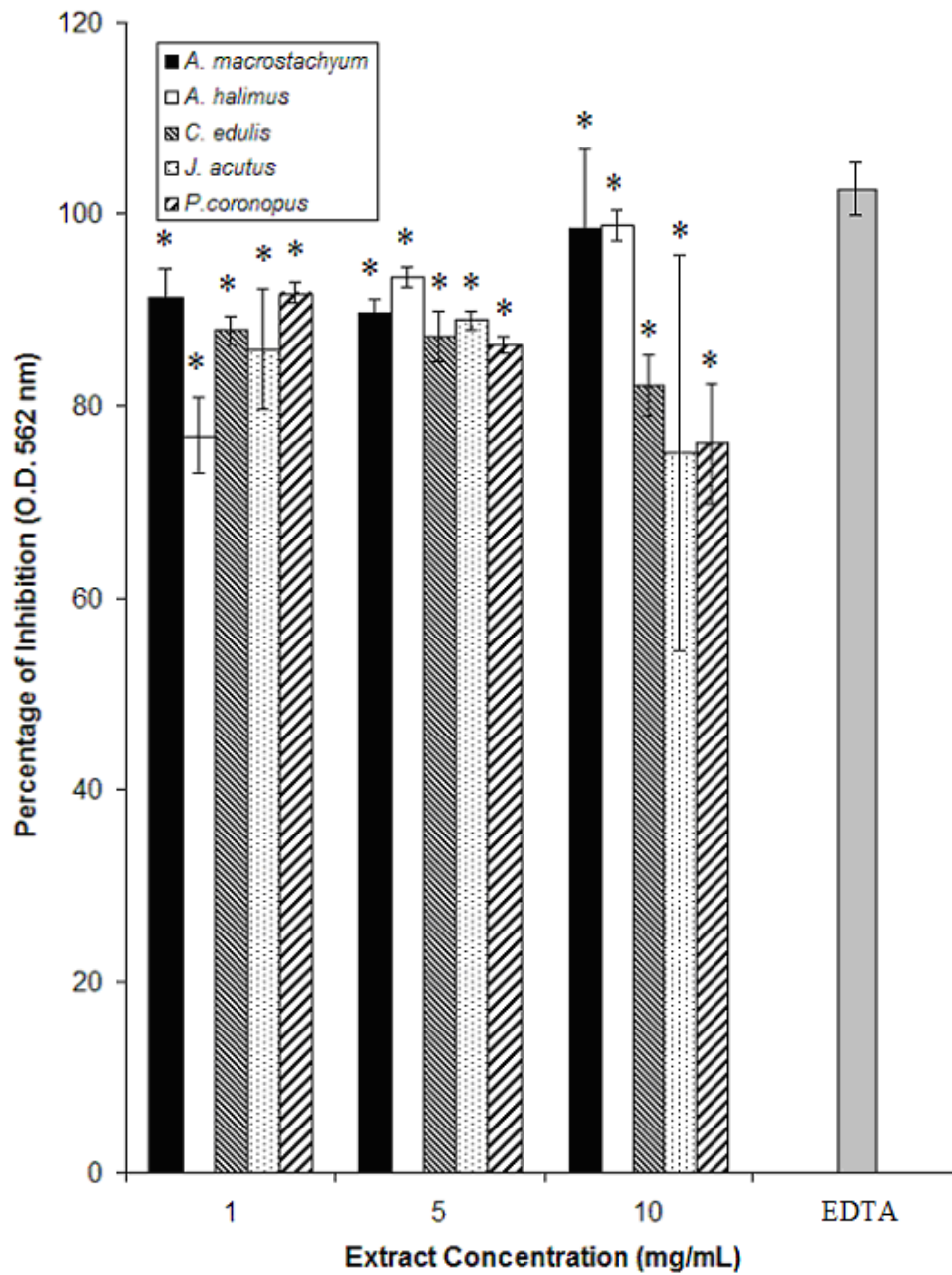


Fig. 10. Iron chelating activity of 1, 5 and 10 mg/mL water based halophyte extracts. The data represents means of 6 trial wells in a multichamber plate. The letter * denotes statistically significant differences ($p < 0.05$) when compared with negative control wells.

