UNIVERSITÀ DEGLI STUDI DI UDINE

Dottorato di Ricerca in Scienze e Biotecnologie Agrarie
Ciclo XXVII

Coordinatore: prof. Mauro Spanghero

TESI DI DOTTORATO DI RICERCA

ANTIBACTERIAL AND IMMUNOMODULATORY PROPERTIES OF NATURAL COMPOUNDS FOR THE CONTROL OF INFECTIOUS DISEASES IN FARMED FISH

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ANNO ACCADEMICO 2014/2015

SUMMARY

Recently, a relevant number of studies recognised that medicinal plants and marine algae possess antibacterial activity against fish bacterial pathogens and demonstrated that their administration in fish species can enhance growth, immune response and disease resistance.

The present PhD thesis consists of five contributions inherent to these topics. The first chapter is a detailed review entitled "CURRENT RESEARCH ON THE USE OF PLANT-DERIVED PRODUCTS IN FARMED FISH", published in the journal Aquaculture Research (2015). It is a paper in which the effects of medicinal plants on fish growth, haematological profile, immune response and disease resistance are fully described. Moreover, this work provides useful information regarding the most common methods for the administration of plant-derived products in fish, and describes the factors that may influence their effectiveness. The literature search was conducted using some popular scientific sources of references (CAB Direct, Web of Knowledge and PubMed) and panels of keywords that allowed an high specificity of research. Data were then collected in Microsoft Excel tables and analysed in order to extrapolate the necessary information.

The second chapter of the dissertation represents the research paper entitled "*IN VITRO* ANTIBACTERIAL ACTIVITY OF PLANT ETHANOLIC EXTRACTS AGAINST FISH PATHOGENS", published in the Journal of the World Aquaculture Society (2014). *In vitro* assays were performed to assess the antibacterial activity of 15 commercial ethanolic extracts derived from medicinal plants against *Listonella anguillarum* (serotypes O1 and O2), *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*, and *Lactococcus garvieae*. Their antimicrobial potential was determined by the disc diffusion assay, then minimal inhibitory (MIC) and bactericidal (MBC) concentrations were established by the broth micro-dilution method. The medicinal plants *Lavandula officinalis*, *Melissa officinalis*, *Ocimum basilicum*, *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia officinalis*, and *Vaccinium vitis idaea* showed a broad spectrum of inhibitory effects. On the contrary, the extracts of *Achillea millefolium*, *Arnica montana*, *Calendula officinalis*, *Cetraria islandica*, *Equisetum arvense*, *Grindelia robusta*, *Orthosiphon stamineus*, and *Thymus vulgaris* were less or not active. *P. damselae* subsp. *piscicida* was the most susceptible bacterial strain, while *Y. ruckeri* was the most resistant.

The third chapter of the thesis, entitled "MODULATION OF RESPIRATORY BURST AND PROLIFERATION ACTIVITY OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) HEAD KIDNEY LEUKOCYTES BY MEDICINAL PLANT EXTRACTS", is dedicated to the *in vitro* evaluation of the immunomodulatory effects of some medicinal plant extracts on rainbow trout head kidney (HK) leucocytes. In teleosts, researches are currently based on *in vivo* experiments which are only occasionally preceded by *in vitro* approaches, leading to high costs and to the

sacrifice of a large number of fish. Nevertheless, the strict recommendations of the European Union Commission (2010/63/EU) and the recent Italian guidelines (D.L. 26/2014) impose to limit the use of animals for experimental and other scientific purposes and to minimize suffering and pain, consequently a preliminary *in vitro* evaluation is recommended. In our study, the cells were cultured in a medium containing increasing doses of extracts, then they were tested for reactive oxygen species (ROS) production after stimulation with phorbol myristate acetate (PMA) and proliferation in the presence or absence of phytohaemagglutinin from *Phaseolus vulgaris* (PHA). The extracts of *L. officinalis*, *O. vulgare* and *Rheum officinale* strongly reduced the oxidative burst activity of PMA-stimulated leukocytes, in a dose-dependent manner. *Aloe vera*, *Curcuma longa*, *Echinacea purpurea* and *Panax ginseng* extracts demonstrated antioxidant effects, although with lower efficacy and especially at lower concentrations. On the contrary, the highest concentration of ginseng extract stimulated the respiratory burst activity of leukocytes. Moreover, the extracts of *C. longa*, *E. purpurea*, *P. ginseng*, *L. officinalis* and *R. officinale* had a clear dose-dependent stimulatory effect on leukocyte proliferation.

The research described in the fourth chapter of the thesis, entitled "*PANAX GINSENG* AS FEED ADDITIVE FOR RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): EFFECTS ON GROWTH, BLOOD BIOCHEMICAL PROFILE, NON-SPECIFIC IMMUNE RESPONSE AND DISEASE RESISTANCE AGAINST *YERSINIA RUCKERI*", was aimed to investigate the possible inclusion of ginseng in the diet of rainbow trout, as growth promoter or immunostimulant. Four experimental diets were obtained by adding 0.0, 0.1, 0.2, 0.3 g/kg of ginseng ethanolic extract to a commercial basal feed and triplicate groups of fish were fed for 10 weeks. The inclusion of ginseng in the diet did not significantly affect growth performance. At the end of the trial, plasma albumin was significantly lower in fish fed ginseng enriched diets compared to fish fed the basal diet, while triglycerides and non esterified fatty acids levels were higher. A decrease, although not significant, in cholesterol and glucose levels was measured in fish receiving the supplemented feed. Negligible effects on plasma total proteins, globulins, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity were detected. The dietary administration of ginseng extract slightly increased the immune response (serum lysozyme and HK leukocytes respiratory burst activity) and reduced the mortality of rainbow trout infected with *Y. ruckeri* compared to controls, but no significant differences between the experimental groups were observed.

The study described in the fifth chapter of the thesis, entitled "EFFECTS OF DIETARY SUPPLEMENTATION WITH THE MARINE MICROALGAE *TETRASELMIS SUECICA* AND *ISOCHRYSIS GALBANA* ON GROWTH, BLOOD BIOCHEMICAL PROFILE AND INNATE IMMUNE RESPONSE OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)", was aimed to investigate the possibility to include the marine microalgae *T. suecica* and *I. galbana* in the diet of sea bass, as immunostimulants. Three iso-nitrogenous and iso-lipidic diets were

formulated to contain 0, 60 and 180 g/kg of microalgae and triplicate groups of fish were fed for 15 weeks. No significant effect on sea bass growth was registered after feeding with *T. suecica* and *I. galbana* supplemented diets. Similarly, at the end of the trial there were no marked changes in the biochemical parameters in fish fed diets supplemented with microalgae when compared with fish fed the control diet, although a decrease of blood cholesterol and triglycerides levels with increasing doses of microalgae was observed. The administration of *T. suecica* and *I. galbana* stimulated the serum lysozyme and antiprotease activity, especially in fish fed the diet with 60 g/kg of microalgae. No significant differences were found in the serum peroxidase activity, respiratory burst activity, and myeloperoxidase content of HK leukocytes among the experimental groups.

The researches described in the last three chapters, properly drafted, will be part of three scientific papers that will be submitted to selected journals for the publication.

CONTENTS

PREFACE

Infectious diseases are still the most serious problem for the aquaculture industry, causing heavy economic losses. The need to drastically reduce the widespread application of antibiotics in the treatment of diseases is driving new emphasis on the development of alternative strategies for the disease management in aquaculture. On the other hand, though vaccination is the most effective method for preventing disease outbreaks, the development of effective formulations for a number of pathogens is often hindered by high production costs and antigenic heterogeneity of microbial strains. In this context, currently there is a considerable and growing interest in the screening of products derived from medicinal plants or marine algae for their inhibitory activity against fish pathogens, in order to exploit new antimicrobial compounds of natural origin that could be employed in the control of infectious diseases. Moreover, the interest of researchers, aqua-feed mills and pharmaceutical companies has increased in recent years with regard to the possible use of plant/algae-derived products as immunostimulants, capable of strengthening the immune responses (immunocompetence) and consequently the resistance of farmed fish to pathogens.

Medicinal plants are known to synthesize many bioactive secondary metabolites with antimicrobial and biological properties, like alkaloids, terpenoids (triterpenes and steroid saponins), phenolic compounds, glycosides, flavonoids, tannins, and polysaccharides. In addition, plant-derived products are eco-friendly, inexpensive, easily prepared and do not show many of the side-effects that are often associated with synthetic antibiotics.

Marine microalgae received increasing attention in recent years as ingredients in fish feeding, due to their high nutritional value. Single microalgae or combinations of different microalgal species are currently utilized in fish larval nutrition and are also included in the diets of juvenile and adult molluscs (e.g. oysters, scallops, clams and mussels), crustacean and fish, as well as for raising the zooplankton required for feeding larval stages of finfish. In particular, certain marine microalgae currently deserve a growing interest in aquaculture as alternative to fish meal and oil due to their high content of proteins and LC-PUFAs, and the studies performed so far have proven the possibility to use microalgae in relatively small amounts in the diet, without hampering growth performance and major quality traits of fish. Moreover, apart from being potential sources of macronutrients, marine microalgae could also be considered as natural "nutraceutic packages" as they supply vitamins, bio-active compounds and, as single-cell organisms, they are a rich source of nucleotides. Therefore, the use of microalgal biomass and its derivatives is an interesting and innovative approach also to improve fish immune response and disease resistance.

Recently, a number of studies recognised that medicinal plants and marine algae possess antibacterial activity against fish bacterial pathogens and demonstrated that their

administration in fish species can enhance growth, immune response and disease resistance. For these reasons, the application of medicinal plants and algae in aquaculture represents a challenge for the near future. However their beneficial effects in fish species different from those considered to date (mostly freshwater ones, such as salmonids and carps, or tropical species, such as tilapia, grouper, olive flounder and yellow croaker) need to be further investigated. In particular, the possible application of natural compounds obtained from herbs or algae has not yet been adequately evaluated in terms of the integrated response of rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*), that are economically important farmed fish species for the Italian and Mediterranean aquaculture.

Thus, the main objectives of the present PhD thesis were:

1) summarize and discuss the results of the scientific pubblications available in literature since 1998 up to 2014, concerning the administration of medicinal plants in fish species, in order to provide useful information for the application of herbal products in finfish culture;

2) investigate the *in vitro* antibacterial activity of ethanolic extracts obtained from medicinal plants against relevant bacterial fish pathogens, in order to identify novel natural antimicrobial compounds that could be used in aquaculture for the control of bacterial infections;

3) assess and compare the *in vitro* effects of medicinal plant extracts on some immune functions of leukocytes purified from head kidney of rainbow trout (*O. mykiss*), in order to explore whether they contain bioactive compounds of potential interest as antioxidant agents or immunostimulants in aquaculture;

4) evaluate the possible modulation of growth performance, blood biochemical profile, non-specific immune parameters and resistance to *Yersinia ruckeri* infection in rainbow trout (*O. mykiss*) by the dietary administration of *Panax ginseng* root extract, in order to examine the possibility to use this medicinal plant as feed additive and immunostimulant in aquaculture;

5) investigate the effects of the dietary inclusion of the marine microalgae *Tetraselmis suecica* and *Isochrysis galbana* on growth performance, blood biochemical profile and non-specific immune response of European sea bass (*D. labrax*), in order to examine the potential use of these microalgae as supplements in aquafeeds.

CHAPTER I

CURRENT RESEARCH ON THE USE OF PLANT-DERIVED PRODUCTS IN FARMED FISH

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Aquaculture Research (2015), 46: 513-551, doi:10.1111/are.12238.

Abstract

Over the years, aquaculture has shown increasing development in terms of production. However, due to intensive farming practices, infectious diseases represent the main problem in fish farms, causing heavy economic losses. The use of antibiotics for controlling diseases is widely criticized for its negative impact, including selection of antibiotic resistant bacterial strains, immunosuppression, environmental pollution and accumulation of chemical residues in fish tissues. On the other hand, though vaccination is the most effective prophylactic method of preventing disease outbreaks, the development of effective formulations is often hindered by high production costs and the antigenic heterogeneity of the microbial strains. Recently, there has been increased interest in the possibility of using medicinal herbs as immunostimulants, capable of enhancing immune responses and disease resistance of cultured fish. Plant-derived products seem to represent a promising source of bioactive molecules, being at the same time readily available, inexpensive and biocompatible. The aim of this article is to provide an overview of recent research dealing with the use of medicinal plants in aquaculture. Special attention is given to the information about the effects of plant extracts/products on fish growth, haematological profiles, immune responses and resistance to infectious diseases.

Keywords: medicinal plants, feed additives, immunostimulants, natural antibiotics, disease resistance, aquaculture.

Riassunto

Nel corso degli anni l'acquacoltura ha mostrato un crescente sviluppo in termini tecnologici e produttivi. Tuttavia, a causa di pratiche di allevamento intensivo, le malattie infettive rappresentano il problema principale negli allevamenti ittici, causando rilevanti perdite economiche. L'uso di antibiotici per il controllo delle malattie è ampiamente criticato in quanto associato a selezione di ceppi batterici antibiotico-resistenti, immunosoppressione, inquinamento ambientale e accumulo nei tessuti dei pesci di residui chimici potenzialmente pericolosi per il consumatore. D'altra parte, sebbene la vaccinazione sia il metodo di profilassi più efficace per prevenire la comparsa di malattie, lo sviluppo di formulazioni efficaci è spesso ostacolato dagli elevati costi di produzione e dalla eterogeneità antigenica dei ceppi microbici. Per questi motivi, recentemente in acquacoltura è aumentato l'interesse verso l'impiego di piante medicinali come immunostimolanti, in grado di migliorare la risposta immunitaria e la resistenza alle malattie dei pesci allevati. I prodotti di origine vegetale sembrano rappresentare una fonte promettente di molecole bioattive, essendo allo stesso tempo facilmente reperibili, poco costose e biocompatibili.

Lo scopo di questo lavoro è quello di fornire una panoramica delle recenti ricerche riguardanti l'uso di piante medicinali in acquacoltura. Particolare attenzione viene data alle informazioni relative agli effetti della somministrazione di estratti/prodotti vegetali su crescita, profilo ematologico, risposta immunitaria e resistenza alle malattie infettive delle specie ittiche.

Parole chiave: piante medicinali, additivi alimentari, immunostimolanti, antibiotici naturali, resistenza alle malattie, acquacoltura.

1.1 Introduction

Over the years world aquaculture has grown rapidly in terms of production. However, intensive and stressful rearing conditions make farmed fish highly susceptible to different infectious diseases which are now the most serious problem for the aquaculture industry, causing heavy economic losses. The use of antibiotics for controlling diseases is widely criticized because it is often very expensive and leads to the selection of antibiotic-resistant bacterial strains, immunosuppression, environmental pollution and the accumulation of chemical residues in fish tissues which can be potentially harmful to public health (FAO/WHO/OIE 2006). In U.S.A. and Europe governmental restrictions [U.S. Food and Drug Administration (FDA) and U.S. Environmental Protection Agency (EPA) guidance; CE Regulations Nos. 1804/1999, 37/2000, 82/2001, 178/2002, 74/2003, 28/2004, 726/2004 and 834/2007; Italian Decrees Nos. 119/1992, 47/1997, 336/1999 and 71/2003] have limited the number of drugs that can be currently used in aquaculture and even in Asian countries a strict demand for fish products free of pollutants/antibiotics is steeply increasing (Ji *et al.*, 2007a). On the other hand, though vaccination is the most effective method of preventing disease outbreaks, the production of effective formulations for a number of pathogens is often hindered by high production costs and the antigenic heterogeneity of the microbial strains (Le Breton, 2009; Toranzo *et al.*, 2009).

Given the problems and limitations listed above, the interest of researchers, aqua-feed mills and pharmaceutical companies has increased in recent years with regard to the development of alternative strategies for the disease management in aquaculture, capable of strengthening the immune responses (immunocompetence) of fish and consequently their resistance to pathogens. Synthetic or natural immunostimulants (probiotics, complex carbohydrates, nutritional factors, hormones, cytokines, products derived from animal, plants and algae) are able to effectively promote fish growth, the innate/non-specific immune response (lysozyme, complement, phagocyte activity, respiratory burst and microbial activities of phagocytes) and, to a lesser extent, the adaptive/specific immune response (immunoglobulin production) (Anderson, 1992; Galeotti, 1998; Sakai, 1999). However, the use of hormones, vitamins and chemicals is often not recommended because they may produce side effects in fish and could leave potentially dangerous residues for consumers. Set against this, natural products like plant-derived products could represent a promising approach complementary to vaccination and traditional drugs, since they provide a useful source of biologically active secondary metabolites, being at the same time easily available, inexpensive and biocompatible (Mohamad & Abasali, 2010).

The use of medicinal plants in aquaculture has attracted a lot of attention globally and has become a subject of active scientific investigations (Jeney *et al.*, 2009; Chakraborty & Hancz, 2011; Harikrishnan *et al.*, 2011a). In this manuscript 105 scientific pubblications available in

literature since 1998 up to 2011 concerning the administration of phytomedicines in fish species were examined. Such studies were performed mainly over the past five years. In particular, 83% were made during the period 2006-2011, 15% were performed between 2001 and 2005, relatively few in number are those conducted before 2001 (Fig. 1). The majority of these experiments were carried out in Asia [the countries with the highest percentages of documents are India (30.2%), Korea (19.8%), Thailand (7.5%) and China (5.7%)], some experiments were performed in Middle East [Iran (6.6%), Egypt (6.6%), Turkey (3.8%), Israel (2.8%)], and just a few in Europe [UK (4.7%), Greece (0.9%) and Hungary (0.9%)]. No data are available about experiments conduced in America.

The purpose of this article is to summarize and discuss the results of these studies, in order to provide useful information for the application of herbal products in finfish culture. This is the first detailed review in which the effects of plants on fish growth, haematological profile, immune response and disease resistance are fully described.

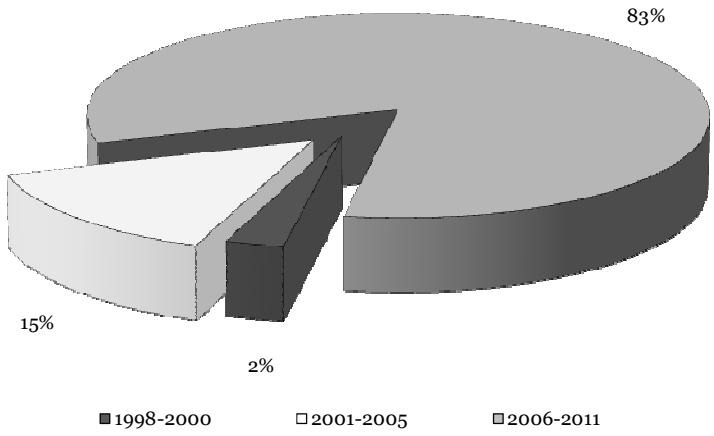

Figure 1 Distribution between the years 1998 and 2011 of the literature concerning the use of plants and plant-derived products in fish species. Reference publications = 105.

1.2 Plant species and plant-derived products currently under investigation

Medicinal plants have been reported as having a broad spectrum of growth promotion, appetite stimulation, antimicrobial, immunostimulant, antinflammatory, antistress, anticancer properties and their use in traditional medicine has been known for thousands of years around the world. In some Asian countries such as China, India, Japan, Thailand, Korea and some countries in South and Central America, different herbal preparations are used in human and veterinary medicine in order to prevent and treat bacterial, fungal and viral diseases as well as in the therapy of several disorders (Briskin, 2000; Lovkova *et al.*, 2001; Wynn & Fougere, 2007; Sher, 2009; Hashemi & Davoodi, 2011; Sakarkar & Deshmukh, 2011; Sharma *et al.*, 2011; Wallace *et al.*, 2011). In aquaculture the application of medicinal herbs has been reported in

various Asian countries (Direkbusarakom, 2004).

Recently, more than 60 different plant species have been studied for the improvement of fish health and disease management in aquaculture (Tables 1-5). The most investigated herbs are those widely used in folk medicine in China, India, Thailand and Korea, such as *Achyranthes aspera*, *Aegle marmelos*, *Andrographis paniculata*, *Angelica sinensis*, *Artemisia capillaries*, *Astragalus membranaceus*, *Azadirachta indica*, *Cnidium officinale*, *Crataegi fructus*, *Cynodon dactylon*, *Echinacea purpurea*, *Eclipta alba*, *Lonicera japonica*, *Massa medicata*, *Nyctanthes arbortristis*, *Punica granatum*, *Scutellaria baicalensis*, *Solanum nigrum*, *Solanum trilobatum*, *Tinospora cordifolia*, *Toona sinensis*, *Whitania somnifera* and *Zataria multiflora*. Nevertheless, other plants which are used all over the world for both curative and culinary purposes such as garlic (*Allium sativum*), garlic chives (*Allium tuberosum*), green tea (*Camellia sinensis*), cinnamom (*Cinnamomum verum* or *zeylanicum*), turmeric (*Curcuma longa*), Sundial lupine (*Lupinus perennis*), mango (*Mangifera indica*), peppermint (*Mentha piperita*), nutmeg (*Myristica fragrans*), basil (*Ocimum basilicum* and *sanctum*), oregano (*Origanum vulgare*), rhubarb (*Rheum officinale*), rosemary (*Rosmarinus officinalis*) and ginger (*Zingiber officinale*) have been also screened. The herbal remedies have been generally administered as plant materials (seeds, bulbs, leaves) or plant-derived products, including extracts obtained using a range of extraction procedures and different aqueous or organic solvents (ethanol, methanol, ethyl acetate, hexane, butane, acetone, benzene, petroleum ether, etc.), or other preparations such as essential oils, concoctions and decoctions.

1.3 Fish species under investigation

The fish species widely reared in Asian aquaculture have been the most studied ones. In fact, 29.9% of the research has been conducted in tilapias (*Oreochromis mossambicus* and *niloticus*), 25.2% in carps (*Cyprinus carpio, Labeo rohita* and *Catla catla*), 7.5% in the goldfish (*Carassius auratus*), 7.5% in groupers (*Epinephelus tauvina* and *bruneus*), 5.6% in the olive flounder (*Paralichthys olivaceus*). The remaining studies dealt with other Asian fish species such as yellow croaker (*Pseudosciaena crocea*), rockfish (*Sebastes schlegeli*), Japanese eel (*Anguilla japonica*), rock bream (*Oplegnathus fasciatus*), spotted snakehead (*Channa punctatus*). Among fish species that are cultured in western countries, the rainbow trout (*Oncorhynchus mykiss*) has been the most investigated one with 10.3% of references. Other studies have been performed on the channel catfish (*Ictalurus punctatus*), sharpsnout sea bream (*Diplodus puntazzo*), red sea bream (*Pagrus major*), sea bass (*Dicentrarchus labrax*) have been little studied, while no data exist on gilthead sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), solea (*Solea* spp.) and Atlantic salmon (*Salmo salar*) (Fig.2).

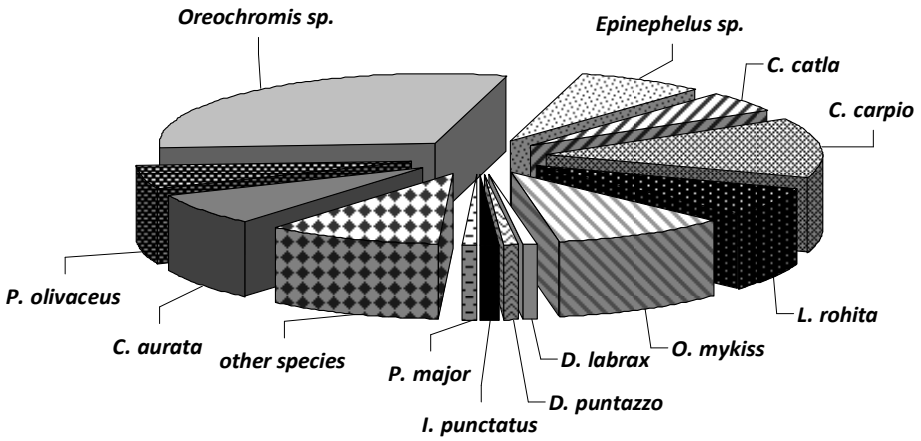

Figure 2 Fish species treated with plant-derived products. Reference publications = 105.

1.4 Route of plant administration and dosage

Medicinal plants have been applied almost exclusively via oral administration as immunostimulants and less frequently via injection or immersion for preventive purposes or disease treatment.

Oral delivery, although it is the least effective method for immunostimulants administration, since the product is slowly absorbed by the fish (Harikrishnan *et al.*, 2009b), is regarded as the most suitable method for fish farming. It is non stressful for fish and allows a large number of subjects to be treated with the minimum cost and effort (Sakai, 1999). Different strategies have been used for the oral administration of plants and plant-derived products in fish (Tables 1 and 3). Very frequently plant extracts/plant parts have been included as ingredients of the diet (pellet /extruded feed) at doses ranging from 0.1% to 2% and fish were fed for a period ranging from 2 to 5 weeks, according to a dietary regime (% BW/day) calibrated for fish species and size (Dügenci *et al.*, 2003; Vasudeva *et al.*, 2004; Vasudeva & Chakrabarti, 2004; 2005a; 2005b; Vasudeva *et al.*, 2006; Christybapita *et al.*, 2007; Choi *et al.*, 2008; Awad & Austin, 2010; Harikrishnan *et al.*, 2010b; Kirubakaran *et al.*, 2010; Rattanachaikunsopon & Phumkhachorn, 2010a; 2010c; Awad *et al.*, 2011; Harikrishnan *et al.*, 2011d; 2011f; 2011g). In other experiments similar percentages of herbs have been included in the diet but fish were fed until satiation or for a longer time period (6 to 12 weeks) (Kim *et al.*, 1999; Jian & Wu, 2003; 2004; Shalaby *et al.*, 2006; Yin *et al.*, 2006; Ji *et al.*, 2007a; Ji *et al.*, 2007b; Sahu *et al.*, 2007a; Sahu *et al.*, 2007b; Xie *et al.*, 2008; Immanuel *et al.*, 2009; Nya & Austin, 2009a; 2009b; Yin *et al.*, 2009; Aly & Mohamed, 2010; Bilen & Bulut, 2010; Mohamad & Abasali, 2010; Sharma *et al.*, 2010; Bilen *et al.*, 2011; Harikrishnan *et al.*, 2011b; Nya & Austin, 2011). Otherwise fish were fed diets supplemented with smaller percentages (0.002%-0.08%) of plants (Sivaram *et al.*, 2004; Aly *et*

al., 2008a; 2008b; Goda, 2008; Rattanachaikunsopon & Phumkhachorn, 2009a; Zheng *et al.*, 2009; Harikrishnan *et al.*, 2010a; Sheikhzadeh *et al.*, 2011). Occasionally diets supplemented with higher doses (between 4% to 16%) of plants have been used (Abutbul *et al.*, 2004; Shalabay *et al.*, 2006; Xie *et al.*, 2008; Dorucu *et al.*, 2009; Sharma *et al.*, 2010; Zilberg *et al.*, 2010).

When plant administration has been performed by intraperitoneal injection (Tables 2 and 4), fish were treated with low doses of extracts, between 0.8 and 800 mg/kg of BW (Sudhakaran *et al.*, 2006; Divyagnaneswari *et al.*, 2007; 2008; Harikrishnan *et al.*, 2009b; Alexander *et al.*, 2010; Harikrishnan, *et al.*, 2010e; Harikrishnan *et al.*, 2010f). Intraperitoneal injection has been proved to be the most effective way of administration because enables the immunostimulant to be quickly absorbed and functional (Harikrishnan *et al.*, 2009b), but it is stressful, labour intensive, relatively time-consuming and becomes impractical when the fish weigh is less than 10-15 g (Sakai, 1999; Galindo-Villegas & Hosokawa, 2004).

The administration of plant extracts to fish by immersion (Table 5) has been tested in the treatment of diseases as alternative to traditional drugs and the protocols adopted included single or repeated treatments (Harikrishnan *et al.*, 2003; Harikrishnan *et al.*, 2005; Harikrishnan & Balasundaram, 2008; Rajendiran *et al.*, 2008; Harikrishnan *et al.*, 2009c; Harikrishnan *et al.*, 2010g; Rattanachaikunsopon & Phumkhachorn, 2010b). Dip treatment is logistically more practical for a large number of small fish (weighting less than 5 g) but dilution, exposure time and levels of efficacy are usually not well defined for the majority of immunostimulants (Sakai, 1999; Galindo-Villegas & Hosokawa, 2004).

1.5 Effects on fish growth and survival

In aquaculture various growth-promoting additives are commonly added to the diets to improve the nutrient utilization, growth performance and survival of cultured fish. Dietary supplements include probiotics, yeast, amino acids, antioxidants, carnitine, colorants, enzymes, lipid derivatives, nutraceutics, vitamins, hormones, aromatic compounds, plant extracts and certain organic acids/salts (Goda, 2008). The beneficial effects of the inclusion of plants or their derived products in fish feed are well documented (Table 1). Diets enriched with *A. sativum* have been reported to significantly increase survival (Aly & Mohamed, 2010), weight gain (WG), specific growth rate (SGR), feed efficiency ratio (FER) and decrease the feed conversion ratio (FCR) in Nile tilapia (Shalaby *et al.*, 2006; Aly *et al.*, 2008a; Aly & Mohamed, 2010). Similar effects have been observed in this species fed diets including extract of ginseng (Goda, 2008) or *E. purpurea* (Aly *et al.*, 2008b; Aly & Mohamed, 2010). Tilapia *O. mossambicus* fed a diet supplemented with acetone extract of *A. marmelos, C. dactylon*, *W. somnifera* or *Z. officinale* showed increased SGR (Immanuel *et al.*, 2009). Ji *et al.* (2007b) found increased FBW and survival rates in red sea bream fed diet supplemented with *A. capillaries, C. officinale* or *C.*

fructus. An improvement of the growth parameters WG, SGR and FCR was observed in rainbow trout fed diets containing *A. sativum* (Nya & Austin, 2009a) or *Z. officinale* (Nya & Austin, 2009b). Catfish fed a diet including *O. vulgare* essential oil showed reduced FCR and elevated WG, SGR and survival (Zheng *et al.*, 2009). The oral administration of *A. aspera* (Vasudeva *et al.*, 2006) increased the SGR value and decreased the FCR value in *L. rohita* and similar results have been reported in *C. carpio* fed *Rheum officinale* enriched diets (Xie *et al.*, 2008). Moreover, the survival in *L. rohita* was improved by feeding diets with *C. longa* (Sahu *et al.*, 2008). A mixture of *A. sinensis* and *A. membranaceus* in the diet increased the FBW value in *C. carpio* var. *Jian* (Jian & Wu, 2004). In greasy groupers, the dietary incorporation of *O. sanctum* or *W. somnifera* extract induced a significant increase in both WG and SGR values (Sivaram *et al.*, 2004). In the same fish species increased SGR value was also observed after feeding a mixture of *C. dactylon, Piper longum*, *Phyllanthus niruri*, *Tridax procumbens* and *Z. officinale* extracts (Punitha *et al.*, 2008). Diets containing *A. capillaries*, *C. officinale*, *C. fructus* or *M. medicata* increased survival rates, FBW, WG and SGR in olive flounder juveniles (Ji *et al.*, 2007a).

Some authors suggest that a dietary supplementation with plants could improve lipid metabolism and modulate the activities of trypsin-like enzymes during digestive processes, resulting in an efficient protein deposition and growth performance. Further, it has been reported that alcohol extracts of herbs might inhibit the colonization and proliferation of pathogenic bacteria in fish digestive tract and preferentially maintain the intestinal bacterial flora, improving consequently the digestibility of feeds and subsequent nutrient absorption (Ji *et al.*, 2007a; Goda, 2008; Xie *et al.*, 2008; Aly *et al.*, 2008a; Aly & Mohamed, 2010). In support of these hypotheses there is some evidence of an increase in the protein efficiency ratio (PER), protein productive value (PPV) or energy retention (ER) in different fish species fed herbs supplemented diets (Shalabay *et al.*, 2006; Goda, 2008; Nya & Austin, 2009a; 2009b).

1.6 Effects on fish haematological profile

Although the reference values for haematological and biochemical blood parameters of fish species have not been fully defined and the correlation between changes in these parameters and the occurrence of specific diseases or metabolic disorders has not been well characterized, haematological analysis may prove to be an important tool to diagnose diseases and to monitor the fish physiological status in response to therapeutic and dietary treatments, environmental changes and stress (Shalabay *et al.*, 2006).

1.6.1 Haematological indices

The red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) are haematological indices which indicate the erythrocyte status and oxygen-carrying capability in fish (Houston, 1997). The amount of oxygen received by tissues depends on the maturity of the erythrocytes and their haemoglobin content. Therefore these parameters can be helpful in detecting any abnormal changes in fish health conditions, for example during the use of immunostimulants (Goda, 2008). The leukocrit (Lct) and white blood cell count (WBC) are indicators of the leukocyte percentages in the blood. A sharp increase in the total number of leukocytes is known to be correlated to the activation of a protective response against infectious agents and chemicals (Harikrishnan *et al.*, 2010a). Several authors have observed positive changes in these cell parameters following the administration of plants to fish (Tables 1, 3 and 5), probably as a result of the stimulation of erythropoiesis and leukopoiesis by plant active compounds. Nya & Austin (2009a; 2011) reported that the dietary administration of garlic in rainbow trout enhanced the maturation and haemoglobin content of erythrocytes as well as the number of total leukocytes and lymphocytes, leading to an improvement in fish health status and immune competence. Similarly, diets with *L. perennis*, *M. indica* or *Urtica dioica* increased the RBC, Hct and WBC values (Awad & Austin, 2010), with *Z. officinale* elevated RBC, Hct, WBC, lymphocytes, monocytes and neutrophils (Nya & Austin, 2009b), with *Nigella sativa* increased Lct and Hct (Dorucu *et al.*, 2009). Diets supplemented with garlic (Sahu *et al.*, 2007a), mango (Sahu *et al.*, 2007b) or *C. dactylon* extract (Kaleeswaran *et al.*, 2012) significantly increased the blood indices RBC, Hb and WBC in Indian major carp. Similar increases in RBC and WBC values in this fish species were described by Sahu *et al.* (2008) after the administration of turmeric. Moreover, common carps fed diets containing a mixture of *Brussica nigra*, *Chelidonium majus*, *E. purpurea*, *Inula helenium* and *Tussilago farfara* extracts (Mohamed & Abasali, 2010), a mixture of *C. zeylanicum*, *Juglans regia*, *M. piperita* and *O. basilicum* extracts (Abasali & Mohamad, 2010), *A. marmelos* extract (Pratheepa *et al.*, 2010) or *Euphorbia hirta* extract (Pratheepa & Sukumaran, 2011) had significantly higher haemoglobin content, RBC and WBC indices. Common carps fed diets with *Z. multiflora* essential oil (Soltani *et al.*, 2010) showed higher WBC. The dietary administration of garlic (Shalabay *et al.*, 2006; Aly *et al.*, 2008a; Aly & Mohamed, 2010) and ginseng root extract (Goda, 2008) in Nile tilapia induced significant increases in RBC, Hb and Hct, although these plants did not seem to have an evident positive effect on WBC. On the other hand, the extract of *E. purpurea* increased Hct as well as WBC, lymphocytes and eosinophils counts (Aly *et al.*, 2008b; Aly & Mohamed, 2010). Tilapia *O. mossambicus* fed diets enriched with acetone extract of *A. marmelos*, *C. dactylon*, *W. somnifera* or *Z. officinale* had higher Hct and Lct values (Immanuel

et al., 2009). Higher Hb concentration was observed in greasy grouper fed diets containing *O. sanctum* extract or *W. somnifera* extract (Sivaram *et al.*, 2004), kelp grouper fed diets including *E. japonica* extract (Kim *et al.*, 2011) or *Kalopanax pictus* extract (Harikrishnan *et al.*, 2011f), red sea bream fed a diet supplemented with *A. capillaries*, *C. officinale*, *C. fructus* or *M. medicata* (Ji *et al.*, 2007b). Interestingly, the use of traditional herbal medicines has been reported to restore the altered haematological parameters to nearly normal values and to heal lesions caused by *Aeromonas hydrophila* and *Aphanomyces invadans* in *C. carpio* and *C. auratus* (Harikrishnan *et al.*, 2003; 2005; Harikrishnan &Balasundaram 2008; Harikrishnan *et al.*, 2010a; 2010b).

1.6.2 Biochemical parameters

Serum/plasma proteins include albumin, globulins and various humoral elements of the non-specific immune system, such as transferrin, precipitins, agglutinins, antimicrobial peptides, complement factors, lysozyme and antiproteases (Ellis, 1999; Magnadottir, 2006). Albumin is essential for maintaining the osmotic pressure needed for the proper distribution of body fluids and acts as a plasma carrier or non-specific ligand with many binding domains (Nya & Austin, 2009a). Serum globulins, such as gamma globulins, are the source of immunoglobulins, so their level in blood reflects the concentration of antibodies and consequently the immune status of fish (Goda, 2008). Certain herbal medicines have been reported to increase serum total proteins, albumin and globulin (Table 1), suggesting a stimulation of humoral immune response and the improvement in health status. In Indian major carp fed diets enriched with *A. sativum* (Sahu *et al.*, 2007a), *C. longa* (Sahu *et al.*, 2008), *C. dactylon* extract (Kaleeswaran *et al.*, 2012) or *M. indica* (Sahu *et al.*, 2007b) significant increases in serum total proteins, albumin and globulins were noted. Higher levels of albumin (Vasudeva *et al.*, 2006) and globulins (Vasudeva & Chakrabarti, 2004; Vasudeva *et al.*, 2004; Vasudeva & Chakrabarti, 2005a; 2005b) were found in carps fed diets containing *A. aspera*. Positive variations in total proteins, albumin and globulins were observed in common carp fed diets supplemented with a mixture of ethanolic extracts from *B. nigra*, *C. majus*, *E. purpurea*, *I. helenium* and *T. farfara* (Mohamad & Abasali, 2010) or *C. zeylanicum*, *J. regia*, *M. piperita* and *O. basilicum* (Abasali & Mohamad, 2010). Goda (2008) reported an increase of these parameters in *O. niloticus* fed diets including ginseng root extract while Shalabay *et al.* (2006) described only an increase of proteins after feeding diets with *A. sativum*. Total proteins, albumin and globulins were elevated in *O. mossambicus* after the dietary administration of *C. dactylon*, *W. somnifera* or *Z. officinale* acetone extract (Immanuel *et al.*, 2009). Higher serum proteins level was reported in rainbow trout fed diets supplemented with *Cotinus coggyria* (Bilen *et al.*, 2011), *M. indica* (Awad & Austin, 2010) or *N. sativa* (Dorucu *et al.*, 2009) as well as with aqueous extract of *U. dioica* or *Viscum album* (Dügenci *et al.*, 2003). Furthermore, in the same fish species a significant increase in both

protein and globulin concentrations was detected after feeding diets enriched with *A. sativum* (Nya & Austin, 2009a) or *Z. officinale* (Dügenci *et al.*, 2003; Nya & Austin, 2009b). Kim *et al.* (2011) reported an increase of total proteins, albumin and globulins in kelp grouper fed diets containing *Eriobotrya japonica* ethanolic extract, similarly other authors observed an increase of proteins using diets enriched with ethanolic extract of *K. pictus* (Harikrishnan *et al.*, 2011f) or *Styrax japonica* (Harikrishnan *et al.*, 2011g). The dietary administration of *O. sanctum* extract or *W. somnifera* extract increased globulins in greasy grouper (Sivaram *et al.*, 2004).

Blood glucose concentration is often used as an indicator of non-specific stress in fish rather than raised cortisol and adrenaline levels. In fact, during stressful situations there is an abrupt increase in blood cortisol which causes a breakdown of glycogen from the liver through glycogenolysis and, consequently, a rise in blood glucose levels (Shalabay *et al.*, 2006; Xie *et al.*, 2008; Kaleeswaran *et al.*, 2012). Several studies (Table 1) indicated that the administration of medicinal plants in fish can significantly reduce blood glucose and cortisol concentrations, limiting the effects of environmental stressors or infections, which normally cause their increase. Blood glucose significantly decreased in *O. niloticus* (Shalabay *et al.*, 2006) and *L. rohita* (Sahu *et al.*, 2007a) after the dietary administration of garlic. In *L. rohita* this parameter was also reduced after feeding diets containing *M. indica* (Sahu *et al.*, 2007b). In common carp both blood cortisol and glucose decreased after feeding diets including rhubarb anthraquinone extract (Xie *et al.*, 2008), while lower levels of glucose were induced by diets enriched with *B. nigra*, *C. majus*, *E. purpurea*, *I. helenium*, *T. farfara* (Mohamad & Abasali, 2010), *C. zeylanicum*, *J. regia*, *M. piperita* and *O. basilicum* (Abasali & Mohamad, 2010) extracts. Diets with extract of *A. marmelos*, *C. dactylon*, *W. somnifera* or *Z. officinale* significantly reduced plasma glucose in *O. mossambicus* (Immanuel *et al.*, 2009). Harikrishnan *et al.*, (2010a) reported an effective resistance to stress caused by *A. hydrophila* infections in *C. auratus* treated with diets containing different herbal extracts. Similarly, *E. bruneus* infected with *Vibrio carchariae* and fed diets supplemented with *E. japonica* ethanolic extract had lower glucose level in comparison with control untreated group (Kim *et al.*, 2011).

It is suggested that several medicinal herbs promote lipid metabolism that catabolizes body fatty acids as a main energy expenditure, resulting in an efficient protein accumulation and growth performance (Ji *et al.*, 2007a). However, information concerning the effects of the administration of plants on blood lipids are limited (Tables 1 and 3). Diets containing *A. sativum* significantly decreased total lipids in Nile tilapia (Shalabay *et al.*, 2006). Triglycerides and cholesterol have been reported as being reduced in tilapia *O. mossambicus* fed a diet with acetone extract of *A. marmelos*, *C. dactylon*, *W. somnifera* or *Z. officinale* (Immanuel *et al.*, 2009). Moreover, lower plasma triglycerides and higher plasma HDL-cholesterol were observed in red sea bream fed a fishmeal diet supplemented with *C. officinale* (Ji *et al.*, 2007b) and in juvenile flounder fed a diet containing a mixture of *A. capillaries*, *C. fructus*, *C. officinale* and *M.*

medicata (Ji *et al.*, 2007a).

Alkaline phosphatase (ALP) and acid phosphatase (ACP) are important enzymes that regulate a number of essential functions in organisms. An increase in serum ALP activity indicates higher breakdown of the energy reserves which are then used for the growth and survival of fish (Sahu *et al.*, 2008). ACP is widely considered to be a valuable parameter of macrophage activation (Pratheepa *et al.*, 2010). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are ubiquitous aminotransferases that represent indices for the diagnosis of hepatopancreas injury. Liver is rich in ALT and AST and hepatic damage (induced by chemical, infectious and physiological factors) or a disturbance in the Kreb's cycle may result in their increase (Shalabay *et al.*, 2006; Ji *et al.*, 2007b). A range of evidences suggests that the administration of medicinal herbs in fish can modulate the activities of these enzymes (Table 1). A diet including leaf extract of *A. marmelos* significantly enhanced ACP and ALP activity in *C. carpio*, probably as a consequence of the macrophage activation (Pratheepa *et al.*, 2010). Shalabay *et al.* (2006) found lower serum ALT and AST activities in Nile tilapia fed diets with *A. sativum*. Similarly, a diet enriched with *A. capillaries*, *C. officinale*, *C. fructus* or *M. medicata* has been reported to reduce the activity of these enzymes in red sea bream (Ji *et al.*, 2007b) and olive flounder (Ji *et al.*, 2007a). Diets supplemented with *A. aspera* (Vasudeva *et al.*, 2006; Vasudeva & Sunil, 2009) or *C. longa* (Sahu *et al.*, 2008) increased ALP activity and reduced both ALT and AST concentrations in the serum of Indian major carp.

1.7 Effects on fish immune response

The immunomodulatory effects of herbal medicines have been well reported in various fish species. The products derived from plants improved mainly the innate/non specific immune response, being able to stimulate both humoral and cellular defence mechanisms, as reported by Galindo-Villegas & Hosokawa (2004) also for other immunostimulant substances. Immunostimulants probably interact with specific receptors on cells surface and promote the expression of intracellular genes encoding for antimicrobial molecules (Raa, 1996; Bricknell & Dalmo, 2005). The research findings demonstrate also positive effects of plants on fish specific immune response.

1.7.1 Non-specific immune response

Plant products enhance various components of the innate immunity such as serum lysozyme, complement, antiproteases, phagocytes microbicidal activities (Tables 1-4).

Lysozyme is a bactericidal enzyme that hydrolyzes the β-1,4 glycosidic linkage between N-

acetyl glucosamine and N-acetyl muramic acid of bacterial cell wall peptidoglycan, thereby causing bacteriolysis and preventing the growth of bacteria. Lysozyme is also known to activate the complement system and phagocytes by acting as an opsonin, as well as to display anti-viral and anti-inflammatory properties (Magnadottir, 2006; Saurabh & Sahoo, 2008). The serum lysozyme activity was reported to be enhanced in rainbow trout fed diets with *A. sativum* bulbs (Nya & Austin, 2009a; 2011), *C. sinensis* extract (Sheikhzadeh *et al.*, 2011), *C. coggyria* (Bilen *et al.*, 2011), *L. perennis* seeds, *M. indica* fruits, *U. dioica* leaves (Awad & Austin, 2010) or *Z. officinalis* root (Nya & Austin, 2009b). A similar immunostimulatory effect was observed in tilapia *O. niloticus* fed diets supplemented with extract of *A. membranaceus* (Yin *et al.*, 2006; Ardò *et al.*, 2008), *Cratoxylum formosum* (Rattanachaikunsopon & Phumkhachorn, 2010c), *E. purpurea* (Aly *et al.*, 2008b) or *L. japonica* (Ardò *et al.*, 2008) as well as in tilapia *O. mossambicus* fed diets enriched with extract of *C. dactylon*, *W. somnifera*, *Z. officinale* (Immanuel *et al.*, 2009), *E. alba* (Chhristybapita *et al.*, 2007) or *N. arbortristis* (Kirubakaran *et al.*, 2010). The lysozyme activity significantly increased in *O. mossambicus* also after intraperitoneal injection of water and hexane soluble fractions of *S. trilobatum* leaves (Divyagnaneswari *et al.*, 2007; 2008) and water soluble fraction of *T. cordifolia* leaves (Alexander *et al.*, 2010). A significant increase of serum lysozyme activity was reported in Indian major carp after feeding diets with *A. aspera* seeds (Vasudeva *et al.*, 2006; Vasudeva & Sunil, 2009), *A. sativum* bulbs (Sahu *et al.*, 2007a), *C. dactylon* extract (Kaleeswaran *et al.*, 2011a), *C. longa* (Sahu *et al.*, 2008), *M. indica* kernels (Sahu *et al.*, 2007b) or *W. somnifera* roots (Sharma *et al.*, 2010). Besides, the medicinal herbs *A. aspera* (Vasudeva & Chakrabarti, 2005b), *A. membranaceus* (Yin *et al.*, 2009), *Rheum officinale* (Xie *et al.*, 2008), *B. nigra, C. majus, E. purpurea, I. helenium, T. farfara* (Mohamad & Abasali, 2010), *C. zeylanicum*, *J. regia*, *M. piperita*, *O. basilicum* (Abasali & Mohamad, 2010), *A. marmelos* (Pratheepa *et al.*, 2010) and *E. hirta* (Pratheepa & Sukumaran, 2011) enhanced lysozyme activity in common carps. This immune parameter was higher also in channel catfish fed a diet with *O. vulgare* essential oil (Zheng *et al.*, 2009), in red sea bream fed a diet with *C. officinale* root (Ji *et al.*, 2007b), in goldfish fed a diet with triherbal extracts of *A. indica, C. longa* and *O. sanctum* (Harikrishnan *et al.*, 2009b), in kelp grouper fed diets with extract from green tea (Harikrishnan *et al.*, 2011b), *K. pictus* (Harikrishnan *et al.*, 2011f), *S. japonica* (Harikrishnan *et al.*, 2011g), *V. album* (Harikrishnan *et al.*, 2011c) or *E. japonica* (Kim *et al.*, 2011), in yellow croaker fed diets with *A. sinensis* and *A. membranaceus* (Jian & Wu, 2003), in rock bream fed diets with *S. baicalensis* extract (Harikrishnan *et al.*, 2011h), in Japanese eel fed diets with *V. album* extract (Choi *et al.*, 2008), in olive flounder fed diets with *P. vulgaris* extract (Harikrishnan *et al.*, 2011e). The lysozyme activity was promoted in juvenile flounders also by dietary/intraperitoneal administration of aqueous, methanolic, ethanolic thriherbal extracts of the traditional Korean medicinal plants *Crysanthemum cinerariaefolium*, *P. granatum*,

Zanthoxylum schinifolium (Harikrishnan *et al.*, 2010d; 2010f).

The alternative complement pathway is known to be one of the powerful non-specific defense mechanisms which protects fish from a wide range of bacteria (Ellis, 1999; 2001). The serum natural haemolytic complement activity was improved by oral administration of *A. sinensis* and *A. membranaceus* mixture in yellow croaker (Jian & Wu, 2003) and Jian carp (Jian & Wu, 2004), *E. alba* (Christybapita *et al.*, 2007) or *N. arbortristis* (Kirubakaran *et al.*, 2010) extract in tilapia, *C. sinenis* (Harikrishnan *et al.*, 2011b), *K. pictus* (Harikrishnan *et al.*, 2011f), *S. japonica* (Harikrishnan *et al.*, 2011g) or *E. japonica* (Kim *et al.*, 2011) extract in kelp grouper, *S. baicalensis* extract in rock bream (Harikrishnan *et al.*, 2011h), *C. dactylon* extract in *C. catla* (Kaleeswaran *et al.*, 2011a), *P. vulgaris* extract (Harikrishnan *et al.*, 2011e) or mixed *C. cinerariafolium*, *P. granatum*, *Z. schinifolium* extracts (Harikrishnan *et al.*, 2010d) in olive flounder. Similarly, the alternative complement pathway was enhanced by intraperitoneal injection of aqueous, methanolic, ethanolic extracts of *A. indica*, *C. longa*, *O. sanctum* in goldfish (Harikrishnan *et al.*, 2009b), aqueous extract of *T. cordifolia* in tilapia (Alexander *et al.*, 2010), aqueous, methanolic, ethanolic extracts of *C. cinerariafolium*, *P. granatum*, *Z. schinifolium* (Harikrishnan *et al.*, 2010f) in olive flounder.

