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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES FROM THE RED SEAWEED *CERAMIUM RUBRUM* (HUDSON) (RHODOPHYTA, FLORIDEOPHYCEAE) AGAINST *YERSINIA RUCKERI* AND *SAPROLEGNIA PARASITICA*

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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“ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES FROM THE RED SEAWEED *CERAMIUM RUBRUM* (HUDSON) (RHODOPHYTA, FLORIDEOPHYCEAE) AGAINST *YERSINIA RUCKERI* AND *SAPROLEGNIA PARASITICA*”

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To Matilda…

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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES FROM THE RED SEAWEED *CERAMIUM RUBRUM* (HUDSON) (RHODOPHYTA, FLORIDEOPHYCEAE) AGAINST *YERSINIA RUCKERI* AND *SAPROLEGNIA PARASITICA*

Abstract

Enteric red mouth disease and Saprolegniasis, which are caused by the bacteria *Yersinia ruckeri* and the oomycete *Saprolegnia parasitica*, respectively, are illnesses that affect salmonid farming. Sanitary problems in farms are addressed by the prevention of disease outbreaks or by the treatment of diseases with chemicals. Environmental and governmental restrictions, toxicity and high treatment costs limit the use of drugs. Marine organisms, such as algae, sponges and corals, have developed an antimicrobial defense strategy based on the production of bioactive metabolites. Among these organisms, seaweeds offer a particularly rich source of potential new drugs. Hence, many pharmacologically active substances have been isolated from seaweeds. In the *Ceramium* genus, *C. rubrum* has been emphasized by several authors for its antimicrobial properties. Based on this background, the present study focused on the antimicrobial activity of a lipophilic extract of *C. rubrum* on *Y. ruckeri* and *S. parasitica*. The alga, collected from the Pacific coast of Chile, underwent an ethanol extraction, and the concentrated extract was partitioned between water and dichloromethane. From the dichloromethane extract, fatty acids, fatty acid esters, one hydrocarbon and phytol were identified by gas chromatography - mass spectrometry analysis (GC/MS). The antimicrobial study showed that the whole extract was more active than the individual

components, which suggests a strong synergistic effect among the components. In a column chromatographic, the ethyl acetate extract was partitioned in 20 extracts. By means of bioautographic assays, 9 extracts and 15 spots were active on *Y. ruckeri*. The minimal inhibitory concentration of each active spot was determined by microdilution broth test. Further analysis of these spots includes HPLC, HPLC/MS, IR and NMR analysis to determine metabolites or group of metabolites with inhibitory activity on the bacterium. These results may constitute a basis for promising future applied research that could investigate the use of *C. rubrum* seaweed as a source of antimicrobial compounds against fish pathogens.

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CHAPTER I

General Introduction

GENERAL INTRODUCTION

1.1. Bioactive metabolites production in marine environment

Approximately 70% of our planet is covered by oceans and marine species comprising almost one half of the total global biodiversity. Marine animals and plants, such as sponges, mollusks and algae live in a complex habitat and are subject to sometimes ecological pressures (e.g., competition for space, the fouling of the surface, predation, extreme ambient conditions changes) very different in many aspects from the terrestrial environment (De Carvalho and Fernandes, 2010). Because of this have evolved potent biological mechanisms to survive in this environment, such as epibiosis (Adnan *et al.*, 2018) and biofouling (Dobretsov *et al.*, 2006), are crucial for the evolutionary success of marine organisms. In fact, the production of the bioactive metabolites from oceans organisms is a source of natural products with unique characteristics (Bibi *et al.*, 2017; Habbu *et al.*, 2016; Kiran *et al.*, 2018; Tingting *et al.*, 2019).

Although most natural drugs currently used have their origin terrestrial plants, marine organisms have not been well explored as potential sources of new drugs. The sea offers an enormous resource of novel compounds and there are until now over 29,000 marine natural products, of which approximately 41% discovered in the last 10 years. There are 12 phyla, among which are Chlorophyta, Phaeophyta and Rhodophyta seaweeds, are sources for 36% of the total marine natural products inventory (Carroll *et al.*, 2019). These metabolites are characterized by a broad spectrum of biological activities, such as antimicrobial (e.g., viruses, bacteria, fungi, parasitic) (Abdelmohsen *et al.*, 2017), anticoagulant, anti-inflammatory (Barzkar *et al.*, 2019) and others therapeutic properties (Huang *et al.*, 2019; Vizetto-Duarte

et al., 2020; Sisir and Sharman, 2019; Wali et al; 2019), even toxins (Chekan *et al.*, 2020; Van Dolah, 2000; Ysumoto and Murata, 1993).

As shown at the global marine pharmaceutical pipeline website, https://www.midwestern.edu/departments/marinepharmacology.xml, there are currently 14 approved marine-derived pharmaceuticals, and an additional 23 compounds are either in Phase I, II, and III of clinical pharmaceutical development.