Table 9. Iron chelating activity IC50 values of water based halophyte extracts.

Halophytes	IC50 Values (mg/mL)
<i>A. macrostachyum</i>	0.61 ± 0.03
<i>A. halimus</i>	0.74 ± 0.19
<i>C. edulis</i>	0.67 ± 0.22
<i>J. acutus</i>	0.79 ± 0.12
<i>P. coronopus</i>	0.53 ± 0.14

3.3 Testing of immunostimulant activity

3.3.1 Determination of respiratory burst activity by Cytochrome c and Griess assays

The fish immune system is responsible for protection against pathogens, suppressing as well the emergence and growth of tumors. The immune system also maintains homeostasis following tissue damage, inflammatory processes and during development. It is divided into adaptive and innate immune systems. The adaptive immune system comprises antibody defences (a tetrameric IgM immunoglobulin type in the case of teleosts) for pathogen recognition and elimination. In the adaptive immune response, lymphocytes (B-lymphocytes and T-lymphocytes) recognize pathogens by specific antigens in soluble form or in association with a MHC marker on antigen presenting cells. As a response, lymphocytes differentiate into memory and plasma cells, which produce the specific antibody. However this immune response needs time to develop after exposure to the pathogen (Wilson *et al.*, 1997; Ewart, Johnson & Ross, 2001; Randelli, Buonocore & Scapigliati, 2008).

The innate immune system is more important in fish when compared with higher vertebrates, is independent of prior exposure to pathogens and generally rapid. (Saurabh & Sahoo, 2008). It is divided in epithelial, humoral and cellular components.

The epithelial component of the innate immune system covers the most exposed areas in fish, providing physical, mechanical and biological barriers. This lymphoid tissue is associated with a layer of mucus which contains antimicrobial agents such as lysozyme, peptides, phosphatases and trypsin. The humoral component of the innate immune system consists of cell receptors, and serum type biomolecules, for pathogen recognition and elimination. These include antibacterial peptides, cytokines, chemokines, growth inhibitors (transferrin, interferon), lectins (C-type lectins and pentraxins), lytic enzymes (such as lysozyme and chitinase) and protease inhibitors (such as $\alpha 2$ -macroglobulin and $\alpha 1$ -antitrypsin). The humoral innate immune response includes the complement system, which is divided in the alternative, classical and lectin pathways (Ardo *et al.*, 2008; Saurabh & Sahoo, 2008).

The cellular component of the innate immune system (collectively called phagocytes) comprises granulocytes (mainly neutrophils), leukocytes, macrophages, monocytes and cytotoxic cells. These defense cells search for pathogens and signals marked by pattern, recognizing proteins or receptors. Reactive oxygen species and nitric oxide are produced by phagocytes, during the respiratory burst to kill invading pathogens and abnormal cells. The respiratory burst activity is conditioned by factors such as body condition, stress, nutrition and water quality (Shoemaker, Evans & Klesius, 2000; Saurabh & Sahoo, 2008).

The immunostimulant properties of halophyte extracts were tested on sea bream phagocytes, and assayed by cytochrome *c* and Griess (Babior, Kipnes & Curnutte, 1973; Secombes, 1990).

The cytochrome *c* and griess assays did not yield significant results ($p < 0.05$) when compared with negative control wells, for any extract (**Fig 11.**). Its possible that there is extract toxicity to the seabream phagocytes. The use of phenoxyethanol as an anesthetic also affects phagocytes. A study made to understand the effects of phenoxyethanol in the innate immune system of seabream, revealed that the anesthetic itself produces a stress response and causes a decrease in phagocytic activity (Ortuño, Esteban & Meseguer, 2002).

Future research is suggested to study the effects of halophyte extracts, with further *in vitro*, as well as *in vivo* testing. Different components of the innate immune system can be stimulated, without necessarily affecting the respiratory burst activity of phagocytes. In Díaz-Rosales *et al.*, 2006, two strains of heat-inactivated pathogenic bacteria (Vibrionaceae) were introduced in seabrem diet for probiotic porpouses. Blood samples

were taken each week and measured for innate humoral and cellular parameters. During the 4 weeks of the experiment the haemolytic complement activity, the serum peroxidase content, the phagocytic activity and the cytotoxic activity of treatment specimens, significantly increased when compared with control animals (fed a non enriched diet). However the respiratory burst of phagocytes remained unaffected by both treatments during the whole course of the procedure. This suggests that different components of the innate immune system can be affected by immunomodulatory substances.