Antiproteases, principally α2-macroglobulin, α1-antiprotease and α2-antiplasmin, are protease inhibitors that restrict the ability of bacteria to invade and grow in fish, by inhibiting their extracellular enzymes (Ellis, 2001). Serum antiprotease activity was elevated in different species of carp treated with *A. aspera* (Vasudeva *et al.*, 2004; Vasudeva & Chakrabarti, 2004; 2005a; 2005b; Vasudeva &, Sunil 2009) or *C. dactylon* extract (Kaleeswaran *et al.*, 2012; 2011a; Kaleeswaran *et al.*, 2011b). Positive effects on these enzymes have been also exhibited by *E. alba* (Christybapita *et al.*, 2007) or *T. cordifolia* (Alexander *et al.*, 2010) extract in tilapia, *C. sinensis* extract (Sheikhzadeh *et al.*, 2011) and *Z. officinale* (Nya & Austin, 2009b) in rainbow trout, *C. sinensis* (Harikrishnan *et al.*, 2011b), *K. pictus* (Harikrishnan *et al.*, 2011f) or *S. japonica* (Harikrishnan *et al.*, 2011g) extract in kelp grouper, *S. baicalensis* extract in rock bream (Harikrishnan *et al.*, 2011h).

Phagocytosis is the most important cellular mechanism of the non-specific immune system of teleosts, and together with humoral components it constitutes the first line of defence against invading pathogens. Phagocytes (monocytes/macrophages and neutrophils) engulf the microorganisms and kill them by degranulation, metabolic activation and release of microbicidal oxygen and nitrogen reactive species (ROS and NOS) (Neumann *et al.*, 2001). In rainbow trout, phagocytosis and respiratory burst activity of leukocytes have been significantly promoted by *A. sativum* (Nya & Austin, 2009a; 2011), *C. coggyria* (Bilen *et al.*, 2011), *Laurus nobilis* (Bilen & Bulut, 2010) or *Z. officinale* (Dügenci *et al.*, 2003; Nya & Austin, 2009b) enriched diets, while *L. perennis*, *M. indica* or *U. dioica* enriched diets enhanced only the ROS production (Awad & Austin, 2010). Diets including extract form *A. membranaceus* (Yin *et al.*,

2006; Ardò *et al.*, 2008), *L. japonica* (Ardò *et al.*, 2008), *C. dactylon*, *W. somnifera*, *Z. officinalis* (Immanuel *et al.*, 2009), *C. formosum* (Rattanachaikunsopon & Phumkhachorn, 2010c) or *T. sinensis* (Wu *et al.*, 2010) effectively enhanced the phagocytic efficiency of white blood cells in tilapia. Furthermore, the killing capability of tilapia leukocytes, evaluated by measuring ROS, NOS and myeloperoxidases, has been improved after feeding diets containing *A. sativum* (Aly *et al.*, 2008a) or extracts from *A. membranaceus*, *L. japonica* (Ardò *et al.*, 2008), *C. formosum* (Rattanachaikunsopon & Phumkhachorn, 2010c), *E. alba* (Christybapita *et al.*, 2007), *N. arbortristis* (Kirubakaran *et al.*, 2010) as well as after intraperitoneal injection of extracts from *O. sanctum* (Logambal *et al.*, 2000), *S. trilobatum* (Divyagnaneswari *et al.*, 2007), *T. cordifolia* (Sudhakaran *et al.*, 2006; Alexander *et al.*, 2010) or *T. sinensis* (Wu *et al.*, 2010). In Indian major carp, diets with *A. aspera* (Vasudeva *et al.*, 2006; Vasudeva & Sunil, 2009), *A. sativum* (Sahu *et al.*, 2007a), *C. longa* (Sahu *et al.*, 2008), *M. indica* (Sahu *et al.*, 2007b) or *W. somnifera* (Sharma *et al.*, 2010) induced a significant stimulation of blood phagocytes respiratory burst, diets with *A. marmelos* enhanced the phagocytosis activity (Pratheepa *et al.*, 2011), diets with *C. dactylon* promoted the oxidative burst, NOS production and myeloperoxidase activity (Kaleeswaran *et al.*, 2011a). Common carps showed an improvement of phagocytosis after feeding diets with *A. membranaceus* and *A. sinensis* mixture (Jian & Wu, 2004), an increase of both phagocytosis and respiratory burst activity after feeding diets with extract of *A. membranaceus* (Yin *et al.*, 2009), *A. marmelos* (Pratheepa *et al.*, 2010) or *E. hirta* (Pratheepa & Sukumaran, 2011), an increasing trend in the respiratory burst activity after feeding diets with *B. nigra*, *C. majus*, *E. purpurea*, *I. helenium*, *T. farfara* ethanolic extracts (Mohamad & Abasali, 2010) or *C. zeylanicum*, *J. regia*, *M. piperita* and *O. basilicum* ethanolic extracts (Abasali & Mohamad, 2010). Oral, immersion and intraperitoneal treatments with *A. indica*, *C. longa* and *O. sanctum* extracts enhanced phagocytosis and ROS generation of goldfish leukocytes (Harikrishnan *et al.*, 2009b). The antimicrobial mechanisms of phagocytes were improved also in greasy grouper fed diets enriched with *O. sanctum* extract, *W. somnifera* extract (Sivaram *et al.*, 2004) or *C. dactylon*, *P. longum*, *P. niruri*, *T. procumbens*, *Z.officinale* extracts mixture (Punitha *et al.*, 2008), in kelp grouper fed diets enriched with extract from *C. sinensis* (Harikrishnan *et al.*, 2011b), *E. japonica* (Kim *et al.*, 2011), *K. pictus* (Harikrishnan *et al.*, 2011f), *Lactuca indica* (Harikrishnan *et al.*, 2011d), *S. japonica* (Harikrishnan *et al.*, 2011g) or *V. album* (Harikrishnan *et al.*, 2011c), in yellow croaker fed diets supplemented with *A. membranaceus* and *A. sinensis* (Jian & Wu, 2003), in Japanese eel fed diets with *V. album* extract (Choi *et al.*, 2008), in rock bream fed diets with *S. baicalensis* leaves extract (Harikrishnan *et al.*, 2011h). The function of phagocytic cells was promoted in olive flounder by diets with *Prunella vulgaris* ethanolic extract (Harikrishnan *et al.*, 2011e) or mixed *C. cinerariafolium*, *P. granatum*, *Z. schinifolium* extracts (Harikrishnan *et al.*, 2010d). The release of myeloperoxide enzymes by azurophilic granules of neutrophils is measured also through the

serum peroxidase activity. A significant increase of this parameter was detected in *C. carpio* fed diets including *A. marmelos* leaf extract (Pratheepa *et al.*, 2010), in *O. mykiss* fed diets containing decaffeinated green tea extract (Sheikhzadeh *et al.*, 2011), in *E. bruneus* fed diets with extract of *K. pictus* (Harikrishnan *et al.*, 2011f) or *S. japonica* (Harikrishnan *et al.*, 2011g).

1.7.2 Specific immune response

The administration of herbal medicines in fish in association with immunization trials or infections has been reported to enhance the adaptive/specific immune response by improving the synthesis of specific antibodies (Tables 1, 2 and 4). Colorni *et al.* (1998) observed an enhancing effect on antibody level in *D. labrax* infected with *M. marinum* and i.p. treated with *A. sativum* extract. Moreover, it has been demonstrated that the ethanolic extract of the medicinal herb *C. dactylon* induces a significant increase of serum specific antibody titre and an aggregation of spleen melanomacrophage centres in *C. catla* vaccinated against *A. hydrophila* (Kaleeswaran *et al.*, 2012). Similarly, extract from astragalus (Yin *et al.*, 2009) or *A. vera* (Alishahi *et al.*, 2010) and the essential oil of *Z. multiflora* (Soltani *et al.*, 2010) promoted the production of anti-*A. hydrophila* immunoglobulins in vaccinated *C. carpio*. Moreover, *O. mossambicus* intraperitoneally/orally treated with *O. sanctum* (Logambal *et al.*, 2000) or *S. trilobatum* (Divyagnaneswari *et al.*, 2008) and immunized against *A. hydrophila* showed a higher serum antibody response compared to controls. Similar immunostimulatory effects were observed also in tilapia injected with *T. cordifolia* ethanol or petroleum ether extract (Sudhakaran *et al.*, 2006). Feeds with aqueous extract from *A. marmelos* or *E. hirta* strongly enhanced the primary antibody response of *C. carpio* experimentally infected with *A. hydrophila* (Pratheepa *et al.*, 2010) and *Pseudomonas fluorescens* (Pratheepa & Sukumaran, 2011) respectively.

Table 1 Impact of plant-derived immunostimulants used *per os* on growth, survival, haematological profile, immune response and disease resistance of cultured fish

Immunostimulants – oral administration									
Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Achyranthes aspera	Seeds	0.5%	3% BW day^{-1} 9 weeks	*C. catla*	20 ± 4	n.a.	TP (↔), ALB (↔), GLB (↔), AST (↑), ALT (↑), LYZ (↑), AP (↑), BA (↑), RB (↑)	*A. hydrophila*	Vasudeva and Sunil (2009)
	Seeds	0.5%	2% BW day^{-1} 4 weeks	*C. carpio*	90 ± 17	n.a.	TP (↔), ALB (↔), GLB (↑), LYZ (↑), AP (↑), RNA/DNA (↑)	n.a.	Vasudeva and Chakrabarti (2005b)
	Seeds	0.01%, 0.1%, 0.5%	3% BW day^{-1} 5 weeks	*L. rohita*	3.0 ± 0.4	SGR (↑), FCR (↓)	TP (↔), ALB (↑), GLB (↔), AST (↓), ALT (↓), ALP (↑), LYZ (↑), BA (↑), RB (↑)	*A. hydrophila*	Vasudeva *et al.* (2006)
	Seeds extract (water)	0.5%	1% BW day^{-1} 4 weeks	*C. catla*	150 ± 20	n.a.	TP (↔), ALB (↔), GLB (↑), AP (↑), RNA/DNA (↑)	n.a.	Vasudeva and Chakrabarti (2005a)
	Roots extract (water)	0.5%	1% BW day^{-1} 4 weeks	*L. rohita*	200 ± 17	n.a.	TP (↔), GLB (↑), AP (↑), RNA/DNA (↑)	n.a.	Vasudeva *et al.* (2004); Vasudeva & Chakrabarti (2004)
Aegle marmelos	Leaves extract (water)	0.5%, 1%, 1.5%, 2%, 2.5%, 3%	30 d	*C. catla*	16 ± 5	n.a.	↑PHAG (↑)	n.a.	Prattheepa *et al.* (2011)
	Leaves extract (water)	0.5%, 1%, 2%, 2.5%, 5%	2% BW day^{-1} 50 d	*C. carpio*	45.9 ± 1.5	n.a.	↑RBC (↑), Hb (↑), WBC (↑), ALP (↑), ACP (↑), LYZ (↑), PO (↑), PHAG (↑), RB (↑), specific Ig (↑)	*A. hydrophila*	Prattheepa *et al.* (2010)

Table 1 Continued

Immunostimulants – oral administration									
Single plant/plant mixture	**Parts/ products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance***	**References**
	Plant extract (acetone)	1%	5% BW day^{-1} 45 d	*O. mossambicus*	7.46 ± 0.11	FBW (↑), SGR (↑)	Hct (↑), Lct (↔), TP (↑), ALB (↑), GLE (↔), GLU (↓), CHO (↓), TRIG (↓), Ca (↔), LYZ (↔), PHAG (↔)	*V. vulnificus*	Immanuel *et al.* (2009)
Allium sativum	Bulbs	0.1%, 0.5%, 1%	4% BW day^{-1} 60 d	*L. rohita*	10 ± 2	SGR (↔), FCR (↔)	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), BA (↑), RB (↑)	*A. hydrophila*	Sahu, Das, Mishra *et al.* (2007a)
	Bulbs	0.05%, 0.1%, 0.5%, 1%	To satiety 2 weeks	*O. mykiss*	14	WG (↑), SGR (↑), FCR (↓), PER (↑)	RBC (↑), Hct (↑), Hb (↔), MCV (↓), MCH (↓), MCHC (↑), WBC (↑), LYM (↑), MON (↓), NEU (↓), TP (↑), ALB (↔), GLE (↑), LYZ (↑), AP (↔), BA (↑), PHAG (↑), Hbl (↑)	*A. hydrophila*	Nya and Austin (2009a)
	Bulbs	0.5%, 1%	To satiety 2 weeks	*O. mykiss*	14	WG (↔), SGR (↔), CF (↔)	RBC (↑), Hct (↔), Hb (↔), WBC (↑), LYM (↑), MON (↓), NEU (↓), TP (↔), Ca (↑), Mg (↑), Fe (↑), K (↑), LYZ (↑), PO (↔), RB (↑)	*A. hydrophila*	Nya and Austin (2011)
	Bulbs	1%, 2%	1–3% BW day^{-1} 4/8 weeks	*O. niloticus*	6.5 ± 1.0	WG (↑)	Hct (↔), WBC (↓), LYM (↓), MON (↑), NEU (↔), EOS (↔), BAS (↔), PHAG (↑)	*A. hydrophila*	Aly *et al.* (2008a)

Table 1 Continued

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Immunostimulants – oral administration									
	Bulbs	0.5%, 1%	To satiety 4 weeks	*O. niloticus x O. aureus*	25.5 ± 1.0	WG (↔)	WBC (↑), LYZ (↑), COMP (↑), PHAG (↑)	n.a.	Ndong and Fall (2007)
	Plant powder	2%, 3%, 4%	30 d	*O. mykiss*	50 ± 5	n.a.	RBC (↔), Hct (↔), Hb (↔), MCV (↓), MCH (↔), MCHC (↔), WBC (↑), LYM (↑), NEU (↓), AST (↔), ALT (↔)	n.a.	Fazlolahzadeh, Keramati, Nazifi, Shirian and Seifi (2011)
	Plant powder	1%, 2%, 3%, 4%	3% BW day^{-1} 90 d	*O. niloticus*	7 ± 1	FBW (↑), WG (↑), SGR (↑), FCR (↓), FER (↑), PER (↑), HIS (↔), SR (↔)	RBC (↑), Hct (↑), Hb (↑), MCV (↔), MHC (↔), MCHC (↔), TP (↑), GLU (↓), TL (↓), AST (↓), ALT (↓)	*A. hydrophila*	Shalaby *et al.* (2006)
	Plant extract (chic. acid)	3%	3% BW day^{-1} 12 weeks	*O. niloticus*	0.8 ± 0.2	WG (↑), SGR (↑), SR (↑)	Hct (↑), WBC (↔), LYM (↔), MON (↔), NEU (↓), EOS (↔), BAS (↔)	*A. hydrophila*	Aly and Mohamad (2010)
Allium tuberosum	Essential oil	0.02%, 0.04%, 0.06%, 0.08%	5% BW day^{-1} 19 d	*O. niloticus*	10	WG (↔), SGR (↔), FCR (↔), SR (↔)	n.a.	*F. columnare*	Rattanachaikunsopon and Phumkhachorn (2009a)
Alnus firma	Plant extract (ethanol)	0.01%, 0.1%, 1%	5% BW day^{-1} 8 weeks	*P. olivaceus*	22.3 ± 1.3	n.a.	↑RBC (↑), Hct (↑), Hb (↑), WBC (↑), TP (↑), GLU (↑), CHO (↑), Ca (↑), LYZ (↑), RB (↑)	*T. maritimum*	Harikrishnan, Kim, Kim *et al.* (2011i)
Aloe vera	Plant extract (solvent n.a.)	0.5%	5% BW day^{-1} 6 weeks	*C. carpio*	108 ± 11.4	n.a.	RBC (↔), WBC (↑), TP (↑), GLB (↑), LYZ (↑), COM (↔), BA (↑), specific Ig (↑)	*A. hydrophila*	Alishahi *et al.* (2010)
	Plant powder	0.1%, 0.5%	To satiety 6 weeks	*S. schlegeli*	25	n.a.	LYZ (↔), RB (↓)	n.a.	Kim *et al.* (1999)

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Andrographis paniculata	Leaves	5%, 10%, 15%, 20%, 25%	5% BW day^{-1} 2 weeks	*O. niloticus*	10	SR (↔)	n.a.	*S. agalactiae*	Rattanachaikunsopon and Phumkhachorn (2009b)
	Plant extract (water)	2.5%, 5%, 7.5%, 10%, 12.5%							
	Plant extract (methanol)	0.05%, 0.1%, 0.2%, 0.3%	*ad libitum*, 45 d	*O. mossambicus*	20-40	FBW (↔), WG (↑), SGR (↑)	RBC (↑), Hb (↑), MCV (↓), MHC (↓), MCHC (↔), WBC (↑), TRB (↑)	n.a.	Prasad and Mukthiraj (2011)
Artemisia capillaries	Leaves	0.5%	To satiety, 12 weeks	*P. major*	24.0 ± 0.2	FBW (↑), SGR (↑), DFI (↔), FER (↔), CF (↔), SR (↑) HSI (↔), VSI (↔)	Hct (↔), Hb (↑), HDL-CHO (↔), AST (↓), ALT (↓), LYZ (↔), COM (↔)	*V. anguillarum*	Ji, Takaoka *et al.* (2007b)
Artemisia cina	Plant powder	1%, 3%, 5%	3% BW day^{-1} 30 d	*C. gariepinus*	22	WG (↔), SGR (↔), FCR (↓), CF (↓), SR (↑), HSI (↑), SSI (↑)	Hct (↑), Hb (↑), WBC (↔), RB (↑)	n.a.	Abdelhadi, Saleh and Sakr (2010)
Astragalus membranaceus	Plant extract (solvent n.a.)	0.5%	*ad libitum* 5 weeks	*C. carpio*	62.8 ± 5.4	n.a.	LYZ (↑), PHAG (↑), RB (↑), specific Ig (↑)	*A. hydrophila*	Yin *et al.* (2009)
	Plant extract (solvent n.a.)	0.1%, 0.5%, 1%	*ad libitum* 4 weeks	*O. niloticus*	62.8 ± 5.4	n.a.	LYZ (↑), PHAG (↑), RB (↔)	n.a.	Yin *et al.* (2006)
	Plant extract (solvent n.a.)	0.1%	4 weeks	*O. niloticus*	n.a.	n.a.	TP (↔), LYZ (↑), PHAG (↑), RB (↑), total Ig (↔)	*A. hydrophila*	Ardó *et al.* (2006)
Camellia sinensis	Plant extract (water)	0.01%, 0.1%, 1%	To satiety 6 weeks	*E. bruneus*	14.5 ± 2.1	n.a.	†LYZ (↑), COMP (↑), AP (↑), RB (↓), RNI (↑), MPO (↑)	*V. carchariae*	Harikrishnan *et al.* (2011b)
	Plant extract (eth. acid)	0.002%, 0.01%, 0.05%	n.a.	*O. mykiss*	35 ± 3	n.a.	LYZ (↑), AP (↑), PO (↑), BA (↑)	n.a.	Sheikhzadeh *et al.* (2011)

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Cinnamomum verum	Barks Barks extract (water)	4.8%, 10%, 15.8% 3.2%, 6.7%, 10.3%	3% BW day^{-1} 2 weeks	*Oreochromis* sp.	11 ± 2	n.a.	n.a.	*S. agalactiae*	Alsaid, Daad, Bejo and Abuselliana (2010)
	Essential oil	0.1%, 0.2%, 0.3%, 0.4%	5% BW day^{-1} 15 d	*O. niloticus*	10 ± 1	WG (↔), SGR (↔), FCR (↔), SR (↔)	n.a.	*S. iniae*	Rattanachaikunsopon and Phumkhachorn (2010a)
Cnidium officinale	Roots	0.5%	To satiety 12 weeks	*P. major*	24.0 ± 0.2	FBW (↑), SGR (↑), DFI (↔), FER (↑), CF (↔), SR (↑), HSI (↔), VSI (↔)	Hct (↔), Hb (↑), HDL-CHO (↑), AST (↓), ALT (↓), LYZ (↑), COMP (↔)	*V. anguillarum*	Ji, Takaoka *et al.* (2007b)
Cotinus coggyria	Plant powder	0.5%, 1%	*ad libitum* 3 weeks	*O. mykiss*	89.25 ± 0.12	SGR (↔)	TP (↑), LYZ (↑), PHAG (↑), RB (↑)	n.a.	Bilen *et al.* (2011)
Crataegi fructus	Fruits	0.5%	To satiety 12 weeks	*P. major*	24.0 ± 0.2	FBW (↑), SGR (↑), DFI (↔), FER (↔) CF (↔), SR (↑), HSI (↔), VSI (↔)	Hct (↔), Hb (↔), HDL-CHO (↔), AST (↓), ALT (↓), LYZ (↔), COMP (↔)	*V. anguillarum*	Ji, Takaoka *et al.* (2007b)
Craloxylum formosum	Plant extract (water)	0.1%, 1%, 1.5%	2% BW day^{-1} 30 d	*O. niloticus*	30 ± 2	WG (↔), SGR (↔), FCR (↔), SR (↔)	LYZ (↑), PHAG (↑), RB (↑)	*S. agalactiae*	Rattanachaikunsopon and Phumkhachorn (2010c)
Curcuma longa	Plant powder	0.01%, 0.05%, 0.1%, 0.5%	60 d	*L. rohita*	10 ± 2	SGR (↔), FCR (↔), SR (↑)	RBC (↑), Hb (↔), WBC (↑), TP (↑), ALB (↑), GLB (↑), AST (↓), ALT (↓), ALP (↑), LYZ (↑), BA (↑), RE (↑)	*A. hydrophila*	Sahu *et al.* (2008)
	Rhizomes extract (ethanol)	0.0005%, 0.01%, 0.002%, 0.004%, 0.008%	30 d	*O. niloticus*	40 ± 5	n.a.	IL-1β (↑), IL-8 (↑), TGF-β (↑)	n.a.	Panpiemmin, Vanichkul, Panpiemmin and Areechon (2011)

Table 1 Continued

Immunostimulants – oral administration									
Single plant/plant mixture	**Parts/ products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance***	**References**
Cynodon dactylon	Plant extract (ethanol)	0.05%, 0.5%, 5%	2% BW day^{-1} 45-60 d	*C. catla*	88.05 ± 4.75	FBW (↔), WG (↔), SGR (↑), FCR (↑), SR (↔)	RBC (↑), Hb (↑), WBC (↑), TP (↑), GLB (↑), ALB/GLB (↑), GLU (↑), CHO (↑), LYZ (↑), COMP (↑), AP (↑), RB (↑), RNI (↑), MPO (↑), RNA/DNA (↑), specific Ig (↑), APC (↑), MMC (↑)	*A. hydrophila*	Kaleeswaran *et al.* (2010, 2011a,b)
	Plant extract (acetone)	1%	5% BW day^{-1} 45 d	*O. mossambicus*	7.46 ± 0.11	FBW (↑), SGR (↑)	Hct (↑), Lct (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), CHO (↓), TRIG (↓), Ca (↔), LYZ (↑), PHAG (↑)	*V. vuhificus*	Immanuel *et al.* (2009)
Echinacea purpurea	Plant extract (chic. acid)	0.025%	3% BW day^{-1} 24 weeks	*O. niloticus*	4.5 ± 0.2	WG (↑), SGR (↑), CF (↔)	Hct (↑), WBC (↑), LYM (↑), MON (↔), NEU (↔), EOS (↑), BAS (↔), LYZ (↑), RB (↔)	*P. fluorescens*	Aly *et al.* (2008b)
	Plant extract (chic. acid)	0.1%	3% BW day^{-1} 12 weeks	*O. niloticus*	0.8 ± 0.2	WG (↑), SGR (↑), SR (↑)	Hct (↑), WBC (↑), LYM (↑), MON (↔), NEU (↓), EOS (↔), BAS (↔)	*A. hydrophila*	Aly and Mohamed (2010)
Eclipta alba	Leaves extract (water)	0.01%, 0.1%, 1%	2% BW day^{-1} 3 weeks	*O. mossambicus*	50 ± 5	n.a.	LYZ (↑), COMP (↑), AP (↑), RB (↑), RNI (↑), MPO (↑)	*A. hydrophila*	Christybapita *et al.* (2007)
Epilobium hirsutum	Plant extract (ethanol)	0.5%, 1%, 3%	2% BW day^{-1} 8 weeks	*C. carpio*	20 ± 2	SGR (↔), FCR (↔), CF (↔), SR (↔)	†RBC (↔), Hct (↔), Hb (↔), WBC (↑), LYM (↔), MON (↔), NEU (↔)	*A. hydrophila*	Pakravan *et al.* (2011)

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Eriobotrya japonica	Plant extract (ethanol)	0.1%, 1%, 2%	5% BW day^{-1} 8 weeks	*E. bruneus*	25.4 ± 1.2	n.a.	↑WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), COMP (↑), BA (↑), PHAG (↑), RB (↑), LYMK (↑)	*V. carchariae*	Kim *et al.* (2011)
Euphorbia hirta	Leaves extract (water)	0.5%, 1%, 2%, 2.5%, 5%	2% BW day^{-1} 50 d	*C. carpio*	45.9 ± 1.5	n.a.	↑RBC (↑), Hb (↑), WBC (↑), LYZ (↑), PHAG (↑), RB (↑), specific Ig (↑)	n.a.	Pratheepa and Sukumaran (2011)
Garcinia kola	Seeds extract (ethanol)	0.025%, 0.05%, 0.1%, 0.2%	3% BW day^{-1} 56 d	*C. gariepinus*	245.20–255.00	FBW (↑), WG (↑), SGR (↑) FCR (↑)	RBC (↔), Hb (↔), WBC (↑)	n.a.	Dada and Ikuerowo (2009)
Ginseng	Roots extract (solvent n.a.)	0.005%, 0.01%, 0.015%, 0.02%, 0.025%	3% BW day^{-1} 6 weeks	*O. niloticus*	24.4 ± 0.2	FBW (↑), WG (↑), SGR (↑), CF (↔), FCR (↓), DFI (↑), PER (↑), PPV (↑), FR (↑), ER (↑)	RBC (↑), Hct (↑), Hb (↑), MCV (↓), MCH (↓), MCHC (↔), WBC (↔), LYM (↔), MON (↔), NEU+EOS+BAS (↔), TP (↑), ALB (↑), GLB (↑)	n.a.	Goda (2008)
Kalopanax pictus	Plant extract (ethanol)	0.1%, 1%, 2%	5% BW day^{-1} 30 d	*E. bruneus*	26.1 ± 1.4	n.a.	↑RBC (↑), Hct (↑), Hb (↑), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↑), MON (↑), NEU (↔), TRB (↔), TP (↑), LYZ (↑), COMP (↑), AP (↑), PO (↑), BA (↑), PHAG (↑), RB (↑)	*V. alginolyticus* *P. dicentrarchi*	Harikrishnan *et al.* (2011f)
Lactuca indica	Plant extract (ethanol)	0.1%, 1%, 2%	5% BW day^{-1} 30 d	*E. bruneus*	27.7 ± 1.4	n.a.	↑LYZ (↔), PHAG (↑), RB (↑), total Ig (↑)	*S. iniae*	Harikrishnan *et al.* (2011d)
Laurus nobilis	Plant powder	0.5%, 1%	To satiety 3 weeks	*O. mykiss*	89.25 ± 0.12	n.a.	TP (↔), LYZ (↔), PHAG (↑), RB (↑)	n.a.	Bilen and Bulut (2010)

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Lonicera japonica	Plant extract (solvent n.a.)	0.1%	4 weeks	*O. niloticus*	n.a.	n.a.	TP (↔), LYZ (↑), PHAG (↑), RB (↑), total Ig (↔)	*A. hydrophila*	Ardò *et al.* (2008)
Lupinus perennis	Seeds	1%	3% BW day^{-1} 2 weeks	*O. mykiss*	15	n.a.	RBC (↑), Hct (↑), Hb (↔), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↔), MON (↔), NEU (↔), TRB (↑) TP (↔), LYZ (↑), COMP (↔), AP (↔), PO (↔), BA (↓), PHAG (↔), RB (↑)	n.a.	Awad and Austin (2010)
	Seeds	1%, 2%	n.a.	*O. mykiss*	18.0 ± 0.2	n.a.	IL-1β (↑), IL-8 (↑), TGF-β (↑)	n.a.	Awad *et al.* (2011)
Mangifera indica	Kernels	0.1%, 0.5%, 1%	60 d	*L. rohita*	10 ± 2	SGR (↔), FCR (↔)	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), BA (↓), RB (↑)	*A. hydrophila*	Sahu, Das, Pradhan *et al.* (2007b)
	Fruits	1%	3% BW day^{-1} 2 weeks	*O. mykiss*	15	n.a.	RBC (↑), Hct (↑), Hb (↔), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↔), MON (↔), NEU (↔), TRB (↔), TP (↑), LYZ (↑), COMP (↔), AP (↔), PO (↔), BA (↓), PHAG (↔), RB (↑)	n.a.	Awad and Austin (2010)
	Fruits	1%, 2%	n.a.	*O. mykiss*	18.0 ± 0.2	n.a.	IL-1β (↔), IL-8 (↔), TGF-β (↑)	n.a.	Awad *et al.* (2011)

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance[a]	References
Massa medicata	Fruits	0.5%	to satiety 12 weeks	*P. major*	24.0 ± 0.2	FBW (↔), SGR (↔), DFI (↔), FER (↔), CF (↔), SR (↑), HSI (↔), VSI (↔)	Hct (↔), Hb (↑), HDL-CHO (↔), AST (↓), ALT (↔), LYZ (↔), COMP (↔)	*V. anguillarum*	Ji, Takaoka *et al.* (2007b)
Matricaria chamomilla	Plant powder	1%, 3%, 5%	3% BW day^{-1} 30 d	*C. gariepinus*	22	WG (↔), SGR (↔), FCR (↓), CF (↓), SR (↑), HSI (↔), SSI (↔)	Hct (↔), Hb (↔), WBC (↑), RS (↔)	n.a.	Abdelhadi *et al.* (2010)
Myristica fragans	Leaves extract (ethanol)	0.01%, 0.02%, 0.04%, 0.08%	5% BW day^{-1} 12 weeks	*E. tauvina*	30.0 ± 0.5	WG (↔), SGR (↔), FCR (↔)	Lct (↔), ALB (↔), GLB (↔), LYZ (↔), BA (↔), PHAG (↔)	No effect against *V. harveyi* infection	Sivaram *et al.* (2004)
Nigella sativa	Seeds	1%, 2.5%, 5%	2% BW day^{-1} 3 weeks	*O. mykiss*	34.43 ± 3.56	n.a.	Hct (↑), WBC (↔), Lct (↑), TP (↑), RB (↔), total Ig (↑)	n.a.	Dorucu *et al.* (2009)
Nyctanthes arbortristis	Seeds extract (chloroform)	0.01%, 0.1%, 1%	2% BW day^{-1} 3 weeks	*O. mossambicus*	30 ± 5 50 ± 5	n.a.	LYZ (↑), COMP (↑), RB (↑), RNI (↑), MPO (↑)	*A. hydrophila*	Kirubakaran *et al.* (2010)
Ocimum sanctum	Leaves	0.00005%, 0.0005%, 0.005%, 0.05%, 0.25%	4 d	*O. mossambicus*	25	n.a.	specific Ig (↑)	*A. hydrophila*	Logambal *et al.* (2000)
	Leaves extract (ethanol)	0.01%, 0.02%, 0.04%, 0.08%	5% BW day 12 weeks	*E. tauvina*	30.0 ± 0.5	WG (↑), SGR (↑), FCR (↑)	Lct (↑), ALB (↔), GLB (↑), LYZ (↔), BA (↑), PHAG (↑)	*V. harveyi*	Sivaram *et al.* (2004)
Origanum vulgare	Essential oil	0.05%	To satiety 8 weeks	*I. punctatus*	50	WG (↑), SGR (↑), CF (↑), FCR (↓), PER (↑), HSI (↓), VSI (↓), SR (↑)	LYZ (↑), SOD (↑), CAT (↑)	*A. hydrophila*	Zheng *et al.* (2009)
Pennella vulgaris	Plant extract (ethanol)	0.01%, 0.1%, 1%	*ad libitum* 8 weeks	*P. olivaceus*	28.2 ± 1.3	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	*V. marinum*	Harikrishnan *et al.* (2011a)
Psidium guajava	Leaves	25%	5% BW day^{-1} 5 d	*O. niloticus*	10 ± 1	n.a.	n.a.	*A. hydrophila*	Pachanawan *et al.* (2008)
	Leaves extract (ethanol)	4%							

Table 1 Continued

Immunostimulants – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Rheum officinale	Plant extract (n.a.)	0.5%, 1%, 2%, 4%	2.4% BW day^{-1} 10 weeks	*C. carpio*	5.30 ± 0.72	SGR (↑), FCR (↓)	GLU (↔), COR (↓), LYZ (↑), SOD (↑), CAT (↔), MDA (↓)	*A. hydrophila*	Xie *et al.* (2008)
Scutellaria baicalensis	Plant extract (solvent n.a.)	0.1%, 0.5%, 1%	*ad libitum* 4 weeks	*O. niloticus*	62.8 ± 5.4	n.a.	LYZ (↔), PHAG (↓), RB (↓)	n.a.	Yin *et al.* (2006)
Styrax japonica	Flowers extract (ethanol)	0.1%, 1%, 2%	5% BW day^{-1} 30 d	*E. tuneus*	29.6 ± 1.1	n.a.	TP (↑), LYZ (↑) COMP (↑), AP (↑), PO (↑), BA (↑), PHAG (↑), RB (↑)	*V. harveyi* *U. marinum*	Harikrishnan *et al.* (2011g)
Urtica dioica	Leaves	1%	3% BW day^{-1} 2 weeks	*O. mykiss*	15	n.a.	RBC (↑), Hct (↑) Hb (↔), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↔), MON (↔), NEU (↔), TRB (↔), TP (↔), LYZ (↑), COMP (↔), AP (↔), PO (↔), BA (↓), PHAG (↔), RB (↑)	n.a.	Awad and Austin (2010)
	Leaves	1%, 2%	n.a.	*O. mykiss*	18.0 ± 0.2	n.a.	IL-1β (↑), IL-8 (↑), TGF-β (↑)	n.a.	Awad *et al.* (2011)
	Leaves extract (water)	0.1%, 1%	2% BW day^{-1} 3 weeks	*O. mykiss*	41	SGR (↔), CF (↔)	TP (↑), PHAG (↔), RB (↔)	n.a.	Dügenci *et al.* (2003)
Viscum album	Leaves, fruits, stems extract (water)	0.1%, 0.5%, 1%	1% BW day^{-1} 4 weeks	*A. japonica*	200	n.a.	LYZ (↑), PHAG (↑), RB (↑)	*A. hydrophila*	Choi *et al.* (2008)
	Leaves extract (water)	0.5%, 1%, 2%	5% BW day^{-1} 30 d	*E. tuneus*	28.7 ± 1.3	n.a.	TP (↑), LYZ (↑), PHAG (↑), RB (↑)	*P. dicentrarchi*	Harikrishnan, Balasundaram & Heo (2011c)
	Leaves extract (water)	0.1%, 1%	2% BW day^{-1} 3 weeks	*O. mykiss*	41	SGR (↔), CF (↔)	TP (↑), PHAG (↔), RB (↔)	n.a.	Dügenci *et al.* (2003)
Withania somnifera	Roots	1%, 2%, 3%	3% BW day^{-1} 6 weeks	*L. rohita*	18.1 ± 0.5	n.a.	LYZ (↑), PHAG (↑), RB (↑), Ig (↑)	*A. hydrophila*	Sharma *et al.* (2010)

Table 1 Continued

Immunostimulants – oral administration									
Single plant/plant mixture	**Parts/ products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance***	**References**
	Leaves extract (methanol)	0.01%, 0.02%, 0.04%, 0.08%	5% BW day 12 weeks	*E. tauvina*	30.0 ± 0.5	WG (↑), SGR (↑), FCR (↑)	Lct (↑), ALB (↔), GLB (↑), LYZ (↔), BA (↔), PHAG (↑)	*V. harveyi*	Sivaram *et al.* (2004)
	Plant extract (acetone)	1%	5% BW day^{-1} 45 d	*O. mossambicus*	7.46 ± 0.11	FBW (↑), SGR (↑)	Hct (↑), Lct (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), CHO (↓), TRIG (↓), Ca (↔), LYZ (↑), PHAG (↑)	*V. vulnificus*	Immanuel *et al.* (2009)
Zataria multiflora	Essential oil	30, 60, 120 ppm	1% BW day^{-1} 8 d	*C. carpio*	30-35	n.a.	WBC (↑), TP (↔), ALB (↔), GLB (↔), LYZ (↔), BA (↑), specific Ig (↑)	n.a.	Soltani *et al.* (2010)
Zingiber officinale	Rhizomes	0.05%, 0.1%, 0.5%, 1%	To satiety 2 weeks	*O. mykiss*	14	WG (↑), SGR (↑), FCR (↓), PER (↑)	RBC (↑), Hct (↑), Hb (↔), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↑), MON (↑), NEU (↑), TRB (↔), TP (↑), ALB (↔), GLB (↑), LYZ (↑), COMP (↓), AP (↑), BA (↓), PHAG (↑), RB (↑)	*A. hydrophila*	Nya and Austin (2009b)
	Leaves extract (water)	0.1%, 1%	2% BW day^{-1} 3 weeks	*O. mykiss*	41	SGR (↔), CF (↔)	TP (↑), PHAG (↑), RB (↑)	n.a.	Dügenci *et al.* (2003)
	Plant extract (acetone)	1%	5% BW day^{-1} 45 d	*O. mossambicus*	7.46 ± 0.11	FBW (↑), SGR (↑)	Hct (↑), Lct (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), CHO (↓), TRIG (↓), Ca (↔), LYZ (↑), PHAG (↑)	*V. vulnificus*	Immanuel *et al.* (2009)

Table 1 Continued

Immunostimulants – oral administration									
Single plant/plant mixture	**Parts/ products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance***	**References**
Angelica sinensis + *Astragalus membranaceus*	Roots	1%, 1.5%	To satiety 30 d	*C. carpio* var. *Jian*	101 ± 7.38	FBW (↑)	LYZ (↑), COMP (↑), PHAG (↑)	n.a.	Jian and Wu (2004)
	Roots	0.5%, 1%, 1.5%	To satiety 30 d	*P. crocea*	120 ± 3.4	n.a.	LYZ (↑), COMP (↑), PHAG (↑)	*V. alginolyticus*	Jian and Wu (2003)
Chrysanthemum cinerariaefolium + *Punica granatum* + *Zanthoxylum schinifolium*	leaves extracts (ethanol)	0.0005%, 0.005%, 0.01%	*ad libitum* 4 weeks	*P. olivaceus*	68.3 ± 2.9	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	*P. dicentrarchi*	Harikrishnan, Balasundaram Kim *et al.* (2010d)
Artemisia capillaries + *Cnidium officinale* + *Crataegi fructus* + *Massa medicata*	Leaves/roots/fruits	0.5%	To satiety 12 weeks	*P. major*	24.0 ± 0.2	FBW (↑), SGR (↑), DFI (↔), FER (↑), CF (↑), SR (↑), HSI (↔), VSI (↔)	Hct (↔), Hb (↑), HDL-CHO (↓), AST (↓), ALT (↓), LYZ (↓), COMP (↓)	*V. anguillarum*	Ji, Takaoka *et al.* (2007b)
Artemisia capillaries + *Cnidium officinale* + *Crataegi fructus* + *Massa medicata*	Plant powder	0.1%, 0.3%, 0.5%, 1%	To satiety 8 weeks	*P. olivaceus*	15.2 ± 0.4	FBW (↑), WG (↑), SGR (↑), DFI (↔), FER (↑), CF (↔), SR (↑), HSI (↔), VSI (↔)	Hct (↔), Hb (↔), TP (↔), CHO (↔), TRIG (↔), HDL-CHO (↑), GLU (↔), AST (↓), ALT (↔)	n.a.	Ji, Jeong *et al.* (2007a)

Table 1 Continued

Immunostimulants – oral administration									
Single plant/plant mixture	**Parts/ products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance***	**References**
Cinnamomum zeylanicum + *Juglans regia* + *Mentha piperita* + *Ocimum basilicum*	Barks/leaves extracts (ethanol)	0.025%, 0.05%, 0.075%, 0.1%, 0.125%	3% BW day^{-1} 45 d	*C. carpio*	n.a.	n.a.	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), BA (↑), RB (↑)	*A. hydrophila*	Abasali and Mohamad (2010)
Brassica nigra + *Chelidonium majus* + *Echinacea purpurea* + *Inula helenium* + *Tussilago farfara*	Whole plants/seeds/ roots/rhizomes/flowers extracts (ethanol)	0.1%, 0.25%, 0.5%, 0.75%, 1%	3% BW day^{-1} 60 d	*C. carpio*	n.a.	n.a.	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), BA (↑), RB (↑)	*A. hydrophila*	Mohamad and Abasali (2010)
Cynodon dactylon + *Piper longum* + *Phyllanthus niruri* + *Tridax procumbens* + *Zingiber officinale*	Whole plants/seeds/ rhizomes extracts (petroleum ether)	0.01%, 0.02%, 0.04%, 0.08%	5% BW day^{-1} 60 d	*E. tauvina*	20 ± 2	SGR (↑)	ALB/GLB (↑), BA (↑), PHAG (↑)	*V. harveyi*	Punitha *et al.* (2008)

ACP, acid phosphatase; ALB, albumin; ALP, alkaline phosphatase; ALT, alaninaminotransferase; AP, antiprotease activity; APC, antibody producing cells; AST, aspartate aminotransferase; BA, serum bactericidal activity; BAS, basophils; BW, body weight; Ca, calcium; CAT, catalase activity; CF, condition factor; CHO, cholesterol; COMP, complement activity; COR, cortisol; d, days; DFI, daily feed intake; EOS, eosinophils; ER, energy retention; FBW, final body weight; FCR, feed conversion ratio; Fe, iron; FER, feed efficiency ratio; FR, fat retention; GLB, globulins; GLU, glucose; Hb, haemoglobin concentration; Hct, haematocrit value; HDL-CHO, high density lipoprotein cholesterol; HSI, hepatosomatic index; Ig, immunoglobulins; IL-1β, interleukin 1β; IL-8, interleukin 8; K, potassium; Lct, leucocrit value; LYM, lymphocytes; LYMK, lymphokines; LYZ, lysozyme activity; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MDA, hepatic malondialdehyde content; Mg, magnesium; MMC, spleen melanomacrophage centres; MON, monocytes; MPO, myeloperoxidase of leucocytes; NEU, neutrophils; PHAG, phagocytosis; PER, protein efficiency ratio; PO, serum peroxidase activity; PPV, protein productive value; RBC, red blood cell count; RB, respiratory burst activity; RNI, nitrogen reactive intermediates; SGR, specific growth rate; SOD, superoxide dismutase activity; SR, survival rate; SSI, spleno/somatic index; TGF-β, transforming growth factor-β; TL, total lipids; TNF-α, tumour necrosis factor α; TRB, trombocytes; TRIG, triglycerides; TP, total proteins; VSI, viscerosomatic index; WBC, white blood cell count; WG, weight gain; wk, weeks.

Variation in treated fish compared to controls ↑, significant increase; ↓, significant decrease; ↔, no significant changes.

n.a., not available.

*Evaluated in terms of cumulative mortality reduction after challenge with a pathogen.

†Evaluation performed on samples collected after the challenge.

Table 2 Impact of plant derived immunostimulants used by injection on growth, survival, haematological profile, immune response and disease resistance of cultured fish

Immunostimulants – intraperitoneal administration									
Single plant/plant mixture	Parts/products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Ocimum sanctum	Leaves extract (water)	0.0001%, 0.0 %, 1%	200 μL fish^{-1}	*O. mossambicus*	25	n.a.	RB (↑), specific Ig (↑)	*A. hydrophila*	Logambal *et al.* (2000)
Solanum trilobatum	Leaves extract (water, hexane)	4, 40, 400 mg kg^{-1} BW	200 μL fish^{-1}	*O. mossambicus*	25 ± 5	n.a.	LYZ (↑), RB (↑), RNI (↑)	*A. hydrophila*	Divyagnaneswari *et al.* (2007)
	Leaves extract (water, hexane)	4, 6.4, 32, 40, 160, 400, 800 mg kg^{-1} BW	200 μL fish^{-1}	*O. mossambicus*	25 ± 5	n.a.	LYZ (↑), specific Ig (↑)	*A. hydrophila*	Divyagnaneswari *et al.* (2008)
Tinospora cordifolia	Leaves extract (water)	6, 60, 600 mg kg^{-1} BW	200 μL fish^{-1}	*O. mossambicus*	25 ± 5	n.a.	LYZ (↑), COMP (↑), AP (↑), RB (↑), RNI (↑), MPO (↑)	*A. hydrophila*	Alexander *et al.* (2010)
	Leaves extract (ethanol, petroleum ether)	0.8, 8, 80 mg kg^{-1} BW	200 μL fish^{-1}	*O. mossambicus*	25 – 30	n.a.	RB (↑), specific Ig (↑)	*A. hydrophila*	Sudhakaran *et al.* (2006)
Toona sinensis	Leaves extract (water)	0.2%, 0.4%	0 μL fish^{-1}	*O. mossambicus*	10.7 ± 2.5	n.a.	LYZ (↔), PHAG (↑), RB (↑), Ig (↔)	*A. hydrophila*	Wu *et al.* (2010)
Azadirachta indica + *Curcuma longa* + *Ocimum sanctum*	Leaves extracts (water, ethanol, methanol)	5, 50, 100 mg kg^{-1} BW	50 μL fish^{-1}	*C. auratus*	23 ± 2	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	*A. hydrophila*	Harikrishnan, Balasundaram, Kim *et al.* (2009b)
Chrysanthemum cinerariaefolium + *Punica granatum* + *Zanthoxylum schinifolium*	Leaves extracts (water, ethanol, methanol)	5, 50, 100 mg kg^{-1} BW	50 μL fish^{-1}	*P. olivaceus*	63.2 ± 2.4	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	*U. marinum*	Harikrishnan, Heo, Balasundaram *et al.* (2010f)

AP, antiprotease activity; COMP, complement activity; Ig, immunoglobulins; LYZ, lysozyme activity; MPO, myeloperoxidase of leucocytes; PHAG, phagocytosis; RB, respiratory burst activity; RNI, nitrogen reactive intermediates.

Variation in treated fish compared to controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes.

n.a., not available.

*Evaluated in terms of cumulative mortality reduction after challenge with a pathogen.

Table 3 Effect of plant-derived medicines used *per os* in the therapy of fish infectious diseases

Medicines – oral administration

Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Pathogen	Growth performance and survival	Haematological and immunological parameters	Disease resistance	References
Allium sativum	Plant extract (solvent n.a.)	0.1%, 0.4%, 0.8%	45 d	*O. niloticus*	100 ± 20	*Trichodina* sp. *A. hydrophila*	n.a.	n.a.	Reduction of parasite number and fish mortality	Omima (2010)
Azadirachta indica	Leaves extract (ethanol)	0.2%	3% BW day^{-1} 4 weeks	*C. mrigala*	63 ± 2	*A. invadans*	n.a.	*RBC (↔), Hct (↔), Hb (↔), WBC (↔), LYM (↔), MON (↔), NEU (↔), EOS (↔), TP (↔), GLU (↔), CHO (↔), Ca (↔)	n.a.	Harikrishnan, Balasundaram & Heo (2010c)
Origanum minutiflorum	Essential oil	8 ml · 5 kg^{-1}	30 d	*O. punctatus*	1.5	*Myxobolus* sp.	WG (↔)	LYMP (↑), LYZ (↓), PHAG (↔), RNI (↔)	Reduction of infection prevalence and intensity	Karagouni *et al.* (2005)
Rosmarinus officinalis	Leaves powder, leaves extract (ethyl acetate)	4%, 6%	2% BW day^{-1} 15 d	*Oreochromis* sp.	7.5 ± 1.0	*S. iniae*	n.a.	n.a.	Reduction of fish mortality	Abutbul *et al.* (2004)
	Leaves	4%, 8%, 16%	14-20 d	*Oreochromis* sp.	5.5 ± 0.5 7.0 ± 1.0 44.0 ± 19.0	*S. agalactiae* *S. iniae*	n.a.	n.a.	Reduction of fish mortality	Zilberg *et al.* (2010)
Azadirachta indica + *Curcuma longa* + *Ocimum sanctum*	Leaves extract (water)	0.01%, 0.02%, 0.04%, 0.08%	to satiety 4 weeks	*C. aurata*	47 ± 3	*A. hydrophila*	n.a.	*RBC (↓), Hct (↓), Hb (↔), MCH (↓), WBC (↑), TP (↓), GLU (↓), CHO (↓), TRIG (↓), LYZ (↑), PHAG (↑), RB (↑)	n.a.	Harikrishn *et al.* (2010a)
	Leaves extract (ethanol)	0.25%	3% BW day^{-1} 4 weeks	*C. aurata*	23 ± 2	*A. hydrophila*	n.a.	*LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	Reduction of fish mortality	Harikrishnan *et al.* (2009a)
	Leaves extract (water)	0.1%	2% BW day^{-1} 4 weeks	*C. carpio*	52 ± 2	*A. hydrophila*	n.a.	*RBC (↓), Hct (↓), Hb (↓), MCV (↑), MCH (↑), MCHC (↓), WBC (↑), TP (↓), GLU (↓), CHO (↓), COMP (↔), RB (↑)	Reduction of fish mortality	Harikrishnan *et al.* (2010b)

Ca, calcium; CHO, cholesterol; COMP, complement activity; d, days; EOS, eosinophils; GLU, glucose; Hb, haemoglobin concentration; Hct, haematocrit value; LYM, lymphocytes; LYZ, lysozyme activity; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MON, monocytes; NEU, neutrophils; PHAG, phagocytosis; RBC, red blood cell count; RB, respiratory burst activity; RNI, nitrogen reactive intermediates; TRIG, triglycerides; TP, total proteins; WBC, white blood cell count; WG, weight gain; wk, weeks.
Variation in infected and treated fish compared to infected untreated controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes.
*Statistical differences between infected and treated fish and uninfected untreated controls.
n.a., not available.

Table 4 Effect of plant-derived medicines used by injection in the therapy of fish infectious diseases

Medicines – intraperitoneal administration										
Single plant/plant mixture	Parts/ products	Concentrations	Exposure	Fish species	Weight (g)	Pathogen	Growth performance and survival	Haematological and immunological parameters	Disease resistance	References
Allium sativum	Plant extract (water)	1%	500 μL fish^{-1} biweekly 3 months	*D. labrax*	156.9 ± 37.4	*M. marinum*	n.a.	specific Ig (↑)	No effect	Colorni *et al.* (1998)
Punica granatum	Leaves extract, (water, ethanol, methanol)	5, 50, 100 mg kg^{-1} BW	50 μL fish^{-1}	*P. olivaceus*	547 ± 14.5	lymphocystis virus	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	Reduction of fish mortality	Harikrishnan, Heo, Balasundaram *et al.* (2010e)

COMP, complement activity; Ig, immunoglobulins; LYZ, lysozyme activity; PHAG, phagocytosis; RB, respiratory burst activity.
Variation in infected and treated fish compared to uninfected untreated controls; ↑, significant increase; ↓, significant decrease; ↔, no significant changes.
n.a., not available.

Table 5 Effect of plant-derived medicines used by immersion in the therapy of fish infectious diseases

Medicines – immersion administration										
Single plant/plant mixture	**Parts/products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Pathogen**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance**	**References**
Allium sativum	Plant extract (solvent n.a.)	800 ppm	96 h	*O. niloticus*	3.62 ± 0.06	*Trichodina* sp.	n.a.	n.a.	Reduction of parasite number	Chitmanat *et al.* (2005)
Artemisia annua	Leaves extract (ethanol)	0.005%, 0.01%, 0.015%, 0.02%	30–180 min	*H. longifilis*	n.a.	Monogenean parasites	n.a.	n.a.	Reduction of parasite number	Ekanem and Brisibe (2010)
Azadirachta indica	Leaves extract (water)	1%	5 min day^{-1} 24 d	*C. carpio*	40 ± 10	*A. invadans*	n.a.	*RBC (↔), Hct (↓), Hb (↓), WBC (↑), LYM (↓), MON (↔), NEU (↓), EOS (↔)	Lesions recovery	Harikrishnan *et al.* (2005)
Camellia sinensis	Leaves extract (solvent n.a.)	0.03% 0.3%, 0.6%, 0.9%	30, 60 min 1–10 min	*O. keta*	0.5–0.6	*I. necator*	n.a.	n.a.	Reduction of parasite number	Sutzuki, Misaka and Sakai (2006)
		0.03% 0.6%, 0.9%	30, 60 min 1–5 min	*O. masou*	0.2	*I. necator*	n.a.	n.a.	Reduction of parasite number	Sutzuki *et al.* (2006)
		0.9%	5 min	*O. niloticus*	0.2–0.9	*Trichodina* sp.	n.a.	n.a.	Reduction of parasite number and fish mortality	Noor El-Deen (2010)
		0.05%	15 min							
Carica papaya	Seeds extract (petroleum ether)	0.02%, 0.025%	96 h	*C. auratus*	n.a.	*I. multifilis*	n.a.	n.a.	Reduction of parasite number and fish mortality	Ekanem *et al.* (2004a)
Centella asiatica	Plant extract (water)	0.02%, 0.04%, 0.06%, 0.08%	n.a.	*O. niloticus*	10 ± 1	*F. columnare*	n.a.	n.a.	Reduction of fish mortality	Rattanachaikunsopon and Phumkhachorn (2010b)

Table 5 Continued

Medicines – immersion administration

Single plant/plant mixture	Parts/products	Concentrations	Exposure	Fish species	Weight (g)	Pathogen	Growth performance and survival	Haematological and immunological parameters	Disease resistance	References
Macuna pruriens	Leaves extract (methanol)	0.01%, 0.015%, 0.02%	72 h	*C. auratus*	n.a.	*I. multifiliis*	n.a.	n.a.	Reduction of parasite number and fish mortality	Ekanem *et al.* (2004a)
Piper guineense	Seeds extract (methanol)	0.00005%, 0.0001%, 0.00015%, 0.0002%	96 h	*C. auratus*	n.a.	*G. elegans* *D. extensus*	n.a.	n.a.	Reduction of parasite number and fish mortality	Ekanem *et al.* (2004b)
Solanum nigrum	Leaves extract (ethyl acetate)	1%	10 min day^{-1}	*C. punctatus*	25 ± 3	*A. hydrophila*	n.a.	RBC (↑), Hb (↑), MCV (↑), WBC (↓)	Lesions recovery	Rajendiran *et al.* (2008)
Terminalia catappa	Plant extract (solvent n.a.)	800 ppm	96 h	*O. niloticus*	3.62 ± 0.06	*Trichodina* sp.	n.a.	n.a.	Reduction of parasite number	Chitmanat *et al.* (2005)
Azadirachta indica + *Curcuma longa* + *Ocimum sanctum*	Leaves extract (water)	1%	5 min day^{-1}, two times	*C. auratus*	10 ± 2	*A. hydrophila*	n.a.	*RBC (↔), Hct (↑), Hb (↑), MCV (↑), MCH (↑), MCHC (↑), WBC (↓)	n.a.	Harikrishnan and Balasundaram (2008)
	Leaves extract (water)	1%	5 min day^{-1}, two times	*C. auratus*	15 ± 2	*A. hydrophila*	n.a.	n.a.	Lesions recovery	Harikrishnan, Balasundaram, Moon *et al.* (2009c), Harikrishnan, Moon, Kim *et al.* (2010g)

EOS, eosinophils; Hb, haemoglobin concentration; Hct, haematocrit value; LYM, lymphocytes; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MON, monocytes; NEU, neutrophils; RBC, red blood cell count; WBC, white blood cell count.

n.a., not available.

Variation in infected and treated fish compared to uninfected untreated controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes.

*Statistical differences between infected and treated fish and uninfected untreated controls.

1.8 Effects on fish resistance to infections

1.8.1 Bacterial infections

It has been widely established that certain herbs can improve the resistance of fish to bacterial diseases, as an overall consequence of their immunostimulatory effects (Tables 1-2). Diets supplemented with *A. sativum* (Shalabay *et al.*, 2006; Aly *et al.*, 2008a; Aly & Mohamed, 2010), *A. membranaceus* extract (Ardò *et al.*, 2008), *E. purpurea* extract (Aly & Mohamed, 2010), *L. japonica* extract (Ardò *et al.*, 2008) or *Psidium guajava* (Pachanawan *et al.*, 2008) have been reported to significantly ameliorate the immune competence and the survival of Nile tilapia submitted to a challenge with a virulent strain of *A. hydrophila*. Similarly, diets with extract of *A. paniculata* (Rattanachaikunsopon & Phumkhachorn, 2009b), *C. verum* (Rattanachaikunsopon & Phumkhachorn, 2010a) or *C. formosum* (Rattanachaikunsopon & Phumkhachorn, 2010c) enhanced the disease resistance of this fish species to S*treptococcus* sp. while *A. tuberosum* essential oil (Rattanachaikunsopon & Phumkhachorn, 2009a) or *Centella asiatica* extract (Rattanachaikunsopon & Phumkhachorn, 2010b) improved its resistance to *Flavobacterium columnare*. Oral administration of *E. alba* aqueous extract (Christybapita *et al.*, 2007) or *N. arbortristis* chloroform extract (Kirubakaran *et al.*, 2010) significantly increased the resistance of tilapia *O. mossambicus* to haemorrhagic septicaemia by enhancing lysozyme, complement, antiproteases activity and phagocyte microbicidal capability. Similarly, the intraperitoneal injection of *O. sanctum* aqueous extract (Logambal *et al.*, 2000), *S. trilobatum* water and hexane soluble fractions (Divyagnaneswari *et al.*, 2007; 2008), *T. cordifolia* water, ethanol and petroleum ether fractions (Sudhakaran *et al.*, 2006; Alexander *et al.*, 2010) or *T. sinensis* hot-water extract (Wu *et al.*, 2010) reduced the susceptibility of *O. mossambicus* to this disease due to the improvement of the non-specific/specific humoral and cellular immune response. In other researches, diets enriched with acetone extract from *A. marmelos*, *C. dactylon*, *W. somnifera* or *Z. officinale* have been reported to reduce mortality in *O. mossambicus* challenged with *Vibrio vulnificus*, and were able to enhance blood leukocrit, phagocytic and lysozyme activity (Immanuel *et al.*, 2009). Long term dietary administration of *A. aspera* (Vasudeva *et al.*, 2006; Vasudeva & Sunil, 2009), *A. sativum* (Sahu *et al.*, 2007a), *C. dactylon* extract (Kaleeswaran *et al.*, 2011a), *C. longa* (Sahu *et al.*, 2008), *M. indica* (Sahu *et al.*, 2007b) or *W. somnifera* (Sharma *et al.*, 2010) largely prevented *A. hydrophila* infections in Indian major carp, enhancing serum and phagocyte bactericidal activities. Similarly, diet supplementation with *Rheum officinale* (Xie *et al.*, 2008), *A. membranaceus* (Yin *et al.*, 2009), *A. marmelos* extract (Pratheepa *et al.*, 2010), *B. nigra*, *C. majus*, *E. purpurea*, *I. helenium*, *T. farfara* extracts (Mohamad & Abasali, 2010), *C. zeylanicum*, *J. regia*, *M. piperita* and *O. basilicum* extracts (Abasali & Mohamad, 2010) or *A. vera* extract (Alishahi *et al.*, 2010)

improved the resistance of *C. carpio* to *A. hydrophila*. Nya and Austin (2009a; 2009b; 2011) demonstrated the potential value of garlic and ginger in terms of conferring protection against *A. hydrophila* in rainbow trout whilst Harikrishnan *et al.* (2009a) found equivalent results in goldfish preventively fed *A. indica*, *C. longa* and *O. sanctum*. Resistance to *A. hydrophila* was also increased in channel catfish fed a diet supplemented with oregano essential oil (Zheng *et al.*, 2009) and in Japanese eel fed diets enriched with Korean mistletoe extract (Choi *et al.*, 2008). Ji *et al.* (2007b) reported that red sea bream juveniles fed a diet with *A. capillaries*, *C. fructus*, *C. officinale* or *M. medicata* showed reduced mortality when infected with *Vibrio anguillarum*. Moreover, diets supplemented with *O. sanctum* or *W. somnifera* methanolic extract increased the survival rates in *E. tauvina* juveniles during *Vibrio harveyi* infections (Sivaram *et al.*, 2004) and similar results were obtained using a mixture of *C. dactylon*, *P. longum*, *P. niruri*, *T. procumbens* and *Z. officinale* petroleum ether extracts (Punitha *et al.*, 2008). Diet with *Alnus firma* ethanol extract protected olive flounder against *Tenacibaculum maritimum* (Harikrishnan *et al.*, 2011i) while a dietary mixture containing *A. sinensis* and *A. membranaceus* significantly reduced the cumulative mortality of yellow croaker infected with *Vibrio alginolyticus* (Jian & Wu, 2003). *S. baicalensis* extract showed a protective effect against *Edwardsiella tarda* in rock bream, improving the haematological and immune status (Harikrishnan *et al.*, 2011h). Green tea (Harikrishnan *et al.*, 2011b) and *E. japonica* (Kim *et al.*, 2011) extracts incorporated into the diet yielded high survival rate of *E. bruneus* against *V. carchariae*, *L. indica* extract enhanced its resistance against *Streptococcus iniae* (Harikrishnan *et al.*, 2011c) while *S. japonica* extract enhanced its resistance against *V. harveyi* (Harikrishnan *et al.*, 2011g). However, further studies are required to elucidate how long the plant-based treatments must be provided to fish in order to get a long term protection. Aly *et al.* (2008a), Aly & Mohamad (2010) suggested that the longest lasting protection can be obtained by increasing the period of plant application. In fact, *O. niloticus* fed diets supplemented with garlic or echinacea extract for 1 month were protected against an immediate challenge with *A. hydrophila*, while a protection lasting 8 months was ensured by giving the same diets for 2 or 3 months.

On the other hand, Harikrishnan *et al.* demonstrated that different extracts of *A. indica*, *C. longa* and *O. sanctum* can be used as therapeutic agents to control *A. hydrophila* infections in goldfish (Harikrishnan *et al.*, 2009a; 2009c; 2010a; 2010g) and common carp (Harikrishnan *et al.*, 2010b), being able to induce gradual reduction of the clinical signs, complete recovery of health status and increased survival (Tables 3 and 5). Similar results were described by Rajendiran *et al.* (2008) in spotted snakehead dip-treated with *S. nigrum* ethyl acetate leaf extract (Table 5). In *Tilapia* spp., the dietary application of rosemary provided good results in the treatment of streptococcal infections (Abutbul *et al.*, 2004; Zilberg *et al.*, 2010) whereas bath treatment with *C. asiatica* aqueous extract demonstrated therapeutic effects against *F.*

columnare (Rattanachaikunsopon & Phumkhachorn, 2010b) (Tables 3 and 5).

1.8.2 Parasitic infections

Herbal based immunostimulants are also capable of reducing parasitic infections (Tables 1 and 2). Oral administration of *P. vulgaris* extract (Harikrishnan *et al.*, 2011e) and intraperitoneal administration of traditional Korean medicinal (TKM) triherbal extracts (Harikrishnan *et al.*, 2010f) clearly enhanced the resistance of olive flounder *P. olivaceus* against the parasite *Uronema marinum*. Moreover, a diet with mixed *C. cinerariafolium*, *P. granatum* and *Z. schinifolium* extracts protected olive flounder against *Philasterides dicentrarchi* (Harikrishnan *et al.*, 2010d). These results are in agreement with the significantly increased survival rates observed in kelp grouper *E. bruneus* fed diets including *S. japonica* extract (Harikrishnan *et al.*, 2011g) or *V. album* extract (Harikrishnan *et al.*, 2011c). Furthermore, in this species the dietary supplementation with *K. pictus* extract conferred protection from a mixed infection by *V. alginolyticus* and *P. dicentrarchi* (Harikrishnan *et al.*, 2011f).

Other plant extracts showed potential therapeutic effects on parasitic infections in cultured fish (Tables 3 and 5). Methanolic extract of *Mucuna pruriens* leaves and petroleum-ether extract of *Carica papaya* seeds demonstrated their potential in the control of *Ichthyophthirius multifiliis* infections in goldfish, having produced a significant reduction of the parasite burden and the fish recovery (Ekanem *et al.*, 2004a). Similarly, methanolic extracts of *Piper guineense* seeds were active in the treatment of monogenean diseases (Ekanem *et al.*, 2004b). Baths with green tea (Noor El- Deen, 2010), garlic or Indian almond (Chitmanat *et al.*, 2005; Omima, 2010) extract have produced good results in *O.niloticus* against *Trichodina* sp. infestations. Furthermore, oral administration of *O. minutiflorum* essential oil reduced the prevalence of infection caused by *Myxobolus* sp. in sharpsnout sea bream (Karagouni *et al.*, 2005).

1.8.3 Fungal and viral infections

The information concerning the plant-based control of fungal and viral infections is limited. Immersion treatment with *A. indica* extract demonstrated a restorative effect in common carp experimentally infected with the fungus *A. invadans* (Harikrishnan *et al.*, 2005) (Table 5). Intraperitoneal administration of *P. granatum* extracts reduced the mortality of olive flounder infected with the lymphocystis disease virus (LDV) (Harikrishnan *et al.*, 2010e) (Table 4).

1.9 Adverse effects

The application of medicinal herbs in aquaculture have received attention for their promoting effects on growth and immune functions but also because, unlike chemotherapeutic agents, their administration in several animal models including humans is usually associated with few or no side effects (Briskin, 2000). There are few reports with regard to this aspect in fish, still they underline similar evidences. Abutbul *et al.* (2004) found that the oral administration of *Rosmarinus officinalis* in *Tilapia* spp. had no negative effects on fish survival, appearance and behaviour. No apparent toxic effects and mortality were observed in Nile tilapia (*O. niloticus*) fed with *A. paniculata* extract (Rattanachaikunsopon & Phumkhachorn, 2009b), *C. verum* essential oil (Rattanachaikunsopon & Phumkhachorn, 2010a), *C. formosum* extract (Rattanachaikunsopon & Phumkhachorn, 2010c) or *P. guajava* extract (Pachanawan *et al.*, 2008). Further insights and appropriate trials are necessary to evaluate whether plant extracts are toxic to fish at high levels or if administered for long periods, as well as to study how the herbal bio-active compounds are metabolized and/or stored in fish tissues.

1.10 Factors influencing the effectiveness of plants and plant products

1.10.1 Plant/plant products concentration and duration of administration

Amongst factors that may influence the effectiveness of herbal products in fish, their dosage and the duration of administration are crucial. In fact, only an appropriate dosage can significantly induce a stimulation of immune responses increasing disease resistance, without being toxic to animals (Sakai, 1999).

It has been observed that the response of fish after the administration of plants and plant products was usually dose-dependent and therefore more evident at higher concentrations. However, sometimes high doses did not yield the enhanced effects observed at lower concentrations and were occasionally found to be less effective, as has already been demonstrated for many other immunostimulants (Sakai, 1999). For example, Jian & Wu have shown that an increase of Traditional Chinese Medicine (a product composed of several herbs) concentration in the diet did not improve the immune response of yellow croakers (2003) and Jian carps (2004), since they observed that 1.0% and 1.5% dietary levels resulted in similar lysozyme, complement and phagocyte activities. Similarly, Sivaram *et al.* (2004) reported that diets supplemented with 100 and 200 mg/kg of ethanol extract from *O. sanctum* or *W. somnifera* significantly improved growth, immune response and resistance to *V. harveyi* infections of greasy groupers but higher levels (400 and 800 mg/kg) did not provide the same

positive results. Yin *et al.* (2006) observed that the roots of *Astragalus* effectively stimulated phagocytosis and lysozyme activity of Nile tilapia when added to the diet at concentrations of 0.1% and 0.5%, but a dose of 1% was not equally effective. Choi *et al.* (2008) found no significant differences in ROS production of leukocytes when eels were fed 0.5% and 1.0% dietary mistletoe, suggesting that the lower dose is sufficient to induce the maximum response. Sharma *et al.* (2010) reported that carps fed a diet enriched with 2 g/kg of *W. somnifera* root showed higher immune response and relative percentage of survival when compared to carps fed doses of 1 g/kg or 3 g/kg. The absence of a linear relationship between dose and effect has been described by other authors for other plants and other species of fish (Xie *et al.*, 2008; Harikrishnan *et al.*, 2009b; Kirubakaran *et al.*, 2010; Mohamad & Abasali, 2010; Soltani *et al.*, 2010). Pratheepa *et al.* (2011) observed a progressive increase in the immune capability of *C. catla* by increasing the level of *A. marmelos* extract in the diet from 5 g/kg to 30 g/kg, whereas a higher concentration (50 g/kg) induced immunosuppression.

Different effects on fish health status, humoral and cellular immune mechanisms and disease resistance have also been detected following different periods of stimulation. Kirubakaran *et al.* (2010) reported that the administration of 0.1% *N. arbortristis* chloroform extract supplemented diet in *O. mossambicus* for 3 weeks induced a more effective immune response then a feeding lasting 1 or 2 weeks. Harikrishnan *et al.* (2003) observed that an immersion treatment repeated for 30 days with *A. indica* aqueous extract was necessary to improve the haematological parameters in *C. carpio*. However, other authors measured a maximum immune response after just a few days of administration and a decrease of immune competence during the following weeks of treatment. For example, humoral and cellular non-specific immune responses in tilapia (*O. mossambicus*) fed diets including *E. alba* aqueous extract significantly increased after 1 week while no modulation was observed after 3 weeks feeding (Christybapita *et al.*, 2007).

1.10.2 Bioactive compounds contained in plants and plant extracts

Medicinal plants synthesize and accumulate natural bio-active substances responsible for their pharmacological properties in humans and animals. Based on their chemical structure, plant active compounds can principally be categorized into alkaloids, terpenoids (triterpenes and steroid saponins), phenolic compounds, glycosides, flavonoids, tannins and polysaccharides (Lovkova *et al.*, 2001).

The studies regarding the use of plants in aquaculture usually do not provide any data about the chemical composition of the tested plant products (extracts, oils…), hovewer it is thought that the antimicrobial/immunomodulatory properties could be ascribed to secondary metabolites of the above mentioned classes of compounds. For instance, Zheng *et al.* (2009)

considered carvacrol, a phenolic compound, as the main active substance in oregano essential oil administered in *I. punctatus*. Similarly, Dada & Ikuerovo (2009) attributed the effects of *G. cola* to its content of different phenols, including biflavonoids, xanthones and benzophanones while Logambal *et al.* (2000) suggested that the water extract of *O. sanctum* leaves is rich in eugenol, methyl eugenol and caryophyllene. Wu *et al.* (2010) reported that water extract of the Chinese herb *T. sinensis* consists of tritrpenes and phenolic substances including methyl gallate, gallic acid, kaempferol, quercetin, quercitrin, rutin, kaempferol-D-glucoside, catechin, epicatechin, β-sitosterol, stigmasterol, β-sitosteryl-glucoside, stigmasterolglicoside, phytol and toosendanin. *Rosmarinus officinalis* contains terpenes (1,8-cineol, o-pinene, α-pinene, limonene, terpineol-4-ol, α-terpineol), camphor and polyphenols (carnosic acid, rosmarinic acid) (Abutbul *et al.*, 2004). Harikrishnan *et al.* (2003, 2005, 2009a) consider the terpenes azadirachtin, nimbin, nimbinin, nimbinidin, nimbolide and nimbidic acid responsible for antimicrobial and immunostimulant roles of neem (*A. indica*) extracts. Polysaccharides, organic acids, alkaloids, glucosides and volatile oil are the major active components of *A. membranaceous* and *S. baicalensis* extracts that have been found to enhance immune function in fish (Yin *et al.*, 2009; Harikrishnan *et al.*, 2011h). The Korean herb *P. vulgaris* includes flavonoids, triterpenes, phenolic acids, triterpenoids, tannins and polysaccharides (Harikrishnan *et al.*, 2011e), *E. alba* contains eclalbatin, α-amyrin, urosilic acid, oleanolic acid, acliptasaponin, daucosterol, stigmasterol-3-O-glucoside (Christybapita *et al.*, 2007), *T. cordifolia* contains alkaloids, diterpenoid lactones, glycosides, steroids and sesuiterpenoids (Alexander *et al.*, 2010), *C. dactylon*, *A. marmelos*, *W. somnifera* and *Z. officinale* contain different alkaloids, coumarins, triterpenoids, β-sitosterol, steroidal lactones and volatile oils (Immanuel *et al.*, 2009).

On the other hand, Kaleeswaran *et al.* (2011a) performed a preliminary phytochemical screening of *C. dactylon* ethanolic extract, revealing tannins, quinines and phenols as responsible for immunostimulatory activity in *C. catla*. A similar experimental approach, which include both chemical analysis and biological activities determination, should be strongly requested.

Accumulation of plant secondary metabolites varies according to season, temperature, water availability and geographical source (Croteau *et al.*, 2000). The current literature does not provide information on eventual differences in the activity of extracts obtained from various batches of plants, therefore further research is needed to explore this aspect.

1.10.3 Solvent used for plant extraction

The solvent used for the extraction (water, methanol, ethanol, ethyl acetate, hexane, butane, acetone, benzene, petroleum ether, etc.) is another factor which can influence the spectrum of

antibacterial and immunomodulatory properties of plant extracts in fish.

Some studies concerning the effectiveness of extraction methods highlighted that alcoholic or organic solvents always provide a higher efficiency in extracting secondary bioactive metabolites (polar or non polar) with antimicrobial and immunostimulant activity, compared to water-based methods. Divyagnaneswari *et al.* (2007) demonstrated that the hexane soluble fraction of *S. trilobatum* was more protective than the water soluble fraction when administered intraperitoneally to tilapia. Harikrishnan *et al.* (2009b) reported that the injection of triherbal aqueous, ethanol or methanol solvent leaf extracts from *A. indica, O. sanctum* and *C. longa* enhanced non-specific immune parameters and disease resistance against *A. hydrophila* in goldfish, but the ethanol solvent extract appeared more effective as an immunostimulant. On the other hand, the use of aqueous extracts, which contain soluble and particulate components, is more suitable when the plants are being administered by immersion (Rajendiran *et al.*, 2008). Similar results were also obtained in studies performed *in vitro*. For example, Borisutpeth *et al.* (2005) demonstrated that methanol extracts from *Cassia fistula* and *Hibiscus sabdariffa* exhibited slightly higher efficacy against *A. hydrophila* and *S. agalactiae* compared to water extracts. Moreover, Rattanachaikunsopon & Phumkhachorn (2009b) observed that ethanol extracts from *A. sativum*, *Cassia alata*, *Gracinia mangostana* and *P. guajava* had a greater inhibitory activity *in vitro* against *S. agalactiae* when compared to aqueous extracts of the same plant species. Ponnusamy *et al.* (2010) reported that *Clitoria ternatea* extracted using ethyl acetate, ethanol, acetone and petroleum ether showed higher antibacterial effects against a range of fish pathogens than that extracted using water.

Other reports show that there may be also differences in the capability of the alcoholic and organic solvents to recover the antimicrobial compounds, that could lead to different susceptibilities of the target bacterial strains. Abutbul *et al.* (2004) observed that rosemary was more active against *S. iniae* growth when extracted using ethyl acetate compared to others organic solvents (ethanol, methanol, methanol/ethyl acetate). Punitha *et al.* (2008) tested different solvent extracts from *C. dactylon*, *P. longum*, *P. niruri*, *T. procumbens* and *Z. officinalis* against *V. harveyi* and reported that those based on petroleum ether and benzene effectively limited the pathogen viability. Dhayanithi *et al.* (2010) established that ethanol and methanol extracts obtained from neem (*A. indica*) were highly inhibitory towards several bacterial fish pathogens when compared to extracts produced using other solvents (chloroform or acetone).

1.10.4 Sinergism or antagonism between plants

Further investigations are needed to elucidate possible synergistic or antagonistic effects between the plants included in the mixtures. However, some studies demonstrated that the

combined use of medicinal herbs brings a synergistic action on fish physiology, as compared with the use of individual herbs. Ji *et al.* (2007b) reported that a diet supplemented with a mixture of *M. medicata* (fruits), *C. fructus* (fruits), *A. capillaries* (leaves) and *C. officinale* (roots) enhanced growth performance, serum constituents and resistance to *V. anguillarum* in red sea bream to a greater extent than a diet containing a single medicinal herb. Similarly, the results of Harikrishnan and Balasundaram (2008) revealed that a tri-herbal extract of *A. indica, C. longa* and *O. sanctum* is effective *in vitro* against *A. hydrophila* at lower concentrations than the extracts obtained from each single plant.

1.11 Legislation on herbal products in aquaculture

Currently several herbal products are included in a list of feed additives (based on the EC Regulation No 1831/2003) continuously updated by the EU. Within this list, herbal products are classified in category 2 - sensory additives, functional group b - flavouring compounds, subclass - natural products botanically defined. The list provides also information concerning the animal species (sometimes including fish) to which each plant can be administered and the dosages.

As regards the use of plants for curative purposes in fish, the Commision Regulation (EC) No 710/2009 covering "organic aquaculture" allows the use of substances of plant origin at a homeopathic dilution as well as plants and plant extracts with no anaesthetic effects as veterinary treatments for fish. The use of allopathic medicines must be limited to a maximum of two treatments per year or to a single treatment when the production cycle takes less than one year. If these limits are exceeded for allopathic treatment, fish can not be marketed as organic products. In addition, plants may be employed for cleaning and disinfection in fish farms addressed to organic production. In the U.S.A. the use of plant-derived products in the aquaculture industry is under the control of the US Food and Drug Administration (FDA) and the US Environmental Protection Agency (EPA), which also approve drugs and chemicals to be used in fish farming. Among plants, only garlic and onion have undergone review by the Food and Drug Administration as new animal drugs of low regulatory priority. Their administration is permitted in salmonids at all stages of life for the treatment of helminth and lice infestations (PPM n. 1240.4200, 2011).

1.12 Conclusions and future perspectives

Infectious diseases represent the main problem for the development and sustainability of the aquaculture industry as they cause significant economic losses inasmuch as they restrict productivity and require the use of control measures that are often very expensive. However, the

indiscriminate administration of antibiotics or other drugs in fish leads to the selection of antibiotic-resistant bacterial strains as well as to the accumulation of chemical residues in water and fish tissues, which may prove damaging to the environment and be potentially dangerous for consumers. On the other hand, the production of effective vaccine formulations for a number of fish pathogens is usually difficult and not cost-effective for pharmaceutical companies.

In this context, plant-derived products seem to represent a promising tool, complementary to vaccination and traditional drugs, being able to improve growth, survival, health status, innate (lysozyme, complement, antiproteases phagocytosis and microbicidal capacity of phagocytic cells) and adaptive (specific Ig production) immune responses as well as disease resistance in various marine and freshwater fish species. In addition, unlike chemotherapeutics, their administration to fish does not seem to be associated with side effects. Medicinal plants are also easily commercially available, inexpensive and biocompatible.

Further investigations are still strongly recommended to define the optimal doses and timings of administration as well as to isolate, characterize and quantify the bioactive compounds contained in plants and phytoextracts, in order to identify the most effective substances/metabolites that could be included in new natural formulations to be use in fish. Moreover, research on mode of action, stability of plant materials in aquatic environment and digestibility in fish as well as *in vitro* and *in vivo* toxicological tests are prerequisites for their safe application.

Nowadays, only few commercial herbal products are available at a global level for large-scale use in aquaculture. In many countries a review of the current legislation should be undertaken to allow a greater flexibility in their use taking into consideration the benefits that they might have in intensive farming conditions, in terms of fish welfare and public health. Plants and plant bioactives might be proposed in aquaculture primarily as feed additives or immunostimulants, rather that therapeutics, since the registration of herbal remedies to be used in this field is a time-consuming process and implies higher economic costs.

1.13 Acknowledgments

The authors are grateful to Ministero delle Politiche Agricole e Forestali (Italy) for the financial support through the project ‘Azione Concertata per l’identificazione di contributi scientifici per lo sviluppo dell’acquacoltura biologica in Italia’ n. 7C 09, which made this work possible. They also thank Prof. Emilio Tibaldi (DIAL, University of Udine, Italy) for his guidance in writing and modifying the review article.

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1.15 Literature udate

Since the previous review considered the literature available from 1998 to 2011, a further update of scientific publications until 2014 is provided in the following tables.

Table 6 Impact of plant-derived immunostimulants used *per os* on growth, survival, haematological profile, immune response and disease resistance of cultured fish

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Achyranthes aspera	seeds	0.5%, 1%	5% BW/day for 50 d	*Catla catla*	37.7 ± 6.7	n.a.	#LYZ (↑), BA (↑), RB (↑), RNI (↑), specific Ig (↑), TNFa (↑), IL-10 (↑↓)	n.a.	Chkrabarti et al. (2014)
Adathoda vasica	leaves	3%	*ad libitum* for 2 weeks	*Labeo rohita*	9-15	n.a.	PT (↑), TRIG (↓), CHO, (↑), ALT (↑), AST (↑), ALP (↑) ǂPT (↑), TRIG (↑), CHO, (↑), ALT (↑), AST (↑), ALP (↑)	n.a.	John et al. (2011)
Aloe vera	leaves extract (solvent n.a.)	0.025%, 0.05%, 0.075%	for 30 d	*Cyprinus carpio*	10 ± 1	n.a.	n.a.	*A. hydrophila*	Valsa and Balasubramanian (2014)
Allium sativum	bulbs	1.3%, 4%	3% BW/day for 2 weeks	*Epinephelus coioides*	19.3 ± 3.2	WG (↑), FE (↑)	n.a.	*S. inieae*	Guo et al. (2012)
	bulbs	0.5%, 1%, 1.5%, 2%	5% BW/day for 2 weeks	*Lates calcarifer*	20 ± 2	WG (↑), SGR (↑), FCR (↓)	RBC (↑), Hb (↑), Hct (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), EOS (↑), BAS (↑), TP (↑), ALB (↑), GLB (↑), TL (↓), TRIG (↓), CHO (↓), GLU (↓) ǂRBC (↑), Hb (↑), Hct (↑), WBC (↑), LYM (↔), MON (↔), NEU (↔), EOS (↑), BAS (↑), TP (↑), ALB (↔), GLB (↑), TL (↔), TRIG (↔), CHO (↔), GLU (↓)	*V. harveyi*	Talpur et al. (2012)
	bulbs	1%, 2%, 3%, 4%, 5%	4% BW/day for 8 weeks	*Oncorhynchus mykiss*	7 ± 1	n.a.	CHO (↓), TRIG (↓), AST (↑), ALT, (↑), HDL-CHO (↑), LDL-CHO (↓), SOD (↑), GPX (↔), CAT (↑), MDA (↓)	n.a.	Mohebbi et al. (2012)
	extract bulbs	0.4% 0.5%, 1%, 2%, 3%, 5%	to satiety for 8 weeks	*Paralychthys olivaceus*	5.1	WG (↑), FI (↓), FER (↓), PER (↓), CF (↔), HSI (↓), SR (↔)	TP (↔), GLU (↔), CHO (↔), TRIG (↔), GOT (↔), GPT (↑), LYZ (↓)	n.a.	Cho et al. (2010)
Andrographis paniculata	leaves extract (methanol)	0.05%, 0.1%, 0.2%, 0.3%	*ad libitum* for 45 d	*Oreochromis mossambicus*	20-40	WG (↑), SGR (↑)	RBC (↔), Hb (↑), WBC (↔), MCV (↔), MCH (↑), MCHC (↔), TRB (↑)	n.a.	Prasad and Mukthiraj (2011)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Astragalus membranaceus	plant extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Azadiractha indica	leaves	0.1%, 0.2%, 0.3%, 0.4%, 0.5%	5% BW/day for 2-4 weeks	*Lates calcarifer*	18 ± 2	WG (↑), SGR (↑), FCR (↓)	RBC (↑), Hb (↔), Hct (↑), MCV (↑), MCH (↔), MCHC (↔), WBC (↑), LYM (↑), MON (↑), NEU (↑), EOS (↑), BAS (↑), TRB (↔), TP (↑), ALB (↔), GLB (↑), TL (↓), TRIG (↔), CHO (↔), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑) ǂRBC (↑), Hb (↔), Hct (↑), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↔), MON (↔), NEU (↑), EOS (↑), BAS (↔), TRB (↔), TP (↑), ALB (↔), GLB (↑), TL (↓), TRIG (↔), CHO (↔), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑)	*V. harveyi*	Talpur et al. (2013a)
Bupleurum chinense	roots extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Calendula officinalis	leaves	3%	*ad libitum* for 2 weeks	*Labeo rohita*	9-15	n.a.	PT (↑), TRIG (↓), CHO, (↑), ALT (↑), AST (↑), ALP (↓) ǂPT (↑), TRIG (↑), CHO, (↑), ALT (↑), AST (↑), ALP (↑)	n.a.	John et al. (2011)
Camellia sinensis	leaves	0.002%, 0.01%, 0.05%	2.5% BW/day for 35 d	*Oncorhynchus mykiss*	23.5±2.6	SGR (↔), FCR (↔)	TP (↑), BA (↑), SOD (↑), GPX (↔), MDA (↓), IL-1β (↑), IL-6 (↑), IL-8 (↑), IL-10 (↓), TNF-α (↑)	n.a.	Nootash et al. (2013)
Cinnamomum verum	bark	0.5%, 1%	*ad libitum* for 30 d	*Oreochromis* sp.	40 ± 6	SGR (↑)	RBC (↔), Hb (↑), WBC (↑), LYM (↔), MON (↔), NEU (↔) ǂRBC (↔), Hb (↑), WBC (↑), LYM (↑), MON (↔), NEU (↓)	n.a.	Sivagurunat and Innocent (2014)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Coptis chinensis	rhizomes extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Echinacea purpurea	plant extract (solvent n.a.)	0.025%, 0.05%, 0.075%, 0.1%	4% BW/day for 4 weeks	*Oreochromis niloticus*	50	n.a.	ǂLYM (↑), MON (↑), TP (↑)	*A. hydrophila*	El-Asely et al. (2012)
Epilobium hirsutum	plant extract (ethanol)	0.5%, 1%, 3%	2% BW/day for 8 weeks	*Cyprinus carpio*	20 ± 2	SGR (↔), FCR (↔), CF (↔), SR (↔)	RBC (↔), Hb (↔), Hct (↔), LYM (↔), MON (↔), NEU (↔), WBC (↑) ǂRBC (↓), Hb (↔), Hct (↔), WBC (↑), LYM (↔), MON (↔), NEU (↔)	*A. hydrophila*	Pakravan et al. (2012)
Ficus benghalensis	roots	5%	5% BW/day for 7 weeks	*Channa punctatus*	120-200	n.a.	TP (↑), ALT (↔), AST (↔), LYZ (↑), PHAG (↑), RNI (↑), SOD (↑), specific Ig (↑)	n.a.	Verma et al. (2012)
	roots	5%	3% BW/day for 20 d	*Clarias gariepinus*	50 ± 5	n.a.	ǂALT(↓), AST (↓), ALP (↑), LYZ (↑), PHAG (↑), RNI (↑), specific Ig (↑), SOD (↑), MDA (↓)	n.a.	Verma et al. (2013)
Forsythia spp	fruits extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Glycyrrhiza glabra	roots/ rhizomes extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Isatis indigotica	roots extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
Leucaena leucocephala	seeds	5%	3% BW/day for 20 d	*Clarias gariepinus*	50 ± 5	n.a.	ǂALT(↓), AST (↓), ALP (↑), LYZ (↑), PHAG (↑), RNI (↑), specific Ig (↑), SOD (↑), MDA (↓)	n.a.	Verma et al. (2013)
Lonicera japonica	flowers extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Ocimum basilicum	leaves extract (ethanol)	0.01%, 0.02%, 0.04%, 0.08%, 0.16%	3% BW/day for 60 d	*Cyprinus carpio*	10.0 ± 0.2	FBW (↑), WG (↑), SGR (↑), FCR (↓), FI (↑), SR (↔)	RBC (↑), Hb (↑), Hct (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓)	*A. hydrophila*	Amirkhani and Firouzbakhsh (2015)
	leaves	3%	*ad libitum* for 2 weeks	*Labeo rohita*	9-15	n.a.	PT (↔), TRIG (↓), CHO, (↑), ALT (↑), AST (↑), ALP (↑) ǂPT (↔), TRIG (↑), CHO, (↑), ALT (↑), AST (↑), ALP (↑)	n.a.	John et al. (2011)
Ocimum sanctum	leaves extract (water)	0.05%, 0.1%, 0.2%, 0.5%, 1%	3% BW/day for 6 weeks	*Labeo rohita*	6.6 ± 0.01	n.a.	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), total Ig (↑), RB (↑) ǂRBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), GLU (↓), LYZ (↑), total Ig (↑), RB (↑)	*A. hydrophila*	Das et al. (2013)
Mentha piperita	leaves	0.1%, 0.2%, 0.3%, 0.4%, 0.5%	5% BW/day for 4 weeks	*Lates calcarifer*	20 ± 1	WG (↑), SGR (↑), FCR (↓)	RBC (↑), Hb (↑), Hct (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), TL (↓), TRIG (↓), CHO (↓), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑) ǂRBC (↑), Hb (↑), Hct (↑), WBC (↑), TP (↑), ALB (↔), GLB (↔), TL (↔), TRIG (↓), CHO (↔), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑)	*V. harveyi*	Talpur et al. (2014)
Nasturtium nasturtium	leaves extract (chloroform–methanol)	0.1%, 1%	for 3 weeks	*Oncorhynchus mykiss*	96 ± 10	FBW (↔), WG (↔), SGR (↔), FCR (↔)	RBC (↔), Hct (↔), Hb (↑), MCV (↔), MCH (↔), MCHC (↑), WBC (↔), TP (↑), ALB (↔), GLB (↑), LYZ (↑), COMP (↑), PO (↔)	n.a.	Asadi et al. (2012)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Nelumbo nucifera	plant extract (ethanol)	1%, 2%	for 40 d	*Cirrhinus mrigala*	45 ± 5	SGR (↑), FCR (↓)	ǂRBC (↑), WBC (↑), LYM (↑), MON (↑), NEU (↔), EOS (↑), BAS (↑), TP (↔), ALB (↔), GLB (↑)	n.a.	Sivagurunat et al. (2012)
Nigella sativa	essential oil	1%, 2%, 3%	for 2 weeks	*Oncorhynchus mykiss*	18 ± 0.2	n.a.	TP (↑), LYZ (↑), AP (↑), BA (↑), PO (↑), total Ig (↑)	n.a.	Awad et al. (2013)
	seeds	3%	*ad libitum* for 30 d	*Oreochromis niloticus*	40 ± 5	n.a.	WBC (↑), GLB (↔), PHAG (↑)	*A. hydrophila*	Elkamel and Mosaad (2012)
Panax ginseng	root extract (ethanol)	0.06%	for 8 weeks	*Oreochromis niloticus*	41.6 ± 0.3	WG (↑), SGR (↑), FCR (↓), FI (↔), CF (↑), SR (↔)	GLB (↑), WBC (↑), LYZ (↑), RB (↑), BA (↔), specific Ig (↑)	*A. hydrophila*	El-Sayed et al. (2014)
Phyllanthus amarus	leaves extract (water)	n.a.	for 2 weeks	*Labeo rohita*	12 ± 2	n.a.	RBC (↑), Hb (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑)	n.a.	Annalakshami et al. (2013)
Pueraria thunbergiana	leaves extract (ethanol)	0.1%, 1%, 2%	5% BW/day for 8 weeks	*Epinephelus bruneus*	28	WG (↑), SGR (↔), FCR (↔), PER (↑)	ǂRBC (↑), Hct (↑), Hb (↑), MCV (↔), MCH (↔), MCHC (↔), WBC (↑), LYM (↑), MON (↑), NEU (↔), TRB (↔), TP (↑), ALB (↑), GLB (↔), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑)	*V. harveyi*	Harikrishnan et al. (2012b)
Rheum officinale	plant extract (solvent n.a.)	0.1%	2-4% BW/day for 7 weeks	*Megalobrama amblycephala*	131.5 ± 4.4	n.a.	TP (↔), ALT (↔), AST (↓), ALP (↔), LYZ (↑), COR (↔), CAT (↔), SOD (↑), MDA (↓), HSP70 (↑) ǂTP (↑), ALT (↔), AST (↔), ALP (↑), LYZ (↑), COR (↓), CAT (↔), SOD (↑) MDA (↓), HSP70 (↑)	*A. hydrophila*	Liu et al. (2012)
Rosmarinus officinalis	plant powder	1%	*ad libitum* for 45 d	*Oreochromis mossambicus*	13.0 ± 0.1	n.a.	RBC (↑), Hb (↔), Hct (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), LYZ (↔), PHAG (↑), RB (↔), PO (↔)	*S. inieae*	Gültepe et al. (2014)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Sargassum cristaefolium	plant extract (water)	0.05%, 0.1%, 0.2%	for 18 d	*Epinephelus coioides*	3.3 ± 0.8 9.0 ± 0.7 35.8 ± 2.3	WG (↑), FER (↑)	LYZ (↑), COMP (↑), PHAG (↑), RB (↑), SOD (↑)	*Streptococcus* sp. grouper Iridovirus	Wong et al. (2013)
Sauropus androgynus	leaves extract (ethanol)	1%, 2.5%, 5%	3% BW/day for 30 or 70 d	*Epinephelus coioides*	11.0 ± 2.0 59.5 ± 4.5	WG (↑), SGR (↑), VSI (↔), HSI (↔)	LYZ (↑), PHAG (↑), RB (↑), SOD (↑)	*V. alginolyticus*	Samad et al. (2014)
Scutellara baicalensis	plant extract (water)	2%	for 4 weeks	*Sciaenops ocellatus*	63.7 ± 1.2	n.a.	LYZ (↑), PHAG (↑)	*V. splendidus*	Pan et al. (2013)
	plant extract (solvent n.a.)	0.5%, 1%, 2%, 3%, 5%	to satiety for 8 weeks	*Silurus asotus*	0.96	WG (↔), SGR (↔), FI (↔), FER (↔), PER (↑), SR (↔)	TP (↔), GLU (↔), CHO (↔), TRIG (↔), GOT (↓), GPT (↑)	*L. anguillarum*, *S. inieae*	Kim et al. (2013)
Siegesbeckia glabrescens	plant extract (ethanol)	0.1%, 1%, 2%	5% BW/day for 8 weeks	*Epinephelus bruneus*	27.6 ± 11.3	n.a.	ǂLYZ (↑), COMP (↑), RB (↑), RNI (↑), MPO (↑)	*V. parahaemolyticus*	Harikrishnan et al. (2012a)
Silybum marianum	plant extract (n.a.)	0.01%, 0.04%, 0.08%	2% BW/day for 30 d	*Oncorhynchus mykiss*	90 ± 15	n.a.	RBC (↑), Hct (↑), Hb (↑), WBC (↑), LYM (↔), MON (↔), NEU (↔), TRB (↑), TP (↑), ALB (↑), GLB (↑), LYZ (↑), COMP (↑), PO (↔)	n.a.	Ahmadi et al. (2012)
Sophora flavescens	root extract (ethanol)	0.025%, 0.05%, 0.1%, 0.2%, 0.4%	2% BW/day for 30 d	*Oreochromis niloticus*	45 ± 5	n.a.	LYZ (↑), COMP (↑), AP (↑), RB (↑), RNI (↑), MPO (↑) ǂLYZ (↑), COMP (↑), AP (↑), RB (↑), RNI (↑), MPO (↑)	*S. agalactiae*	Wu et al. (2013)
Suaeda maritima	plant extract (ethanol)	0.01%, 0.1%, 1%	5% BW/day for 8 weeks	*Paralychthys olivaceus*	16.3 ± 1.2	n.a.	ǂRBC (↑), Hct (↑), Hb (↑), MCV (↔), MCH (↑), MCHC (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), EOS (↔), TP (↑), CHO (↑), GLU (↑), Ca (↑), LYZ (↑), PA (↑), RB (↑)	*M. avidus*	Harikrishnan et al. (2012c)
Thymus vulgaris	plant powder	1%	*ad libitum* for for 45 d	*Oreochromis mossambicus*	13.0 ± 0.1	n.a.	RBC (↑), Hb (↔), Hct (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), LYZ (↔), PHAG (↑), RB (↔), PO (↔)	*S. inieae*	Gültepe et al. (2014)

Table 6 Continued

Immunostimulants - oral administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Trigonella foenum	plant powder	1%	*ad libitum* for for 45 d	*Oreochromis mossambicus*	13.0 ± 0.1	n.a.	RBC (↑), Hb (↔), Hct (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), LYZ (↑), PHAG (↑), RB (↔), PO (↑)	*S. inieae*	Gültepe et al. (2014)
Urtica dioica	plant extract (solvent n.a.)	0.1%, 0.5%, 1%	for 2 weeks	*Oncorhynchus mykiss*	18.0 ± 0.2	n.a.	TP (↑), LYZ (↑), AP (↑), BA (↑), PO (↑), total Ig (↑)	n.a.	Awad et al. (2013)
Viscum album coloratum	leaves/ trunks extracts	0.001%, 0.005%, 0.02%	3% BW/day for 80 d	*Oreochromis niloticus*	30 ± 4	n.a.	LYZ (↑), COMP (↑), PHAG (↑), RB (↑)	*A. hydrophila*	Park et al. (2012)
Zingiber officinale	rhizomes	0.1%, 0.2%, 0.3%, 0.5%, 1%	to satiety for 2 weeks	*Lates calcarifer*	18 ± 1	WG (↑), SGR (↑), FCR (↓)	RBC (↑), Hb (↑), Hct (↑), WBC (↑), LYM (↑), MON (↑), NEU (↑), TP (↑), ALB (↑), GLB (↑), TL (↓), TRIG (↓), CHO (↓), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑) ǂRBC (↑), Hb (↑), Hct (↑), WBC (↑), LYM (↑), MON (↔), NEU (↑), TP (↑), ALB (↓), GLB (↑), TL (↓), TRIG (↓), CHO (↓), GLU (↓), LYZ (↑), AP (↑), BA (↑), PHAG (↑), RB (↑)	*V. harveyi*	Talpur et al. (2013b)
Ocimum sanctum, Solanum trilobatum	plant extract (ethanol)	0.0003%, 0.003%, 0.03%	3% BW/day for 60 d	*Mystus keletius*	n.a.	n.a.	RBC (↑), Hb (↑), WBC (↑), TP (↑), ALB (↑), GLB (↑), LYZ (↑), BA (↑), RB (↑)	*A. hydrophila*	Begum and Navaraj (2012)
Radix coptidis, Radix scutellaria, Herba andrographis, Sophora flavescentis	plant powder	0.5%, 1%, 2%	1% BW/day for 3 weeks	*Ctenopharyngodon idellus*	27.1 ± 4.2	n.a.	TP (↔), BA (↑), RB (↑), total Ig (↑)	*A. hydrophila*	Choi et al. (2014)
astragalus, angelica, hawthorn, licorice, honeysuckle	plant powder	0.5%, 1%, 1.5%, 2%	for 4 weeks	*Oreochromis niloticus*	20 ± 2	n.a.	LYZ (↑), PO (↑), TNF-α (↑), IL-1β (↑), SOD (↑), MDA (↓)	*A. hydrophila*	Tang et al. (2014)

ALB, albumin; ALP, alkaline phosphatase; ALT, alaninaminotransferase; AP, antiprotease activity; AST, aspartate aminotransferase; BA, bactericidal activity; BAS, basophils; BW, body weight; CF, condition factor; CHO, cholesterol; COMP, complement activity; d, days; FI, feed intake; EOS, eosinophils; FBW, final body weight; FCR, feed conversion ratio; FER, feed efficiency ratio; GLB, globulins; GLU, glucose; Hb, haemoglobin concentration; Hct, haematocrit value; HDL-CHO, high density lipoprotein cholesterol; HSI, hepatosomatic index; Ig, immunoglobulins; IL-1β, interleukin 1β; IL-8, interleukin 8; LYM, lymphocytes; LYZ, lysozyme activity; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MDA, malondialdehyde content; MON, monocytes; MPO, myeloperoxidase of leucocytes; NEU, neutrophils; PHAG, phagocytosis; PER, protein efficiency ratio; PO, serum or plasma peroxidase activity; RBC, red blood cell count; RB, respiratory burst activity; RNI, nitrogen reactive intermediates; SGR, specific growth rate; SOD, superoxide dismutase activity; SR, survival rate; TL, total lipids; TNF-α, tumour necrosis factor α; TRB, trombocytes; TRIG, triglycerides; TP, total proteins; VSI, viscerosomatic index; WBC, white blood cell count; WG, weight gain.

Variation in treated fish compared to controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes.

n.a., not available.

*Evaluated in terms of cumulative mortality reduction after challenge with a pathogen. #Evaluation performed on samples collected from immunized fish. ǂEvaluation performed on samples collected after the challenge.