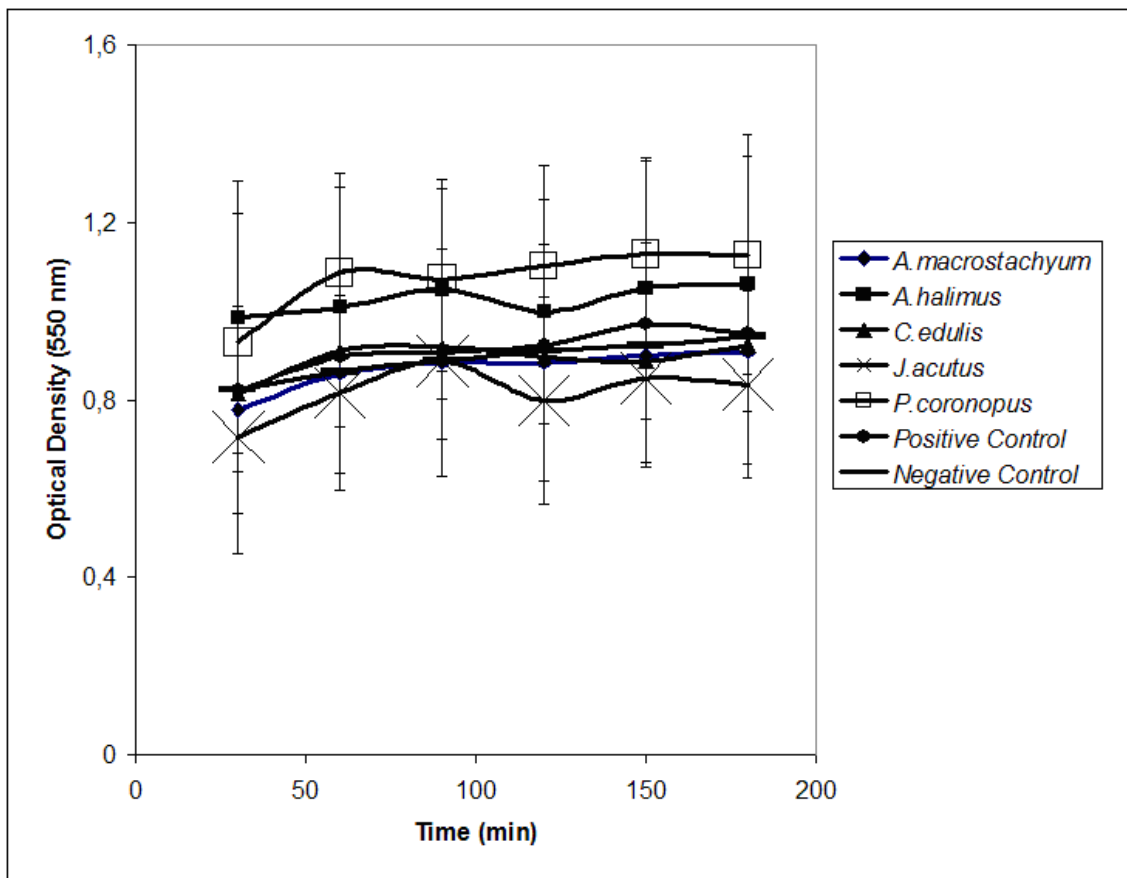


Fig 11. -Respiratory burst activity (O.D. 550 nm) of sea bream macrophages (individual fish head-kidney cells kept separated) after incubation for 3 hours in 10mg/ml halophyte extracts. The data are means of 5 different individuals.

4. Conclusion

Water extracts of the halophyte species *A. macrostachyum*, *A. halimus*, *C. edulis*, *J. acutus* and *P. coronopus* displayed antioxidant, copper chelating and iron chelating properties. *J. acutus* inhibited the growth of *P. d. piscicida*. The biomolecules present in these species are promising for aquaculture research. The techniques for head-kidney extraction were developed and optimised, leading to better prospects at future trials.

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