Table 7 Impact of plant-derived immunostimulants used by injection on growth, survival, haematological profile, immune response and disease resistance of cultured fish

Immunostimulants - intraperitoneal administration

Single plant/plant mixture	Parts/ Products	Concentrations	Exposure	Fish species	Weight (g)	Growth performance and survival	Haematological and immunological parameters	Disease resistance*	References
Liriope platyphylla	leaves extract (water, ethanol, methanol)	0.1, 1, 2 mg	100 µl fish^{-1}	*Paralychthys olivaceus*	29 ± 1.2	n.a.	‡RBC (↑), Hb (↑), Hct (↑), WBC (↑), MCV (↔), MCH (↑), MCHC (↑), LYM (↑), MON (↑), NEU (↑), TRB (↔), TP (↑), ALB (↑), GLB (↑), LYZ (↑), COMP (↑), RB (↑)	n.a.	Harikrishnan et al. (2012d)
Muscari comosum	plant extract (ethyl alcohol)	0.5, 2 mg fish^{-1}	100 µl fish^{-1}	*Sparus aurata*	120 ± 2	SGR (↑)	Hct (↔), WBC (↑), LYM (↓), MON (↑), NEU (↑), EOS (↑), TP (↔), LYZ (↑), RB (↑)	n.a.	Baba and Uluköy (2014)
Nyctanthes arbortristis	leaves extract (methanol)	3.2, 16, 80, 400 mg kg^{-1} BW	200 µl fish^{-1}	*Oreochromis mossambicus*	25 ± 30 50 ± 5	n.a.	#LYZ (↑), RB (↑), specific Ig (↑)	*A. hydrophila*	Devasree et al. (2014)
Ocimum sanctum	leaves extract (petroleum ether)	n.a.	200 µl fish^{-1}	*Catla catla*	18 ± 1	n.a.	#specific Ig (↑)	n.a.	Chitra and Krishnaveni (2011)
Prunella vulgaris	plant extract (water)	1, 3, 5 mg kg-1 BW	500 µl fish^{-1}	*Oreochromis niloticus*	100-150	n.a.	LYZ (↑), PHAG (↑), RB (↑), specific Ig (↑)	n.a.	Park and Choi (2014)
Solanum nigrum	leaves extract (toluene, methanol, ethanol, chloroform, water)	4 ppm	200 µl fish^{-1}	*Etroplus suratensis*	25 ± 5	n.a.	#RB (↑), specific Ig (↑)	*A. invadans*	Haniffa (2011)

ALB, albumin; BW, body weight; COMP, complement activity; EOS, eosinophils; GLB, globulins; Hb, haemoglobin concentration; Hct, haematocrit value; Ig, immunoglobulins; LYM, lymphocytes; LYZ, lysozyme activity; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MON, monocytes; NEU, neutrophils; PHAG, phagocytosis; RBC, red blood cell count; RB, respiratory burst activity; SGR, specific growth rate; TRB, trombocytes; TP, total proteins; WBC, white blood cell count.
Variation in treated fish compared to controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes. n.a., not available. *Evaluated in terms of cumulative mortality reduction after challenge with a pathogen. #Evaluation performed on samples collected from immunized fish. ‡Evaluation performed on samples collected after the challenge.

Table 8 Impact of plant-derived medicines used *per os* in the therapy of fish infectious diseases

Medicines - oral administration										
Single plant/plant mixture	**Parts/ Products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Pathogen**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance**	**References**
Acanthopannax korearum, Glycyrrhiza vralensis, Panax ginseng	plant extract (water)	n.a.	5% of BW/day for 12 d	*Oplegnathus fasciatus*	27.2 ± 1.6	*E. tarda*	FBW (↑), WG (↑)	TP (↑), GLU (↑), AST (↑), ALT (↑), LYZ (↑), RB (↑)	Reduction of mortality	Kim et al. (2012)
	plant extract (water)	n.a.	5% of BW/day for 12 d	*Paralichthys olivaceus*	26.4 ± 1.8	*E. tarda*	FBW (↑), WG (↑)	TP (↑), GLU (↑), AST (↑), ALT (↑), LYZ (↑), RB (↑)	Reduction of mortality	

ALT, alaninaminotransferase; AST, aspartate aminotransferase; BW, body weight; d, days; FBW, final body weight; GLU, glucose; LYZ, lysozyme activity; RB, respiratory burst activity; TP, total proteins; WG, weight gain.
Variation in treated fish compared to controls: ↑, significant increase; ↓, significant decrease; ↔, no significant changes.
n.a., not available.

Table 9 Impact of plant-derived medicines used by immersion in the therapy of fish infectious diseases

Medicines - immersion administration										
Single plant/plant mixture	**Parts/ Products**	**Concentrations**	**Exposure**	**Fish species**	**Weight (g)**	**Pathogen**	**Growth performance and survival**	**Haematological and immunological parameters**	**Disease resistance**	**References**
Dioscorea zingiberensi, Ginko biloba	rhizome/ sarcotesta extract (ethanol)	0-9 mg l^{-1}	for 48 h	*Carassius aurata*	3.5 ± 0.5	*Dactylogyrus spp.*	n.a.	n.a.	Reduction of parasite number	Jian et al. (2014)

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CHAPTER II

IN VITRO ANTIBACTERIAL ACTIVITY OF PLANT ETHANOLIC EXTRACTS AGAINST FISH PATHOGENS

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Journal of the World Aquaculture Society (2014), 45: 545-557, doi: 10.1111/jwas.12151.

Abstract

Fifteen commercial ethanolic extracts from medicinal plants were *in vitro* screened for antibacterial activity against *Listonella anguillarum* (serotypes O1 and O2), *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*, and *Lactococcus garvieae*. Their antimicrobial potential was assessed by the disc diffusion assay, then minimal inhibitory (MIC) and bactericidal (MBC) concentrations were established by the broth micro-dilution method. The extracts of lavender (*Lavandula officinalis*), lemon balm (*Melissa officinalis*), basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), common sage (*Salvia officinalis*), and red bilberry (*Vaccinium vitis idaea*) showed a broad spectrum of inhibitory effects. The extracts of yarrow (*Achillea millefolium*), arnica (*Arnica montana*), marigold (*Calendula officinalis*), Icelandic lichen (*Cetraria islandica*), horsetail (*Equisetum arvense*), grindelia (*Grindelia robusta*), java tea (*Orthosiphon stamineus*), and thyme (*Thymus vulgaris*) were less or not active. *P. damselae* subsp. *piscicida* was the most susceptible bacterial strain, while *Y. ruckeri* was the most resistant. These results can be considered for further investigations aimed to identify novel natural antimicrobial compounds that could be used in aquaculture for the control of bacterial infections.

Keywords: medicinal plants, ethanolic extracts, bacterial fish pathogens, antibacterial activity.

Riassunto

Quindici estratti etanolici commerciali ottenuti da piante medicinali sono stati sottoposti a screening *in vitro* per studiarne l'attività antibatterica nei confronti di *Listonella anguillarum* (sierotipi O1 e O2), *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida* e *Lactococcus garvieae*. Il loro potenziale antimicrobico è stato valutato mediante test di diffusione su agar, quindi la minima concentrazione inibente (MIC) e la minima concentrazione battericida (MBC) sono state determinate mediante metodo delle micro-diluizioni in brodo. Gli estratti di lavanda (*Lavandula officinalis*), melissa (*Melissa officinalis*), basilico (*Ocimum basilicum*), origano (*Origanum vulgare*), rosmarino (*Rosmarinus officinalis*), salvia comune (*Salvia officinalis*) e mirtillo rosso (*Vaccinium vitis idaea*) hanno mostrato un ampio spettro di effetti inibitori. Gli estratti di achillea (*Achillea millefolium*), arnica (*Arnica montana*), calendula (*Calendula officinalis*), lichene islandico (*Cetraria islandica*), equiseto (*Equisetum arvense*), grindelia (*Grindelia robusta*), tè java (*Orthosiphon stamineus*) e timo (*Thymus vulgaris*) sono risultati meno attivi o non inibenti. *P. damselae* subsp. *piscicida* è risultata la specie batterica più sensibile, mentre *Y. ruckeri* è risultata quella più resistente. Questi risultati rappresentano la base di partenza per ulteriori indagini dedicate ad identificare nuovi composti antimicrobici naturali che potrebbero essere utilizzati in acquacoltura per il controllo delle infezioni batteriche.

Parole chiave: piante medicinali, estratti etanolici, batteri patogeni per i pesci, attività antibatterica.

2.1 Introduction

Currently, there is a considerable and growing interest in the screening of extracts and essential oils derived from medicinal plants for their antibacterial activity against fish pathogens, in order to exploit new antimicrobial compounds of natural origin that could be employed in the control of bacterial diseases. In fact, the use of antibiotics in aquaculture needs to be drastically reduced, due to the emergence of multidrug-resistant bacterial strains and the accumulation of residues in environment and aquaculture products (FAO/WHO/OIE 2006). For thousands of years, traditional medicines based on plants and plant extracts have been extensively utilized in humans and animals as remedies to control bacterial, viral and fungal diseases. A variety of plant species are known to synthesize many bioactive secondary metabolites with antimicrobial and biological properties, like alkaloids, terpenoids (triterpenes and steroid saponins), phenolic compounds, glycosides, flavonoids, tannins and polysaccharides (Lovkova *et al.*, 2001). In addition, phytomedicines are eco-friendly, inexpensive, easily prepared and mitigate many of the side-effects that are often associated with synthetic antibiotics (Punitha *et al.*, 2008).

Regarding the antibacterial activity of plant extracts/essential oils, the researchers have described several mechanisms of action, including cell membrane damage resulting in increased permeability, changes in intracellular pH and membrane potential, dissipation of cellular components, decrease in the cytoplasmic ATP concentration, which together induce bacterial death. Secondary effects that may be involved seem to be the inhibition of enzymes, loss of turgor pressure, alterations in macromolecules synthesis, and other cellular processes (Burt, 2004; Sánchez *et al.*, 2010).

In recent years, the ability of some herbs and seaweeds extracts to inhibit the growth of bacterial fish pathogens has been widely documented (Muniruzzaman & Chowdhury 2004; Abutbul *et al.*, 2005; Choudhury *et al.*, 2005; Bansemir *et al.*, 2006; Bhuvaneswari & Balasundaram, 2006; Palavesam *et al.*, 2006; Castro *et al.*, 2008; Dubber & Harder 2008; Najiah *et al.*, 2008; Punitha *et al.*, 2008; Turker *et al.*, 2009a; 2009b; Rattanachaikunsopon & Phumkhachorn 2009a; 2009b; Alsaid *et al.*, 2010; Arivuselvan *et al.*, 2011; Najiah *et al.*, 2011; Haniffa & Kavitha, 2012; Haniffa & Shanthi, 2012). In particular, plant alcoholic extracts were found to be more active than plant aqueous extracts, since alcoholic solvents provide a higher efficiency in extracting the active compounds responsible for antimicrobial effects, compared to water-based methods (Borisutpeth *et al.*, 2005; Turker *et al.*, 2009a; Rattanachaikunsopon & Phumkhachorn 2009b; Ponnusamy *et al.*, 2010).

The present study aimed to investigate the *in vitro* antibacterial activity of ethanolic extracts obtained from 15 medicinal plants against five relevant bacterial fish pathogens (*Listonella anguillarum* serotypes O1 and O2, *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*

and *Lactococcus garvieae*). These bacterial species are frequently responsible of severe disease outbreaks in a variety of economically important fish species (Toranzo *et al.*, 2005). *In vitro* and *in vivo* studies concerning the antimicrobial potential of herbal extracts against these pathogens are still limited (Abutbul *et al.*, 2005; Bansemir *et al.*, 2006; Turker *et al.*, 2009a; 2009b). The extracts studied in this research were selected based on their known antimicrobial and anti-inflammatory properties (Campanini, 2012).

2.2 Materials and Methods

2.2.1 Chemicals

Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA), Brain Heart Infusion (BHI) and Muller Hinton Agar (MHA) were purchased from Biomerieux Italia S.p.a. (FI, Italy). NaCl and oxytetracycline were obtained from Sigma-Aldrich (St. Louis, MO, USA). Throughout the study, water was collected from a Milli-Q system from Millipore (Bedford, MA, USA).

2.2.2 Plant extracts

The 15 ethanolic extracts which were selected for the present study derived from the following medicinal plants: yarrow (*Achillea millefolium*); arnica (*Arnica montana*); marigold (*Calendula officinalis*); Icelandic lichen (*Cetraria islandica*); horsetail (*Equisetum arvense*); grindelia (*Grindelia robusta*); lavender (*Lavandula officinalis*); lemon balm (*Melissa officinalis*); basil (*Ocimum basilicum*); oregano (*Origanum vulgare*); java tea (*Orthosiphon stamineus*); rosemary (*Rosmarinus officinalis*); common sage (*Salvia officinalis*); thyme (*Thymus vulgaris*); and red bilberry (*Vaccinium vitis-idaea*) (Table 1). They were produced and provided by a commercial pharmacotherapeutic institute (EPO S.r.l.-Piante officinali, Milano, Italy). Extracts were sterilized through a 0.22-μm filter prior to be used for the antimicrobial assays.

2.2.3 Bacterial strains and growth media

The biological assays were carried out on a panel of bacterial fish pathogens isolated during spontaneous outbreaks of disease, belonging to the collection of the Division of Veterinary Pathology (DIAL) of Udine University or kindly provided by Dr. Manfrin (IZSVe, Italy). The bacteria included the Gram negative *Listonella* (*Vibrio*) *anguillarum* serotype O1 (strain 77/I03), *Listonella anguillarum* serotype O2 (strain 32/ITT), *Yersinia ruckeri* (strain B05/18),

Photobacterium damselae subsp. *piscicida* (strain B07/8), and the Gram positive *Lactococcus garvieae* (strain O41). The bacterial strains were identified by standard microbiological methods and stored at -80 °C. After thawing, they were grown at 22 ± 2 °C in appropriate nutrient broth medium (*L. anguillarum* and *P. damselae* subsp. *piscicida* in TSB amended with 1.5% NaCl, *Y. ruckeri* in TSB, *L. garvieae* in BHI). Exponentially-growing bacteria were collected by centrifugation at 2000 *x g* for 30 min and resuspended in sterile phosphate buffer saline pH 7.2 (PBS). The inoculum size of each test strain was standardized prior to each antimicrobial test by adjusting spectrophotometrically the optical density (O.D. at 610 nm) of the bacterial suspension to 1.0, then confirmed by spreading serial 10-fold dilutions of each suspension onto TSA duplicate plates and counting the number of colony-forming units (CFU) following incubation at 22 ± 2 °C. The reference concentrations were $1x10^9$ CFU/ml for *L. anguillarum* serotypes O1 and O2, $3x10^9$ CFU/ml for *Y. ruckeri*, $8x10^8$ CFU/ml for *P. damselae* susbsp. *piscicida* and $3x10^9$ CFU/ml for *L. garvieae*.

2.2.4 Screening for antibacterial activity

The susceptibility of the tested strains to plant ethanolic extracts was screened by using the agar disc diffusion assay (Alsaid *et al.*, 2010; partially modified). Each bacterial suspension was adjusted to a concentration of $1x10^8$ CFU/ml in sterile PBS, then inoculated on the surface of MHA plates and spread with a sterilized glass L rod. Subsequently, sterile filter paper discs (6 mm diameter, BioMerieux) were impregnated with 150 µl of each extract to give a final concentration of 15 mg/disc, dried in a biological hood for 1 h to allow solvent evaporation, then placed aseptically onto the surface of inoculated MHA test plates. Discs impregnated with ethanol 55% were prepared as negative controls, whereas discs embedded with oxytetracycline (30 µg/disc) were used as positive controls. The plates were incubated at 22 ± 2 °C. Two replicates in each plate and two plates for each extract were tested for each strain. The diameters (mm) of the inhibition zones around the discs were measured to an accuracy of 0.5 mm after 24-48 h of incubation. The antibacterial activity of plant extracts (mean ± standard deviation [SD]) was interpreted as proposed by Bansemir *et al.* (2006): inhibition zones > 15 mm were categorized as strong activity, from 8 to 15 mm as moderate activity and from 1 to 8 mm as weak activity.

2.2.5 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of plant extracts against the bacterial strains was determined using the broth micro-dilution method in microtiter plate (Manfrin *et al.*, 2008).

Briefly, the bacterial suspension was diluted in broth medium to obtain a concentration of $5x10^5$ CFU/ml. Duplicate two-fold serial dilutions of each extract in culture medium (100 µl/well) were performed in 96-U bottom wells microplates, then 50 µl/well of bacterial suspension were added. The final concentrations of extracts ranged from 33.6 to 0.02 mg/ml. Wells containing twofold serial dilutions of 55% ethanol were used to exclude the effect of the solvent on tested organisms, while twofold serial dilutions of oxytetracycline, ranging from 667 to 0.03 µg/ml, were used to compare the effectiveness of plant extracts with that of a reference antibiotic. Controls (without tested compounds or without tested bacteria) were also included in each plate. After 24 h incubation at 22 ± 2 °C, bacterial growth was examined by observing the turbidity of the wells. Wells that did not show any turbidity were interpreted as negative (absence of growth) and the presence of turbidity was interpreted as positive. The minimum inhibitory concentration (MIC) was defined as the lowest concentration (mg/ml) of each extract dilution series that completely inhibited the visible growth of pathogen, when compared to the control (Konè *et al.*, 2004). All tests were performed in a minimum of two independent assays.

2.2.6 Mode of action and minimum bactericidal concentration

To examine whether the studied plant extracts exhibited bacteriostatic or bactericidal effects on tested fish pathogens at a final concentration equal to or greater than the MIC value, sub-cultures of the bacteria pre-exposed to extracts were prepared. Ten µl of medium from wells with no visible growth were transferred to sterile culture broth (240 µl/well) in 96-wells microtiter plates and incubated for 24 h at 22 ± 2 °C. Positive and negative growth controls were included. The recovery of bacteria (turbidity) in fresh medium indicated that plant extracts had a bacteriostatic mode of action against the tested fish pathogens whereas the absence of visible growth (no turbidity) indicated that the extracts had a bactericidal mode of action. The minimum bactericidal concentration (MBC) was defined as the lowest concentration (mg/ml) of each extract associated with no visible bacterial growth on subcultures.

2.2.7 Statistical analysis

One-way ANOVA and *post hoc* Duncan test were used in order to evaluate the differences of the inhibition zones (mean ± SD) among plant extracts and pathogens. All the statistical analysis were performed using the software SPSS Statistic 20. Statistical significance was designated as a P value < 0.05

Table 1 Chemical and physical characteristics of ethanolic plant extracts used in this study

Botanical name	Botanical family	Plant parts used	Ethanol content	Density (g/ml)	Physical characteristics	pH	Secondary metabolites*
Achillea millefolium	Compositae	inflorescence	18.0-22.0%	1.00-1.03	clear liquid, brown, aromatic	4.5-5.5	essential oil (terpenes, camazulene), flavonoids (apigenin, ..), caffeic and salicylic acids, tannins
Arnica montana	Compositae	flowers	28.0-32.0%	0.99-1.01	clear liquid, brown, aromatic	4.5-5.5	sesquiterpenes (elenalin), essential oil, triterpenes, flavonoids (isoquercitin, astragalin, ..), coumarins
Calendula officinalis	Compositae	flowers	18.0-22.0%	1.01-1.03	clear liquid, brown, balsamic	4.5-6.0	calendulosides, flavonoids, polysaccharides, carotenoids (lycopene, celendulin, lutein, xanthophylls), essential oil (terpenes, phytosterols, mucilages)
Cetraria islandica	Parmeliaceae	thallus	18.0-22.0%	0.99-1.02	clear liquid, brown, distinctive odour	3.5-4.5	polysaccharides (lichenin and isolichenin), lichenic acids (including usnic acid)
Equisetum arvense	Equisetaceae	whole plant	18.0-22.0%	1.00-1.02	clear liquid, brown	4.5-6.0	mineral components (salicylic acid), flavonoids, alkaloids
Grindelia robusta	Compositae	inflorescence	18.0-22.0%	1.00-1.02	clear liquid, brown, balsamic	4.5-5.5	diterpenic acids (grindelic acid), flavonoids, triterpenoid saponins, essential oil
Lavandula officinalis	Labiatae	flowers	47.0-52.0%	0.92-0.96	clear liquid, brown, aromatic	5.0-6.0	essential oil (linalyl acetate), tannins, coumarins, flavonoids, phytosterols
Melissa officinalis	Labiatae	leaves	28.0-32.0%	0.99-1.01	clear liquid, brown, aromatic	4.0-6.0	rosmarinic acid, essential oil (citral, citronellal, β-caryophyllen), caffeic acid and chlorogenic acid derivatives
Ocimum basilicum	Labiatae	flowering plant	28.0-32.0%	1.00-1.02	clear liquid, brown, aromatic	5.0-6.0	essential oil (linalool, estragol, camphor, eugenol, ocimene, cineol, sesquiterpenes), tannins, favonoids, caffeic acid, esculoside
Origanum vulgare	Labiatae	inflorescence	28.0-32.0%	0.99-1.02	clear liquid, brown, aromatic	4.5-5.0	carvacrol, thymol, γ-terpinene, p-cymene, limonene, linolool, borneol
Orthosiphon stamineus	Labiatae	leaves	18.0-22.0%	1.00-1.02	clear liquid, green-brown, aromatic	5.0-6.5	essential oil (sesquiterpenes), flavones, triterpenoid saponins, vitamines, organic salts (potassium)
Rosmarinus officinalis	Labiatae	leaves	28.0-32.0%	0.99-1.02	clear liquid, brown, aromatic	4.5-5.5	essential oil (eucalyptol, α-pinene, camphor, borneol), flavonoids, rosmarinic acid, terpenes
Salvia officinalis	Labiatae	leaves	28.0-32.0%	0.99-1.01	opalescent liquid, brown, aromatic	4.8-6,5	essential oil (thujone, monoterpenes, sesquiterpenes), tannins, bitter substances, flavonoids
Thymus vulgaris	Labiatae	leaves	18.0-22.0%	1.00-1.03	clear liquid, brown, aromatic	4.5-6.0	essential oil (thymol, carvacrol, p-cimol, terpinene), tannins, flavonoids, triterpenes
Vaccinium vitis-idaea	Ericaceae	leaves	18.0-22.0%	0.99-1.02	clear liquid, brown, distinctive odour	4.5-5.0	phenolic glycosides (arbutin, hydroquinone), tannins, flavonoids (iperoside,avicularin, isoquercitrin), terpenic acids (ursolic and oleanolic acids), organic acids, mineral salts

* percentage unknown.

2.3 Results

2.3.1 Zone of inhibition

The results of the antimicrobial screening assays are presented in Table 2. There were no inhibition zones in the negative controls (ethanol) while the positive controls (reference antibiotic) showed strong antibacterial activity against *L. anguillarum* O1 (20.0 ± 1.4 mm), *L. anguillarum* O2 (25.0 ± 1.4 mm), *Y. ruckeri* (20.8 ± 1.1 mm), *L. garvieae* (20.0 ± 0.0 mm) and weak antibacterial activity against *P. damselae* subsp. *piscicida* (1.5 ± 0.7 mm).

Significant differences in the antimicrobial potential of plant extracts, depending on herbal source and on bacterial strain, were detected. The extracts of *A. montana*, *C. officinalis*, *G. robusta*, *L. officinalis*, *M. officinalis*, *O. basilicum*, *O. vulgare*, *R. officinalis*, *S. officinalis* and *V. vitis-idaea* exhibited a broad spectrum of inhibitory effects while the other extracts displayed limited antimicrobial activity.

P. damselae subsp. *piscicida* was the most susceptible species, being inhibited by the plant extracts (with the exception of *C. islandica*) with the largest zones of inhibition. *L. officinalis* and *O. vulgare* showed a moderate activity against this pathogen (11.1 ± 2.5 mm and 8.8 ± 1.8 mm, respectively). *E. arvense*, *O. stamineus*, *T. vulgaris*, *G. robusta*, *A. millefolium*, *C. officinalis*, *O. basilicum*, *A. montana*, *S. officinalis*, *R. officinalis*, *V. vitis-idaea*, and *M. officinalis* displayed lower antibacterial activities with inhibition zones between 0.5 mm and 7.8 mm.

The growth of *L. anguillarum* serotype O1 was inhibited mainly by the extracts of *O. stamineus*, *G. robusta*, *C. officinalis*, *A. montana*, *S. officinalis*, *O. basilicum*, *V. vitis-idaea*, *O. vulgare*, *M. officinalis*, *R. officinalis*, and *L. officinalis*, which determined inhibition zones between 1.4 mm and 6.5 mm (weak activity). The extracts of *E. arvense*, *A. millefolium*, *C. islandica*, and *T. vulgaris* showed inhibition zones between 0.5 mm and 0.8 mm (weak activity). Similar antimicrobial activities (0.5-6.8 mm) of plant extracts were detected against *L. anguillarum* serotype O2.

Only five extracts (*S. officinalis*, *C. officinalis*, *O. basilicum*, *O. vulgare*, and *L. officinalis*) exhibited inhibitory effects (1.0-3.8 mm) against *Y. ruckeri* that are worth mentioning.

The growth of *L. garvieae* was weakly inhibited by the plant extracts. Inhibition zones between 1.0 mm and 6.8 mm were measured for *C. officinalis*, *A. montana*, *O. basilicum*, *V. vitis-idaea*, *G. robusta*, *M. officinalis*, *L. officinalis*, *S. officinalis*, *R. officinalis*, and *O. vulgare* and inhibition zones between 0.3 mm and 0.6 mm were measured for *O. stamineus*, *E. arvense*, *C. islandica*, and *A. millefolium*.

Table 2 Antimicrobial activity of the plant extracts (15 mg per disc) as measured by agar diffusion assay. Different lowercase letters indicate differences between plant extracts within each pathogen and different capital letters indicate differences between pathogens within each plant extract ($P<0.05$).[1]

		Mean diameter of the inhibition zones[2] (mm±SD)				
		P. damselae subsp. *piscicida*	*L. anguillarum O1*	*L. anguillarum O2*	*Y. ruckeri*	*L. garvieae*
Plant species (15 mg per disc)	Botanical family					
Achillea millefolium	Compositae	2.9 ± 0.3 $^{fg\ A}$	0.5 ± 0.6 $^{g\ B}$	0.6 ± 0.5 $^{hi\ B}$	0.3 ± 0.3 $^{ef\ B}$	0.6 ± 0.8 $^{ghi\ B}$
Arnica montana	Compositae	4.3 ± 0.5 $^{ef\ A}$	2.5 ± 0.4 $^{de\ B}$	2.3 ± 1.0 $^{f\ B}$	0.3 ± 0.3 $^{ef\ C}$	1.6 ± 0.8 $^{efgh\ A}$
Calendula officinalis	Compositae	4.0 ± 0.8 $^{ef\ A}$	2.3 ± 0.4 $^{def\ AB}$	2.0 ± 0.0 $^{fgh\ AB}$	1.3 ± 1.8 $^{cd\ B}$	1.0 ± 0.0 $^{fghi\ B}$
Cetraria islandica	Parmeliaceae	0.0 ± 0.0 $^{h\ B}$	0.8 ± 0.3 $^{fg\ A}$	0.5 ± 0.6 $^{i\ AB}$	0.1 ± 0.3 $^{ef\ AB}$	0.5 ± 0.7 $^{hi\ AB}$
Equisetum arvense	Equisetaceae	0.5 ± 1.0 $^{h\ A}$	0.5 ± 0.4 $^{g\ A}$	0.9 ± 0.8 $^{ghi\ A}$	0.0 ± 0.0 $^{f\ A}$	0.5 ± 0.6 $^{hi\ A}$
Grindelia robusta	Compositae	2.6 ± 0.5 $^{fg\ A}$	1.4 ± 0.8 $^{efg\ AB}$	2.1 ± 0.3 $^{fg\ A}$	0.3 ± 0.3 $^{ef\ B}$	2.1 ± 1.4 $^{ef\ A}$
Lavandula officinalis	Labiatae	11.1 ± 2.5 $^{a\ A}$	6.6 ± 1.3 $^{b\ B}$	6.8 ± 1.0 $^{b\ B}$	3.8 ± 0.5 $^{b\ C}$	3.5 ± 1.1 $^{cd\ C}$
Melissa officinalis	Labiatae	7.8 ± 2.1 $^{bc\ A}$	6.1 ± 2.1 $^{b\ AB}$	4.5 ± 1.0 $^{de\ BC}$	0.4 ± 0.3 $^{ef\ D}$	2.9 ± 0.3 $^{de\ C}$
Ocimum basilicum	Labiatae	4.0 ± 0.9 $^{ef\ A}$	3.9 ± 0.3 $^{cd\ A}$	3.9 ± 0.3 $^{de\ A}$	1.5 ± 0.8 $^{c\ B}$	1.9 ± 0.6 $^{efg\ B}$
Origanum vulgare	Labiatae	8.8 ± 1.8 $^{b\ A}$	5.0 ± 0.8 $^{bc\ B}$	6.4 ± 1.4 $^{bc\ B}$	3.1 ± 0.9 $^{b\ C}$	6.8 ± 0.5 $^{b\ B}$
Orthosiphon stamineus	Labiatae	1.1 ± 0.5 $^{gh\ A}$	1.4 ± 1.3 $^{efg\ A}$	0.6 ± 0.8 $^{hi\ A}$	0.3 ± 0.3 $^{ef\ A}$	0.3 ± 0.3 $^{i\ A}$
Rosmarinus officinalis	Labiatae	5.8 ± 1.0 $^{de\ A}$	6.3 ± 0.6 $^{b\ A}$	5.1 ± 0.6 $^{cd\ AB}$	0.3 ± 0.3 $^{ef\ C}$	4.3 ± 1.2 $^{c\ B}$
Salvia officinalis	Labiatae	5.6 ± 0.6 $^{de\ A}$	3.5 ± 1.5 $^{cd\ AB}$	3.3 ± 0.3 $^{ef\ B}$	1.0 ± 0.0 $^{cde\ C}$	4.3 ± 0.3 $^{c\ AB}$
Thymus vulgaris	Labiatae	1.9 ± 0.5 $^{gh\ A}$	0.8 ± 0.3 $^{fg\ B}$	0.6 ± 0.5 $^{hi\ B}$	0.5 ± 0.0 $^{def\ B}$	0.0 ± 0.0 $^{i\ C}$
Vaccinium vitis-idaea	Ericaceae	6.3 ± 1.9 $^{cd\ A}$	5.0 ± 0.8 $^{bc\ AB}$	3.6 ± 1.1 $^{e\ BC}$	0.0 ± 0.0 $^{f\ D}$	2.0 ± 0.9 $^{ef\ C}$
Controls						
Oxytetracycline 30 µg per disc		1.5 ± 0.7 $^{gh\ C}$	20.0 ± 1.4 $^{a\ B}$	25.0 ± 1.4 $^{a\ A}$	20.8 ± 1.1 $^{a\ B}$	20.0 ± 0.0 $^{a\ B}$
ethanol 55%		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

SD = standard deviation.

[1]Inhibition zones > 15 mm were declared as strong, from 8 to 15 mm as moderate and from 1 to 8 as weak activities.

[2]Measurement of the inhibition zones did not include the diameter of 6 mm paper disc.

2.3.2 Minimum inhibitory concentration

The MIC values of plant extracts against *P. damselae* subsp. *piscicida* were globally lower than those observed for the other bacterial species (Table 3). *L. officinalis* and *O. vulgare* extracts inhibited this pathogen with a MIC equal to 1.1 mg/ml, *M. officinalis*, *O. basilicum*, *R. officinalis*, *S. officinalis*, and *V. vitis-idaea* extracts showed a MIC of 4.2 mg/ml, *A. millefolium*, *A. montana*, and *T. vulgaris* extracts displayed a MIC of 8.4 mg/ml. The MIC value established for *C. officinalis*, *G. robusta*, and *O. stamineus* extracts was 16.8 mg/ml while *C. islandica* and *E. arvense* extracts did not exhibit antibacterial properties against this pathogen within the range of tested concentrations. The MIC value of oxytetracycline against *P. damselae* subsp. *piscicida* was 21 µg/ml.

Almost all the tested plant extracts demonstrated inhibitory effects against *L. anguillarum* serotypes O1 and O2 in the broth micro-dilution method, with the exception of *E. arvense* extract, that did not inhibit the growth of *L. anguillarum* serotype O1 (Tables 4, 5). The extracts of *L. officinalis* and *O. vulgare* displayed the greatest antimicrobial activity (MIC=2.1 mg/ml). The MIC values of *M. officinalis*, *S. officinalis*, *O. basilicum*, *R. officinalis*, *O. stamineus*, and *V. vitis-idaea* extracts ranged from 4.2 mg/ml to 8.4 mg/ml. The other extracts showed lower inhibitory activities, with MICs ≥ 16.8 mg/ml. In general, the growth of the serotype O2 was

inhibited by most of the extracts at concentrations lower than those requested for the serotype O1. The MIC value of oxytetracycline was 0.13 µg/ml against *L. anguillarum* serotype O1 and 0.5 µg/ml against *L. anguillarum* serotype O2.

Only eight plant extracts showed inhibitory activities against *Y. ruckeri* in microplate (Table 6). The MIC values were 8.4 mg/ml for *L. officinalis* and *O. vulgare* extracts, 16.8 mg/ml for *M. officinalis*, *O. basilicum*, *R. officinalis*, and *S. officinalis* extracts, 33.6 mg/ml for *C. officinalis* and *V. vitis-idaea* extracts. All the other extracts were not active. The MIC value of oxytetracycline against *Y. ruckeri* was 1.0 µg/ml.

A weak inhibitory action of plant extracts was also observed against *L. garvieae* (Table 7). The MICs were 4.2 mg/ml for *L. officinalis*, *O. vulgare*, and *V. vitis-idaea* extracts, 8.4 mg/ml for *M. officinalis*, *R. officinalis*, and *S. officinalis* extracts, 16.8 mg/ml for *O. basilicum* extract, 33.6 mg/ml for *O. stamineus* extract, whereas the other plant extracts did not produce any inhibitory effect on bacterial growth. The MIC value of oxytetracycline against *L. garvieae* was 1.0 µg/ml.

2.3.3 Mode of action and minimum bactericidal concentration

Among the 13 extracts active against *P. damselae* subsp. *piscicida*, the extracts of *A. millefolium*, *C. officinalis*, *G. robusta*, *M. officinalis*, *O. basilicum*, *O. stamineus*, and *S. officinalis* showed a bactericidal effect at concentrations ≥ MIC (MIC=MBC) while the other six extracts displayed MICs with bacteriostatic effect. The MBC values were 2.1 mg/ml for *L. officinalis* and *O. vulgare* extracts (MBC/MIC=2), 8.4 mg/ml for *R. officinalis* and *V. vitis-idaea* extracts (MBC/MIC=2), 16.8 mg/ml for *A. montana* and *T. vulgaris* extracts (MBC/MIC=2) (Table 3).

All the 14 extracts active on *L. anguillarum* serotype O1 exhibited a bactericidal activity at a concentration ≥ MIC (MIC=MBC) (Table 4). *A. millefolium*, *A. montana*, *C. officinalis*, *G. robusta*, *L. officinalis*, *M. officinalis*, *O. vulgare*, *O. stamineus*, *R. officinalis*, *S. officinalis*, *T. vulgaris*, and *V. vitis-idaea* extracts demonstrated a similar bacterial killing action (MIC=MBC) also on *L. anguillarum* serotype O2, whereas the extracts of *C. islandica*, *E. arvense*, and *O. basilicum* exhibited against this pathogen only a growth inhibitory effect at their MIC. The MBC for *C. islandica* and *O. basilicum* extracts was 33.6 mg/ml (MBC/MIC=2 and MBC/MIC=4, respectively) while the MBC for *E. arvense* extract was not detectable (Table 5).

Among the eight extracts active against *Y. ruckeri*, the extracts of *O. vulgare*, *M. officinalis*, and *R. officinalis* showed a bactericidal effect at concentrations ≥ MIC (MIC=MBC) while the other extracts demonstrated a bacteriostatic action at MICs. The MBCs were equal to 16.8 mg/ml for *L. officinalis* extract (MBC/MIC=2), not detectable in the range of concentrations tested for the extracts of *C. officinalis*, *V. vitis-idaea* (MBC/MIC>1), *O. basilicum*, and *S. officinalis* (MBC/MIC>2) (Table 6).

Table 3 MIC and MBC values of plant extracts on *P. damselae* subsp. *piscicida*.

Plant species	Botanical family	Range (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Achillea millefolium	Compositae	33.6-0.02	8.4	8.4	1
Arnica montana	Compositae	33.6-0.02	8.4	16.8	2
Calendula officinalis	Compositae	33.6-0.02	16.8	16.8	1
Cetraria islandica	Parmeliaceae	33.6-0.02	ud	ud	ud
Equisetum arvense	Equisetaceae	33.6-0.02	ud	ud	ud
Grindelia robusta	Compositae	33.6-0.02	16.8	16.8	1
Lavandula officinalis	Labiatae	33.6-0.02	1.1	2.1	2
Melissa officinalis	Labiatae	33.6-0.02	4.2	4.2	1
Ocimum basilicum	Labiatae	33.6-0.02	4.2	4.2	1
Origanum vulgare	Labiatae	33.6-0.02	1.1	2.1	2
Orthosiphon stamineus	Labiatae	33.6-0.02	16.8	16.8	1
Rosmarinus officinalis	Labiatae	33.6-0.02	4.2	8.4	2
Salvia officinalis	Labiatae	33.6-0.02	4.2	4.2	1
Thymus vulgaris	Labiatae	33.6-0.02	8.4	16.8	2
Vaccinium vitis-idaea	Ericaceae	33.6-0.02	4.2	8.4	2
Oxytetracycline		667-0.03 µg/ml	21 µg/ml		

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

Table 4 MIC and MBC values of plant extracts on *L. anguillarum* O1.

Plant species	Botanical family	Range (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Achillea millefolium	Compositae	33.6-0.02	33.6	33.6	1
Arnica montana	Compositae	33.6-0.02	16.8	16.8	1
Calendula officinalis	Compositae	33.6-0.02	33.6	33.6	1
Cetraria islandica	Parmeliaceae	33.6-0.02	33.6	33.6	1
Equisetum arvense	Equisetaceae	33.6-0.02	ud	ud	ud
Grindelia robusta	Compositae	33.6-0.02	33.6	33.6	1
Lavandula officinalis	Labiatae	33.6-0.02	2.1	2.1	1
Melissa officinalis	Labiatae	33.6-0.02	4.2	4.2	1
Ocimum basilicum	Labiatae	33.6-0.02	8.4	8.4	1
Origanum vulgare	Labiatae	33.6-0.02	2.1	2.1	1
Orthosiphon stamineus	Labiatae	33.6-0.02	8.4	8.4	1
Rosmarinus officinalis	Labiatae	33.6-0.02	8.4	8.4	1
Salvia officinalis	Labiatae	33.6-0.02	4.2	4.2	1
Thymus vulgaris	Labiatae	33.6-0.02	33.6	33.6	1
Vaccinium vitis-idaea	Ericaceae	33.6-0.02	8.4	8.4	1
Oxytetracycline		667-0.03 µg/ml	0.13 µg/ml		

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

Table 5 MIC and MBC values of plant extracts on *L. anguillarum* O2.

Plant species	Botanical family	Range (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Achillea millefolium	Compositae	33.6-0.02	16.8	16.8	1
Arnica montana	Compositae	33.6-0.02	16.8	16.8	1
Calendula officinalis	Compositae	33.6-0.02	33.6	33.6	1
Cetraria islandica	Parmeliaceae	33.6-0.02	16.8	33.6	2
Equisetum arvense	Equisetaceae	33.6-0.02	33.6	ud	>1
Grindelia robusta	Compositae	33.6-0.02	16.8	16.8	1
Lavandula officinalis	Labiatae	33.6-0.02	2.1	2.1	1
Melissa officinalis	Labiatae	33.6-0.02	4.2	4.2	1
Ocimum basilicum	Labiatae	33.6-0.02	8.4	33.6	4
Origanum vulgare	Labiatae	33.6-0.02	2.1	2.1	1
Orthosiphon stamineus	Labiatae	33.6-0.02	8.4	8.4	1
Rosmarinus officinalis	Labiatae	33.6-0.02	8.4	8.4	1
Salvia officinalis	Labiatae	33.6-0.02	8.4	8.4	1
Thymus vulgaris	Labiatae	33.6-0.02	16.8	16.8	1
Vaccinium vitis-idaea	Ericaceae	33.6-0.02	8.4	8.4	1
Oxytetracycline		667-0.03 µg/ml	0.5 µg/ml		

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

Table 6 MIC and MBC values of plant extracts on *Y. ruckeri*.

Plant species	Botanical family	Range (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Achillea millefolium	Compositae	33.6-0.02	ud	ud	ud
Arnica montana	Compositae	33.6-0.02	ud	ud	ud
Calendula officinalis	Compositae	33.6-0.02	33.6	ud	>1
Cetraria islandica	Parmeliaceae	33.6-0.02	ud	ud	ud
Equisetum arvense	Equisetaceae	33.6-0.02	ud	ud	ud
Grindelia robusta	Compositae	33.6-0.02	ud	ud	ud
Lavandula officinalis	Labiatae	33.6-0.02	8.4	16.8	2
Melissa officinalis	Labiatae	33.6-0.02	16.8	16.8	1
Ocimum basilicum	Labiatae	33.6-0.02	16.8	ud	>2
Origanum vulgare	Labiatae	33.6-0.02	8.4	8.4	1
Orthosiphon stamineus	Labiatae	33.6-0.02	ud	ud	ud
Rosmarinus officinalis	Labiatae	33.6-0.02	16.8	16.8	1
Salvia officinalis	Labiatae	33.6-0.02	16.8	ud	>2
Thymus vulgaris	Labiatae	33.6-0.02	ud	ud	ud
Vaccinium vitis-idaea	Ericaceae	33.6-0.02	33.6	ud	>1
Oxytetracycline		667-0.03 µg/ml	1 µg/ml		

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

All the eight extracts active against *L. garvieae* demonstrated a bacteriostatic mode of action at concentration equal to the MIC. The MBCs were 8.4 mg/ml for *L. officinalis* extract (MBC/MIC=2), 33.6 mg/ml for the extracts of *O. vulgare* (MBC/MIC=8), *M. officinalis*, and *S. officinalis* (MBC/MIC=4). The MBC values for the extracts of *O. basilicum* (MBC/MIC>2), *O. stamineus* (MBC/MIC>1), *R. officinalis* (MBC/MIC>4), and *V. vitis-idaea* (MBC/MIC>8) were not detectable (Table 7).

Table 7 MIC and MBC values of plant extracts on *L. garvieae*.

Plant species	Botanical family	Range (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Achillea millefolium	Compositae	33.6-0.02	ud	ud	ud
Arnica montana	Compositae	33.6-0.02	ud	ud	ud
Calendula officinalis	Compositae	33.6-0.02	ud	ud	ud
Cetraria islandica	Parmeliaceae	33.6-0.02	ud	ud	ud
Equisetum arvense	Equisetaceae	33.6-0.02	ud	ud	ud
Grindelia robusta	Compositae	33.6-0.02	ud	ud	ud
Lavandula officinalis	Labiatae	33.6-0.02	4.2	8.4	2
Melissa officinalis	Labiatae	33.6-0.02	8.4	33.6	4
Ocimum basilicum	Labiatae	33.6-0.02	16.8	ud	>2
Origanum vulgare	Labiatae	33.6-0.02	4.2	33.6	8
Orthosiphon stamineus	Labiatae	33.6-0.02	33.6	ud	>1
Rosmarinus officinalis	Labiatae	33.6-0.02	8.4	ud	>4
Salvia officinalis	Labiatae	33.6-0.02	8.4	33.6	4
Thymus vulgaris	Labiatae	33.6-0.02	ud	ud	ud
Vaccinium vitis-idaea	Ericaceae	33.6-0.02	4.2	ud	>8
Oxytetracycline		667-0.03 µg/ml	1 µg/ml		

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

2.4 Discussion

In this research we studied the *in vitro* antibacterial activity of commercial ethanolic extracts derived from 15 medicinal plants against the fish pathogens *L. anguillarum* (serotypes O1 and O2), *Y. ruckeri*, *P. damselae* subsp. *piscicida*, and *L. garvieae*, with the aim to investigate their possible use in aquaculture in the control of bacterial infections. To the best of our knowledge, this is the first report on the antibacterial properties of these medicinal plants on a wide range of fish bacterial pathogens.

The results obtained in this study revealed some discrepancies between the antibacterial activity of plant extracts measured by using the agar disc diffusion assay or by using the broth micro-dilution method in microtiter plate. This evidence is probably related to the different diffusion ability of the extracts in the solid culture medium, which can mainly influence the diameter of the inhibition zones, as suggested by Niculae *et al.* (2009).

We found significant differences in the antimicrobial potential of the investigated plant

extracts, which were dependent on herbal species and on bacterial strain. The extracts of *L. officinalis*, *M. officinalis*, *O. basilicum*, *O. vulgare*, *R. officinalis*, and *S. officinalis*, which belong to the Labiatae botanical family, were the most effective. These extracts exhibited a broad spectrum of inhibitory effects both on Gram negative (*L. anguillarum* serotypes O1 and O2, *Y. ruckeri*, and *P. damselae* subsp. *piscicida*) and Gram positive bacteria (*L. garvieae*), displaying the largest zones of growth inhibition and the lowest MIC and MBC values among the tested strains. On the contrary, although belonging to the Labiatae botanical family, *T. vulgaris* and *O. stamineus* displayed a limited antibacterial activity against the tested pathogens.

It is well documented that Labiatae are one of the most diverse and widespread botanical families in terms of ethnomedicine. Several plant species belonging to this family possess various biological activities and antimicrobial properties against Gram negative and Gram positive bacteria, fungi, parasites, and virus (Naghibi *et al.*, 2005), mainly based on their content of phenolic derivatives, such as carvacrol and thymol, and other minor compounds, including γ-terpinene, p-cymene, tannins, flavonoids, triterpenes, camphor, and limonene (Burt, 2004; Naghibi *et al.*, 2005). The chemical analysis performed by the manufacturer confirmed that the commercial extracts used in this study contain these active compounds; however there is no specific indication about their amounts. Changes in the composition among different batches of plant products (essential oils or extracts) and consequently in their antimicrobial activity are very common and are due to many factors, such as plant part, season or site of collection, nature of soil, water availability, temperature and day length, postharvest handling and extraction processes (Croteau *et al.*, 2000). The limited antibacterial properties displayed by the extracts of thyme and java tea may be due to these aspects.

Even the extract of red bilberry *V. vitis idaea* (Ericaceae) displayed an evident antibacterial activity on *L. anguillarum* serotypes, *P. damselae* subsp. *piscicida*, and *L. garvieae*, while it was less active on *Y. ruckeri*. The plant species *A. millefolium*, *A. montana*, *C. officinalis*, *C. islandica*, *E. arvense*, and *G. robusta* showed lower or negligible effects.

Gram negative bacteria commonly are less susceptible to the inhibitory effects of plant extracts/essential oils compared to Gram positive bacteria, since they possess an outer membrane surrounding the cell wall, which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering (Burt, 2004; Turker *et al.*, 2009b). In our experiments, *Y. ruckeri* was the most resistant pathogen and *P. damselae* subsp. *piscicida* was the most susceptible, but *L. garvieae* appeared to be less sensible to the investigated plant extracts compared to *P. damselae* subsp. *piscicida* and *L. anguillarum* serotypes. The observed resistance of this *L. garvieae* strain is probably due to the presence of a bacterial capsule (confirmed by specific staining), which may have limited the penetration of the substances. Furthermore, our results revealed a different sensitivity of the two serotypes of *L. anguillarum* to plant extracts. No literature reports about the antibacterial activity of medicinal plants

against this fish pathogen were found. Only Bansemir *et al.* (2006) have investigated the ability of some cultivated seaweed species to inhibit the *in vitro* growth of *L. anguillarum* (serotype not known), so further studies are needed to elucidate this aspect.

According to Koné *et al.* (2004) plant extracts can be classified into two categories, considering the MBC/MIC ratio. The extracts which exhibited bacterial killing effects with MBC/MIC ratio ≤ 4 are denoted as bactericidal for tested bacteria, while the extracts which demonstrated inhibitory effects with MBC/MIC ratio > 4 are denoted as bacteriostatic. All the extracts active against *P. damselae* subsp. *piscicida* and *L. anguillarum* serotypes were bactericidal for these strains. We could not exactly define the inhibitory effects of *E. arvense* extract on *L. anguillarum* O2. Among the eight extracts active against *Y. ruckeri*, the extracts of *L. officinalis*, *M. officinalis*, *O. vulgare*, and *R. officinalis* were bactericidal while we were unable to define if the extracts of *C. officinalis*, *O. basilicum*, *S. officinalis*, and *V. vitis idaea* were bactericidal or bacteriostatic. Among the eight extracts active against *L. garvieae*, the extracts of *L. officinalis*, *M. officinalis*, and *S. officinalis* were bactericidal, the extracts of *O. vulgare*, *R. officinalis*, and *V. vitis idaea* were bacteriostatic, while the extracts of *G. robusta* and *O. stamineus* could not be characterized.

The MIC values of the studied extracts were much higher than those of the antibiotic oxytetracycline, used as positive control. This is not surprising because plant extracts are complex mixtures containing various bioactive compounds at low concentrations. In agreement with our results, several authors reported similar MIC values (ranging from 12.5 mg/ml to 100 mg/ml) for methanolic extracts of other Labiatae plants, such as Mexican mint (*Coleus aromaticus*), basil (*Ocimum basilicum* and *Ocimum sanctum*), wild mint (*Mentha arvensis*), Malabar catmint (*Anisomeles malabarica*), and Thumbai (*Leucas aspera*) against the fish pathogen *Aeromonas hydrophila* (Haniffa & Kavitha, 2012; Haniffa & Shanthi, 2012). On the other hand, lower MIC values (between 25 µg/ml and 3.13 mg/ml) were detected for methanolic or ethanolic extracts obtained from some of the tested Labiatae species against human pathogens (Nascimento *et al.*, 2000; Ceyhan *et al.*, 2012; Alnamer *et al.*, 2013). Therefore, stronger antibacterial activities could be obtained by modifying the extraction protocols.

Our study highlights that the plant species belonging to Labiatae botanical family may be promising sources of natural antimicrobial compounds that could be used in aquaculture to control fish diseases. However, further studies will be carried out to purify, characterize and quantify the bioactive compounds contained in phytoextracts as well as to investigate the effects of herbal extracts and their metabolites on fish cells or in *in vivo* fish models. For example, a diet containing *R. officinalis* ethyl acetate extract has been reported to reduce mortality in tilapia infected with *Streptococcus iniae* (Abutbul *et al.*, 2005). Similarly, we demonstrated that the dietary administration of carvacrol, a phenolic compound contained in Labiatae species, induce antioxidant effects and a significant reduction of susceptibility to vibriosis in sea bass,

Dicentrarchus labrax (Volpatti *et al.*, 2014). Moreover, additional experiments aimed to evaluate the stability of plant materials in the aquatic environment, their digestibility for fish and their metabolism/toxicity are strongly recommended.

2.5 Acknowledgments

This research was supported by the Italian Ministero delle Politiche Agricole e Forestali (project No. 7C 09 "Azione concertata per l'identificazione di contributi scientifici per lo sviluppo dell'acquacoltura biologica in Italia"). The authors wish to thank Amedeo Manfrin (Istituto Zooprofilattico Sperimentale delle Venezie, Italy) for providing the bacterial isolates and EPO S.r.l.-Piante Officinali (Milano, Italy) for providing the ethanolic plant extracts. The authors are also grateful to Andrea Caboni (University of Udine, Italy) for his support in statistical analysis.

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CHAPTER III

MODULATION OF RESPIRATORY BURST AND PROLIFERATION ACTIVITY OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) HEAD KIDNEY LEUKOCYTES BY MEDICINAL PLANT EXTRACTS

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Article in preparation.

Abstract

The use of medicinal plants for pharmaceutical purposes has received increasing attention both in human and veterinary medicine, due to their antioxidant and immune-enhancing properties. Even in aquaculture, the possible application of natural herbal-based immunostimulants is attracting considerable interest, in order to reduce the use of antibiotics in the treatment of infectious diseases, so as to limit the constant emergence of antibiotic-resistant pathogens and to ensure eco-friendly productions for the consumer. A number of *in vivo* trials were carried out to investigate the effect of the administration of plant-derived products on the immune response and disease resistance of different fish species, while *in vitro* studies aimed to the evaluation of immunomodulatory properties of plant extracts or bioactive compounds on fish immune cells are very scarce. The recent European Union recommendations (Directive 2010/63/EU) and Italian guidelines (D.L. 26/2014) imposed to limit the use of animals for scientific purposes, therefore a preliminary *in vitro* approach is strongly recommended.

In the present study, ethanolic extracts of *Aloe vera*, *Curcuma longa*, *Echinacea purpurea*, *Panax ginseng*, *Lavandula officinalis*, *Origanum vulgare* and *Rheum officinale* were assessed for their effects on immune responses of leukocytes purified from head kidney of rainbow trout (*Oncorhynchus mykiss*). Six healthy fish were selected and used for the evaluation of each parameter investigated. The freshly isolated cells were cultured in a medium containing increasing concentrations of extracts for various times (2-72 h), then they were tested for reactive oxygen species (ROS) production after stimulation with phorbol myristate acetate (PMA) and proliferation in the presence or absence of phytohaemagglutinin from *Phaseolus vulgaris* (PHA). The extracts of *L. officinalis*, *O. vulgare* and *R. officinale* strongly reduced the oxidative burst activity of PMA-stimulated leukocytes, in a dose-dependent manner ($P \leq 0.05$). Also *A. vera*, *C. longa*, *E. purpurea* and *P. ginseng* extracts demonstrated antioxidant effects, although with lower efficacy and especially at lower concentrations ($P \leq 0.05$). The highest concentration of ginseng extract stimulated the respiratory burst activity of leukocytes compared to untreated control cells. Moreover, the extracts of *C. longa*, *E. purpurea*, *P. ginseng*, *L. officinalis* and *R. officinale* had a clear dose-dependent stimulatory effect on leukocyte proliferation ($P \leq 0.05$).

The results suggest that these medicinal plants are able to modulate the immune responses of rainbow trout leukocytes, emphasizing their potential as a source of new natural antioxidant or immunostimulant agents to be used in aquaculture.

Keywords: rainbow trout, leukocytes, respiratory burst, proliferation, medicinal plants, immunostimulants, antioxidant agents.

Riassunto

L'impiego di piante medicinali a scopo farmaceutico ha ricevuto una crescente attenzione sia in medicina umana che veterinaria, in particolare per le loro proprietà antiossidanti e immunostimolanti. Anche in acquacoltura, l'applicazione di immunostimolanti naturali a base di erbe sta suscitando notevole interesse, allo scopo di ridurre l'impiego di antibiotici nel trattamento delle malattie infettive, così da limitare la costante comparsa di batteri patogeni resistenti e garantire produzioni ecocompatibili e sicure per il consumatore. Numerose prove sperimentali *in vivo* sono state effettuate per indagare l'effetto della somministrazione di prodotti ottenuti da piante su risposta immunitaria e resistenza alle malattie di diverse specie ittiche, mentre sono limitati gli studi *in vitro* dedicati alla valutazione delle proprietà immunomodulatorie di fitoestratti o composti bioattivi sulle cellule immunitarie dei pesci. Le recenti linee guida comunitarie (Direttiva 2010/63/UE) e italiane (D.L. 26/2014) impongono di limitare l'uso di animali a fini scientifici, di conseguenza un approccio preliminare *in vitro* viene sempre più raccomandato.

L'obiettivo di questo studio è stato quello di indagare *in vitro* gli effetti degli estratti etanolici di *Aloe vera*, *Curcuma longa*, *Echinacea purpurea*, *Lavandula officinalis*, *Origanum vulgare*, *Panax ginseng* e *Rheum officinale* sulle risposte immunitarie di leucociti purificati da rene anteriore di trota iridea (*Oncorhynchus mykiss*). Sei pesci sani sono stati selezionati e utilizzati per la valutazione di ciascun parametro studiato. Le cellule sono state incubate in terreno con concentrazioni crescenti di estratti per diversi tempi (2 o 72 h), quindi sono state saggiate per la loro attività di "burst respiratorio" dopo stimolazione con PMA e per la loro proliferazione in presenza o assenza di PHA. Gli estratti di *L. officinalis*, *O. vulgare* e *R. officinale* hanno fortemente ridotto l'attività di "burst respiratorio" dei leucociti PMA-stimolati, in modo dose-dipendente ($P \leq 0.05$). Anche gli estratti di *A. vera*, *C. longa*, *E. purpurea* e *P. ginseng* hanno mostrato effetti inibitori, ma meno efficaci e più evidenti alle concentrazioni minori ($P \leq 0.05$). La più alta concentrazione di estratto di ginseng ha invece stimolato l'attività di "burst respiratorio" dei leucociti rispetto alle cellule di controllo non trattate. Gli estratti di *C. longa*, *E. purpurea*, *P. ginseng*, *L. officinalis* e *R. officinale* hanno mostrato, inoltre, un evidente effetto stimolante dose-dipendente sulla proliferazione dei leucociti ($P \leq 0.05$).

I risultati suggeriscono che queste piante medicinali sono in grado di modulare le funzioni immunitarie dei leucociti di trota iridea e possono essere considerate come potenziale fonte di nuovi agenti antiossidanti o immunostimolanti naturali da impiegare in acquacoltura.

Parole chiave: trota iridea, leucociti, "burst respiratorio", proliferazione, piante medicinali, immunostimolanti, agenti antiossidanti.

3.1 Introduction

The use of medicinal plants for pharmaceutical purposes has received an increased attention in human and animal medicine, particularly due to their antioxidant and immune-enhancing properties. Herbs are known to contain many bioactive components, such as ascorbic acid, carotenoids, vitamins, flavonoids and phenolic compounds, which are capable of exerting protective effects against oxidative stress in biological systems with little or no side effects (Sharma *et al.*, 2013; Rashed, 2014). Likewise plants are rich in polysaccharides, organic acids, alkaloids, glycosides, glucans and tannins, which can enhance immune functions (Kumar *et al.*, 2011; Shukla *et al.*, 2014).

The need to reduce the widespread application of antibiotics in the treatment of infectious diseases, due to the constant emergence of antibiotic-resistant microbial pathogens and the increasing demand for eco-friendly productions (FAO/WHO/OIE 2006), is driving new emphasis on the use of natural immunostimulants also in aquaculture. The current European and national legislation that authorize the temporary or experimental use of veterinary drugs (Regulation EC 726/2004 and Italian D.L. 193/2006 in execution of the Directive 2004/28/EC) does not guarantee a wide availability of drugs for the treatment of diseases in farmed fish species. In Italy the number of therapeutis currently registered for aquaculture is very limited, especially when compared with the number of drugs registered and used in other livestock sectors. Moreover, starting from January 2006, the European Community has also banned the use of synthetic antibiotics as growth promoters in animal feed (Directive 1831/2003 EC). On the other hand, commercial vaccines are expensive for fish producers and may not be available for all species against emerging diseases, therefore the use of immunostimulants seem to be an alternative way of reducing disease risk in fish culture (Le Breton, 2009; Toranzo *et al.*, 2009).

In this context, the potential use of herbal remedies in fish recently became the focus of numerous *in vivo* scientific studies. Plant products and active compounds (extracts, essential oils, anthraquinone, azadiractin, glycyrrhizin, saponins, polysaccharides) have demonstrated the capability to enhance innate (lysozyme, complement, antiprotease, myeloperoxidase, phagocytosis, respiratory burst activity, reactive nitrogen species production, cytokines synthesis) and adaptive (antibody titre, hemagglutination) immune response in different fish species (Harikrishnan *et al.*, 2011a; Bulfon *et al.*, 2015), including rainbow trout (*Oncorhynchus mykiss*) (Dügenci *et al.*, 2003; Nya and Austin, 2009a; 2009b; 2011; Dorucu *et al.*, 2009; Award and Austin, 2010; Bilen and Bulut, 2010; Award *et al.*, 2011; Bilen *et al.*, 2011; Sheikhzadeh *et al.*, 2011; Asadi *et al.*, 2012; Award *et al.*, 2013; Nootash *et al.*, 2013). In many instances, the heightened immunity was also coincident with an increase of the resistance to infections (Harikrishnan *et al.*, 2011a; Bulfon *et al.*, 2015). Furthermore, it has been reported that plant derivatives can protect fish cells against oxidative stress through the inhibition of reactive

oxygen species (ROS) production or the activation of antioxidant enzymes (Xie *et al.*, 2008; Zheng *et al.*, 2009; Liu *et al.*, 2012; Volpatti *et al.*, 2014).

On the other hand, *in vitro* studies evaluating the immune modulatory effects of medicinal plants or phytochemicals on fish immune cells are still particularly scarce. Treatment of rainbow trout leukocytes with glycyrrhizin, a saponin present in the aqueous extract of *Glycyrrhiza glabra*, enhanced their respiratory burst activity, proliferation and macrophage activating factor (MAF) release (Jang *et al.*, 1995). Similarly, the blood leukocytes of pacu (*Piaractus mesopotamicus*) incubated with the extract of *Aloe vera* exhibited an increased respiratory burst activity (Zanuzzo *et al.*, 2012).On the other hand resveratrol, a polyphenol purified from the roots of *Veratrum grandiflorum*, exerted a significant inhibitory effect on the migratory response, production of ROS, intracellular and extracellular myeloperoxidase (MPO) activity, and synthesis of the pro-inflammatory prostaglandin E2 (PGE2) in head kidney leukocytes of turbot (*Psetta maxima*) (Castro *et al.*, 2008). *Astragalus radix* water extract stimulated the proliferation and nitric oxide production of head kidney macrophages and granulocytes in common carp (*Cyprinus carpio*) (Yin *et al.*, 2004). Picchietti *et al.* (2013) examined the properties of *Aloe arborescens* extract on the *Sparus aurata* fibroblast cell line SAF-1 stimulated with lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (poly I:C). Their results claimed the ability of *A. arborescens* to modulate the transcription of some genes crucially involved in fish immune response (IL-1β, TGF-α, TNF-α, COX-2, IFN-I, Mx and MHCI-α).

The design and development of specific vaccines, immunomodulatory molecules or drugs require species-specific testing, due to differences in the structure and function of the immune system (Haley, 2003). In teleosts, researches are currently based on *in vivo* experiments which are only occasionally preceded by *in vitro* approaches, leading to high costs and the sacrifice of a large number of fish. Nevertheless, the strict recommendations of the European Union Commission (Directive 2010/63/EU) and the recent Italian guidelines (D.L. 26/2014) impose to limit the use of animals for experimental and other scientific purposes in order to minimize suffering and pain, consequently a preliminary *in vitro* approach is strongly recommended. In fact, in animal testing should be applied the cardinal principle of the 3Rs ("*Replacement, Reduction and Refinement*") in accordance with the protocol on animal welfare: 1) replacement of animal models with *in vitro* models, when possible; 2) reduction of animal number used in the experiments; 3) use of less invasive techniques when the experiments involve the use of animals (Marino *et al.*, 2009; Midtlyng *et al.*, 2011; Romberg *et al.*, 2012).

In vitro assays can be performed using primary cell cultures (e.g. leukocytes purified from lymphatic organs) or cell lines developed for immunological research. They provide more repetitive results under highly controlled experimental conditions and allow to screen simultaneously a large number of products at different concentrations. Thereby *in vitro*

methods may constitute a cost-effective alternative to *in vivo* experimentation in accordance with the animal testing regulations and may be used to elucidate different aspects of fish haematopoietic and immune system functions as well as to select new immunostimulant candidates to be exploited in aquaculture. Information from the *in vitro* studies can be analyzed prior to proceed with more expensive *in vivo* tests (Galeotti, 1998; Fierro-Castro *et al.*, 2012; 2013). Several promising immunostimulant compounds, including β-glucans, alginates, vitamins or synthetic substances have been tested *in vitro* on fish immune cells (Mulero *et al.*, 1998; Castro *et al.*, 1999; Peddie *et al.*, 2002; Castro *et al.*, 2006; Caipang *et al.*, 2010; Chettri *et al.*, 2010; Małaczewska *et al.*, 2010; Caipang *et al.*, 2011; Chettri *et al.*, 2011; Abarca *et al.*, 2012; Fierro-Castro *et al.*, 2012; 2013).

The main objective of this study was to assess and compare *in vitro* the effects of *Aloe vera*, *Curcuma longa*, *Echinacea purpurea*, *Lavandula officinalis*, *Origanum vulgare*, *Panax ginseng* and *Rheum officinale* extracts on different functions of leukocytes purified from head kidney of rainbow trout (*Oncorhynchus mykiss*).

A. vera is a succulent herb belonging to the Alliaceae family. Various studies revealed that it possesses many pharmaceutical activities, including antimicrobial, anti-inflammatory, antioxidant, anticancer, antidiabetic, antiulcer, hepatoprotective and immunomodulatory. It is a popular folk medicine used in treating stomach disorders, gastrointestinal problems, skin diseases, constipation, ulcer and diabetes, wounds and burns caused by radiation injury. Currently, the plant is widely used in skin care, cosmetics and as nutraceutical (Singh *et al.*, 2011; Sahu *et al.*, 2013; Manvitha & Bidya, 2014).

C. longa is a perennial herb, member of the Zingiberaceae family. The pharmacological properties of turmeric are anti-inflammatory, antioxidant, hepatoprotective, antitumor, antidiabetic, antimicrobial, immune-modulatory. In addition, it is used against cardiovascular disease, gastrointestinal and neurological disorders. Turmeric is commonly applied for the treatment of acne, wounds, boils, bruises, blistering, ulcers, eczema, insect bites, parasitic infections, hemorrhages, skin allergy, viral hepatitis, inflammatory conditions of joints, sore throat (Luthra *et al.*, 2001; Krup *et al.*, 2013; Labban, 2014).

E. purpurea is a perennial, herbaceous plant of the Asteraceae botanical family. Pharmaceutically, its preparations are used for the treatment of colds, toothaches, headache, wound infections, chronic infections of respiratory and lower urinary tract (viral and bacterial origin). It possesses antioxidant, anti-inflammatory, immunoenhancing effects and it is considered the most popular herbal immunostimulant (Percival, 2000; Barrett, 2003; Kumar & Ramaiah, 2011).

L. officinalis and *O. vulgare* are aromatic perennial herbs belonging to the Labiatae botanical family. Extracts and essential oils obtained from these plants have shown considerable antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant properties, resulting

successful in the treatment of respiratory disorders, rheumatoid arthritis, bacterial and fungal infections, and recent studies suggested that they could become helpful agents in the therapy of cancer, heart disease and high blood pressure (Cavanagh & Wilkinson, 2002; Yusufolu *et al.*, 2004; Ličina *et al.*, 2013; Teixeira *et al.*, 2013).

P. ginseng belongs to the Araliaceae family and it is used primarily in the treatment of weakness and fatigue. It is also reported to possess beneficial activities as antioxidant and immunostimulant, for diabetes, gastritis, neurasthenia, fever, asthma, coagulation disorders, loss of appetite, vomiting, colitis, neurodegenerative diseases, cardiovascular disorders and cancer (Helms, 2004; Lakshmi *et al.*, 2011; Cho, 2012; Kim, 2012).

R. officinale is a medicinal plant of the Polygalaceae family which possess antibacterial, antiviral, anti-inflammatory, antioxidant and anticancer effects. Traditionally, rhubarb is used for remedies against the digestive system diseases, such as constipation, dysentery, gastritis, enteritis, gastric ulcer as well as for the treatment of hepatitis, hemorrhages and trauma (Cai *et al.*, 2004).

In view of the proven activities of these medicinal plants in humans and the lack of studies in fish, we tested *in vitro* for the first time their properties on reactive oxygen species (ROS) production and proliferation of rainbow trout leukocytes, in order to explore whether they contain bioactive compounds of potential interest as antioxidant agents or immunostimulants in aquaculture.

3.2 Materials and Methods

3.2.1 Reagents

Benzocaine, Bradford reagent, cetyltrimethylammoniumbromide (CTAB), dimethylsulphoxide (DMSO), 3 – [4, 5 – dimethlythiazol-2yl] - 2, 5 – di phenyltetrazolium bromide (MTT), fetal calf serum (FCS), Hank's Balanced Salt Solution without phenol red, Ca^{2+} and Mg^{2+} (HBSS), heparin (5 KU ml^{-1}), Histopaque®1077, L-glutamine, luminol (5-amino-2,3-dihydro-1,4-pathalazinedione), Leibovitz medium (L-15), penicillin/streptomycin solution, phorbol myristate acetate (PMA), phytohaemagglutinin from *Phaseolus vulgaris* (PHA-P), sulphuric acid (H_2SO_4), 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB), trypan blue, Triton X-100 were purchased from Sigma-Aldrich (St. Louis, USA). May-Grunwald-Giemsa stain was obtained from Kaltek S.r.l (PD, Italy).

3.2.2 Plant extracts

Commercial hydroethanolic extracts from *Aloe vera* (Liliaceae), *Curcuma longa* (Zingiberaceae), *Echinacea purpurea* (Asteraceae), *Lavandula officinalis* (Labiatae), *Origanum vulgare* (Labiatae), *Panax ginseng* (Araliaceae) and *Rheum officinale* (Polygonaceae) were kindly provided by EPO S.r.l.-Piante officinali, Milan, Italy (Table 1). Prior to be tested in the experiments, they were desiccated using a rotary evaporator under a vacuum (UNIVAPO) at 37°C for 3 h, then the recovered dry residues were suspended in distillated water at the concentration of 2 mg/ml. This concentration was chosen based on previous published data (Zanuzzo *et al.*, 2012; Pichietti *et al.*, 2013). Then, the extracts were sterilized by filtration through a 0.22 μm filter (Sarsted) and stored in the dark at -20°C for the following assays.

Table 1 Chemical and physical characteristics of ethanolic plant extracts used in this study

Botanical name	Botanical family	Plant parts used	Ethanol content	Density (g/ml)	Physical characteristics	pH	Secondary metabolites*
Aloe vera	Liliaceae	juice	18.0-22.0%	≥ 0,900	clear liquid, brown, sweetish odor, bitter taste	4.0-6.0	hydroxyanthracene derivatives (aloin A and B, aloinoside A and B), resins
Curcuma longa	Zingiberaceae	rhizome	48.0-52.0%	0.93-0.95	clear liquid, yellow-orange, characteristic odor and taste	5.5-7.5	phenols (curcumin), essential oil (rich in terpenes derivatives), starch, flavonoids
Echinacea purpurea	Asteraceae	root	47.0-52.0%	0.94-0.97	opalescent liquid, yellow, characteristic odor and taste	5.0-6.0	caffeic acid derivatives (chicoric acid and chlorogenic acid), essential oil, polisacharides, echinacein, flavonoids
Lavandula officinalis	Labiatae	inflorescence	48.0-52.0%	0.92-0.96	clear liquid, brown, characteristic odor and taste	5.0-6.0	essential oil (linalyl acetate), tannins, coumarins, flavonoids, phytosterols
Origanum vulgare	Labiatae	inflorescence	28.0-32.0%	0.99-1.02	clear liquid, brown, characteristic odor and taste	4.0-5.0	carvacrol, thymol, γ-terpinene, p-cymene, limonene, linolool, borneol
Panax ginseng	Araliaceae	root	47.0-52.0%	0.93-0.95	clear liquid, brown, root characteristic odor, bitter taste	5.0-6.5	triterpene saponins (ginsenosides Rg1, Rc, Rd, Rb1, Rb0), essential oil, flavonoids, tannins, alkaloids, aminoacids, pectins, vitamins, sterols, fatty acids
Rheum officinale	Polygonaceae	root	48.0-54.0%	1.00-1.50	clear liquid, brown-yellow, characteristic odor, bitter taste	5.0-6.0	anthraquinones, tannins and gallic acid

* percentage unknown (except ginsenosides in ginseng extract).

3.2.3 Fish

Adult rainbow trout (*Oncorhynchus mykiss*) were originally obtained from a local fish farm, confirmed free of pathogens and maintained in the aquarium facilities at the University of Udine (Department of Food Science, DIAL), Italy. Fish were kept in squared fiberglass tanks of 500 l capacity in an indoor open system supplied with filtered freshwater and fed daily with a commercial pelleted diet at a level recommended by the manufacturer. Water physico-chemical parameters were periodically measured throughout the experimental period and maintained at the optimum level for this fish species [temperature 12.5°C, pH 8.0, dissolved oxygen 9.5 mg/l, NH_4-N 0.02-0.03 mg/l, NO_2-N below the detection limit of the method (<0.015mg/l), natural photoperiod]. All experimental procedures were conducted in compliance with the guidelines of the European Union Commission (Directive 2010/63/EU) and the Italian legislation (D.L. 26/2014) concerning the use of animals for experimental purposes, in order to minimize suffering and pain.

3.2.4 Isolation of head kidney leukocytes

To avoid possible mixed lymphocytes reaction (MLR) (Heiger *et al.*, 1977; Caspi and Avtalion, 1984; Miller *et al.*, 1986; Meloni *et al.*, 2006), we used head kidney leucocytes from 6 individual fish for each parameter evaluated (3 fish for the cytotoxicity assay). Rainbow trouts with an average weight of 400-500 g were euthanized throughout an overdose of benzocaine (0.1 g/l) and immediately submitted to blood sampling from the caudal vein. Head kidney (HK) was aseptically removed, placed in a Petri dish containing sterile HBSS with 0.25% heparin, then gently pressed by a sterile syringe piston. The resultant cell suspension was carefully layered onto a Histopaque-1077 gradient and centrifuged (300 x *g* for 25 min at 4°C) to obtain a fraction enriched in leukocytes. Thereafter, cells at the interface were collected and washed twice with sterile HBSS (200 x *g* for 10 min at 4°C). Cell viability was determined with the trypan blue exclusion method and cell concentration was estimated using a Thoma counting chamber. Cell viabilities were routinely higher than 99%. Leukocytes were adjusted to $5x10^6$ viable cells/ml in L-15 medium containing 2 mM L-glutamine, 10% FCS, penicillin 100 U/ml and streptomycin 100 µg/ml (culture medium) or alternatively in HBSS according to the needs of the following assays, and kept on ice until assay performance. Cells from individual fish were not pooled but maintained as individual cell cultures throughout the experiments, and tested in duplicates. Aliquots (100 µl) of the cell suspension in culture medium were dispensed into each well of 96-well tissue culture plates and were incubated with the extracts on the same day that the cells were obtained, as describe in the following paragraphs.

3.2.5 Leukocytes differential count and protein content

Small samples of purified cells in culture medium were smeared onto glass slides. The smears were air dried at room temperature (RT), fixed with methanol, stained with Giemsa and observed under oil immersion objective (magnification of 1000x) with an optical microscope (Leica DMRB, Wien, Austria). One hundred leucocytes were differentiated morphologically and counted.

The protein content of HK leukocytes was determined as described by Enane *et al.* (1993) with minor modifications. Cells in HBSS were lysed with 0.2% Triton X-100, then the cell content was mixed with Bradford reagent (Bradford, 1976) in a 96-well microtiter plate (Sarstedt). The absorbance was measured spectrophotometrically at 595 nm using a microplate reader (Sunrise Tecan) against a standard curve prepared with bovine serum albumin (BSA).

3.2.6 Cytotoxicity assay

Preliminary studies were performed to evaluate any cytotoxicity of plant extracts on leukocytes and define appropriate experimental doses to be used in the following assays. Cytotoxicity was quantified by using the colorimetric CytoTox 96®Non-Radioactive kit (Promega, USA), that measures the release of cytosolic lactate dehydrogenase (LDH) from cells with a loss of membrane integrity. Leukocytes in L-15 (100 µl/well) were plated into flat-bottom 96-well culture plates (Sarstedt) and allowed to adhere at 18°C overnight before exposure to plant extracts. Then, the non adherent cells were removed and the adherent cells were incubated with plant extracts (100 µl/well) at different dilutions (1:2, 1:4, 1:8). Untreated cells served as negative controls. After 1 h exposure at 18°C, plates were centrifuged at 300 x *g* for 5 min and the LDH activity was measured at 490 nm using a microplate reader (Tecan Sunrise). According to the manufacturer's instructions, the LDH activity was determined both in culture supernatants and in the remaining cells after their lysis. The percentage of cytotoxicity was calculated as 100 x (LDH release/total LDH), where LDH release is the amount of LDH activity in the supernatant of cells and total LDH is the sum of LDH activity in the supernatant and LDH activity in cell lysates.

3.2.7 Leukocytes respiratory burst activity

Leukocytes in L-15 (100 µl/well) were placed into sterile black 96-well plates (Nunc) and treated with plant extracts dilutions (1:2-1:64, 100 µl/well) at 18°C for 2 h. Untreated control cells were incubated with the same volume of culture medium without the extracts. Afterwards,

the plates were centrifuged at 300 x *g* for 10 min to allow the cells to adhere and the supernatant was removed. The respiratory burst activity of phagocytes was detected using the chemiluminescence (CL) assay according to the method of Coteur *et al.* (2002) with minor modifications. Luminol, prepared in DMSO just before use and diluted in L-15 medium to obtain a final concentration of 2 mM, was used as a luminescent probe. The cells were incubated with 100 µl/well of HBSS, 50 µl/well of luminol and 100 µl/well of PMA (2 µg/ml) as respiratory burst stimulatory agent. Wells without PMA were included as blank. The CL emission was measured in terms of relative luminescence units (RLU) using a luminometer (Tecan), every 3 min for 21 min at RT.

3.2.8 Leukocytes proliferation

Leukocytes in L-15 (100 µl/well) were distributed into sterile flat-bottom 96-well plates with plant extracts at serial dilutions (1:2-1:64, 100 µl/well), in presence or absence of the mitogen PHA-P (10 µg/ml). Leukocytes incubated only with culture medium or only with PHA were included as relative controls. After 72 h incubation at 18°C, the microplates were centrifuged at 300 x *g* for 10 min at 18°C and the supernatant was discarded. Subsequently, the proliferation of HK leucocytes was determined by the indirect colorimetric MTT assay (Galeotti *et al.*, 1999 partially modified), which is based on the cellular uptake of the yellow substrate MTT and its subsequent reduction into purple colored formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in live cells (dead cells do not cause this change). Forty µl/well of MTT solution (dissolved in HBSS at a concentration of 1 mg/ml and sterilized by filtration) were added to each well and the plates were incubated for 3 h at 18°C. Then, 150 µl of DMSO were added and gently mixed with a micropipette to solubilize the dark blue formazan crystals. After incubation at RT for 10 min, the absorbance was read at 570 nm.

3.2.9 Statistical analysis

Each extract was tested on leukocytes isolated from six fish in duplicate for each dose. The mean values of duplicates were calculated and six individual data (one for each fish) were obtained for each dose and compound. Since, primary cell cultures, if compared to fish stable cell lines, might show considerable fluctuations in their responses depending on the physiological status of the donor fish and/or the quality of the isolation procedure giving rise to potentially relevant variations, the results [mean ± standard error (SE)] were expressed as percentage of the response of treated cells in comparison to the response of control untreated cells. This approach allows to standardize the data from the different experimental runs. Values

higher than 100% reflect cell activation, while lower values reflect cell inhibition. Comparisons were done among cells treated with different concentrations of extracts for each immune parameter. All statistical analysis were conducted using the software SPSS Statistic version 20 (SPSS, Inc, Chicago, IL, USA). Data were compared by one-way analysis of variance (ANOVA) after testing for normality and homogeneity of variances using the Kolmogorov-Smirnov test and Levene test, respectively. If results were significant, Duncan's post hoc test was applied for multiple comparisons. If the variances were not normally distributed, the Kruskal-Wallis non-parametric test and the relative post hoc test (Mann-Whitney *U* test) were used. Differences were considered statistically significant when *P* value was ≤ 0.05.

3.3 Results

3.3.1 Leukocytes differential count and protein content

The microscopic investigation showed that the leukocytes populations purified from adult rainbow trout HK consisted of different cell types, whose identification was rather difficult due to the lack of morphological data concerning the immature/mature stages present in this organ. The main fraction of cells was ascribable to the lymphocyte lineage (about 70-80%), including both small and large lymphocytes. Monocyte-like cells were lower than 10% and none of them resembled mature macrophages. In fact, the nucleus of these cells was kidney bean-shaped but the cytoplasm was not aboundant and did not present vacuolisations. The remaining cells, about 10-20%, were characterized by a large size, a round nucleus and a cytoplasm containing acidophilic or basophilic granules. These leukocytes could be considered as belonging to the granulocyte lineages.

The total protein content of leukocytes was 204.50 ± 31.68 μg/5 x 10^6 cells.

3.3.2 Plant extracts cytotoxicity

In order to evaluate any cytotoxic effect of plant extracts on rainbow trout leukocytes, the release (%) of the cytoplasmic enzyme LDH in culture medium was measured after 1 h incubation in the presence or absence of extracts. The plant extracts caused a negligible release of LDH, suggesting that they are not toxic at the concentrations used (Table 2). Moreover, no evident alterations in cell morphology or cellular damage were observed under the inverted microscope (data not shown).

Table 2 Lactate dehydrogenase release (%) by rainbow trout HK leukocytes treated with plant extracts.

Plant extract	**Dilutions**		
	1:2	**1:4**	**1:8**
Aloe vera	6,49	6,34	5,42
Curcuma longa	6,10	5,55	6,61
Echinacea purpurea	4,62	3,83	4,46
Lavandula officinalis	6,14	4,88	4,51
Origanum vulgare	3,11	1,73	3,96
Panax ginseng	3,58	2,84	4,53
Rheum officinale	3,10	2,43	7,21
Untreated cells	7,84		

Percent value of LDH release, referred as percent value of the LDH activity in the supernatant with respect to total LDH activity. The results of one independent fish assessed in duplicate are reported as example.

3.3.3 Leukocytes respiratory burst activity

The extracts of *L. officinalis* (Fig. 1A), *O. vulgare* (Fig. 2A) and *R. officinale* (Fig. 3A) markedly affected the respiratory burst activity of HK leukocytes post-stimulated with PMA compared to untreated control cells. The ROS production significantly decreased in a dose-dependent manner ($P \leq 0.05$) in the presence of these plant extracts. A very significant inhibition ($P \leq 0.01$) was observed with the highest concentrations of all these extracts. In particular, the treatment of leukocytes with *L. officinalis*, *O. vulgare* and *R. officinale* at the 1:2 dilution induced a reduction of respiratory burst activity, reaching 84.2 ± 2.70%, 85.8 ± 0.98% and 83.6 ± 2.10% respectively.

Also the CL response of cells exposed to the extracts of *A. vera* (Fig. 4A), *C. longa* (Fig. 5A), *E. purpurea* (Fig. 6A) and *P. ginseng* (Fig. 7A) was reduced compared to untreated control cells, although less effectively and especially at lower concentrations. Specifically, *C. longa* and *E purpurea* at dilution 1:64 caused an inhibition of respiratory burst activity reaching 59.4 ± 6.71% and 62.2 ± 2.30% respectively, *A. vera* reaching 36.8 ± 7.47 % and *P. ginseng* reaching 23.9 ± 7.94%. Significant differences in ROS production between leukocytes exposed to different concentrations of extract were observed in the case of *C. longa* and *P. ginseng* ($P \leq 0.05$). Otherwise, the highest concentration of ginseng extract stimulated the respiratory burst activity of leukocytes (about 30%) compared to untreated control cells.

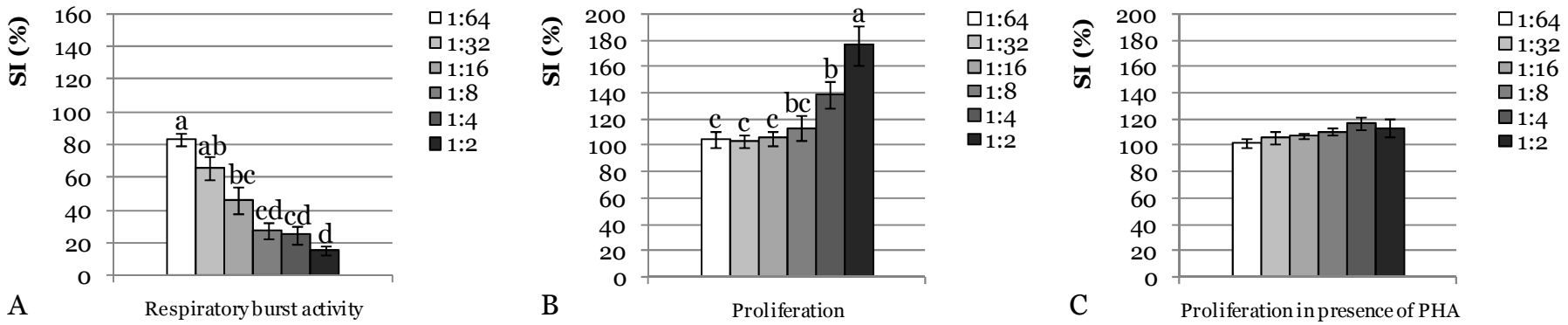

Fig. 1 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Lavandula officinalis*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

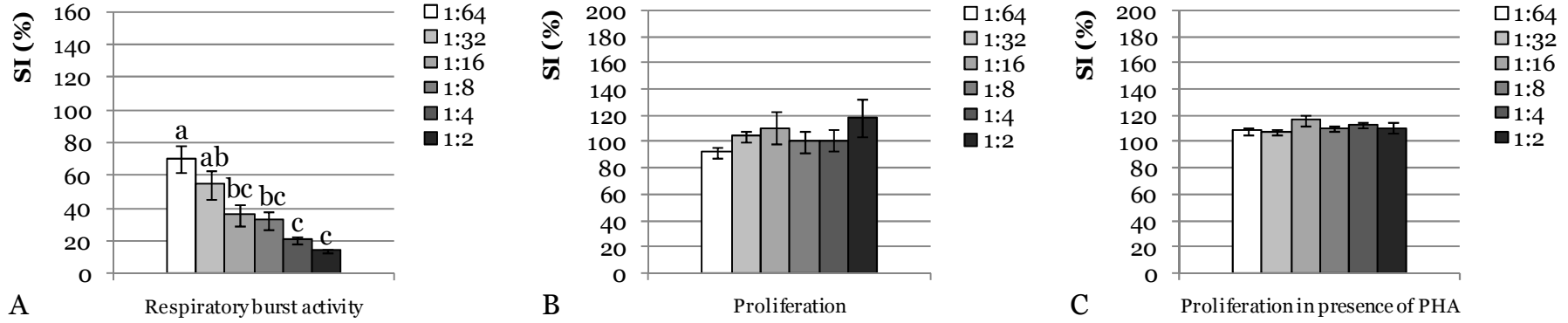

Fig. 2 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Origanum vulgare*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

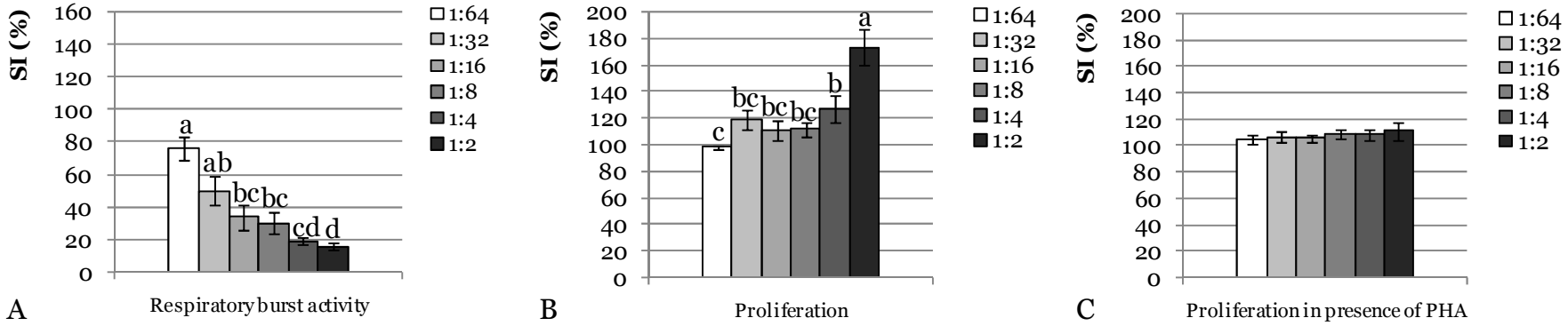

Fig. 3 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Rheum officinale*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

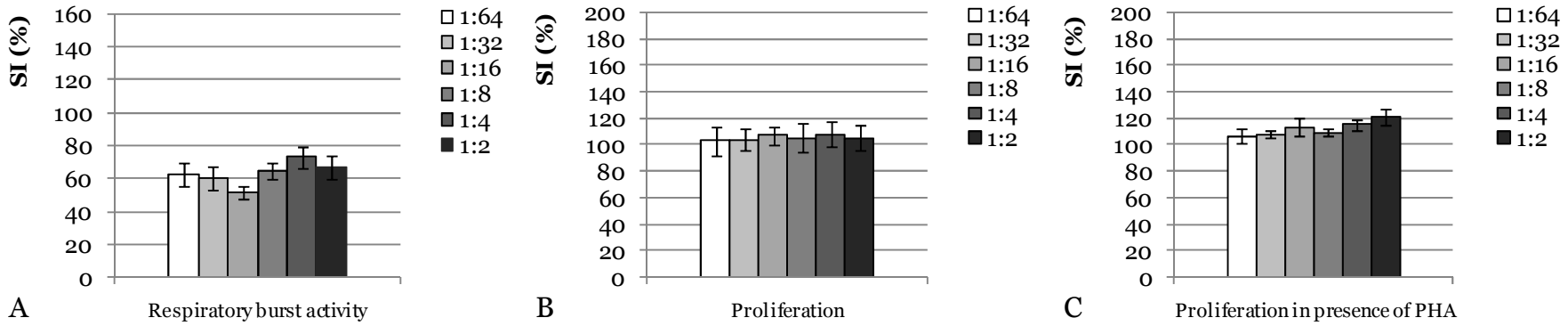

Fig. 4 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Aloe vera*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

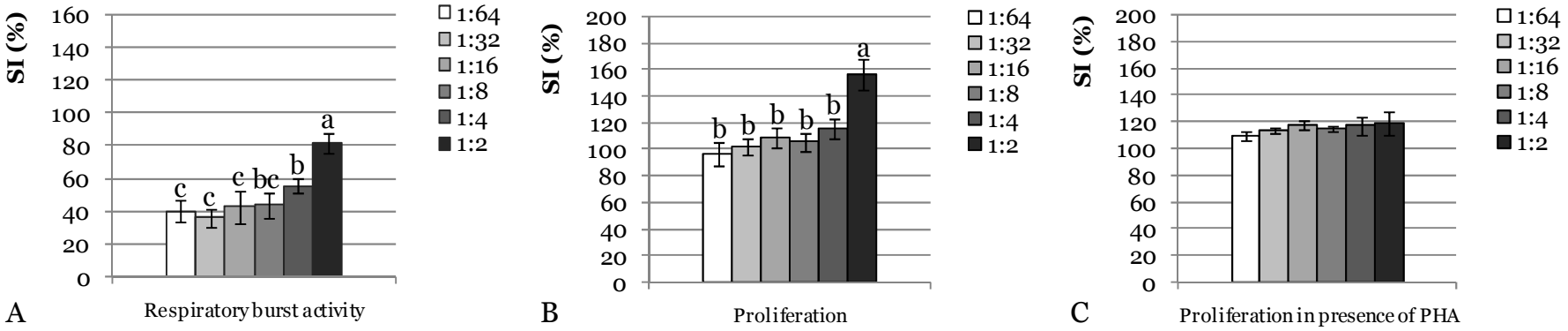

Fig. 5 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Curcuma longa*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

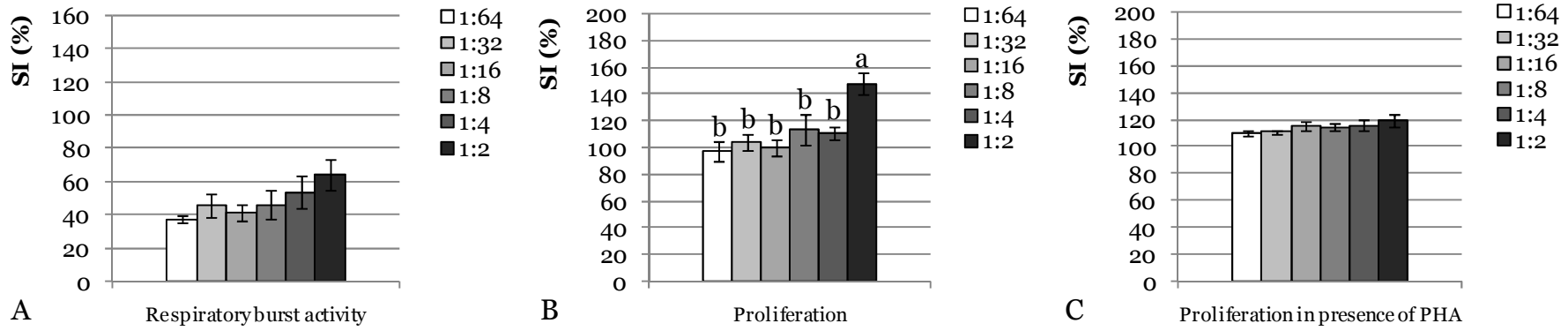

Fig. 6 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Echinacea purpurea*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

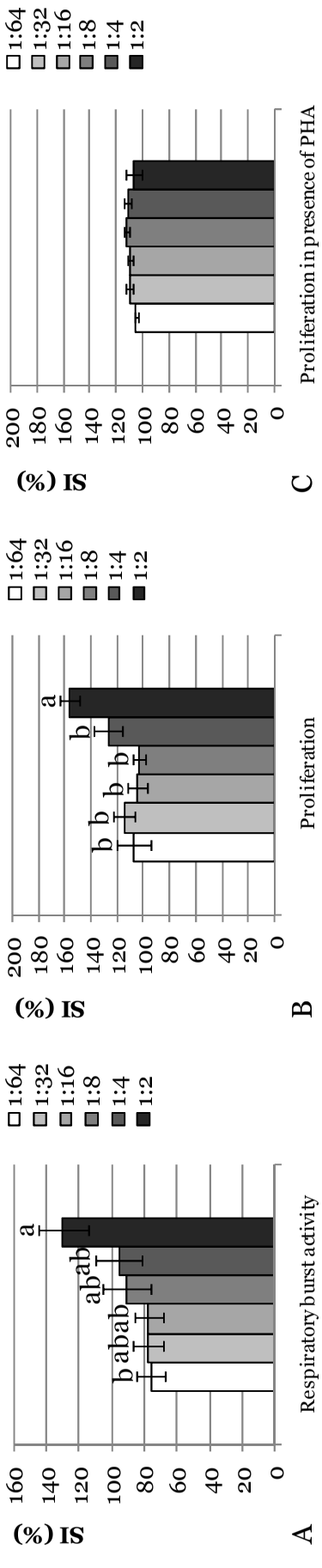

Fig. 7 Respiratory burst activity (A), proliferative response in absence of PHA (B) and proliferative response in presence of PHA (C) of HK leucocytes exposed to different dilutions of *Panax ginseng*, referred as percent values with respect to untreated control cells (control values are 100%). Data are expressed as mean ± SE from n=6 independent fish (in duplicate). Different lowercase letters indicate significant differences between concentrations within each extract ($P \leq 0.05$).

3.3.4 Leukocytes proliferation

The extracts of *C. longa* (Fig. 5B), *E. purpurea* (Fig. 6B), *P. ginseng* (Fig. 7B), *L. officinalis* (Fig. 1B) and *R. officinale* (Fig. 3B) had a clear dose-dependent stimulatory effect on leukocyte proliferation when incubated with the cells in the absence of PHA for 72 h, compared to untreated control cells. Maximal increases of this response were detected with the highest concentration tested of each extract ($P \leq 0.05$). *L. officinalis* and *R. officinale* extracts showed higher levels of stimulation (reaching 76.5 ± 15.33% and 73.3 ± 13.44% respectively) if compared to *C. longa* (reaching 57.2 ± 11.30%), *P. ginseng* (reaching 56.5 ± 7.29%) and *E. purpurea* (reaching 47.9 ± 8.31%) extracts.

O. vulgare extract less clearly enhanced the proliferation of HK leukocytes (about 19%) and no differences between different concentrations were recorded (Fig. 2B).

The addition of *Aloe vera* extract to cells had no effects on their proliferation (Fig. 4B).

The addition of extracts, at different dilutions, to cell cultures in the presence of the mitogen PHA did not induced an evident enhancement of the leukocyte response, compared to cells incubated with PHA alone (Fig 1C-7C).

The proliferative response of leukocytes stimulated only with PHA was 1-2 times higher than that of cells stimulated only with plant extracts (data not shown).

3.4 Discussion

The stimulation of immune response by plant extracts has been recently tested in various fish species, primarily after the administration of dietary treatments and the subsequent evaluation of related target parameters (Dügenci et al., 2003; Christybapita *et al.*, 2007; Ardò *et al.*, 2008; Immanuel *et al.*, 2009; Kirubakaran *et al.*, 2010; Harikrishnan *et al.*, 2011b; 2011c; 2011d; Kaleeswaran *et al.*, 2011; Sheikhzadeh *et a*l., 2011; Asadi *et al.*, 2012; Harikrishnan *et al.*, 2012a; 2012b; Park & Choi, 2012; Awad *et al.*, 2013; Das *et al.*, 2013; Baba *et al.*, 2014; Samad *et al.*, 2014).

In the present study we investigated the *in vitro* immune modulatory effects of seven medicinal plants (*Aloe vera*, *Curcuma longa*, *Echinacea purpurea*, *Panax ginseng*, *Lavandula officinalis*, *Origanum vulgare*, and *Rheum officinale*) on head kidney (HK) leucocytes of rainbow trout (*O. mykiss*). The main fraction of purified leukocytes consisted of cells belonging to the lymphocyte lineage (about 70-80%), including both small and large lymphocytes. The remaining cells were monocyte-like cells and cells with a granular cytoplasm, probably ascribable to granulocytes. The full identification of different populations was rather difficult due to the lack of morphological data concerning the immature/mature stages physiologically colonizing head kidney. This mixed population showed a total protein content of 204.50 ± 31.68 μg/5 x 10^6 cells,

in agreement with the data reported by Enane *et al.* (1993) for rainbow trout. Based on its composition in terms of leukocytes sub-populations, this suspension was considered suitable as *in vitro* model to study the role of plant extracts as stimulants on cellular mechanisms such as respiratory burst and proliferation.

In order to investigate and to avoid possible cytotoxic effect of plant extracts, we tested preliminarily their impact on cell viability, using the lactate dehydrogenase (LDH) assay. LDH is a cytosolic enzyme present in all metabolically active cells which is released due to a loss of membrane integrity, therefore its measurement can provide reliable information about the possible toxicity of a substance. The extracts did not affect the leukocytes LDH release after 1 h treatment, suggesting that plant extracts have not cytoxic effects at the concentrations used. Moreover, no evident alterations in cell morphology or cellular damage were observed under the inverted microscope (data not shown).

Subsequently, we analyzed the respiratory burst activity and proliferation of rainbow trout HK leukocytes exposed to the extracts in order to assess the effect of medicinal plants on the activation of cellular immune processes.

The respiratory burst is a potent antimicrobial defense mechanism in phagocytic cells, which play a central role in the non-specific immune response of fish, as in mammals. It is activated after the stimulation of plasma membrane during phagocytosis and triggers the production of ROS. Briefly, the multi-component enzyme NADPH oxidase is assembled on the inner surface of the plasma membrane via protein kinase C (PKC), producing superoxide anion (O_2^-), which dismutates into hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase. This reactive compound is a substrate for myeloperoxidase (MPO) of neutrophils which produce hypochlorous acid (HOCl), a strong oxidant and antimicrobial compound at the origin of the highly reactive hydroxyl radical ($^{\cdot}OH$) (Neumann *et al.*, 2001; Rieger & Barreda, 2011). Although an evolutionary divergence and a consequent relatively low sequence homology has been observed for NADPH oxidase between fish and mammals, the functional domains remained highly conserved. Moreover, in teleosts the enzymatic subunits have been shown to have a similar expression pattern to that of mammals, suggesting that fish and mammal NADPH oxidase may have similar modes of activation and functional activities (Rieger & Barreda, 2011).

ROS released by activated neutrophils and macrophages are capable of destroying the invading pathogens, nevertheless the excessive ROS production is deleterious for cells and tissues, since they change the structure and function of biomolecules and may be involved in the pathogenesis of many inflammatory diseases (Tsumsu *et al.*, 2012). Various medicinal plants have got growing interest as antioxidants, and are widely used in human and animal medicine to boost health and reduce the risk of inflammatory diseases, cancer and cardiovascular disorders (Kaplan *et al.*, 2007; Rubió *et al.*, 2013).

In the present work, the ROS production was measured by luminol-enhanced

chemiluminescence in HK leukocytes activated with phorbol 12-myristate 13-acetate (PMA). PMA is a fast activator of PKC, so it activates the enzymes responsible for ROS production bypassing all previous events involved in the signal transduction cascade. Luminol can cross biological membranes due to its chemical structure, allowing the detection of extracellular and intracellular production of oxygen radicals (Vera-Jimenez *et al.*, 2013). Our results demonstrated that there is a modulation of respiratory burst activities in the HK leukocytes pre-incubated with plant extracts. In particular, the extracts of *O. vulgare*, *L. officinalis* and *R. officinale* markedly reduced the respiratory burst activity of cells stimulated with PMA compared to untreated control cells, in a dose-dependent manner. In particular, *L. officinalis*, *O. vulgare* and *R. officinale* induced a reduction of respiratory burst activity above 80%. Also *A. vera*, *C. longa*, *E. purpurea* and *P. ginseng* extracts displayed inhibitory effects on leukocytes ROS production, although less effectively and especially at lower concentrations. Specifically, *C. longa* and *E purpurea* caused a reduction of respiratory burst activity up to 60%, *A. vera* up to 40% and *P. ginseng* up to 20%. On the other hand, the highest dose of ginseng extract stimulated the CL response of leukocytes (about 30%) compared to untreated control cells.

The antioxidant properties exhibited by *O. vulgare*, *L. officinalis* and *R. officinale* are commonly ascribable to their content of phenolic compounds, including carvacrol and thymol in the case of oregano (Kačániová *et al.*, 2012; Licina *et al.*, 2013; Teixeira *et al.*, 2013), phenolic acids, anthocyanins, flavonoids and tannins in the case of lavender (Blažeković *et al.*, 2010), anthraquinones, tannins and gallic acid in the case of rhubarb (Cai *et al.*, 2004). The mechanism of action of polyphenols has been partially elucidated in mammals. *In vitro* studies indicate that polyphenols can act on mammalian leukocytes stimulated *in vitro* with PMA in several ways, either by scavenging and neutralizing the ROS produced by the cells on a direct stoichiometric relationship or by modifying their reactivity to PMA. Authors showed that polyphenols can enter into the cells and may inhibit the phorbol ester-induced assembly of the NADPH oxidase subunits, probably by interfering with the activation of molecules involved in the signal transduction or with the calcium regulation (Leiro *et al.*, 2002; Kohnen *et al.*, 2007; Franck *et al.*, 2008; Tsumbu *et al.*, 2012). In our experiments plant extracts were incubated for 2 h with rainbow trout leukocytes, then they were removed before the stimulation of cells with PMA. Thereby, it is expected that polyphenols contained in oregano, lavender and rhubarb penetrate into the cells and this may explain an intracellular ROS scavenging activity or an interference with the signal transduction pathway (after the activation of PKC).

Polyphenols have been reported to *in vitro* and *in vivo* inhibit the production of ROS also in leukocytes of fish species different from rainbow trout. Resveratrol incubated with turbot (*P. maxima*) leukocytes caused a significant reduction of PMA-induced respiratory burst activity (Castro *et al.*, 2008), moreover we demonstrated that diet supplemented with carvacrol decreased the generation of ROS in HK leukocytes of sea bass (*Dicentrarchus labrax*) (Volpatti

et al., 2014). In addition, the dietary administration of green tea (*Camelia sinensis*) polyphenols to rainbow trout significantly increased superoxide dismutase (SOD) activity in liver and muscle, and decreased malondialdehyde (MDA) content in muscle and plasma, reducing the potential oxidative damage (Xu *et al.*, 2008). Similarly, anthraquinones extracted from *R. officinale* increased hepatic catalase (CAT) and SOD activity, and decreased MDA content in Wuchang bream (*M. amblycephala*) (Liu *et al.*, 2012) and in common carp (*Cyprinus carpio* var. *Jian*) (Xie *et al.*, 2008). Moreover, the activities of these antioxidant enzymes in the plasma of channel catfish (*Ictalurus punctatus*) were markedly enhanced in fish fed a diet containing *Origanum heracleoticum* essential oil and were slightly increased in fish fed diets containing carvacrol and thymol (Zheng *et al.*, 2009).

The extracts of *A. vera*, *C. longa*, *E. purpurea* and *P. ginseng* contain a wide variety of chemical bioactive compounds, so we can assume that they act on different cellular targets at different optimal concentrations by interfering in different ways with the activity of respiratory burst. Less consistent data are available in literature about the *in vitro* effects of extracts obtained from these medicinal plants or their active compounds on ROS production by fish or mammalian phagocytes. Blood leukocytes of pacu (*Piaractus mesopotamicus*) incubated with the extract of *A. vera* exhibited an increased respiratory burst activity (Zanuzzo *et al.*, 2012). However, it has been reported that *A. vera* possess antioxidant activity that might be attributed to its content of polyphenols, flavonoids and polysaccharides (Hu *et al.*, 2003; Patel *et al.*, 2012). Polysaccharides, glycoproteins and alkylamides isolated from *Echinacea* sp. produced *in vitro* and *in vivo* stimulatory effects on macrophages functions (phagocytosis, respire burst, NO production, TNF-α, IL-1, IL-6, IL-10, INF-α synthesis) in mice, rats and humans (Rininger *et al.*, 2000; Percival, 2000; Barrett, 2003). In contrast, *Echinacea* phenolic acid or echinocaside displayed anti-inflammatory and radical scavenging properties (Rininger *et al.*, 2000). Similarly, neutrophils and macrophages treated with ginseng polysaccharides were found to enhance CL and phagoocytic capacity (Hu *et al.*, 1995; Shin *et al.*, 2002), but saponins, flavonoids and polyphenolic compounds demonstrated also antioxidant activities (Kang *et al.*, 2006; Saumya & Mahaboob, 2011). According to Franck *et al.* (2008) curcuminoids, the principal phenolic compounds in turmeric, showed dose-dependent inhibitory effects on reactive oxygen and myeloperoxidase release by activated equine neutrophils. Farinacci *et al.* (2009) reported that *C. longa* and *Echinacea angustifolia* extracts strongly reduced the adhesion and superoxide production of PMA-stimulated ovine neutrophils.

On the other hand, the administration of diets containing aloe, turmeric or ginseng have been reported to increase leukocytes respiratory burst activity in different fish species. Rainbow trout fed 1% *A. vera* extract supplemented diet showed a significant increase in ROS production of blood leukocytes compared with untreated control group (Haghighi *et al.*, 2014a; 2014b), although Zanuzzo *et al.* (2015) noted that the inclusion of *A. vera* in the diet failed to influence

respiratory burst activity in this fish species. Similar positive effects on the activity of HK leukocytes have been also described in carp (*Labeo rohita*) fed diets enriched with 0.01%, 0.05%, 0.1% and 0.5% *C. longa* powder (Sahu *et al.*, 2008) or in olive flounder (*Paralichthys olivaceus*) fed diet incorporated with 3% Siberian ginseng (*Eleutherococcus senticosus*) extract (Won *et al.*, 2008). Similarly, Behera *et al.* (2011) demonstrated that the intraperitoneal injection of curcumin (1.5 and 15 μg) in *L. rohita* significantly increased respiratory burst activity of blood leukocytes. On the contrary, Aly *et al.* (2008) did not found a significant increase of this immune parameter in Nile tilapia (*Oreochromis niloticus*) fed 0.025% *E. purpurea* extract supplemented diet.

Afterwards, we investigated the effects of plant extracts on the proliferation of rainbow trout HK leukocytes in order to test if they can act directly on immune cells as mitogens. In literature, there is evidence that some of these medicinal plants enhanced the proliferation of lymphocytes in *in vitro* studies. For instance, *L. officinalis* and *P. ginseng* extracts have been reported to enhance the proliferative response of human peripheral blood lymphocytes in the presence of the mitogen PHA (Wu *et al.*, 1991; Azadmehr *et al.*, 2011). In addition, ginsenosides from *P. ginseng* stimulated T cell proliferation as well as NK cell activity in mouse (Kenarova *et al.*, 1990; Lee *et al.*, 2004). Similarly, an aqueous extract of *Echinacea* was able to stimulate the proliferation of human lymphocytes incubated with the lectins ConA, PHA and PWM up to 4,3 times (Chaves *et al.*, 2007). Even, *A.vera* has been reported to stimulate lymphocytes activities in mammals (Tan & Vanitha, 2004). In the present study, the extracts effect on proliferative response was assessed both in absence and in presence of PHA co-stimulation (specific for T lymphocytes). The incubation of rainbow trout leukocytes with all the extracts, except *A. vera* and *O. vulgare*, induced a dose-dependent proliferation of cells compared to unstimulated control cells. In particular, *L. officinalis* and *R. officinale* extracts showed higher levels of stimulation (about 77% and 73% respectively), whereas *C. longa* extract increases leukocytes proliferation of about 57%, *P. ginseng* extract of about 57% and *E. purpurea* extract of about 48%. On the other hand, the addition of extracts to cell cultures in the presence of the mitogen PHA did not improve the proliferative response of leukocyte in a significant manner compared to cells incubated only with PHA. We can speculate that the activation of rainbow trout leukocytes is due primarily to the polysaccharides present in medicinal plant extracts, which act as polyclonal activators on cell surface, as proposed by other authors (Chaves *et al.*, 2007). The results obtained with mammalian cells also indicated that one or several compounds present in plant extracts can act as costimulatory molecules, potentiating the mitogenic effects induced by the lectins ConA, PHA or PWM (Chavez *et al.*, 2007). The fact that in our experiments the plant extract/PHA combination gives only a slightly enhancement of leukocyte proliferation could be due to a non optimal ratio of the specific concentrations used. Similar *in vitro* studies aimed to investigate the effects of phytoextracts or plant compounds on fish leukocyte proliferation are

very scarce. For comparison we can mention the study of Jang *et al.* (1995), who examined the effects of glycyrrhizin on the proliferation of *O. mykiss* peripheral blood lymphocyte in response to T and B cell mitogens. They found that the addition of glycyrrhizin alone to rainbow trout leucocytes had no effect upon their proliferation, whereas the addition of such compound to cultures in the presence of PHA but not of LPS gave a significant enhancement of the response with an inverse correlation with the dose. Similarly, Yin *et al.* (2004) demonstrated that *Astragalus radix* extract stimulated the proliferation of head kidney macrophages of common carp (*C. carpio*).

In conclusion, this *in vitro* study speculated that *A. vera*, *C. longa, E. purpurea*, *L. officinalis*, *O. vulgare*, *P. ginseng* and *R. officinale* can modulate some innate immune functions of rainbow trout leukocytes, emphasizing their potential as sources of natural antioxidants and immunostimulants, which might be useful as preventive agents against oxidative stress and as promoters of the immune response in fish. Nevertheless, further studies should be carried out with isolate active plant principles to elucidate the prime source of beneficial antioxidant/immunomodulatory properties. Moreover, it is important to note that these *in vitro* tests bring insufficient information about the real effect of these medicinal plants on rainbow trout physiology. However, the results of this investigation provide the baseline indications for further *in vivo* trials that must be performed in order to verify the effects of plant incorporation into the diet of rainbow trout on fish hematological parameters, antimicrobial activity of the serum, phagocytic activity of leukocytes, liver enzyme levels and disease resistance.

3.5 Acknowledgements

This work was supported by CRITA, project financed by Friuli Venezia Giulia Region and coordinated by Dr. F. Tulli (Aquaculture Section, DIAL, University of Udine). The authors would like to thank EPO S.r.l.-Piante Officinali (Milano, Italy) for providing the plant extracts, Dr. A. Sepulcri (DISA, University of Udine) for technical help in the preparation of extracts and are grateful to Prof. E. Tibaldi and Dr. M. Bruno (Aquaculture Section, DIAL, University of Udine) for providing the rainbow trouts used in this study.

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CHAPTER IV

PANAX GINSENG AS FEED ADDITIVE FOR RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): EFFECTS ON GROWTH, BLOOD BIOCHEMICAL PROFILE, NON-SPECIFIC IMMUNE RESPONSE AND DISEASE RESISTANCE AGAINST *YERSINIA RUCKERI*

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Article in preparation.

Abstract

Asian ginseng (*Panax ginseng*) is known to have a broad spectrum of positive properties, since it increases resistance against exogenous stressors, improves vitality and longevity, enhances mental capacity and immune response, therefore it has been used in traditional medicine for many centuries. In the present work, a feeding trial was conducted to evaluate the effects of a dietary supplementation with *P. ginseng* extract on growth, blood biochemical profile, innate immune response and resistance against *Yersinia ruckeri* infection in rainbow trout (*Oncorhynchus mykiss*). Four experimental diets were obtained by adding four different levels of ginseng ethanolic extract to a commercial basal feed (0.0, 0.1, 0.2, 0.3 g/kg) and triplicate groups of fish (mean initial body weight 30.5 ± 0.15 g) were fed at 1% of body weight/day for 10 weeks. The results of this study showed that the inclusion of ginseng in the diet did not significantly affect rainbow trout growth performance ($P > 0.05$). At the end of the feeding trial, plasma total proteins and globulins did not significantly change in fish fed ginseng enriched diets compared to fish fed the basal diet ($P > 0.05$) while albumin level was significantly lower ($P < 0.05$). Triglycerides and non esterified fatty acids (NEFA) levels were higher in rainbow trout receiving the supplemented feed compared to control group ($P < 0.05$), while a decrease although not significant in cholesterol and glucose levels was measured ($P > 0.05$). Dietary ginseng led to negligible effects on plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels ($P > 0.05$). Serum lysozyme and respiratory burst activity of leukocytes stimulated with phorbol myristate acetate (PMA) were slightly increased in fish fed diets containing ginseng compared to controls, although no significant differences were noted among different dietary treatments ($P > 0.05$). Serum antiprotease activity and leukocytes myeloperoxidase content did not significantly changed in fish fed ginseng enriched diets compared with the control group ($P > 0.05$). The dietary administration of ginseng extract induced a reduction of mortality in rainbow trout infected with *Y. ruckeri* compared to the controls, but no significant differences between the experimental groups were observed ($P>0.05$). Fish fed the control diet (0% herbal extract) suffered the maximum mortality of 93%. Rainbow trout fed 0.01%, 0.02% and 0.03% ginseng supplemented diets registered a cumulative mortality of 83% (RPS = 10.71%), 90% (RPS = 3.57%) and 87% (RPS = 7.14%), respectively.

Keywords: *Panax ginseng*, rainbow trout, growth performance, blood biochemical profile, non-specific immune response, disease resistance.

Riassunto

Il ginseng (*Panax ginseng*) è noto per avere un ampio spettro di proprietà, dal momento che aumenta la resistenza contro fattori esogeni di stress, migliora la vitalità, la longevità, la capacità mentale e la risposta immunitaria, quindi viene impiegato nella medicina tradizionale da molti secoli. Nel presente lavoro, è stata condotta una prova di alimentazione al fine di valutare gli effetti dell'inclusione nella dieta dell'estratto di *P. ginseng* su crescita, profilo ematochimico, risposta immunitaria innata e resistenza ad infezione con *Yersinia ruckeri* in trota iridea (*Oncorhynchus mykiss*). Quattro diete sperimentali sono state ottenute aggiungendo quattro diversi livelli di estratto etanolico di ginseng ad un mangime commerciale (0.0, 0.1, 0.2, 0.3 g/kg) e gruppi di pesci in triplicato (peso corporeo medio iniziale di 30.5 ± 0.15 g) sono stati alimentati all' 1% del peso vivo/giorno per 10 settimane. I risultati di questo studio mostrano che l'aggiunta di ginseng alla dieta non ha influenzato in modo significativo le performance zootecniche delle trote ($P > 0.05$). Alla fine della prova, le proteine totali e le globuline nel plasma non sono cambiate in modo significativo nei pesci alimentati con le diete contenenti ginseng rispetto ai pesci alimentati con la dieta di controllo ($P > 0.05$) mentre il livello di albumina è risultato significativamente minore ($P < 0.05$). I livelli di trigliceridi e NEFA sono risultati più elevati nelle trote che hanno ricevuto le diete con ginseng rispetto al gruppo di controllo ($P < 0,05$), mentre è stata misurata una diminuzione, anche se non significativa, dei livelli di colesterolo e glucosio ($P > 0.05$). La somministrazione del ginseng ha avuto effetti trascurabili sui livelli plasmatici di ALT, AST e ALP ($P > 0.05$). L'attività del lisozima nel siero e di "burst respiratorio" dei leucociti stimolati con PMA sono risultati leggermente aumentati nei pesci alimentati con le diete contenenti ginseng rispetto ai soggetti di controllo, anche se non sono state rilevate differenze significative tra i vari trattamenti dietetici ($P > 0.05$). L'attività delle antiproteasi nel siero e il contenuto di mieloperossidasi dei leucociti non sono cambiati in maniera significativa nei pesci alimentati con le diete arricchite con ginseng rispetto al gruppo di controllo ($P > 0.05$). La somministrazione alimentare del ginseng ha indotto una riduzione della mortalità nelle trote infettate con *Y. ruckeri* rispetto ai controlli, ma non sono state osservate differenze significative tra i gruppi sperimentali ($P > 0.05$). I pesci alimentati con la dieta di controllo (0% estratto) hanno registrato la mortalità massima, pari a 93%. Le trote iridee alimentate con le diete integrate con 0.01%, 0.02% e 0.03% di ginseng hanno registrato una mortalità cumulativa pari a 83% (RPS = 10,71%), 90% (RPS = 3,57%) e 87% (RPS = 7,14%), rispettivamente.

Parole chiave: *Panax ginseng*, trota iridea, performance zootecniche, profilo ematochimico, risposta immunitaria non specifica, resistenza alle malattie.

4.1 Introduction

Nowadays, aquaculture is one of the fastest food production sectors and an increasingly important option of animal protein sources in many countries of the world. Sustainable aquaculture depends on a perfect balance between growth and health condition of fish, therefore there is a constant need to increase productivity but also fish resistance to infections. Consequently, the diets should contain not only high quality and levels of essential dietary nutrients such as amino acids, fatty acids, vitamins and minerals but also complementary feed additives to improve growth performance and nutrient utilization as well as to keep fish healthy. Moreover, in aquaculture it is expected to reduce the use of synthetic drugs to control fish diseases (Regulation EC 726/2004 and Italian D.L. 71/2003) and starting from January 2006 the European Community has also banned the use of antibiotics in animal feed (Regulation EC 1831/2003). On the other hand, commercial vaccines are expensive for fish farming practices and are not specific against all species of pathogens (Le Breton, 2009; Toranzo *et al.*, 2009).

In this context, a range of feed additives have been investigated as growth promoters and immunostimulants in fish (Galeotti, 1998; Sakai, 1999; Galindo-Villegas & Hosokawa, 2004; Li & Gatlin, 2006; Nayak, 2010; Trichet, 2010; Dimitrouglou *et al.*, 2011; Viswanath, 2012; Barman *et al.*, 2013). However, some of these additives are chemical products, hormones, cytokines and antibiotics, which may cause unfavourable side effects, including the selection of resistant microbial strains, environmental pollution and bioaccumulation potentially harmful for human health. Natural substances, such as probiotics, bacterial products, complex carbohydrates, animal/plant extracts, essential oils and some organic acids/salts, are interesting candidates as alternatives to synthetic compounds since they allow to obtain more ecological and acceptable products to the consumer.

In particular, medicinal herbs have recently received an increasing interest because of their biological activities related to high content of secondary metabolites, low costs and minimal side effects (Reverter *et al.*, 2014). The inclusion of herbal remedies or botanicals in terrestrial animal diets has been largely proposed due to their antimicrobial action and positive effects on appetite and digestion, immune system, and oxidative stress (Greathead, 2003; Frankič *et al.*, 2009; Wallace *et al.*, 2010; Hashemi & Davoodi, 2011). Similarly, to date many studies have pointed out that the dietary administration of plant active compounds (anthraquinone, azadiractin, glicyrizin, saponins, polysaccharides) and products (extracts, essential oils) can significantly enhance innate (lysozyme, complement, antiproteases, myeloperoxidase, phagocytosis, respiratory burst activity, nitric oxide production, cytokines synthesis) and adaptive (antibody production, lymphocytes aggregation) immune response in different fish species and consequently their resistance to diseases (Jeney *et al.*, 2009; Chakraborty & Hancz, 2011; Harikrishnan *et al.*, 2011a; Bulfon *et al.*, 2015; Reverter *et al.*, 2014). In addition, it has

been demonstrated that medicinal plants can improve fish growth, probably through an improvement in nutrient utilization that may be due to a modulation of trypsin-like enzymes activities during the digestive processes (Bulfon *et al.*, 2015; Chakraborty *et al.*, 2014; Reverter *et al.*, 2014). It has been reported that herbal extracts might inhibit the colonization and proliferation of potential pathogenic bacteria in fish gastrointestinal tract and maintain the intestinal beneficial bacterial flora, improving consequently feed digestibility and nutrient absorption (Bulfon *et al.*, 2015; Reverter *et al.*, 2014). Phytochemicals can be also effectively used as endocrine modulating agents (Chakraborty *et al.*, 2014). On the other hand, diets enriched with plant derivatives have protected fish against oxidative stress through the inhibition of reactive oxygen species production or the activation of antioxidant enzymes (Xie *et al.*, 2008; Zheng *et al.*, 2009; Liu *et al.*, 2012; Volpatti *et al.*, 2014).

In the present study, we used Asian ginseng (*Panax ginseng*) as a feed additive in rainbow trout diet. *P. ginseng* belongs to the Araliaceae botanical family and it is the most widely used ginseng. This species has been a popular and widely-used herbal remedy in traditional Chinese medicine for thousands of years, primarily in the treatment of weakness and fatigue (Lakshmi *et al.*, 2011). The main active compounds in *P. ginseng* roots are triterpene saponins, commonly named ginsenosides, but many other active compounds can be found in all parts of the plant, including amino acids, alkaloids, phenols, polysaccharides, polypeptides, vitamins B1 and B2 (Lakshmi *et al.*, 2011; Kim, 2012a). *P. ginseng* is referred to have a number of positive actions in humans, since it increases resistance against exogenous stressors, improves vitality and longevity, enhances mental capacity, affects the hypothalamic-pituitary-adrenal (HPA) axis and the immune system (Lakshmi *et al.*, 2011). Animal models and *in vitro* experiments have indicated that ginseng extracts enhance phagocytosis, reactive nitrogen species (NOS) production by macrophages, lymphocytes proliferation, both B and T-cell mediated immune responses, natural killer (NK) cell activity, cytokines and chemokines (IL-1α, TNF- α, GM-CSF, IFN-γ, IL-2) synthesis (Tan & Vanitha, 2004; Kang & Min, 2012). Moreover, beneficial effects of ginseng on neurodegenerative diseases, cardiovascular disorders and cancer have been also described, due to the antioxidant properties of its active components (Helms, 2004; Radad *et al.*, 2006; Cho, 2012; Kim, 2012b).

Few studies have assessed the efficacy of ginseng as a possible immunostimulant or growth promoter in fish and shellfish. An improvement of haematological indices, growth performance and nutrient utilization was observed in Nile tilapia (*Oreochromis niloticus*) fingerlings fed diets supplemented with different levels of *P. ginseng* extract (Ginsana G115) (Goda, 2008). Similarly, the inclusion of American ginseng (*Panax quinquefolium*) root powder or capsules containing ginseng root extract in the diet of Nile tilapia stimulated growth and immune response against *Aeromonas hydrophila* (Abdel-Tawwab, 2012; El-Sayed *et al.*, 2014). Furthermore, olive flounder (*Paralichthys olivaceus*) fed diet enriched with Siberian ginseng

Eleutherococcus senticosus exhibited enhanced non-specific humoral and cellular immunity as well as higher resistance to *Edwardsiella tarda* and *Vibrio anguillarum* infections (Won *et al.*, 2008). On the other hand, the dietary administration of *P. ginseng* root polysaccharide extract in white shrimp (*Litopenaeus vannamei*) increased immune enzyme activities (AKP activity, ACP activity, SOD activity, CAT activity, GSHPx) and up-regulated the mRNA expression of immune genes cyt-SOD, CAT, GSH-Px and Prx (Liu *et al.*, 2011).

The objective of the present study was to evaluate the possible modulation of growth performance, blood biochemical profile, non-specific immune parameters and resistance to *Yersinia ruckeri* infection in rainbow trout (*Oncorhynchuss mykiss*) by the dietary administration of *P. ginseng* root extract. Moreover, *P. ginseng* extract was preliminarily *in vitro* screened for its inhibitory activity against the bacterial fish pathogens *Y. ruckeri* and *L. garvieae.*

4.2 Materials and methods

4.2.1 Chemicals

Acetic acid, 5-amino-2,3-dihydro-1,4-pathalazinedione (luminol), benzocaine, cetyltrimethylammoniumbromide (CTAB), Hank's Balanced Salt Solution without phenol red, Ca^{2+} and Mg^{2+} (HBSS), heparin (5 KU ml-1), Histopaque®1077, hydrogen peroxide (H_2O_2), lysozyme from chicken egg white, *Micrococcus lysodeikticus*, phorbol myristate acetate (PMA), sodium-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), sodium phosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB), trypan blue, Tris-HCl were purchased from Sigma-Aldrich (St. Louis, USA). Trypsin (Type 1 from bovine pancreas) was purchased from VWR International (England). Ethanol 99% was purchased from Carlo Erba Reagents S.r.l. (Cornaredo, MI, Italy). Clove oil from *Eugenia caryophyllata* was obtained from Vitalis Dr. Joseph Natural Intelligence (Brunico, BZ, Italy). Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA) were purchased from Biomerieux Italia S.p.a. (FI, Italy). Throughout the study, water was collected from a Milli-Q system from Millipore (Bedford, MA, USA).

4.2.2 Antibacterial activity of ginseng extract

The antibacterial activity of ginseng extract was evaluated against the bacterial fish pathogens *Yersinia ruckeri* (strain B11/8) and *Lactococcus garvieae* (strain O41), isolated from spleen of diseased rainbow trout during spontaneous outbreaks of disease (collection of the Division of

Veterinary Pathology, DIAL, Udine University). Bacteria were identified with the biochemical API 20E or API Strep (Biomerieux) kit following the instructions of the manufacturer (*Y. ruckeri* profile 5307100 and *L. garvieae* profile 4041110) and by standard molecular tests, then stored in PBS with 15% sterile glycerol at −80 °C (collection of the Veterinary Pathology Section, DIAL, University of Udine). After thawing, they were cultured overnight in an appropriate nutrient broth medium (TSB or BHI respectively) at 22 ± 2 °C. Exponentially-growing bacteria were pelleted by centrifugation at 2,000 *x g* for 30 min and resuspended in sterile phosphate buffer saline pH 7.2 (PBS). The inoculum size of each test strain was standardized prior antimicrobial test by adjusting spectrophotometrically the optical density (O.D. at 610 nm) of the bacterial suspension to 1.0, then confirmed by spreading serial 10-fold dilutions of each suspension onto TSA duplicate plates and counting the number of colony-forming units (CFU) following incubation at 22 ± 2 °C.

P. ginseng root ethanolic extract containing 1% ginsenosides (Table 1) was kindly provided by EPO s.r.l. (Milano, Italy) and sterilized through a 0.22-µm filter prior to be used for the antimicrobial assays. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of ginseng extract were evaluated according to the broth micro-dilution method in microtiter plate described by Bulfon *et al.* (2014). All tests were run in triplicates and were performed in a minimum of three independent assays.

Table 1 Chemical and physical properties of *P. ginseng* root ethanolic extract.

Botanical name	Botanical family	Plant parts used	Ethanol content	Density (g/ml)	Physical characteristics	pH	Secondary metabolites*
Panax ginseng	Araliaceae	root	47.0-52.0%	0,93-0.95	clear liquid, brown, characteristic odor of root, bitter taste	5,0-6.5	triterpene saponins (ginsenosides Rg1, Rc, Rd, Rb1, Rb0), essential oil, flavonoids, tannins, alkaloids, aminoacids, vitamins, sterols, fatty acids

* percentage unknown (except ginsenosides).

4.2.3 Preparation of diets

Four experimental diets (G0, G1, G2, G3) were obtained by adding *P. ginseng* ethanolic extract 1% ginsenosides (EPO s.r.l., Milano, Italy) at a rate of 0.0, 0.1, 0.2 and 0.3 g/kg to a commercial basal pelleted feed (Vita 2, Veronesi, Verona, Italy). These concentrations were chosen based on previous published data (Goda, 2008; El-Sayed *et al.*, 2014). The ingredients and proximate composition of the basal diet are reported in Table 2. *P. ginseng* ethanolic extract for 1 kg of each diet was diluted in 50 mL 99% ethanol and slowly sprayed onto the pellets during the

mixing in a drum mixer. The control diet (0% ginseng extract) was sprayed with the same volume of solvent without herbal extract. Pellets were dried in a heater at 25°C for 48 h, packed and stored under vacuum in plastic bags at 4 ± 1 °C until use.

Table 2 Composition of the basal diet.

Ingredients	Nutrient levels (%)	
Fish meal	Crude protein	50.00
Wheat gluten meal	Oils and fats	21.00
Wheat flour	Crude fibre	1.00
Fish oil	Ash	8.30
Soybean meal	Calcium	1.60
Corn oil	Phosphorus	1.10
Vitamin mix[a]	Sodium	0.64
Mineral mix[b]		

[a]vitamin mix composition: vitamin A; vitamin D3; vitamin E (alpha-tocopherol acetate); vitamin C (ascorbic acid).

[b]mineral mix composition; manganese sulfate monohydrate; zinc sulfate monohydrate; iron sulfate monohydrate; copper sulfate pentahydrate; potassium iodure; sodium selenite.

4.2.4 Fish and experimental design

Rainbow trout (*O. mykiss*) fingelings were obtained from a local commercial fish farm and examined for their health status immediately upon arrival. Fish were acclimatized to the experimental conditions for 2 weeks in 12 conical fibreglass tanks with 150 l capacity (20 fish/tank) being part in an indoor open freshwater system (water renewal = 0.25 l/sec/tank, artificial day length= 12 h) provided with thermostatic control (Department of Food Science, University of Udine). The fish were kept at controlled and optimal water quality during the trial [temperature 12.3 ± 0.4°C, pH 7.7 ± 0.1, dissolved oxygen 10.1 ± 0.2, NH_4-N 0.06 ± 0.00 mg/l, NO_2-N always below the detection limit of the method (<0.015 mg/l)] and fed daily with the basal pelleted diet of 1% of body weight/day. After the acclimatization period, the tanks were assigned to the four test diets according to a random design with triplicate tanks per dietary treatment group. Therefore G0 group was fed with basal diet and acted as the control, G1 group was fed with diet containing 0.1 g/kg (0.01%) ginseng extract, G2 group was fed with diet containing 0.2 g/kg (0.02%) ginseng extract and G3 group was fed with diet containing 0.3 g/kg (0.03%) ginseng extract. At the beginning of the feeding trial, fish average body weight was 30.5 ± 0.15 g. Fish were hand-fed the experimental diets at the rate of 1% of body weight/day, 6 days a week, for 10 weeks. The daily ration was subdivided into two equal meals administered at 9:00 and 16:00 hours. Fish were kept under veterinary control during the trial and any clinical sign of disease or mortality was daily registered. Any dead fish was weighed and removed. All

experimental procedures were carried out according to the Guidelines of the European Union Council (Directive 2010/63/EU) and the Italian legislation (D.L. 26/2014) for the use of laboratory animals. The fish were sedated during handling (i.e. for sampling) and the rearing/experimental procedures were performed in such a way so as to minimize suffering and pain. The number of fish used was kept to a minimum where possible.

4.2.5 Growth performance

Every 3 weeks and at the end of feeding trial, fish on each tank were counted and group-weighted, after 48h-fast and under a moderate anaesthesia with cloves oil (25 ppm, Gholipour kanani *et al.*, 2013). The feed intake (FI), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated per group as follows:

Feed intake (g) = total administered feed during experimental period

WG % = 100 x [final weight (g) – initial weight (g)]

SGR = 100 x [(ln final weight (g) – ln initial weight (g)]/ experimental period (days)

FCR = feed intake (g)/weight gain (g)

4.2.6 Blood and tissue sampling

At the end of the feeding trial, eighteen fish from each dietary treatment (6 fish from each replicate per treatment group) were randomly anaesthetized with cloves oil and submitted to blood collection from the caudal vein. Blood samples were transferred in sterile tubes with or without heparin as anticoagulant and kept on ice. Plasma (2 fish/replicate/group) was separated from heparinised blood after centrifugation at 1500 x *g* for 15 min at 4°C. Serum (4 fish/replicate/group) was obtained from non-heparinised blood after clotting for 2 h at 4°C and centrifugation at 1500 x *g* for 15 min at 4°C. Both samples were immediately stored at -80°C until the biochemical and immunological analysis. After the blood sampling, head kidney (HK) was aseptically removed from 6 fish/group euthanized by an overdose of anesthetic and placed in a Petri dish with HBSS containing 0.25% heparin for leukocytes isolation.

4.2.7 Blood biochemical profile

Plasma samples were analyzed using an automated analyzer system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) based on the use of commercially available kits. The parameters determined were: total proteins (TP, g/dl), albumin (ALB, g/dl), glucose (GLU, mg/dl), cholesterol (CHO, mg/dl), triglycerides (TRIG, mg/dl), non esterified fatty acids (NEFA,

mmol/l), aspartate aminotransferase (AST, U/l), alanine aminotransferase (ALT, U/l), alkaline phosphatase (ALP, U/l). The globulin content (GLB, g/dl) was calculated by subtracting albumin from total proteins.

4.2.8 Humoral immune response

4.2.8.1 Lysozyme activity

The serum lysozyme activity was determined by using a turbidimetric method according to Parry *et al.* (1965). Briefly, 10 µl of fish serum were incubated (in triplicate) in 96-well microtitre plates (Sarsetdt) with 200 µl of 0.02% lyophilized *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.8). The reduction of absorbance was measured at 450 nm every 10 min for 1 h using a microplate reader (Sunrise Tecan S.r.l., Milan, Italy). The lysozyme activity (U/ml) in serum samples was subsequently calculated from a standard curve prepared with serial dilutions of chicken egg white lysozyme.

4.2.8.2 Antiprotease activity

The serum antiprotease activity was determined following the method of Bowden *et al.* (1997) with minor modifications. Ten microliters of serum were incubated in triplicate with 10 µl of 0.3% trypsin in 0.01 M Tris-HCl pH 8.2, then 500 µl of 5 mM BAPNA substrate was added and the volume was made up to 1 ml with 0.1 M Tris-HCl pH 8.2. Samples were incubated at 22°C for 25 min. The reaction was stopped with 150 µl of 30% acetic acid and the mixtures were centrifuged at 400 x *g* for 5 min. Two hundred microliters of the supernatant were transferred in triplicate to a 96-well microplate and the optical density (O.D.) was read at 415 nm using a microplate reader against a blank. The inhibitory activity of antiproteases was expressed in terms of percentage of trypsin inhibition as described by Zuo & Woo (1997): Percent inhibition (%) = [(O.D. trypsin – O.D. sample)/ O.D. trypsin] x 100.

4.2.9 Cellular immune response

4.2.9.1 Isolation of head kidney leukocytes

HK tissue was gently pressed with a sterile syringe piston, then the cell suspension, containing leukocytes and other cells, was layered onto a Histopaque-1077 gradient and centrifuged (300 x *g* for 25 min at 4°C) to obtain a fraction enriched in leukocytes. Thereafter, cells at the interface

were collected and washed twice with HBSS (200 x *g* for 10 min at 4°C). Cell viability was determined with the trypan blue exclusion method and the cell concentration was estimated using a Thoma counting chamber. HK leucocytes were adjusted to 1 x 10^7 cells/ml or to 1 x 10^6 cells/ml in HBSS according to the following assays.

4.2.9.2 Respiratory burst activity

The production of reactive oxygen species (ROS) by rainbow trout HK phagocytes during the respiratory burst was quantified by a luminol-enhanced chemiluminescence method modified from Coteur *et al.* (2002). Black 96-well plates (Nunc) were prepared containing 50 µl/well of HK leukocytes (1 x 10^7 cells/ml in HBSS), then 50 µl/well of 2 mM luminol and 100 µl/well of 2 µg/ml PMA as stimulus were added. Each sample was evaluated in triplicate. The chemiluminescence emission was immediately measured for 15 min at 3 min intervals (T = 20°C), using a luminometer (Sunrise, Tecan) with integration time = 0.5 s and photomultiplier gain = 180. Luminescence backgrounds were calculated using wells containing cells and luminol but without PMA. The ROS production was expressed in terms of cumulative response [relative luminescence units (RLU)/10^7 cells/ml].

4.2.9.3 Myeloperoxidase content

The total MPO content in rainbow trout HK neutrophils was measured as previously described with slight modifications (Quade & Roth, 1997). HK leucocytes at concentration 1 x 10^6 cells/ml in HBSS were lysed with 0.002% CTAB for 5 min in microfuge tubes. After centrifugation at 400 x *g* for 15 min to separate cell debris from supernatant containing peroxidases, 150 µl of cell lysate were transferred in 96-well plates (in triplicate). Twenty-five µl/well of 10 mM TMB and 25 µl/well of 5 mM H_2O_2 were added to produce a colour-change reaction. The reaction was stopped after 2 min with 50 µl/well of 2 M H_2SO_4. The optical density (OD) was measured at 450 nm using a microplate reader. Blank samples without leukocytes were also analysed.

4.2.10 Disease resistance

Challenge experiment was performed at the end of feeding trial using the gram-negative bacterium *Y. ruckeri* (strain B11/8, collection of the Division of Veterinary Pathology, DIAL, Udine University). After thawing, bacteria were grown in TSB for 24 h at 22±2°C, then culture broth was centrifuged at 2000 x *g* for 20 min. The supernatant was discarded and the pellet was resuspended in sterile PBS (pH 7.3). The suspension was adjusted to an optical density (OD) at 610 nm of 1.0 using a spectrophotometer (Tecan Sunrise), corresponding to 1 x 10^9 colony

forming units (CFU)/ml. The bacterial concentration was confirmed by spreading serial 10-fold dilutions of the suspension onto TSA plates in duplicate and counting the number of CFU following 24 h incubation at 22±2°C.

Fish from each treatment group (30 fish/group, 10 fish from each replicate) were anaesthetized by immersion in benzocaine (0.03 g/l) and intraperitoneally (IP) challenged with 0.1 ml/fish of PBS containing 2.2 x10^4 CFU/ml, then were randomly distributed into 3 aerated replicate 140 l tanks (10 fish/tank), being part of a non-circulating system. The infection dose was based on preliminary experiments aimed to determine the DL70 in control unvaccinated fish. A group of fish was IP injected with 0.1 ml/fish of PBS and served as placebo. Mortalities were recorded daily over a period of two weeks (15 days) and any moribund fish was removed and bacteriologically examined to confirm the presence of *Y. ruckeri*. The relative percentage survival (RPS) was calculated as described by Amend (1981) for vaccines: RPS = [1 – (mortality (%) in infected ginseng group/mortality (%) in infected control group)] × 100.

4.2.11 Statistical analysis

Values of each physiological parameter measured were expressed as arithmetic mean ± standard error (SE). Statistical analysis were performed using the Statistical Package for Social Science (SPSS version 20.0, Inc. Chicago, IL, USA) suite for Windows. The assumptions of normality and homogeneity of variances were checked using the Shapiro-Wilk and Levene tests, respectively. Data were statistically analysed by one-way analysis of variance (ANOVA) to test differences between all treatments. If appropriate, Duncan *post hoc* test was applied to compare individual means and to determine significant differences among dietary treatment groups. If the variances were not normally distributed, the Kruskal–Wallis non-parametric test and the relative post hoc test (Mann-Whitney *U* test) were used. The results of the *in vivo* bacterial challenge were analysed using Fisher's exact test (two tailed). Differences were considered statistically significant when $P < 0.05$.

4.3 Results

4.3.1 Antibacterial activity of ginseng extract

The ethanolic extract of *P. ginseng* root demonstrated inhibitory effects when tested for antibacterial activity against both the pathogenic bacterial strains of *Y. ruckeri* and *L. garvieae*, using the broth micro-dilution method (Table 3). Specifically, the MIC value of ginseng extract against *Y. ruckeri* was equal to 4.3 mg/ml and against *L. garvieae* was equal to 8.7 mg/ml. The

MIC value of oxytetracycline used as reference antibiotic against both pathogens was 1.0 μg/ml. The extract of ginseng showed a bacteriostatic action against both pathogens at MICs. The MBC was equal to 17.3 mg/ml against *L. garvieae*, while the MBC against *Y. ruckeri* was not detectable within the range of concentrations tested.

Table 3 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *P. ginseng* root extract against *Y. ruckeri* and *L. garvieae*. Values ar referred to three independent tests performed in triplicate.

Items	***Yersinia ruckeri***		***Lactococcus garvieae***	
	MIC	MBC	MIC	MBC
Panax ginseng	4,3 mg/ml	ud	8,7 mg/ml	17,3 mg/ml
Oxytetracycline (reference)	1,0 μg/ml		1,0 μg/ml	

MBC = minimal bactericidal concentration; MIC = minimal inhibitory concentration; ud = undetectable.

4.3.2 Growth performance

The physiological indices, including feed intake (FI), wight gain (WG%), specific growth rate (SGR) and feed conversion ratio (FCR) are shown in Table 4. Overall, feed intake and growth performance did not significantly change in the groups of rainbow trout fed diets supplemented with ginseng extract compared to the control group ($P > 0.05$).

Table 4 Feed intake and growth performance of rainbow trout fed the experimental diets over 10 weeks. Data are expressed as mean ± SE (3 tanks/group). Data were statistically analysed by one-way ANOVA. *P* values < 0.05 were considered as significant.

Variable	**Ginseng levels**			
	Control (0%)	0.01%	0.02%	0.03%
FI[a]	779.4 ± 2.56	760.9 ± 15.68	744.6 ± 47.89	767.7 ± 18.92
IBW[b]	30.4 ± 0.07	30.6 ± 0.11	30.4 ± 0.04	30.4 ± 0.02
FBW[c]	84.7 ± 0.63	83.0 ± 1.71	83.9 ± 2.34	84.4 ± 0.83
WG%[d]	179.1 ± 1.91	171.4 ± 4.74	175.8 ± 7.98	177.4 ± 2.89
SGR[e]	1.47 ± 0.0098	1.43 ± 0.0248	1.45 ± 0.0417	1.46 ± 0.0150
FCR[f]	0.70 ± 0.0115	0.71 ± 0.0207	0.68 ± 0.0663	0.69 ± 0.0329

[a]Feed intake (g)
[b]Initial body weight (g)
[c]Final body weight (g)
[d]Weight gain %
[e]Specific growth rate
[f]Feed conversion ratio
Data are expressed as mean ± SE (3 tanks/group).

4.3.3 Blood biochemical profile

Plasma biochemical parameters are reported in Table 5. The results show that total proteins (TP) and globulins (GLB) did not significantly change in fish fed ginseng extract enriched diets compared to fish fed the basal diet ($P > 0.05$). Albumin (ALB) level was significantly lower in rainbow trout fed G1 and G3 diets compared to fish fed G0 and G2 diets ($P < 0.05$). In this study, glucose (GLU) was lower in fish fed diets containing ginseng extract compared to controls, but no significant differences were noted between experimental groups ($P > 0.05$). Triglycerides (TRIG) level was found significantly higher in fish receiving G1 and G2 diets compared to the other groups ($P < 0.05$) while a decrease in cholesterol (CHO) was measured in fish fed ginseng enriched diets with respect to fish fed the control diet, although the differences among groups were not statistically significant ($P > 0.05$). The measurement of NEFA in the blood samples showed that they were higher in rainbow trout fed ginseng extract supplemented diets, especially in G2 group ($P < 0.05$). Dietary ginseng led to negligible effects on plasma ALT, AST and ALP levels ($P > 0.05$).

Table 5 Plasma biochemical parameters of rainbow trout fed the experimental diets over 10 weeks. Data are presented as mean ± SE (n = 6). Data were statistically analysed by one-way ANOVA followed by Duncan's multiple comparison test or by Kruskal–Wallis non-parametric test and relative *post hoc* Mann-Whitney U test. *P* values < 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

Parameters	G0	G1	G2	G3
TP (g/dl)	2.4 ± 0.06	2.3 ± 0.08	2.5 ± 0.06	2.3 ± 0.03
ALB (g/dl)	1.6 ±0.04**a**	1.4 ± 0.04**b**	1.6 ± 0.06**a**	1.4 ± 0.06**b**
GLB (g/dl)	0.8 ± 0.03	0.9 ± 0.07	0.8 ± 0.04	0.9 ± 0.09
GLU (mg/dl)	95.5 ± 3.07	84.3 ± 3.21	86.7 ± 4.20	92.2 ± 2.50
CHO (mg/dl)	148.8 ± 6.60	134.3 ± 8.41	140.0 ± 9.50	136.7 ± 7.21
TRIG (mg/dl)	158.2 ± 8.21**b**	219.8 ± 19,16**a**	249.1 ± 26.40**a**	152.6 ± 12.10**b**
NEFA (mmol/l)	0.6 ± 0.02**b**	0.7 ± 0.05**ab**	0.7 ± 0.03**a**	0.6 ± 0.03**b**
AST (U/l)	44.3 ± 4.57	38.7 ± 1,99	46.8 ± 3.53	34.0 ± 3.00
ALT (U/l)	3.4 ± 1.29	3.8 ± 1.24	4.2 ± 1.33	4.2 ± 1.40
ALP (U/l)	9.5 ± 3.08	17.0 ± 3.56	14.3 ± 1.36	14.5 ± 2.08

4.3.4 Innate immune response

The inclusion of ginseng extract in the diet slightly enhanced serum lysozyme activity of rainbow trout, especially in groups receiving 0.01% and 0.02%, although no significant differences were detected between fish fed diets supplemented with herbal extract and fish fed the basal diet ($P > 0.05$). The activity was 635.4 ± 15.61 U/ml, 609.3 ± 9.01 U/ml and 586.1 ± 26.46 U/ml in the groups G1, G2 and G3 respectively, compared to 564.1 ± 28.84 U/ml in G0 group (Fig. 1).

The dietary supplementation with ginseng extract did not show any significant effect on serum antiprotease activity ($P > 0.05$). In particular, serum antiprotease activity expressed as the percentage of trypsin inhibition was 66.3 ± 1.96 in G0 group, 59.7 ± 2.15 in G1 group, 61.5 ± 1.44 in G2 group and 60.7 ± 2.22 in G3 group (Fig. 2). Even if this parameter was lower in fish fed ginseng enriched diets compared to fish fed the control diet, the differences were not significant.

The respiratory burst activity of HK leukocytes stimulated with PMA was higher in fish fed diets containing all doses of ginseng extract compared with that of fish fed the control diet. The chemiluminescence response was 13703 ± 1808 RLU, 13073 ± 1075 RLU and 14197 ± 1140 RLU in the groups G1, G2 and G3 respectively, compared to 11068 ± 1356 RLU in G0 group (Fig. 3). Nevertheless, the differences among different dietary treatments were not statistically significant ($P > 0.05$).

The MPO content of rainbow trout HK leukocytes was not significantly modulated after feeding with ginseng extract supplemented diets compared with the control diet ($P > 0.05$). Specifically, 0.7 ± 0.09 O.D., 0.7 ± 0.10 O.D., 0.7 ± 0.07 O.D. were recorded in fish which receiving diet G1, diet G2, diet G3 respectively, compared to 0.7 ± 0.06 O.D. in the controls (Fig. 4).

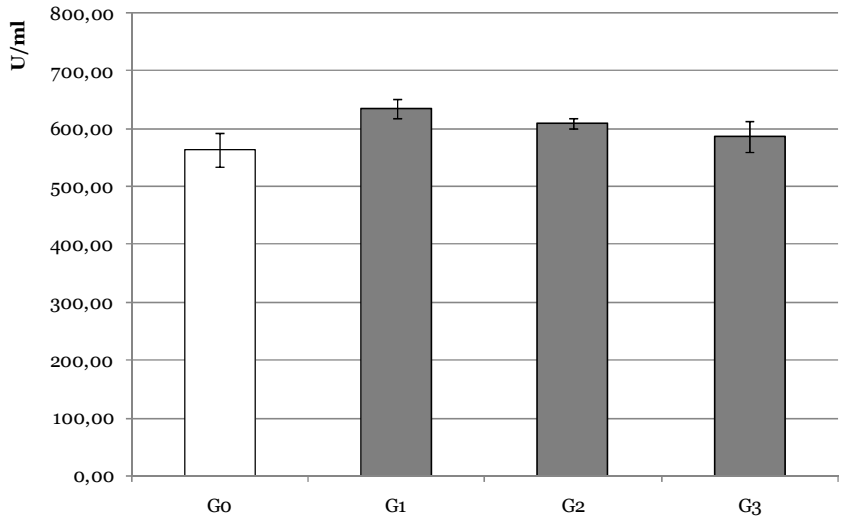

Fig. 1 Lysozyme activity (U/ml) in rainbow trout fed diets supplemented with 0% (G0), 0.01% (G1), 0.02% (G2) and 0.03% (G3) *P. ginseng* extract. Data are expressed as mean ± SE (n = 12). Data were statistically analysed by one-way ANOVA. *P* values < 0.05 were considered as significant.

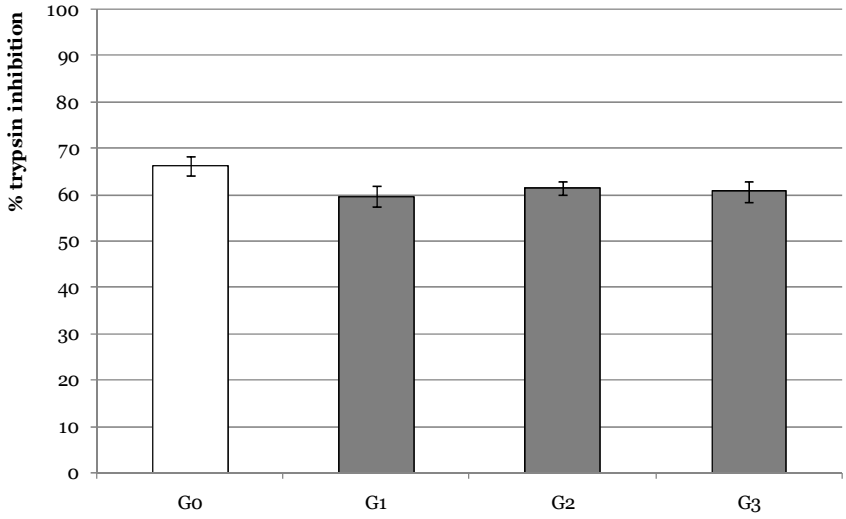

Fig. 2 Antiprotease activity (% trypsin inhibition) in rainbow trout fed diets supplemented with 0% (G0), 0.01% (G1), 0.02% (G2) and 0.03% (G3) *P. ginseng* extract. Data are expressed as mean ± SEM (n = 12). Data were statistically analysed by one-way ANOVA. *P* values < 0.05 were considered as significant.

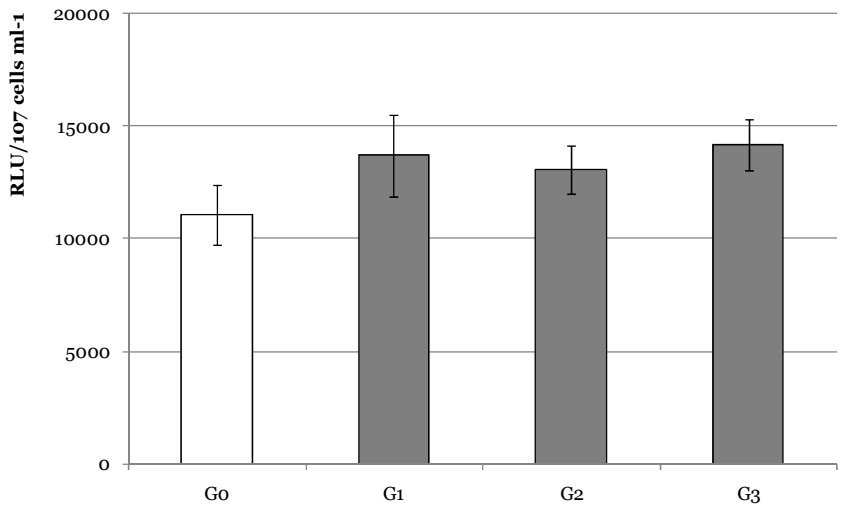

Fig. 3 Respiratory burst activity of leukocytes (RLU/10^7 cells ml^{-1}) in rainbow trout fed diets supplemented with 0% (G0), 0.01% (G1), 0.02% (G2) and 0.03% (G3) *P. ginseng* extract. Data are expressed as mean ± SEM (n = 6). Data were statistically analysed by one-way ANOVA. *P* values < 0.05 were considered as significant.

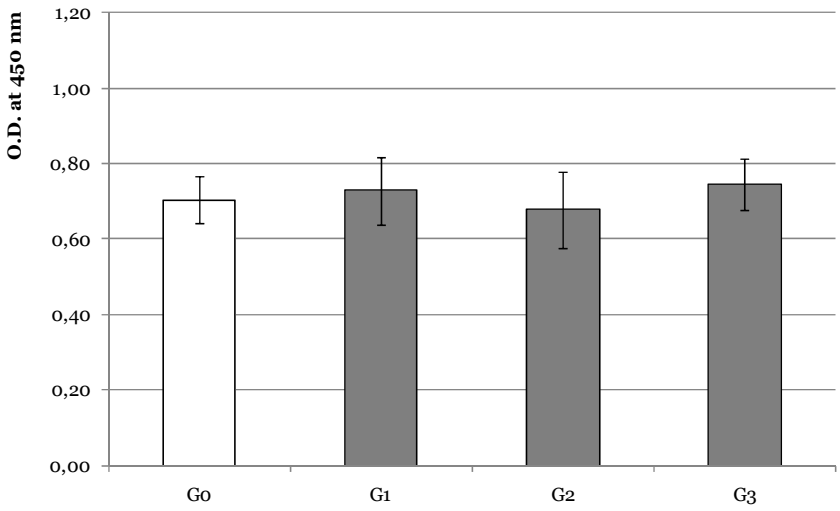

Fig. 4 Myeloperoxidase content of leukocytes (O.D. at 450 nm) in rainbow trout fed diets supplemented with 0% (G0), 0.01% (G1), 0.02% (G2) and 0.03% (G3) *P. ginseng* extract. Data are expressed as mean ± SEM (n = 6). Data were statistically analysed by one-way ANOVA. *P* values < 0.05 were considered as significant.

4.3.5 Disease resistance

The post-challenge symptomatic rainbow trout were lethargic and showed haemorrhages on the body surface, with reddening at the base of the fins and along the lateral line, as well as in the head region and around the oral cavity. Petechial haemorrhage on the surface of the liver and in the lateral musculature were also observed. Fish showed expanded abdomen, the spleen was enlarged and the intestine was inflamed with an opaque and purulent fluid which was released from the anus when slight pressure was applied to the abdomen. Mortalities in fish infected with *Y. ruckeri* were observed from the second day post-challenge in all experimental groups. The greatest increase in cumulative mortality occurred at 6th day post-infection in G0 group (control), at 8th day post-infection in G1, G2 and G3 groups. After the 8th day, there was no mortality up to the 15th day.

Feeding with ginseng extract led to a delay in the onset of disease and to a reduction of cumulative mortality compared to the controls fed the basal diet, albeit the differences between experimental groups were not statistically significant during the trial ($P > 0.05$). At the conclusion of the experiment (15 days post challenge), fish fed the control diet (0% herbal extract) suffered the maximum mortality of 93%. The total registered percentages of cumulative mortality were 83% (RPS = 10.71%), 90% (RPS = 3.57%) and 87% (RPS = 7.14%) in rainbow trout fed 0.01%, 0.02% and 0.03% ginseng supplemented diets, respectively (Fig. 5). In the placebo group (I.P. injected with PBS) no mortality was recorded (data not shown).

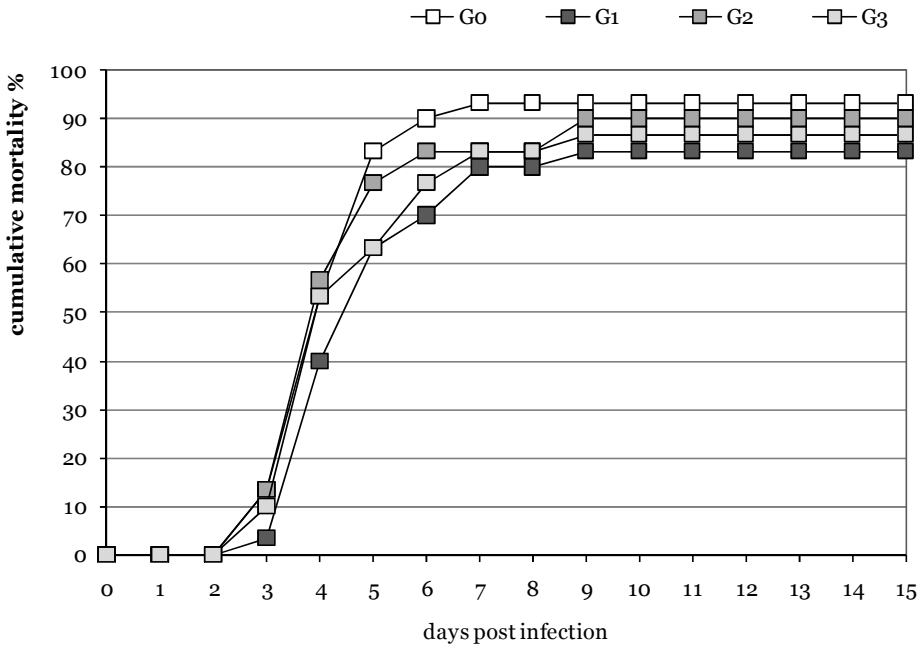

Fig. 5 Cumulative mortality (%) of rainbow trout fed diets supplemented with 0% (G0), 0.01% (G1), 0.02% (G2) and 0.03% (G3) *P. ginseng* extract and infected with *Yersinia ruckeri* 2,2x10^4 UFC/ml (n = 30). Data were statistically analysed by Fisher's exact test (two tailed). *P* values < 0.05 were considered as significant.

4.4 Discussion

The inclusion of medicinal herbs or botanical active compounds in the diet is one of the most promising methods for promoting fish growth and immune response, as well as conferring protection from infectious diseases. The use of plants and plant extracts had been introduced in early centuries for various human and animal health benefits, and recently it has been applied with good results also in aquaculture (Harikrishnan *et al.*, 2011a; Chakraborty *et al.*, 2014; Reverter *et al.*, 2014; Bulfon *et al.*, 2015). *P. ginseng* is a popular and widely-used herbal remedy in traditional Chinese medicine. It is well-considered to possess a broad-spectrum of biological activities including immunostimulant as evidenced by both humoral and cell-mediated responses, antinflammatory, antioxidant, antipyretic, antigastric ulcer, antidiabetes, antibacterial, moreover it demonstrated beneficial effects against neurodegenerative diseases, cardiovascular disorders and cancer (Lakshmi *et al.*, 2011). Although numerous positive effects have been documented on human and animal health, few studies have assessed the efficacy of ginseng as potential immunostimulant or growth promoter in fish and shellfish (Goda, 2008; Won *et al.*, 2008; Liu *et al.*, 2011; Abdel-Tawwab, 2012; El-Sayed *et al.*, 2014). Still, the mechanisms by which the components of this plant exert a positive influence on fish remain unclear. Proximate composition of ginseng includes triterpene saponins (commonly named ginsenosides), aminoacids, alkaloids, polyphenols, polysaccharides, polypeptides, vitamins B1

and B2 (Lakshmi *et al.*, 2011; Kim, 2012a). The bioactive saponins and polysaccharides found in ginseng may directly affect fish health by activating immune mechanisms as reported in mammals and humans (Hofmann, 2003; Tan & Vanitha; Kang & Min, 2012). In general, plant polyphenols and flavonoids display well recognised antioxidant properties, have a role in the prevention of infections and also a hypoglycaemic potential (Scalbert *et al.*, 2005). Moreover, saponins show cholesterol-lowering effects, hypoglycaemic activity, antimicrobial and anti-inflammatory properties (Hofmann, 2003).

Hence, the present study was carried out to investigate the effects of the dietary administration of *P. ginseng* root ethanolic extract on growth, blood biochemical parameters, non-specific immune response and resistance against *Y. ruckeri* in rainbow trout (*O. mykiss*). To the best of our knowledge, the present research is the first report that investigates the inclusion of ginseng extract in the diet as potential immunostimulant for *O. mykiss*.

In this survey, rainbow trout fed diets supplemented with ginseng extract did not show significant differences in feed intake, growth performance and feed utilization compared to the control group. From a nutritional point of view, it has been proposed that the mechanism by which *P. ginseng* can improve growth in fish may consist in the inhibition of the digestive tract colonization of potential pathogens, in enhancing the population of beneficial microorganism and microbial enzyme activity, in improving intestinal microbial balance and consequently feed digestibility and nutrient absorption. Another possible explanation may be the improvement in nutrient utilization throughout a modulation of trypsin-like enzymes activities during the digestive processes (Goda, 2008). In rainbow trout, other medicinal plants such as *Urtica dioica*, *Viscum album* (Dügenci *et al.*, 2003), *Cotinus coggyria* (Bilen *et al.*, 2011), *Camellia sinensis* (Nootash *et al.*, 2013), *Nasturtium nasturtium* (Asadi *et al.*, 2012) did not exhibit evident effects on growth performance. On the other hand, the dietary supplementation with 0.05%-1% *Allium sativum* and *Zingiber officinale* for 2 weeks significantly increased WG, SGR and PER values, and decreased FCR indices in *O. mykiss* (Nya & Austin, 2009a; 2009b).

The analysis of blood biochemical parameters is a diagnostic tool that could be useful for monitoring fish health and physiological status in relation to diet, environmental changes (temperature, salinity, pollutants…), infectious diseases, therapeutic treatments or stress. In this investigation, plasma samples were analysed using an automated analyser system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy), commonly used for human and veterinary medicine. It has already been shown that these biochemical analysers can be also used with good reliability to estimate biochemical parameters in fish blood samples (Coz-Rakovac *et al.*, 2005, 2009; Manera & Britti, 2006).

Total proteins include albumin, globulins and various humoral elements of the non-specific immune system, such as transferrin, precipitins, agglutinins, antimicrobial peptides, complement factors, lysozyme and antiproteases (Magnadottir, 2006). Albumin is essential for

maintaining the osmotic pressure of body fluids and acts as a plasma carrier or non-specific ligand (Nya & Austin 2009a). The globulin fractions, including subunits of α1, α2, β, and γ globulins, are considered as the source of almost all the immunologically active proteins in the blood, which are essential for maintaining fish immune status. Commonly, increases in serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fish (Das *et al.*, 2013). In this study, plasma total proteins and globulins did not significantly change in fish fed diets containing ginseng extract compared to fish fed the basal diet while albumin level was significantly lower in rainbow trout received 0.01% and 0.03% ginseng enriched diets. On the contrary, rainbow trout fed *A. sativum* and *Z. officinale* supplemented diets for 2 weeks showed elevated serum proteins and globulins (Nya & Austin, 2009a; 2009b) and similar results were detected in *O. mykiss* fed *N. nasturtium* enriched diets for 3 weeks (Asadi *et al.*, 2012). These findings are in agreement with other investigations, in which feeding rainbow trout with diets supplemented with 0.1% and 1% *U. dioica* or *V. album* (Dügenci *et al.*, 2003; Awad *et al.*, 2013), 1%-5% *Nigella sativa* (Dorucu *et al.*, 2009; Awad *et al.*, 2013), 0.5% and 1% *C. coggyria* (Bilen *et al.*, 2011), and 1% *Mangifera indica* (Awad & Austin, 2010), 0.002%-0.05% *C. sinensis* (Nootash *et al.*, 2013) led to an increase in serum total proteins. Moreover, Ahmadi *et al.* (2012) reported that plasma total proteins, albumin and globulins significantly increased in rainbow trout fed diets enriched with silymarin extract (*Silybum marianum*) during a 30-day experiment. However, the dietary supplementation with *Laurus nobilis* (Bilen & Bulut, 2010), *Lupinus perennis* or *U. dioica* (Awad & Austin, 2010) did not influence the level of proteins in this fish species.

Glucose is one of the most important sources of energy for the animals. Generally, glycemia is selected in fish as an indicator of stress caused by infections or physical factors. In fact, a rise in the concentration of blood glucose is normally measured in fish almost immediately following stress, inducing the breakdown of glycogen from liver, mainly through glycogenolysis (Das *et al.*, 2013). In the present work, plasma glucose decreased in all groups of rainbow trout treated with *P. ginseng* supplemented diets, although differences were not statistically significant when compared to fish fed the basal diet. These findings suggest that the bioactive compounds such as polyphenols, flavonoids and saponins contained in ginseng extract could have a hypoglycaemic potential in fish and might stimulate the insulin activity, thus reducing the blood glucose level. A significant decrease in blood glucose was measured by several authors after feeding with plant-derived products in different fish species (Sahu *et al.*, 2007a; 2007b; Immanuel *et al.*, 2009; Das *et al.*, 2013; Talpur & Ikhwanuddin, 2013; Talpur *et al.*, 2013; Talpur, 2014).

Ginseng-added feed induced a significant increase of plasma triglycerides and NEFA, especially in rainbow trout fed 0.01% and 0.02% supplemented diets. Instead, cholesterol level was lower in fish treated with ginseng compared to control group, although the differences among experimental groups were not statistically significant. In this regard, other authors noted a

decrease in the levels of blood lipids in fish species fed with medicinal plants (Shalabay *et al.*, 2006; Immanuel *et al.*, 2009; Talpur & Ikhwanuddin, 2013; Talpur *et al.*, 2013; Talpur, 2014). In particular, Mohebbi *et al.* (2012) found lower cholesterol, triglycerides and LDL-CHO in rainbow trout fed diets supplemented with 1%-5% *A. sativum* bulbs for 8 weeks when compared to controls. Although further investigations are needed to clarify the effects of ginseng on rainbow trout blood lipids, our results suggest that ginseng might display cholesterol-lowering effects also in fish, which are probably due to its content of saponins. It has been reported that saponins present in other medicinal plants might be responsible of hypolipidemia in fish. For example, Talpur & Ikhwanuddin (2013) and Talpur *et al.* (2013) attribute to such compounds the reduction of total lipids levels found in the blood of Asian seabass (*Lates calcifer*) fed diets enriched with neem or ginger.

ALT and AST are common indicators of liver or kidney functions and can be used to detect damage of these organs induced by diseases, toxins or a disturbance in the Kreb's cycle that may result in their increase. ALP is an important hydrolase enzyme, which is mainly secreted from liver/bones and helps in dephosphorylation of proteins and nucleotides. In fish its level varies from juveniles to adults, but lower and higher levels of this enzyme are correlated to some harmful diseases (Verma *et al.*, 2013). In the present investigation, through the evaluation of ALT, AST and ALP in the blood, the treated fish were checked for any adverse effects on liver and kidney due to the inclusion of ginseng in feed. There were no marked changes in ALT, AST and ALP levels in plasma of rainbow trout fed with control and supplement feed, indicating that ginseng extract does not have toxic effects on fish. Also, there were no visual symptoms of stress or disease, and feeding behaviour of rainbow trout was normal during the experimental trial. Anyway, a range of evidences demonstrated that the dietary supplementation with medicinal plants reduced ALT and AST, and increased ALP, suggesting beneficial effects on fish liver and kidney status (Shalabay *et al.*, 2006; Vasudeva *et al.*, 2006; Sahu *et al.*, 2008; Pratheepa *et al.*, 2010; Liu *et al.*, 2012; Verma *et al.*, 2013).

In order to assess the effects of ginseng on the immune responses of rainbow trout, serum lysozyme and antiprotease activity as well as leukocyte respiratory burst and MPO content were estimated. It has been shown that herbal extracts-based immunostimulants are capable of enhancing both humoral and cellular immune responses and reducing losses from microbial infections in different fish species (Vasudeva & Chakrabarti, 2005; Christybapita *et al.*, 2007; Ardò *et al.*, 2008; Immanuel *et al.*, 2009; Yin *et al.*, 2009; Kirubakaran *et al.*, 2010; Harikrishnan *et al.*, 2011b; 2011c; 2011d; 2011e; 2011f; Kaleeswaran *et al.*, 2011; 2012; Harikrishnan *et al.*, 2012a; 2012b; Park & Choi, 2012; Das *et al.*, 2013; Wu *et al.*, 2013; Samad *et al.*, 2014).

The results of this study revealed a slightly enhancement of serum lysozyme activity of rainbow trout in all treatment groups after feeding for 10 weeks, especially in fish receiving 0.01% and

0.03% ginseng extract, although the differences among dietary treatments were not statistically significant. Lysozyme is a bactericidal enzyme that hydrolyses the β-1,4 glycosidic linkage between N-acetylglucosamine and N-acetyl muramic acid of bacterial cell wall peptidoglycan, thereby causing bacteriolysis and preventing the growth of bacteria. In fish, lysozyme is present in mucus, lymphoid tissue, plasma and other body fluids, and it is well recognized as activator of complement system and phagocytes, by acting as an opsonin (Magnadottir, 2006; Saurabh & Sahoo, 2008). In rainbow trout, a stimulation of this parameter has been reported after using various herbal immunostimulants. For example, lysozyme activity was significantly increased in rainbow trout fed 0.01% *C. sinensis* extract (Sheikhzadeh *et al.*, 2011) enriched diets for 30 days compared to the control group. A significant increase in plasma lysozyme activity was observed in rainbow trout fed for 21 days with 1% watercress enriched diet (Asadi *et al.*, 2012). Similarly, the oral administration of 0.5% and 1% *C. coggyria* powder for 3 weeks (Bilen *et al.*, 2011), 1% *L. perennis* seeds, *M. indica* fruits or *U. dioica* leaves for 2 weeks (Awad & Austin, 2010), 2% and 3% *N. sativa* oil for 2 weeks (Awad *et al.*, 2013) in rainbow trout significantly enhanced lysozyme activity compared to fish fed the basal diet. Moreover, improved serum lysozyme activity was observed in rainbow trout fed diets enriched with 0.1% and 1% *A. sativum* bulbs (Nya & Austin, 2009a; 2011) or 0.05% and 0.1% *Z. officinale* rhizomes (Nya & Austin, 2009b) for 2 weeks. On the other hand, dietary *Aloe vera* (Zanuzzo *et al.*, 2015) and *L. nobilis* (Bilen & Bulut, 2010) inclusion had no effect on serum lysozyme activity in trout.

In the present investigation, the dietary supplementation with ginseng extract did not significantly affect serum antiprotease activity of rainbow trout. Serum antiproteases, principally α1-antiprotease, α2- antiplasmin and α2- macroglobulin, are protease inhibitors involved in acute phase reactions that may play an important role in restricting the ability of bacteria to invade and grow *in vivo* (Ellis, 2001). Their activity has been reported to be enhanced in rainbow trout fed diets supplemented with 0.002% and 0.01% *C. sinensis* extract for 30 days (Sheikhzadeh *et al.* 2011) and diets enriched with 0.05%-1% *Z. officinale* for 2 weeks (Nya & Austin 2009b). Furthermore, a significant increase of serum antiprotease activity was demonstrated in *O. mykiss* fed 2% and 3% doses of *N. sativa* oil for 2 weeks compared to fish fed the basal diet (Awad *et al.*, 2013). In contrast, no variations in the antiproteases level were observed in rainbow trout after feeding for 2 weeks with garlic (Nya & Austin, 2009a), lupin, mango and nettle (Awad & Austin, 2010) enriched diets.

Phagocytes are the most important cellular components of the innate immune system in fish. Neutrophils and macrophages migrate from the circulating blood into infected tissues in response to inflammatory stimuli, and efficiently bind, engulf, and kill the invading pathogens via the activity of proteolytic enzymes and antimicrobial proteins, coupled with the generation of microbicidal reactive oxygen and nitrogen species (ROS and RNS). ROS, such as superoxide anion, singlet oxygen and hydrogen peroxide, are produced by activated phagocytes during a

process called respiratory burst. The oxidative killing mechanism and the activity of myeloperoxidase (MPO), an enzyme contained in the cytoplasmic azurophilic granules of neutrophils, are considered as indicators of non-specific defense in fish. Their increased in blood and HK phagocytes can be correlated with enhanced pathogen killing capacity and disease resistance in fish (Neumann *et al.*, 2001; Rieger & Barreda, 2011).

In the present work, the respiratory burst activity was measured by luminol-enhanced chemiluminescence in rainbow trout HK leukocytes activated with phorbol 12-myristate 13-acetate (PMA). Luminol can cross biological membranes due to its chemical structure, allowing the detection of extracellular and intracellular production of oxygen radicals (Vera-Jimenez *et al.*, 2013). Rainbow trout fed diets containing ginseng extract showed higher respiratory burst activity compared with fish fed the control diet, albeit the differences were not statistically significant. Enhanced intracellular superoxide anion production by phagoytic cells, as examined by nitroble tetrazolium (NBT), has been reported in rainbow trout after feeding for 2 weeks with 0.1% and 0.5% *A. sativum* (Nya & Austin, 2009a; 2011) or 0.05%-1% *Z. officinale* (Nya & Austin, 2009b). Similar results were also obtained in rainbow trout fed with 1% lupin, *L. perennis*, mango, *M. indica* or stinging nettle *U. dioica* for 2 weeks (Awad & Austin, 2010). A significant increase of extracellular ROS production, as measured using cytochrome c, was found in *O. mykiss* fed 1% *Z. officinale* (Dügenci *et al.*, 2003) and 0.5% *L. nobilis* (Bilen and Bulut, 2010) enriched diets. Moreover, ROS production was significantly improved in rainbow trout fed diets supplemented with 0.5% and 1% *C. coggyria* for 3 weeks (Bilen *et al.*, 2011). Rainbow trout fed 1% *A. vera* extract supplemented diet showed a significant increase in ROS production of blood leukocytes compared with untreated control group (Haghighi *et al.*, 2014a; 2014b). On the other hand, the inclusion of *N. sativa* (Dorucu *et al.*, 2009), *U. dioica* and *V. album* extract (Dügenci *et al.*, 2003) in the diet failed to influence respiratory burst activity in rainbow trout.

The present study revealed that MPO content of rainbow trout HK leukocytes was not significantly modulated after feeding for 10 weeks with ginseng extract supplemented diet. Our results are in agreement with the observations of other researchers, which described negligible effects on serum MPO activity in rainbow trout fed 0.5% and 1% *A. sativum* (Nya & Austin, 2011), 1% *L. perennis*, *M. indica* or *U. dioica* (Awad & Austin, 2010) enriched diets for 2 weeks. Instead, an increase of serum MPO activity, which is correlated to a stimulation of blood neutrophils, was demonstrated in rainbow trout fed diet containing 0.002%-0.05% *C. sinensis* extract (Sheikhzadeh *et al.*, 2011) or 1% *N. sativa* oil (Awad *et al.*, 2013) compared to the control group.

The efficacy of an immunostimulant ultimately depends on its ability to increase the host resistance to infection. At the end of the feeding trial, we tested the resistance of rainbow trout against *Y. ruckeri*. An experimental challenge provides an opportunity to evaluate the

effectiveness of the test diets in terms of protection against pathogens. *Y. ruckeri* is the causative agent of yersiniosis or enteric redmouth disease (ERM) and causes significant economic losses in salmonid farming industry worldwide. Although infections with this bacterium have been reported in other fish species, salmonids and especially rainbow trout are the most susceptible to ERM, which is characterized by a septicaemic condition with haemorrhages on the body surface, in particular around and within the oral cavity, and in the internal organs (Tobback *et al.*, 2007). The cumulative mortality in rainbow trout fed 0.01%, 0.02% and 0.03% ginseng enriched diets after challenge with *Y. ruckeri* was slightly reduced (83%, 90% and 87% respectively) compared to the 93% in infected untreated group, although the differences were not statistically significant. Other authors reported a significant reduction of mortality rate in *O. mykiss* fed with garlic (Nya & Austin, 2009a; 2011), *L. perennis*, *M. indica*, *U. dioica* (Awad & Austin, 2010) and *Z. officinale* (Nya & Austin, 2009b) after *A. hydrophila* infection.

In the present study, *P. ginseng* ethanolic extract was also preliminarily *in vitro* screened for its inhibitory activity against the bacterial fish pathogens *Y. ruckeri* and *L. garvieae*, that commonly affect rainbow trout. The results indicated that ethanolic extract of *P. ginseng* inhibited the growth of both pathogen in broth medium, with a MIC values of 4.3 mg/ml against *Y. ruckeri* and 8.7 mg/ml against *L. garvieae*. This findings are consistent with our previous studies, in which we reported that the extracts of *Lavandula officinalis*, *Melissa officinalis*, *Ocimum basilicum*, *Origanum vulgare*, *Rosmarinum officinalis*, *Salvia officinalis*, and *Vacinum vitis idaea* show inhibitory effects on the growth of these bacteria with MIC value ranging from 4.2 mg/ml and 33.6 mg/ml (Bulfon *et al.*, 2014). The specific components of ginseng extract responsible for this property have not been identified, however the antibacterial activity may be due to its content of saponins, tannins, flavonoids, and alkaloids. Plant saponins have been reported to possess many biological activities and their antibacterial effects seem to involve membranolytic properties, rather than alteration of the surface tension of the extracellular medium. Tannins and flavonoids are polyphenols obtained from various parts of several plants. The antimicrobial action of tannins may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins. The mechanism of antibacterial action of flavonoids is probably due to their ability to complex with extracellular and soluble proteins or with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999). Moreover, recent studies suggested that the inhibition of nucleic acid synthesis may be the primary cause of their antibacterial character (Pakravan *et al.*, 2012). The mode of action of alkaloids remains largely unknown, however, their activity is attributed to their ability to intercalate with DNA and inhibit its synthesis (Cowan, 1999).

Our findings are comparable with the results found by Goda (2008) who studied the effects of diets supplemented with 0.005%, 0.010%, 0.015%, 0.020% and 0.025% Asian ginseng (*P. ginseng*) extract (Ginsana G115) on growth and haematological indices in Nile tilapia (*O.*

niloticus). They reported that fish fed ginseng extract significantly increased growth performance and feed utilization, red blood cells number, haematocrit, haemoglobin, plasma total proteins and globulins with increasing herbal levels compared to the control group. Similarly, El-Sayed *et al.* (2014) noted that tilapia fed 0.06% ginseng extracts significantly increased growth, non-specific (total leukocytic count, total globulins, serum lysozyme and bactericidal activity, respiratory burst activity) and specific (IgM and lymphocyte proliferation) immune parameters, and resistance against *A. hydrophila*. On the other hand, Abdel-Tawwab (2012) demonstrated that the inclusion of 0.2% and 0.5% American ginseng (*Panax quinquefolium*) root powder in the diet of in *O. niloticus* enhanced growth, respiratory burst activity and resistance to *A. hydrophila*. Morover, Won *et al.* (2008) found that olive flounder (*P. olivaceus*) fed diet supplemented with 3% Siberian ginseng (*Eleutherococcus senticosus*) residuum extract for 8 weeks exhibited improved innate immunity, including lysozyme and phagocytes activity, and developed resistance to *E. tarda* and *V. anguillarum* infections.

Our results indicate that the levels of ginseng extract included in the diet are not sufficient to improve growth performance and immune response of rainbow trout, in disagreement with the studies of Goda (2008) and El-Sayed *et al.* (2014) in tilapia, therefore further research need to be performed in order to investigate the effect of higher concentrations of *P. ginseng* extract in rainbow trout diet and to assess the protective effects of ginseng in other farmed fish species, in a view of its potential employ as an immunoprophylactic measure against pathogens. Nevertheless, the application of dietary additives to improve fish growth and immune response should be carefully considered because their favourable roles vary depending on targeting activities of additives, doses, administration method, nutritional value, duration of administration as well as on fish species, age, size, physiological and nutritional status (Kim *et al.*, 2013; Bulfon *et al.*, 2015). Additionally, since some principles contained in *P. ginseng* extract like saponins and polysaccharides have been reported to possess an immunomodulatory potential (Hu *et al.*, 1995; Kenarova *et al.*, 1990; Shin *et al.*, 2002; Lee *et al.*, 2004; Tan & Vanitha, 2004), future researches should include the separation of the bioactive compounds in the extract so as to identify those responsible for possible immunostimulatory properties in fish. Moreover, further investigations should be required to understand the effects of saponins (ginsenosides) and other compounds contained in ginseng extract on rainbow trout intestinal morphology. In fact, it is known that some plant antinutritional factors such as protease inhibitors, amylase and lipase, tannins, saponins, lectins and antivitamins may cause disturbances in the gastrointestinal tract and this condition could impair digestive processes and feeding efficiency (Francis *et al.*, 2001). Furthermore, appropriate studies aimed to explain the variable effects of ginseng extract on rainbow trout intestinal microflora would be recommended. In this regard, recently the effect of diets supplemented with *Thymus vulgaris* essential oil (Navarrete *et al.*, 2010) or with the plant-derived phenols carvacrol and thymol

(Giannenas *et al.*, 2012) on the composition of rainbow trout intestinal microbiota was evaluated, however data concerning this topic in fish are still very scarce.

4.5 Acknowledgements

This work was supported by CRITA project financed by Friuli Venezia Giulia Region and coordinated by Dr. F. Tulli (Aquaculture Section, DIAL, University of Udine). The authors would like to thank EPO S.r.l.-Piante Officinali (Milano, Italy) for providing the ginseng ethanolic extract.

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CHAPTER V

EFFECTS OF DIETARY SUPPLEMENTATION WITH THE MARINE MICROALGAE *TETRASELMIS SUECICA* AND *ISOCHRYSIS GALBANA* ON GROWTH, BLOOD BIOCHEMICAL PROFILE AND INNATE IMMUNE RESPONSE OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

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Article in preparation.

Abstract

In recent years, microalgae received increasing attention as ingredients in animal feeding, due to their high nutritional value. In particular, the inclusion of microalgal biomass or its derivatives in fish diets is an interesting and innovative approach aimed to improve immune response and disease resistance against pathogens. In the present study, a feeding trial was conducted to evaluate the effects of a dietary supplementation with the microalgae *Tetraselmis suecica* and *Isochrysis galbana* on growth, blood biochemical profile and innate immune response of European sea bass (*Dicentrarchus labrax*). Three iso-nitrogenous and iso-lipidic test diets were formulated to contain three different levels of microalgae (0 g/kg, 60 g/kg to replace 6% of fish meal protein and 180 g/kg to replace 18% of fish meal protein) and triplicate groups of fish (mean initial body weight 204 ± 0.7 g) were fed to satiety for 15 weeks. At the end of the feeding trial, growth performance, biochemical (plasma total proteins, albumin, globulins, glucose, cholesterol and tryglicerides) and immunological parameters (serum lysozyme, antiprotease and peroxidase activity, respiratory burst activity and myeloperoxidase content of head kidney leukocytes) were examined. No significant effect on sea bass growth was registered after feeding with *T. suecica* and *I. galbana* supplemented diets compared to fish fed the basal diet ($P > 0.05$). The analysis of blood samples revealed that there were no marked changes in the biochemical parameters evaluated in fish fed diets supplemented with the microalgae when compared with fish fed the control diet ($P > 0.05$), although a decrease in the levels of blood cholesterol and triglycerides with increasing doses of microalgae in the diet was observed. The oral administration of microalgae resulted in an increase of serum lysozyme and antiprotease activity, which was significant in fish fed the diet containing 60 g/kg of microalgae ($P \leq 0.05$). No statistically significant differences were found in the serum peroxidase activity of fish fed *T. suecica* and *I. galbana* enriched diets with regard to control group ($P > 0.05$), however a higher peroxidase activity was measured in fish fed diet containing 60 g/kg of microalgae. The respiratory burst activity and myeloperoxidase content of leukocyte were not significantly affect by the dietary administration of microalgae ($P > 0.05$). These results indicate that *T. suecica* and *I. galbana* can replace up to 18% of fish meal protein in the diet without hampering growth performance and health status of sea bass, and at lower inclusion level are effective in promoting the humoral innate immune response, demonstrating their potential as immunostimulants for fish.

Keywords: *Tetraselmis suecica*, *Isochrysis galbana*, microalgae, sea bass, growth performance, blood biochemical profile, innate immunity.

Riassunto

Recentemente le microalghe hanno ricevuto una crescente attenzione quali ingredienti nell'alimentazione dei pesci, grazie al loro alto valore nutrizionale. In particolare, l'inclusione di microalghe e/o loro derivati nella dieta è un approccio interessante ed alternativo per migliorare la risposta immunitaria e la resistenza alle malattie dei pesci allevati. Nel presente studio, è stata condotta una prova di alimentazione al fine di valutare gli effetti dell'inclusione nella dieta delle microalghe *Tetraselmis suecica* e *Isochrysis galbana* su crescita, profilo biochimico del sangue e risposta immunitaria innata del branzino (*Dicentrarchus labrax*). Tre diete iso-proteiche e iso-lipidiche sono state formulate per contenere tre diversi livelli di microalghe (0 g/kg, 60 g/kg per sostituire il 6% della proteina da farina di pesce e 180 g/kg per sostituire il 18% della proteina da farina di pesce) e gruppi di pesci in triplicato (peso medio iniziale 204 ± 0.7 g) sono stati alimentati a sazietà per 15 settimane. Al termine della prova di alimentazione, sono stati valutati le performance zootecniche e i parametri biochimici (proteine totali, albumina, globuline, glucosio, colesterolo e trigliceridi nel plasma) e immunologici (attività di lisozima, antiproteasi e perossidasi nel siero, attività di "burst respiratorio" e contenuto di mieloperossidasi dei leucociti purificati da rene anteriore). Non sono stati registrati effetti sulle performance di crescita dei branzini a seguito dell'alimentazione con le diete contenenti *T. suecica* e *I. galbana*, rispetto ai pesci alimentati con la dieta di controllo ($P > 0.05$). L'analisi dei campioni di sangue ha rivelato che non ci sono stati cambiamenti significativi dei parametri biochimici nei pesci alimentati con le diete integrate con le microalghe rispetto ai pesci alimentati con la dieta di controllo ($P > 0.05$), anche se una diminuzione dei livelli di colesterolo e trigliceridi nel sangue è stata osservata all'aumentare della dose di microalghe nella dieta. La somministrazione delle microalghe ha determinato un aumento dell'attività sierica di lisozima e antiproteasi, che è risultato significativo nei pesci alimentati con la dieta integrata con una quantità di microalghe pari a 60 g/kg ($P \leq 0.05$). Nessuna differenza significativa è stata rilevata nei livelli sierici di perossidasi nei pesci alimentati con le diete contenenti *T. suecica* e *I. galbana* rispetto al gruppo di controllo ($P > 0.05$), tuttavia una maggiore attività delle perossidasi è stata misurata nei pesci alimentati con la dieta contenente 60 g/kg di microalghe. L'attività di "burst respiratorio" e il contenuto di mieloperossidasi dei leucociti non sono state influenzate in modo significativo dalla somministrazione dietetica di microalghe ($P > 0.05$). Questi risultati indicano che *T. suecica* e *I. galbana* possono sostituire fino al 18% della proteina da farina di pesce nella dieta senza perturbare le performance di crescita e lo stato di salute del branzino, e al livello di inclusione più basso sono efficaci nel promuovere la risposta immunitaria innata, dimostrando il loro potenziale come immunostimolanti per i pesci.

Parole chiave: *Tetraselmis suecica*, *Isochrysis galbana*, microalghe, branzino, performance di crescita, profilo ematochimico, immunità innata.

5.1 Introduction

Aquaculture contributed to 47% of the fish consumed by the world population in the year 2012. During the last three decades (1982-2012) the production of aquaculture has increased from 5 to 90.4 milions tons (including algae), with an average global trend of growth of 8.6% (FAO, 2014), and it is expected to surpass the yield of wild fisheries by 2020–2025 (Tacon, 2003). Increased production is dependent on the adoption of an approach with overall economic management, improved water management, better feeding strategies, more environmentally friendly feeds, genetically fit stocks, improved fish health status and integration with agriculture (Hemaiswarya *et al.*, 2011).

Marine microalgae comprise a vast group of photosynthetic heterotrophic organisms, which are classified according to various criteria, such as cell structure, pigments and substances stored. In recent years, microalgae received increasing attention as novel ingredients in animal feeding due to their high nutritional value, attributed to their content in proteins, essential vitamins (A, B1, B2, B6, B12, C, E, biotin, folic acid and pantothenic acid), long chain polyunsaturated fatty acids LC-PUFAs (e.g. eicosapentanoic acid [EPA], arachidonic acid [AA] and docosahexaenoic acid [DHA]), carbohydrates composed of starch, glucose, sugars and non-digestible polysaccharides (agar, carrageenan, alginate, and β-glucans), minerals (phosphorus, zinc, iron, calcium, selenium, magnesium), pigments, antioxidants, pharmaceutical and biologically active compounds (Brown *et al.*, 1997; Duerr *et al.*, 1998; Gouveia *et al.*, 2008; Christaki *et al.*, 2010; Hemaiswarya *et al.*, 2011; Kim & Kang, 2011; Guedes & Malcata, 2012).

Microalgae are required for larval nutrition during a brief period, either for direct consumption in the case of molluscs and peneid shrimp or indirectly as food for the live prey fed to small fish larvae. Over the last decades, several hundred microalgal species have been tested as feed in larval nutrition, but probably less than twenty have experienced a widespread application. In fact, in order to be used in aquaculture, microalgal strains have to meet various criteria. They must be of an appropriate size and shape for larval ingestion and readily digested to make the nutrients available. They must have rapid growth rates, be amenable to mass culture, and also be stable in culture to any fluctuations in temperature, light and nutrients as may occur in hatchery systems. Finally, they must have a high nutritional value, which is also dependent on culturing conditions, and including an absence of toxins that might be transferred to the food chain. Microalgal species belonging to the genus *Chlorella, Tetraselmis*, *Isochrysis*, *Pavlova*, *Navicula*, *Phaeodactylum*, *Chaetoceros*, *Nanochloropsis*, *Skeletonema* and *Thalassiosira* are the most frequently used in aquaculture feeding (Brown *et al.*, 1997; Hemaiswarya *et al.*, 2011). Single marine microalgae or a combination of different marine microalgal species are currently utilized in larval nutrition and they are also included in the diets of juvenile and adult molluscs (e.g. oysters, scallops, clams and mussels), crustacean and some fish species, as well as for

raising the zooplankton required for feeding larval stages of finfish (Brown *et al.*, 1997; Spolaore *et al.*, 2006; Hemaiswarya *et al.*, 2011). It has been reported that a carefully selected mixture of microalgae provides a well balanced nutrition and effectively improves fish growth better than a diet composed of only one algal species (Hemaiswarya *et al.*, 2011; Guedes & Malcata, 2012).

In particular, certain marine microalgae currently deserve a growing interest in aquaculture as alternatives to fish meal and oil due to their high content of proteins and LC-PUFAs. Fish meals and oils are nearly ideal protein and lipid sources in the diet of farmed carnivorous fish species, especially those with high value like salmon and trout. However, recent estimates indicate that, due to the gap between the high demand for these commodities by the growing aquaculture industry worldwide and the unsustainable room for further exploitation of the ocean resources, their use in aquafeeds will be dramatically cut down in the next 10 years (Tacon & Metian, 2008). Such a realistic forecast stresses the future strategic role of a continuous evaluation and research on sustainable alternatives to fish-based ingredients. Microalgae constitute a unlimited resource which can be produced in industrial quantities under environmentally safe conditions, controlling the species and culture media (pH, temperature, aeration, light intensity, age of culture) to design particular fatty acid and proteins compositions, and are indeed potentially promising ingredients for the feed industry (Brown *et al.*, 1997; Hemaiswarya *et al.*, 2011). The studies performed so far have proven the possibility to use microalgae as fish oil and meal valuable substitutes in relatively small amounts in fish diet, without hampering growth performance and major quality traits of fish (Olvera-Novoa *et al.*, 1998; Miller *et al.*, 2007; Ganuza *et al.*, 2008; Palmegiano *et al.*, 2009; Kiron *et al.*, 2012; Tulli *et al.*, 2012; Stadtlander *et al.*, 2013; Abdulrahman & Ameen, 2014; Tibaldi *et al.*, 2015). However the information on the use of marine microalgae as ingredients in fish diets are still limited and many aspects concerning type, dose and administration timing need to be elucidated.

Moreover, apart from being potential sources of macronutrients, marine microalgae could also be considered as natural "nutraceutic packages" as they supply vitamins, bio-active compounds and, as single-cell organisms, they are a rich source of nucleotides (Coutinho *et al.*, 2006). Therefore, the inclusion of microalgal biomass and/or its derivatives in fish diets is also an interesting and innovative approach to improve fish immune response and disease resistance against pathogens. Infectious diseases are still one of the most significant factors threatening the aquaculture industry, and the wide use of antibiotics for their control has been largely criticized due to the growing concern related to the selection of resistant bacterial strains, the environmental pollution and the accumulation of residues harmful to public health (FAO/WHO/OIE 2006). In this context, an increasing attention has directed towards the use of alternative natural compounds with potent antimicrobial and immunostimulating properties, such as plant and algal-derived products.

In this regard, it has been recognised that dietary microalgae can enhance aspecific/innate

immunity in different fish species, by improving the serum activity of complement and lysozyme, phagocytosis and ROS production, as well as the expression of pro-inflammatory cytokines (Duncan & Klesius 1996; Watanuki *et al.*, 2006; Diaz-Rosalez *et al.*, 2008; Abdel-Tawwab & Ahmad, 2009; Andrews *et al.*, 2009; Das *et al.*, 2009; Venkatesh *et al.*, 2009; Andrews *et al.*, 2011; Cerezuela *et al.*, 2012b; 2012c; Das *et al.*, 2013; Ibrahem *et al.*, 2013; Reyes-Becerril *et al.*, 2013; 2014; Li *et al.*, 2014). Moreover, there is clear evidence that marine microalgae positively influence the specific/adaptive immune response, by enhancing the antibody synthesis (Cerenzuela *et al.*, 2012b; Ragap *et al.*, 2012; Reyes-Becerril *et al.*, 2014; Zhang *et al.*, 2014), and the resistance to bacterial diseases (Abdel-Tawwab & Ahmad, 2009; Andrews *et al.*, 2009; Das *et al.*, 2009; Venkatesh *et al.*, 2009; Andrews *et al.*, 2011; Ragap *et al.*, 2012; Das *et al.*, 2013). On the other hand, although some *in vitro* studies have focused on the evaluation of immunomodulatory properties of macroalgae, by the incubation of fish immune cells with different algal extracts (Castro *et al.*, 2004; Díaz-Rosales *et al.*, 2007; Leiro *et al.*, 2007), no information regarding similar aspects of marine microalgae is available in literature.

Tetraselmis suecica (Prasinophyceae) is a marine green microalga rich in proteins, lipids, essentials fatty acids and sterols, and it also contain chlorophyll a and b, carotene, zeaxanthin and violaxanthin (Brown & Jeffrey 1992). It is widely used in aquaculture facilities as feed for bivalve molluscs, penaeid shrimp larvae and rotifers. This marine genus has been found to have a large spectrum of antimicrobial activity (Austin *et al.*, 1992) and its members have shown a high potential as probiotics (Irianto & Austin, 2002). Because of its high content of vitamin E, *Tetraselmis* sp. has also been proposed as a source of this vitamin for human and animal consumption (Carballo-Cárdenas *et al.*, 2003). *Isochrysis galbana* (Prymnesiophyceae) is a marine microalga which has received special attention due to its ability to produce LC-PUFA, mainly eicosapentaenoic acid and also docosahexaenoic acid, that are accumulated as oil droplets in prominent lipid bodies in the cell, supplying also sterols, tocopherols, colouring pigments and other nutraceuticals (Yu *et al.*, 2010).

Mass cultivation of microalgae has been carried out in different kinds of open ponds, however the main problem in outdoor cultivation open systems is the contamination of the culture by other algal species. Moreover, these systems are inefficient, leading to low productivities and little reliable cultures (Tredici, 1999). In the last decade, research efforts have been directed towards the development of more efficient, high surface-to-volume ratio photobioreactors for microalgae cultivation in laboratory or at small scale level outdoors, including *Tetraselmis* spp. and *Isochrysis* spp. These recent technologies utilized in the massive production of microalgae significantly improve productivity and nutritional quality of the biomass compared to the traditional open culture systems (Tredici, 1999; Chini Zittelli *et al.*, 2003; 2004; Tredici 2004; Chini Zittelli *et al.*, 2006; Michels *et al.*, 2014a, 2014b).

The current study aimed to investigate the effects of the dietary inclusion of *Tetraselmis suecica* and *Isochrysis galbana* on growth performance, blood biochemical profile and innate immune response of European sea bass (*Dicentrarchus labrax*), in order to examine the potential use of these marine microalgae as immunostimulant supplements that can be applicable in aquafeed industry. The inclusion of microalgal extracts or whole microalgae cells in aquafeeds represents a challenge for the near future, however their beneficial effects in fish species different from the few ones which have been considered to date (mostly salmonids, freshwater and tropical species) need to be investigated. In particular, the possible application of microalgae has not yet been evaluated in terms of the integrated response of the European sea bass (*D. labrax*), an economically important cultured fish species for the Italian and Mediterranean Aquaculture but not included among those being investigated in a recent EU project on this subject (AQUAMAX project 2006).

5.2 Materials and methods

5.2.1 Chemicals

Acetic acid, 5-amino-2,3-dihydro-1,4-pathalazinedione (luminol), benzocaine, bovine serum albumin (BSA), Bradford reagent, cetyltrimethylammoniumbromide (CTAB, cod. 52369), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), Hank's Balanced Salt Solution without phenol red, Ca^{2+} and Mg^{2+} (HBSS), heparin (5 KU ml^{-1}, cod. H3149), Histopaque®1077 and 1191, hydrogen peroxide (H_2O_2), lysozyme from chicken egg white, *Micrococcus lysodeikticus*, NaCl, phorbol myristate acetate (PMA), 10 000 kDa polyethylene glycol (PEG), sodium-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), sodium phosphate ($NaHPO_4$), sulphuric acid (H_2SO_4), 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB), trypan blue, Tris-HCl were purchased from Sigma-Aldrich (St. Louis, USA). Trypsin (Type 1 from bovine pancreas) was purchased from VWR International (England). Throughout the study, water was collected from a Milli-Q system from Millipore (Bedford, MA, USA).

5.2.2 Microalgae

The marine microalgae *Tetraselmis suecica* and *Isochrysis galbana* were generously provided by Dr. Chini Zittelli of the Institute of Ecosystem Study, CNR (Sesto Fiorentino, FI, Italy). They were produced in Green Wall Panel photobioreactors at Microalghe Camporosso S.r.l. (Camporosso, Imperia, Italy). The harvested microalgae were concentrated by centrifugation, then freeze lyophilized and stored under vacuum in plastic bags at 4 ± 1 °C until use.

5.2.3 Diets

Three experimental diets, whose composition and proximate analysis are reported in Table 1 and Table 2, were formulated to be grossly isolipidic (crude lipid, CL, 17.4% DM) and isoproteic (crude protein, CP, 43.3% DM). The levels of proteins and lipids were chosen to be optimal for sea bass. Lyophilized *T. suecica* and *I. galbana* were added to the basal diet to obtain the following experimental diets: CTRL diet, non-supplemented diet; TI60 diet containing microalgae at the concentration 60 g/kg; TI180 diet containing microalgae at the concentration 180 g/kg. *T. suecica* and *I. galbana* were incorporated to replace 6% and 18% protein of fish meal which was the major protein source in the control diet, including also gluten meal (20.0%), soybean extract (15.0%), starch (13.0%), cod liver oil (5.6%), palm oil (6.0%). The microalgae concentrations employed were based on previous studies carried out by Tulli *et al.* (2012) and were chosen in order to ensure a high contribution of the different components of microalgae.

Table 1 Composition of the test diets.

Ingredients	CTRL	TI60	TI180
	g/kg	g/kg	g/kg
CPSP90	50.0	50.0	50.0
fish meal, Chile prime	275.0	232.0	150.0
gluten meal	200.0	200.0	200.0
SBM solv. ext.w.out hulls 48%	150.0	150.0	150.0
starch, gelatinized, D500	130.0	121.0	90.0
cod liver oil	56.0	51.0	45.0
palm oil	60.0	57.0	50.0
soy lecithin	25.0	25.0	25.0
freeze dried *Isochrysis galbana*	**0.0**	**40.0**	**120.0**
freeze dried *Tetraselmis suecica*	**0.0**	**20.0**	**60.0**
L-methionine	0.0	0.0	6.0
mineral supplement[a]	4.0	4.0	4.0
vitamin supplement[b]	5.0	5.0	5.0
Na lignosulfite	30.0	30.0	30.0
celite	15.0	15.0	15.0
Total	1000.0	1000.0	1000.0

[a] Mineral supplement composition (% mix): $CaHPO_4*2H_2O$, 78.9; MgO, 2.725 g; KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4*H_2O$, 0.197; $MnSO_4*H_2O$, 0.094; $CuSO_4*5H_2O$, 0.027; Na_2SeO_3, 0.067.

[b] Vitamin supplement composition (% mix): thiamine HCL Vit B1, 0.16; riboflavin Vit B2, 0.39; pyridoxine HCL Vit B6, 0.21; cyanocobalamine B12, 0.21; niacin Vit PP, 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05; myoinositol, 3.15; stay C Roche, 4.51; tocoferol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500UI/kg diet), 0.026; Vit D3 (2400UI/kg diet), 0.05; choline clorure, 83.99.

Table 2 Proximate analysis of the test diets.

	dry matter	N x 6.25	total lipid	LC-n-3 PUFA (EPA + DHA)
	%	%	%	%
CTRL	93,8	43,2	17,4	1,7
TI60	93,8	43,1	17,3	1,6
TI80	93,8	43,6	17,4	1,5

All major ingredients and additives, apart from microalgae, were provided by local providers. The experimental diets were produced in the laboratories of the Aquaculture Division of the Department of Food Science (DIAL), at University of Udine. Briefly, all diets ingredients were ground through a 0.5 mm sieve, then mixed with microalgae species at the desired concentration and dry pelleted through a 3.5 mm dye. Control diet was processed in the same manner. Then, diets were allowed to air dry and stored under vacuum in plastic bags at 4 ± 1 °C until use.

5.2.4 Fish and experimental design

European sea bass (*D. labrax*) were obtained from a local commercial fish farm and examined for their health status immediately upon arrival. Fish were acclimatized for 3 weeks in 9 circular fibreglass tanks with 250 l capacity (12 fish/tank) being part in an indoor partially-recirculating sea water system (total volume = 9 m^3, daily water renewal = 5%, photoperiod = 12 h light/12 h dark, light intensity = 200 lx) provided with thermostatic control, mechanical sand-filter, biological filter and UV lamp apparatus [facilities at the Department of Food Science, University of Udine, Pagnacco (UD), Italy]. The fish were kept at controlled and optimal water quality during the trial (22.8 ± 0.5°C, pH 8.0 ± 0.13, salinity = 25.8 ± 1.3‰, dissolved oxygen 6.8 ± 0.43 mg/l, total ammonia nitrogen 0.2 ± 0.04 mg/l, total nitrite nitrogen 0.2 ± 0.06 mg/l) and fed daily with the control diet. After acclimatization period, the tanks were assigned to the three test diets according to a random design with triplicate tanks per dietary treatment group. Therefore CTRL group was fed with the basal diet and acted as the control, TI60 group was fed with the diet containing 60 g/kg microalgae and TI180 group was fed with the diet containing 180 g/kg microalgae. At the beginning of the feeding trial fish average body weight was 204 ± 0.7 g. Fish were hand-fed the experimental diets to apparent satiety (i.e. until the first feed item was refused), 6 days a week, for 15 weeks. The daily ration was subdivided into two equal meals administered at 9:00 and 16:00 hours. Fish were kept under veterinary control during the trial and any clinical sign of disease was registered. All experimental procedures were carried out according to the guidelines of the European Union Council (Directive 2010/63/EU) and the

Italian legislation (D.L. 26/2014) for the use of laboratory animals. The fish were sedated during handling (i.e. for sampling) and the rearing/experimental procedures were performed in such a way so as to minimize suffering and pain. The number of fish used was kept to a minimum where possible.

5.2.5 Growth performance

After 8 and 12 weeks, and at the end of the feeding trial (15 weeks), fish on each tank were weighted and counted, after a 24-h fasting and under moderate anaesthesia with benzocaine (0.03 g/l). The following parameters were calculated per group at the end of the trial:

Specific growth rate (SGR) = 100 x [(ln final weight (g) – ln initial weight (g)]/experimental period (days)

Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)

5.2.6 Blood and tissue sampling

At the end of the 15 weeks feeding trial, 26 fish from each dietary treatment, were anaesthetized with benzocaine (0.03 g/l) and randomly submitted to blood collection from the caudal vein with a 5-ml syringe. Blood samples were transferred in sterile tubes with or without heparin. Plasma (16 fish/group) was separated from heparinised blood after centrifugation at 1500 x *g* for 15 min using a high-speed refrigerated microcentrifuge (Sigma). Serum (10 fish/group) was obtained from non-heparinised blood after clotting for 2 h at 4°C and centrifugation at 1500 x *g* for 15 min. Both samples were immediately aliquoted and stored at -80°C for subsequent analysis. After blood sampling, head kidney (HK) was aseptically removed from 6 fish/group euthanized throughout an overdose of benzocaine (0.1 g/l), cut into small fragments and placed in sea bass isosmolar HBSS (360 mOsm by adding NaCl) containing 0.25% heparin, for leukocytes isolation.

5.2.7 Blood biochemical profile

Plasma samples were analyzed spectrophotometrically using an automated analyzer system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) based on the use of commercially available kits. The parameters measured were: total proteins (TP, g/dl), albumin (ALB, g/dl), glucose (GLU, mg/dl), cholesterol (CHO, mg/dl), triglycerides (TRIG, mg/dl). The globulin content (GLB, g/dl) was calculated by subtracting albumin from total proteins.

5.2.8 Humoral immune response

5.2.8.1 Serum lysozyme activity

The serum lysozyme activity was determined by using a turbidimetric method according to Parry *et al.* (1965). Briefly, 10 µl of fish serum were incubated (in triplicate) in 96-well microtitre plates (Sarstedt) with 200 µl of 0.02% lyophilized *Micrococcus lysodeikticus* in 0.05 M sodium phosphate buffer (pH 6.3). The reduction of absorbance was measured at 540 nm every 10 min for 1 h using a microplate reader (Sunrise, Tecan S.r.l., Milan, Italy). The lysozyme activity (U/ml) in serum samples was subsequently calculated from a standard curve prepared with serial dilutions of chicken egg white lysozyme.

5.2.8.2 Serum antiprotease activity

The serum antiprotease activity was measured following the method of Bowden *et al.* (1997) with minor modifications. Ten microliters of serum was incubated in triplicate with 10 µl of 0.3% trypsin in 0.01 M Tris-HCl pH 8.2, then 500 µl of 5 mM BAPNA substrate was added and the volume was made up to 1 ml with 0.1 M Tris-HCl pH 8.2. Samples were incubated at 22°C for 25 min. The reaction was stopped with 150 µl of 30% acetic acid and the mixtures were centrifuged at 400 x *g* for 5 min. Two hundred microliters of the supernatant were transferred in triplicate to a 96-well microplate and the OD was read at 415 nm using a microplate reader against a blank. The inhibitory activity of antiproteases was expressed in terms of percentage of trypsin inhibition as described by Zuo & Woo (1997): Percent inhibition (%) = [(O.D. trypsin – O.D. sample)/ O.D. trypsin] x 100.

5.2.8.3 Serum myeloperoxidase activity

Total myeloperoxidase (MPO) activity in serum samples was measured according to Quade & Roth (1997), with slight modifications. Fifteen microliters of serum were diluted in triplicate with 135 µl of HBSS in 96-well plates. Then, 50 µl of 2 mM TMB and 5 mM H_2O_2 solution were added (substrate was prepared immediately before use). The colorimetric reaction was stopped after 5 min by adding 50 µl of 2 M H_2SO_4. The optical density (OD) was read at 450 nm by a microplate reader. Blank samples with HBSS were also included.

5.2.9 Cellular immune response

5.2.9.1 Isolation of head kidney leukocytes

HK leukocytes were isolated as described previously by Volpatti *et al.* (2014). Briefly, the tissue was gently pressed in HBSS with heparin by a sterile syringe piston, then the resultant cell suspension was centrifuged at 200 x *g* for 10 min and carefully layered onto a sea bass isosmolar discontinuous density gradient (Histopaque layers of 1077 and 1191 g/ml). After 25 min centrifugation at 480 x *g* (4°C), the cells were collected from the interface and washed with HBSS (200 x *g* for 10 min at 4°C). Cell viability was determined with the trypan blue exclusion method and the cell concentration was estimated using a Thoma counting chamber. HK leucocytes were adjusted to 10^7 cells/ml or to 10^6 cells/ml in HBSS according to the following assays.

5.2.9.2 Respiratory burst activity

The production of reactive oxygen radical (ROS) by sea bass HK phagocytes during the respiratory burst was quantified by a chemiluminescence method (Coteur *et al.*, 2002, partially modified). Black 96-well plates (Nunc) were prepared containing 50 µl/well of 2 mM luminol in HBSS and 100 µl/well of 20 µg/ml PMA as stimulus, then 50 µl/well of HK leukocytes 10^7 cells/ml in HBSS were added. Each sample was evaluated in triplicate. The luminescent emission was immediately measured for 30 min at 5 min intervals (T = 25°C), using a luminometer (Sunrise, Tecan S.r.l., Milan, Italy) with integration time = 0.5 s and photomultiplier gain = 180. Luminescence backgrounds were calculated incubating the cells with luminol but without PMA. The ROS production was expressed in terms of cumulative response [relative luminescence units (RLU)/10^7 cells/ml throughout the 30 min incubation].

5.2.9.3 Myeloperoxidase content

Total myeloperoxidase (MPO) content of sea bass HK leukocytes was measured according to Salinas *et al.*, (2008), albeit with minor modifications. Briefly, 10^6 cells/ml in HBSS were lysed with 0.002% CTAB for 5 min and centrifuged at 400 x *g* for 10 min to separate cell debris from supernatant containing peroxidases. Then, 150µl of the supernatant were transferred to a 96-well microplate in triplicate wells containing 25 µl of freshly prepared 10 mM TMB and 25 µl of 5 mM H_2O_2. The colour-developing reaction was stopped after 1 min by adding 50 µl of 2 M H_2SO_4 and the optical density (OD) was read at 450 nm in a microplate absorbance reader. Blank samples without leukocytes were also analysed.

5.2.10 Statistical analysis

Statistical analysis were performed using the Statistical Package for Social Science (SPSS version 20.0, Inc. Chicago, IL, USA) suite for Windows. The assumptions of normality and homogeneity of variances were checked using the Kolmogorov-Smirnov and Levene tests, respectively. All treatment groups showed a normal distribution and homogeneity of variances for all the parameters investigated. Data (mean ± standard error [SE]) were statistically analysed by one-way analysis of variance (ANOVA) to test differences between treatments, and individual means were compared using Duncan's multiple comparison *post hoc* test when necessary to determine significant differences among dietary treatment groups. Differences were considered statistically significant when $P \leq 0.05$.

5.3 Results

5.3.1 Growth performance

The results of growth performance in *D. labrax* fed the experimental diets for 15 weeks are presented in Table 3.

Table 3 Growth performance of sea bass fed the experimental diets over 15 weeks.
Data are expressed as mean ± SE (3 tanks/group). Data were statistically analysed by one-way ANOVA.
P values ≤ 0.05 were considered as significant.

Variable	Microalgae levels		
	Control (0 g/kg)	60 g/kg	180 g/kg
IBW[a]	204.3 ± 0.66	203.9 ± 0.66	204.3 ± 0.41
FBW[b]	410.4 ± 5.82	427.3 ± 5.82	419.5 ± 9.47
SGR[c]	0.66 ± 0.0130	0.70 ± 0.0231	0.69 ± 0.0228
FCR[d]	1.75 ± 0.0537	1.66 ± 0.0375	1.69 ± 0.0659

[a]Initial body weight (g)
[b]Final body weight (g)
[c]Specific growth rate
[d]Feed conversion ratio

Fish with an initial weight of ca. 200 g grew 101–110% during the 15-week long feeding trial. At the end of the experiment, FBW and SGR were slightly higher in fish fed microalgae supplemented diets compared to control group. Specifically, the mean FBW value was 410.4 ± 5.82 g in the control group, 427.3 ± 5.82 g in TI60 group and 419.5 ± 9.47 g in TI180 group. The SGR was 0.66 ± 0.0130 in the control group, 0.70 ± 0.0231 in TI60 group and 0.69 ± 0.0228 in TI180 group. Moreover, fish fed TI60 diet consumed more feed than the other treatments giving the lowest FCR (1.66 ± 0.0375). Fish fed the control diet and fish fed TI180 diet consumed less

feed, yielding a higher FCR (1.75 ± 0.0537 and 1.69 ± 0.0659 respectively). However, no significant differences with respect to the control group or between treatment groups were observed in growth performance ($P > 0.05$).

5.3.2 Blood biochemical profile

The results of plasma biochemical analysis performed on *D. labrax* fed with the experimental diets are presented in Table 4. Serum total proteins ranged from 4.5 g/dl to 6.4 g/dl, albumin from 2.0 g/dl to 2.7 g/dl, globulins from 2.4 g/dl to 4.0 g/dl, glucose from 87.0 mg/dl to 325.0 mg/dl, cholesterol from 176.0 mg/dl to 451.0 mg/dl and triglycerides from 260.0 mg/dl to 697.0 mg/dl, but levels of these parameters were not significantly affected by the experimental diets. Plasma total proteins, albumin, globulins and glucose levels remained substantially similar ($P > 0.05$). Plasma cholesterol and triglycerides decreased with increasing doses of microalgae in the diet when compared with those of fish fed the basal diet, however the differences observed were not statistically significant due to wide variation within the same treatments ($P > 0.05$).

Table 4 Plasma biochemical parameters of sea bass fed the experimental diets over 15 weeks. Data are presented as mean ± SE (n = 16). Data were statistically analysed by one-way ANOVA. *P* values ≤ 0.05 were considered as significant.

Parameters	CTRL	TI60	TI180
TP (g/dl)	5.7 ± 0.12	5.5 ± 0.12	5.4 ± 0.13
ALB (g/dl)	2.5 ± 0.04	2.5 ± 0.04	2.4 ± 0.06
GLB (g/dl)	3.3 ± 0.11	3.0 ± 0.09	3.0 ± 0.08
GLU (mg/dl)	142.2 ± 12.83	151.4 ± 14.76	144.8 ± 12.54
CHO (mg/dl)	328.7 ±16.44	291.8 ± 13.51	277.4 ± 11.60
TRIG (mg/dl)	588.4 ± 24.07	521.7 ± 25.58	519.4 ± 35.64

5.3.3 Innate immune response

The humoral and cellular innate immune parameters of sea bass fed microalgae supplemented diets were evaluated at the end of 15 weeks feeding trial. Regarding humoral responses, the serum lysozyme activity was stimulated in fish fed diets supplemented with microalgae with respect to fish fed the control diet (Fig. 1). Among the experimental groups, fish fed TI60 diet showed significantly higher lysozyme activity (847.5 ± 11.36 U/ml) compared to fish fed TI180 diet (820.6 ± 11.91 U/ml) and control group (814.0 ± 5.65 U/ml) ($P \leq 0.05$).

As shown in Fig. 2, the serum antiprotease activity showed a similar increase in fish fed microalgae enriched diets compared to control group. In particular, serum antiprotease activity

expressed as the percentage of trypsin inhibition was 51.6 ± 4.64 in control group, 71.0 ± 1.43 in TI60 group, 57.4 ± 5.61 in TI180 group. The antiprotease activity was significantly higher in fish fed diet containing 60 g/kg of microalgae with respect to other experimental groups ($P \leq 0.05$).

The effect of microalgae on serum myeloperoxidase (MPO) activity is depicted in Fig 3. No statistically significant differences were found in the serum MPO activity of fish fed *T. suecica* and *I. galbana* supplemented diets with regard to the levels obtained in fish of the control group ($P > 0.05$). However, a higher serum MPO activity was measured in fish fed diet enriched with 60 g/kg of microalgae (0.2 ± 0.02 O.D.) compared to fish fed diet enriched with 180 g/kg of microalgae and fish fed the basal diet (0.1 ± 0.02 O.D.).

In terms of cellular immune responses, the respiratory burst activity of HK leukocytes stimulated with PMA and their MPO content were not greatly affect by diets enriched with the microalgae compared to control group ($P > 0.05$). The leukocyte chemiluminescence response was 12536 ± 1291 RLU and 13601 ± 1784 RLU in the groups TI60 and TI180 respectively, compared to 13197 ± 2017 RLU in control group (Fig. 4). An optical density related to leukocyte MPO content of 1.1 ± 0.07 O.D. and 1.2 ± 0.13 O.D. was recorded in fish which received diet TI60 and TI180 respectively, compared to 1.2 ± 0.10 O.D. in fish fed the control diet (Fig. 5).

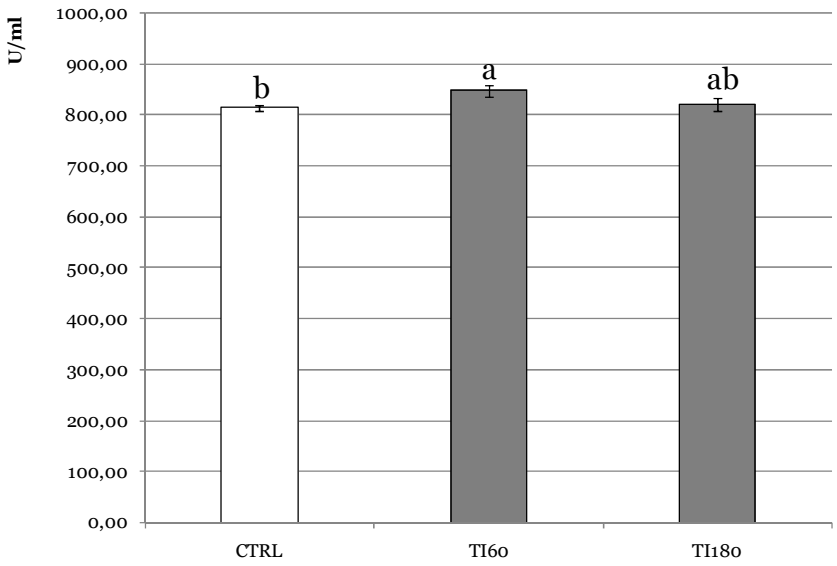

Fig. 1 Serum lysozyme activity (U/ml) of *D. labrax* fed *T. suecica* and *I. galbana* supplemented diets. Data are presented as mean ± SE (n = 10). Data were statistically analysed by one-way ANOVA followed by Duncan's multiple comparison test. *P* values ≤ 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

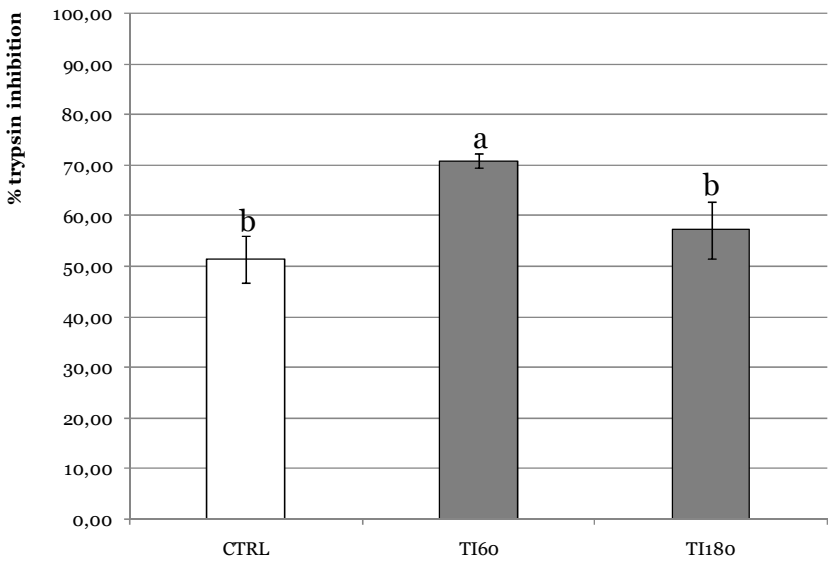

Fig. 2 Serum antiprotease activity (% trypsin inhibition) of *D. labrax* fed *T. suecica* and *I. galbana* supplemented diets. Data are presented as mean ± SE (n = 10). Data were statistically analysed by one-way ANOVA followed by Duncan's multiple comparison test. *P* values ≤ 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

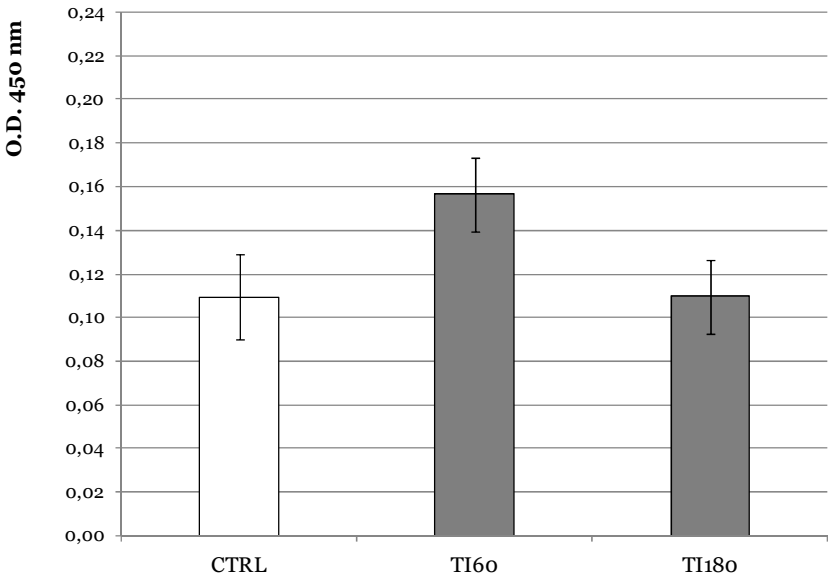

Fig. 3 Serum myeloperoxidase activity (O.D. at 450 nm) of *D. labrax* fed *T. suecica* and *I. galbana* supplemented diets. Data are presented as mean ± SE (n = 10). Data were statistically analysed by one-way ANOVA. *P* values ≤ 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

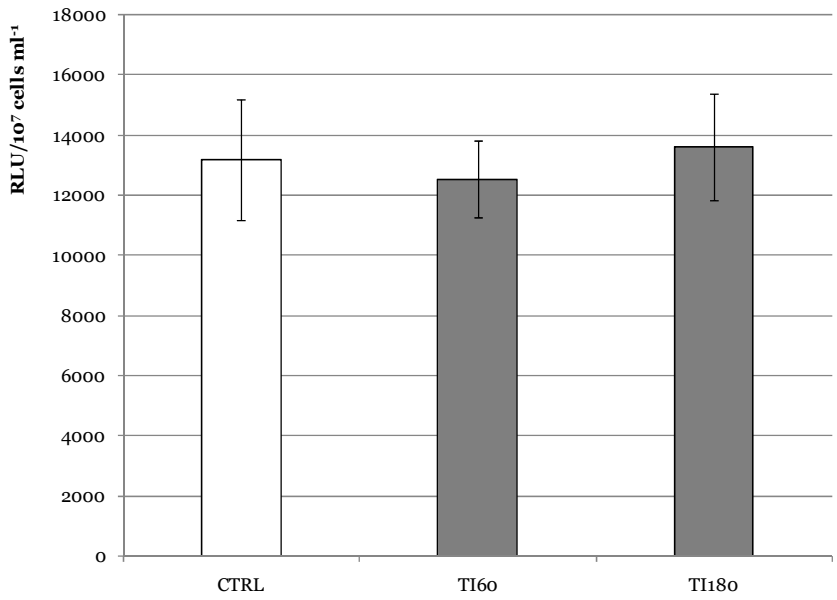

Fig. 4 Leukocyte ROS production (RLU/10^7 cells/ml-30 min cumulative response) of *D. labrax* fed *T. suecica* and *I. galbana* supplemented diets. Data are presented as mean ± SE (n = 6). Data were statistically analysed by one-way ANOVA. *P* values ≤ 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

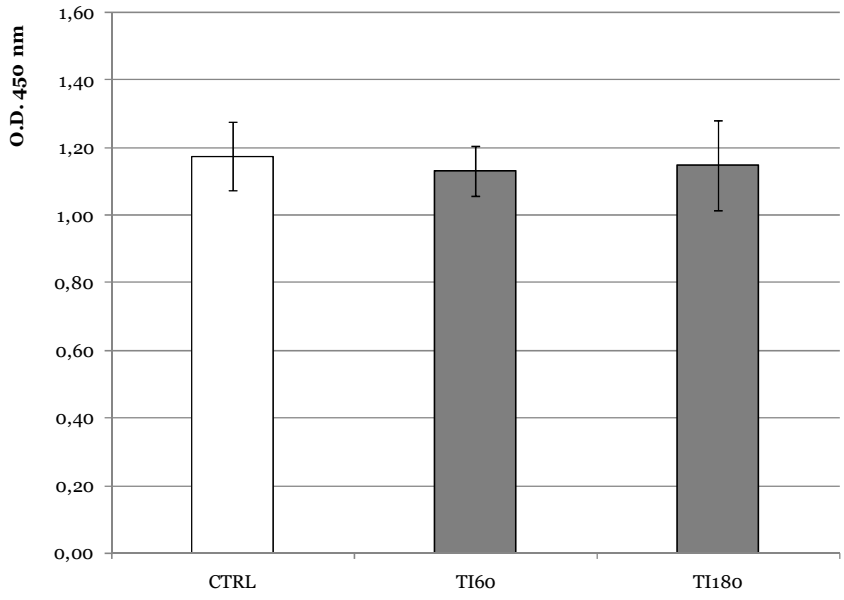

Fig. 5 Leukocyte myeloperoxidase content (O.D. at 450 nm) of *D. labrax* fed *T. suecica* and *I. galbana* supplemented diets. Data are presented as mean ± SE (n = 6). Data were statistically analysed by one-way ANOVA. *P* values ≤ 0.05 were considered as significant. Different superscript letters denote statistical differences among the experimental groups.

5.4 Discussion

Marine microalgae have been recently used as feed ingredients in animal nutrition due to their high nutritional value (Christaki *et al.*, 2010; Hemaiswarya *et al.*, 2011; Guedes & Malcata, 2012). In particular, several microalgal species currently deserve a growing interest in aquaculture as alternative non-conventional protein and lipid sources to fish meal and oil due to their high content of proteins and PUFAs. Moreover, they are gaining high attention in fish feed industry due to their positive effects on growth performance and immune response. Immunostimulation by medicinal plant and algal additives is gaining more popularity in aquaculture as alternative to conventional drugs, the latter resulting in eco-toxicity, accumulation of chemical residues in edible fish tissues and selection of many antibiotic-resistant pathogenic bacteria (FAO/WHO/OIE 2006).

Thereby, the aim of this study was to examine the possible use of the marine microalgae *Tetraselmis suecica* and *Isochrysis galbana* as feed additives in the diet of European sea bass (*Dicentrarchus labrax*). For this purpose, the two microalgae were incorporated in a sea bass diet at concentrations 60 g/kg and 180 g/kg to replace 10% and 30% of fish meal. The effects of the dietary administration of *T. suecica* and *I. galbana* on sea bass growth performance, blood biochemical parameters and innate immune response were investigated after 15 weeks of feeding.

In this study, the oral administration of *T. suecica* and *I. galbana* had no significant effect on sea bass growth and feed utilization, though a trend of higher growth rate was registered in fish fed microalgae supplemented diets compared to fish fed the basal diet. These findings suggest that *T. suecica* and *I. galbana* can replace up to 18% of fish meal protein without hampering growth performance of *D. labrax*. In agreement with our results, previous investigation of Tulli *et al.* (2012) indicated that diets containing dried *T. suecica* as a fish protein substitute to replace (protein basis) 10% and 20% of the control diet did not affect zootechnical performances of European sea bass juveniles. Similarly, Tibaldi *et al.* (2015) demonstrated that replacing up to 20% crude protein from fish meal with a dried *Isochrysis* sp. biomass and up to 36% fish lipid for those supplied by the microalgae in a diet with reduced level of fish oil, did not adversely affect feed intake or growth performance of sea bass relative to controls even if the highest substitution rate resulted in a decline in lipid and energy apparent digestibility coefficients. Other recent studies investigated the potential use of dietary microalgae as fish meal replacement in aquafeed, without underline negative effects on fish growth performance (Olvera-Novoa *et al.*, 1998; Palmegiano *et al.*, 2009; Kiron *et al.*, 2012; Abdulrahman & Ameen, 2014). On the contrary, Walker & Berlinsky (2011) found that diets formulated to replace 15% and 30% of fish meal protein with a combination of dried *Nannochloropsis* sp. and *Isochrysis* sp. reduced feed intake and growth of Atlantic cod (*Gadus morhua*). Similarly, it has been

reported that 30% fish meal replacement with the macroalga *Pyropia yezoensis* in the diet slightly decreased growth of Nile tilapia (*Oreochromis niloticus*) (Stadtlander *et al.*, 2013). Decrease in fish performance can be ascribed to the high carbohydrate and fibre contents in seaweeds, which have been shown to affect digestibility of proteins and dry matter, by reducing the accessibility of intestinal digestive enzymes to food nutrients, thereby resulting in lower availability. Moreover, other natural substances that occur in seaweeds have been observed to block the efficacy of beneficial compounds and inhibit fish digestion. Another contributing factor to the depressed performance may be the relatively low nutritive value of seaweeds (Ragaza *et al.*, 2015).

On the other hand, some authors reported that the supplementation of the diet with a small amount (0.05–10%) of different algae yielded a significant enhancement of growth and feed utilization in various fish species. For example, Abdel-Tawwab & Ahmad (2009) showed that the dietary supplementation of 1.25-10 g/kg live *Spirulina* for 12 weeks could enhance growth performance and feed utilization in Nile tilapia (*O. niloticus*). Similarly, a considerable increase in weight and nutrient utilization was also detected in juvenile olive flounder (*Paralichthys olivaceus*) fed low levels of the seaweeds *Eucheuma denticulatum* (Ragaza *et al.*, 2015) and *P. yezoensis* (Choi *et al.*, 2015) for 8-9 weeks. Even Indian major carp (*Labeo rohita*) fed with 0.5-5 g/kg *Microcystis aeruginosa* for 90 days (Das *et al.*, 2013), Pacific red snapper (*Lutjanus peru*) fed with 100 g/kg *Navicula* sp. for 8 weeks (Reyes-Becerril *et al.*, 2014) and yellow croaker (*Pseudosciaena crocea*) fed 2.8-11.2 g/kg *Haematococcus pluvialis* for 66 days (Li *et al.*, 2014) showed increased growth performance compared with fish fed non supplemented diets. Moreover, Zhang *et al.* (2014) reported that 8 g/kg *Chlorella* supplemente diet could significantly increase the growth of gibel carp (*Carassius auratus gibelio*). Other than as described above, the oral administration of the microalgae *Nannochlorops gaditana*, *Phaeodactylum tricornutum* and *Tetraselmis chuii* at 5% and 10% in *Sparus aurata* (Cerenzuela *et al.*, 2012c), 1%, 2% and 4% *Spirulina platensis* in *L. rohita* (Andrews *et al.*, 2009), 2%, 4%, 6% *Sargassum fusiforme* and 6% *Ecklonia cava* in *P. olivaceus* (Kim & Lee, 2008; Kim *et al.*, 2014) had no significant impact on fish growth. Nevertheless, the application of dietary additives to improve fish growth should be carefully considered because their favourable roles vary depending on targeting activities of additives, doses, administration method, nutritional value, as well as on fish species, physiological and nutritional status (Kim *et al.*, 2013). Some authors speculated that the enhancement of fish growth and feed utilization observed after the dietary administration of algae may be probably due to an improved feed intake and nutrient digestibility, or due to the presence of several compounds in the microalgae, especially vitamins and minerals, that possibly aid growth promotion (Abdel-Tawwab & Ahmad, 2009). It has also been reported that small amounts of inclusion of algae or algal extracts in fish diet may increase growth performance by inducing the activation of growth-related hormones

and metabolic energy saving (Choi *et al.*, 2015). An aspect that should be pursued in future studies is the implication of dietary algal products on intestinal morphology and microbial ecology, since the maintaining of epithelial integrity and microbiota balance is essential for proper gut functionality. In this regard, information available in literature are still very scarce. For example Merrifield *et al.* (2011) demonstrated that Ergosan, an algal extract containing alginic acid extracted from the brown alga *Laminaria digitata*, does not adversely impact the epithelial brush border and the indigenous gastrointestinal microbial balance integrity when administered in Nile tilapia (*O. niloticus*). Similarly, rainbow trout (*Oncorhynchus mykiss*) fed with Ergosan enriched diet displayed normal morphology of proximal intestine and pyloric caeca (Heidariech *et al.*, 2012). On the contrary, Cerenzuela *et al.* (2012a) reported that the dietary administration of the microalgae *T. chuii* and *P. tricornutum* alone or in combination with probiotics compromised gilthead seabream intestinal homeostasis, causing alterations in both intestinal morphology and microbiota. At the end of the feeding trial, we collected samples of fish intestine from each experimental group, so in the future we will investigate the effects of the diets supplemented with *T. suecica* and *I. galbana* on sea bass intestinal morphology.

The analysis of blood biochemical parameters is a powerful tool that can be effective to understand the physiological and pathological changes in fish, so it can provide valuable information in the assessment of fish health status. In this investigation, plasma samples were analysed using an automated analyser for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy), commonly used for human and veterinary medicine. It has already been shown that these apparatuses can be also used with good reliability to estimate biochemical parameters in fish blood samples (Coz-Rakovac *et al.*, 2005; Manera & Britti, 2006; Coz-Rakovac *et al.*, 2009;).

In this study, the analysis of the blood samples revealed that there were no marked changes in the levels of plasma total proteins, albumin and globulins in fish fed diets supplemented with the marine microalgae *T. suecica* and *I. galbana* when compared with fish fed the control diet. Total plasma protein concentration is used as a broad clinical indicator of health, stress, and welfare in both terrestrial and aquatic organisms. Blood proteins include albumin, globulins and various humoral elements of the non-specific immune system (Magnadottir, 2006). Albumin is synthesized by liver and serves as transporter for the delivery of other molecules (Nya & Austin, 2009). Globulins, especially gamma-globulins, play important roles in the immune system. Increase in the serum protein, albumin and globulin levels is thought to be associated with a stronger innate response in fish (Das *et al.*, 2013). Secondly, the dietary supplementation with *T. suecica* and *I. galbana* decreased the levels of blood cholesterol and triglycerides, accordingly to increasing dose of microalgae, although the differences among the experimental groups were not significant. These findings suggest that the marine microalgae might modulate lipid metabolism. Some authors reported that some naturally occurring compounds in seaweeds, such as omega-3 fatty acids or fibres, can decrease blood cholesterol and triglycerides (Ragaza *et*

al., 2015). Thirdly, the administration of *T. suecica* and *I. galbana* in sea bass did not significantly affect the plasma glucose level.

Up to now, to the best of our knowledge few studies were conducted to investigate the effects of the dietary administration of algae on fish blood biochemical parameters. Abdel-Tawwab & Ahmad (2009) found that Nile tilapia fed a diet containing 5 g/kg *Spirulina* exhibited higher plasma proteins, albumin, globulins, glucose and lipids as compared with fish fed the control diet, indicating an improvement in fish health promoted by *Spirulina*-enriched diets. Li *et al.* (2014) measured a decrease of serum glucose, triglyceride and cholesterol but no variation in the levels of total proteins, albumin and globulins in *P. crocea* fed 2.8 g/kg, 5.6 g/kg and 11.2 g/kg *H. pluvialis* enriched diets. Similarly, Ragaza *et al.* (2015) observed that serum total cholesterol and triglicerydes levels were lower in olive flounder (*P. olivaceus*) after feeding with 3%, 6% and 9% *E. denticulatum* supplemented diets. Even juvenile Japanese flounder fed diets supplemented with 2% and 4% *Chlorella* exhibited a significant decrease of serum cholesterol level in comparison to the control fish (Kim *et al.*, 2002). Moreover, *Chlorella* reduced cholesterol and increased total proteins, albumin and globulins in gibel carp (Xu *et al.*, 2014). On the other hand, the dietary administration of *P. yezoensis* extract in olive flounder induced a decrease of glucose and an increase of total proteins in plasma (Choi *et al.*, 2015). Venkatesh *et al.* (2009) found higher levels of serum total proteins, albumin and globulins and lower glucose level in *Etroplus suratensis* fingerlins fed with *S. platensis* for 40 days compared to fish fed the control diet. Significant increases of serum total proteins, albumin and globulins were also reported by Das *et al.* (2009; 2013) in *L. rohita* fed with *Euglena viridis* and *M. aeruginosa*.

In the present study, the dietary supplementation with *I. galbana* and *T. suecica* powder was found to have a stimulatory effect on the sea bass non-specific humoral immune mechanisms assayed, such as serum lysozyme, antiprotease and myeloperoxidase activity, compared to the control group (non-supplemented diet). On the contrary, the non-specific cellular immune responses, such as respiratory burst activity and myeloperoxidase content of HK leukocytes, were not significantly affected by the inclusion of the microalgae in the diet. Though the activity of these and other important immune response components is normally high in fish and hardly modulated even after immunization or infections (Magnadottir, 2006), it has been shown that dietary administration of certain algal-based immunostimulants are capable of enhancing fish immune responses and reducing losses due to microbial infections (Watanuki *et al.*, 2006; Kim & Lee, 2008; Abdel-Tawwab & Ahmad, 2009; Das *et al.*, 2009; 2013; Reyes-Becerril *et al.*, 2013; Choi *et al.*, 2015; Kim *et al.*, 2014; Li *et al.*, 2014; Reyes-Becerril *et al.*, 2014).

Lysozyme is an important component in the innate immune system of fish, present in mucus, lymphoid tissues, plasma, as well as in other fluids, and it is also expressed in a wide variety of tissues. It is a bactericidal cationic enzyme which hydrolyses the β-1,4 glycosidic linkage between N-acetylglucosamine and N-acetyl muramic acid in the peptidoglycan of bacterial cell

walls, thereby resulting in the lysis of certain Gram-positive bacteria and in conjunction with complement and other enzymes even some Gram-negative bacteria. Lysozyme is also involved in opsonization, activating the complement system and phagocytosis (Magnadottir, 2006; Saurabh & Sahoo, 2008). Immunostimulants can increase serum lysozyme activity due to an increase in the number of phagocytes secreting lysozyme or to an increase in the amount of lysozyme synthesized per cell (Engstad *et al.*, 1992). In the present investigation, the serum lysozyme activity was enhanced in all the microalgae treated groups throughout the 15 week-exposure period. Among the experimental groups, sea bass fed diet enriched with 60 g/kg of microalgae showed a significantly higher lysozyme activity compared to fish fed diet supplemented with 180 g/kg of microalgae and the control group. Similar results of elevated serum lysozyme activity were observed in rohu (*L. rohita*) fingerlings fed with the marine microalgae *E. viridis* at 0.1 g/kg, 0.5 g/kg and 1.0 g/kg after 30, 60 and 100 days of feeding (Das *et al.*, 2009) or *M. aeruginosa* at 0.5 g/kg, 1.0 g/kg and 5.0 g/kg after 30 and 60 days of feeding (Das *et al.*, 2013). Serum lysozyme activity was found to be promoted also in large yellow croaker (*P. crocea*) fed diet containing 2.8 g/kg, 5.6 g/kg and 11.2 g/kg of the micro green alga *H. pluvialis* for 66 days (Li *et al.*, 2014) or in Pacific red snapper (*L. peru*) fed diet enriched with 100 g/kg of the microalga *Navicula* sp. at 8 weeks (Reyes-Becerril *et al.*, 2014). Moreover, the activity of serum lysozyme increased significantly in juvenile olive flounder (*P. olivaceus*) fed diets containing different levels (20 g/kg, 40 g/kg and 60 g/kg) of brown seaweed *E. cava* for 6 weeks compared to fish fed the control diet (Kim & Lee, 2008). These results coincide also with the investigation of Promya & Chitmanat (2011), who found that African sharptooth catfish (*Clarias gariepinus*) exhibited higher serum lysozyme activity after feeding with 3% or 5% *S. platensis* and with 5% *Clodophora* sp. The dietary administration of *Spirulina* increased the lysozyme activity also in *O. niloticus* (Ragap *et al.*, 2012) and *E. suratensis* (Venkatesh *et al.*, 2009).

Fish blood contains a number of protease inhibitors, principally α1-antiprotease, α2-antiplasmin and α2-macroglobulin, which have a role in restricting the ability of bacteria to survive *in vivo* (Ellis, 2001). In this work, we reported an increase in serum antiproteases activity in sea bass fed *I. galbana* and *T. suecica* supplemented diets compared to control group. In particular, this parameter was significantly higher in fish fed diet enriched with 60 g/kg of microalgae, as observed for lysozyme activity. Furthermore, in the same experimental group an increase in serum myeloperoxidase activity was also measured, albeit no statistically significant differences were found between fish fed microalgae supplemented diets and fish fed the basal diet. Peroxidases are a large family of enzymes which play an important role as natural antibacterial agent in animal immune system, e.g. the myeloperoxidase (MPO) is released by the azurophilic granules of neutrophils during oxidative respiratory burst and is involved in microorganisms killing. The quantification of MPO activity in serum can be used as indirect

index of the release of this enzyme by activated circulating neutrophils (Quade and Roth, 1997). Few other studies have documented similar findings, e.g. an increase in these parameters as enhanced immune response in fish. However, our findings are in agreement with previous researches concerning Pacific red snapper (*L. peru*), in which the serum total antiprotease and MPO activity were stimulated in fish fed 100 g/kg *Navicula* sp. supplemented diet for 8 weeks compared to the control group (Reyes-Becerril *et al.*, 2014). An enhancement of serum myeloperoxidase activity has also been reported in olive flounder (*P. olivaceus*) fed diets containing 40 g/kg and 60 g/kg of the marine brown alga *E. cava* for 6 weeks (Kim & Lee, 2008).

Regarding the cellular immune response, the phagocytic activity is a key mechanism of fish innate immunity, and together with aspecific humoral components it constitutes the first line of defence against invading pathogens. Phagocytes engulf and kill the invading microorganisms by release of proteolytic enzymes and antimicrobial proteins, metabolic activation and production of microbicidal oxygen reactive species (ROS) during a process called respiratory burst. The ROS produced by activated phagocytes and their content of MPO have been widely used to evaluate the non-specific defense in fish, and their increase can be correlated with an increased bacterial pathogen killing activity of neutrophils and macrophages (Neumann *et al.*, 2001; Rieger & Barreda, 2011). In this study, the respiratory burst activity of sea bass HK leukocytes activated with phorbol 12-myristate 13-acetate (PMA) was evaluated by luminol-enhanced chemiluminescence, which allows to detect both extracellular and intracellular production of ROS (Vera-Jimenez *et al.*, 2013). Our results indicate that respiratory burst activity and MPO content of sea bass HK leukocytes were not affected by the dietary inclusion of the microalgae *T. suecica* and *I. galbana*. On the contrary, several authors reported higher phagocytic and respiratory burst activity in fish leukocytes after the dietary supplementation with algae. Cerezuela *et al.* (2012b; 2012c) showed a significant enhancement of HK phagocytes activities in gilthead seabream (*S. aurata*) fed diets containing 50 g/kg and 100 g/kg of the microalgae *N. gaditana*, *P. tricornutum* and *T. chuii* during a 4 weeks-trial compared to those fed the control diet. Similarly, respiratory burst of HK phagocytes in sole (*Solea senegalensis*) was stimulated after 4 weeks of feeding with the red microalga *Porphyridium cruentum* (10 g/kg) (Diaz-Rosales *et al.*, 2008). The inclusion of the marine microalga *E. viridis* in the diet significantly increased superoxide anion production (NBT values) of blood leukocytes in rohu fingerlings after 30, 60, 90 and 100 days of feeding (Das *et al.*, 2009). Similar results were observed in Nile tilapia (*O. niloticus*) fed *Spirulina* supplemented diets for 12 weeks (Abdel-Tawwab & Ahmad, 2009), in carp (*L. rohita*) fed *Spirulina* or *Chlorella* enriched diets for 60 days (Andrews *et al.*, 2009; 2011) and in juvenile olive flounder (*P. olivaceus*) fed *E. cava* including diets for 6 weeks (Kim & Lee, 2008). Moreover, the dietary administration of *Spirulina* positively modulated phagocytosis and respiratory burst activity of blood or HK leukocytes in *Cyprinus carpio*

(Watanuki *et al.*, 2006), *O. niloticus* (Ragap *et al.*, 2012) and *E. suratensis* (Venkatesh *et al.*, 2009).

The present investigation has provided new insight into the immunostimulatory capacity of *T. suecica* and *I. galbana* in fish and possibly contributes to an enhancement of fish welfare and economic growth in the aquaculture industry. In this study the incorporation of the marine microalgae in the diet at lower inclusion level positively affected sea bass non-specific humoral immune response, whereas it did not improve cellular immune parameters. Based on these findings, appropriate field trials incorporating other doses of microalgae or microalgal derivatives are recomended. Moreover, an *in vitro* approach would be useful to better understand the immunomodulatory properties of *T. suecica* and *I. galbana* on fish immune cells. Further studies aimed to investigate the possible implications of stimulated immune system on the host protective capacity against specific infectious diseases, are also necessary. In this regard, the beneficial effects of algae on fish resistance to certain pathogens has been demonstrated in several recent studies, dealing with various fresh water and marine fish species. For instance, some authors found that *Spirulina* incorporated diets increased protection against *Aeromonas hydrophila* in tilapia (Abdel-Tawwab & Ahmad, 2009; Ragap *et al.*, 2012) and similar findings were observed also in *L. rohita* fed diets containing *E. viridis*, *M. aeruginosa* (Das *et al.*, 2009; 2013) and *Chlorella* (Andrews *et al.*, 2009). Furthermore, *S. platensis* increased survival in *E. suratensis* fingerlings infected with *Aeromonas salmonicida* (Venkatesh *et al.*, 2009). Wong *et al.* (2013) reported that *Epinephelus coiode* fed *Sargassum cristaefolium* hot-water extract enriched diets increased the survival rate when they were infected with *Streptococcus* sp.

In fish, it has been shown that some substances obtained from micro and macroalgae, mainly polysaccharides, can stimulate the immune response and increase resistance against infectious diseases. Sodium alginate, a natural polysaccharide extracted from brown algae, was found to enhance the non-specific immune response and to confer protection in common carp *C. carpio* against *Edwardsiella tarda* (Fujiki *et al.*, 1994; Fujiki & Yano, 1997), in grouper *Epinephelus fuscoguttatus* (Chiu *et al.*, 2008) and *Epinephelus bruneus* (Harikrishnan *et al.*, 2011) against *Streptococcus* sp. Similarly, Ergosan has been reported to increase the non-specific defensive response of snakehead *Channa striata* (Miles *et al.*, 2001), rainbow trout *O. mykiss* (Peddie *et al.*, 2002; Gioacchini *et al.*, 2008; 2010; Sheikhzadeh *et al.*, 2012), and sea bass *D. labrax* (Bagni *et al.*, 2005). Moreover, it demonstrated a protective effect against columnaris disease in rainbow trout (Suomalainen *et al.*, 2009). Cheng *et al.* (2007) established that grouper (*E. coioides*) injected intraperitoneally with κ-carrageenan and sodium alginate had elevated non-specific immune parameters and resistance against *Vibrio alginolyticus*. Several studies evaluated the immunostimulatory effects of β-glucan isolated from *Euglenia gracilis* in rainbow trout (Skov *et al.*, 2012; Chettri *et al.*, 2013) or from *L. digitata* in gilthead seabream (Guzman-

Villanueva *et al.*, 2014). The dietary administration of laminarin improves the immune responses in *O. mykiss* (Morales-Lange *et al.*, 2014) and *E. coioides* (Yin *et al.*, 2014).

On the other hand, the use of marine algae and their extracts as natural "medicines" could be a further novel application in aquaculture as alternatives to conventional drugs, and recently many marine macroalgae demonstrated *in vitro* antibacterial activities against fish pathogens (Choundhury *et al.*, 2005; Bansemir *et al.*, 2006). On the contrary, despite the ample literature on the *in vitro* inhibitory effects of extracts obtained from *Chlorella* sp., *Nannochloropsis* sp., *Dunaliella* sp., *Tetraselmis* sp. e *Isochrysis* sp. against human bacterial pathogens (Srinivasakumar & Rajashekhar, 2009; Kumaran *et al.*, 2010; Amaro *et al.*, 2011; Krishnika *et al.*, 2011), little information is available on the antibacterial activity of marine microalgae against fish pathogens (Austin *et al.*, 1992; Amaro *et al.*, 2011; Pradhan *et al.*, 2011), thereby future research will be performed in this regard.

5.5 Acknowledgements

This work was supported by CRITA project financed by Friuli Venezia Giulia Region and coordinated by Prof. E. Tibaldi (Aquaculture Section, DIAL, University of Udine). The authors would like to thank Dr. Chini-Zittelli (Institute of Ecosystem Study, CNR, Sesto Fiorentino, FI, Italy) for providing the marine microalgae.

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FINAL CONSIDERATIONS

The present PhD thesis was aimed to investigate the possible application of medicinal plants and microalgae as antimicrobial agents or immunostimulants in aquaculture, for the control of infectious diseases.

The use of medicinal plants in farmed fish has attracted a lot of attention worldwide and has become a subject of numerous scientific studies. Plant-derived products were found to improve growth, health status, innate and adaptive immune response as well as disease resistance in several marine and freshwater species, therefore they represent promising tools, complementary to vaccination and traditional drugs, to be employed in the control of diseases. In addition, phytocompounds are eco-friendly, inexpensive, easily prepared and do not show many of the side-effects that are often associated with synthetic antibiotics. Similarly, marine microalgae demonstrated to possess positive properties both in terms of nutritional value and fish health improvement.

To date, the beneficial effects of medicinal plants and microalgae have been investigated principally in freshwater species, such as salmonids and carps, or in tropical species, such as tilapia, grouper, olive flounder and yellow croaker, consequently further studies in fish species different from those which have been considered are strongly needed.

This thesis represents a bibliographic and experimental approach finalized to fill a gap concerning the exploitation of these products in two fish species relevant to European aquaculture: rainbow trout (*O. mykiss*) and sea bass (*D. labrax*). The experiments designed and described in chapters from 2 to 5 were based on the knowledge deriving from the literature insights reported in chapter 1. Specifically, the medicinal plants and microalgae under study, the experimental protocols (*in vitro* and *in vivo*), the doses and timings of administration, have been chosen considering the results of previous scientific papers dealing with other fish species.

Our *in vitro* findings revealed that some of the selected medicinal plants possess a noticeable antibacterial and immunomodulatory activity, whereas the *in vivo* trials, aimed to investigate the possible use of ginseng and microalgae as immunostimulants in fish, did not provide equally positive results, and deserve future in-depth analysis.

Further investigations are still strongly recommended to define the optimal doses and timings of administration as well as to purify, characterize and quantify the single bioactive compounds contained in plants, algae and phytoextracts, in order to identify the most effective substances/metabolites that could be included as novel natural additives in aquafeeds. Moreover, researches on biological mechanism of action, stability of plant/algae materials in aquatic environment and digestibility in fish, as well as *in vitro* and *in vivo* toxicological tests, are prerequisites for their effective and safe application.

Nowadays, a limited number of natural products is available at a global level for large-scale use in aquaculture. In Europe, feed companies can include new ingredients in a formulation only if they are approved by the list of feed additives (EC Regulation No 1831/2003). As consequence the current use of these products in Italy is still limited. On the contrary, we have examples of several foreigner countries, such as India or China, where the application of herbs is a common approach in human and veterinary medicine, and moreover their laws are less strict. In western countries, a review of the current legislation should be undertaken in order to allow a greater flexibility in their use, taking into consideration the benefits that they might provide in intensive farming conditions. Medicinal plants and microalgae might be proposed primarily as feed additives or immunostimulants, rather than therapeutics. In fact, we can not reasonably ascribe them an efficacy similar to that exerted by conventional antibiotics and moreover their registration as medicines is a time-consuming and expensive process.

APPENDIX

Scientific papers published between 2012 and 2014, concerning arguments not included in the PhD thesis topic

MOSCA F., CIULLI S., VOLPATTI D., ROMANO N., VOLPE E., **BULFON C.**, MASSIMINI M., CACCIA E., GALEOTTI M., TISCAR P.G. (2014). Defensive response of European sea bass (*Dicentrarchus labrax*) against *Listonella anguillarum* or *Photobacterium damselae* subsp. *piscicida* experimental infection. *Veterinary Immunology and Immunophatology*, 162: 83-95.

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Scientific presentations at National and International Conferences

BULFON C., BERALDO P., VOLPATTI D., GALEOTTI M. Estratti di aloe, curcuma, echinacea, ginseng, lavanda, origano e rabarbaro modulano il rilascio di ROS e la proliferazione dei leucociti di trota iridea (*Oncorhynchus mykiss*). XX Convegno Nazionale della Società Italiana di Patologia Ittica, poster. Certosa di Calci (PI), September 18-19, 2014.

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BULFON C., VOLPATTI D., GALEOTTI M. I parametri biochimici del sangue come indicatori di benessere in trote iridee (*Oncorhynchus mykiss*) allevate presso gli impianti coinvolti nel progetto I.R.IDEA. Convegno finale del progetto I.R.IDEA. Villa Manin di Passariano (UD), April 14, 2014.

VOLPATTI D., **BULFON C.**, BASSIGNANA D., TIBALDI E., BRUNO M., SCOCCHI M., FURLAN M., COCCHIETTO M., SAVA G., GALEOTTI M. Valutazione preliminare del lisozima microincapsulato come immunostimolante per somministrazione orale in trota iridea (*O.*

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BULFON C., VOLPATTI D., BOSCHETTO F., GALEOTTI M. Vaccinazione polivalente contro *Listonella anguillarum* (O1 e O2) e *Photobacterium damselae* subsp. *piscicida* in branzino (*Dicentrarchus labrax*). XIX Convegno Nazionale della Società Italiana di Patologia Ittica, poster. Siracusa, November 14-16, 2013.

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