1.2. Bioactive metabolites from seaweeds

Seaweed is a term encompassing photosynthetic, macroscopic, multicellular, benthic and littoral marine organisms. Seaweeds are classified into three major division; the green algae (Chlorophyta), the brown algae (Phaeophyta) and the red algae (Rhodophyta) based on their pigments and coloration (Garson, 1989). The red algae owe their pigmentation to the presence of the photosynthetic accessory pigments phycobiliproteins (phycocyanin and phycoerythrin). The brown color of Phaeophyta algae results from the dominance of the xantophyll and fucoxanthin pigments. The division of green algae contains high amounts of chlorophylls a and b in the same proportions like higher plants (Bold and Wynne, 1985). Seaweeds are sessile organisms, so should not only tolerate the adverse conditions of the marine environment and predators, but also the presence of bacteria, fungi, viruses, protozoa and parasites, that they can be symbiotes or not. Because of this, the seaweeds are interesting by its positive or negative biological properties (Claverie *et al.,* 2020; Khalid *et al.,* 2018).

The biological activities of seaweeds have been studied since many years ago, especially their antiviral and antibacterial properties (Ruggieri 1976; Ehresmann *et al.*, 1977; Richards *et al.*, 1978; Fuhrman 1979). Many investigations have identified the bioactive compounds

against numerous pathogens of animal, vegetal and human diseases. The division most studied and with more class of compound or active metabolites elucidated are Rhodophyta seaweeds, followed by Phaeophyta and Chlorophyta, the least analyzed, due to the number of species in each division (Venugopal, 2011).

The algae have lipids, proteins, essentials minerals, dietary fibers, fatty acids, essential amino acids, omega-3, vitamins A, B, C, and E, and polysaccharides (Černá, 2011; MacArtain *et al.*, 2007; Misurcova *et al.*, 2012). In addition, seaweeds also contain biologically active phytochemicals that play important roles in health-promoting effects such as polyunsaturated fatty acids, terpenoids, xanthophylls, carotenoids, phycobilins, chlorophylls, polysaccharides, vitamins, sterols, tocopherol, phycocyanins and polyphenols, among others (De Almeida *et al.*, 2011).

1.2.1. Bioactivity in Rhodophyta seaweeds

The search for the keywords "activity" "biological" "Rhodophyta" in the browser yielded 27,600 results approximately, with research carried out in the 1960s up to studies published today. This shows the great attention of the scientific community due to the bioactive substances produced by macroalgae. From these results, approximately 4,920 correspond to studies about antibacterial activity and 3,230 about antifungal activity.

Several compounds were isolated and identified from the extracts of red algae with potent antimicrobial activity against food, human and animal pathogens, among others. Thus, were found sesquiterpenes (Bansemir *et al.*, 2004; Vairappan *et al.*, 2008), C-15 acetogenins (Vairappan and Lee 2009), brominated diterpenes (Etahiri *et al.*, 2001; Vairappan *et al.* 2010), triterpenes (Abdel-Raouf *et al.*, 2015), cholesterol derivatives (Kavita et al, 2014)

brominated indole related alkaloid (Li *et al.*, 2016), sulfur-containing polybromoindoles (Fang *et al.*, 2014), sulfated polysaccharides (Seedevi *et al.*, 2017), bromophenols (Oh *et al.*, 2008; Xu *et al.*, 2003) with antibacterial activity, among others. Also, secondary metabolites with antifungal bioactivity have been found in Rhodophyta seaweeds, bromophenols (Oh *et al.*, 2008) and carrageenans (Soares, *et al.*, 2016) were reported with strong activity against human fungi.

An important discovery in the natural products chemistry of the red seaweeds, were the isolation of the acyclic monoterpene halomon in *Portieria hornemannii*, which exhibits selective antitumor activity. Halomon ((3S, 6R)-6-bromo3-(bromomethyl)-7-methyl-2,3,7-trichlorooct-1-ene) was tested at the National Cancer Institute against in vitro human tumor cell line screening panel, brain tumor and exhibited selective antitumor activity (Fuller et al., 1994, 1992; Gunatilaka et al., 1999). Subsequently, the NCI Decision Network Committee selected halomon for preclinical drug development (Wise et al., 2002).

1.2.2. Chilean seaweeds situation

Chilean seaweed studies date from late eighteenth century. Over the next 200 years, the taxonomic knowledge increased due to the activity of foreign expeditions mainly from Europe and America. Then, in a period of 40 years, Chilean scientists completed taxonomic studies of algae in Chile. The knowledge accumulated up to the 90s' allowed to identify 750 species belonging to 230 genera of marine algae in continental Chile, Easter Island and Juan Fernández Archipelago. In Antarctic and subantarctic islands territory of Chile about 500 species have been estimated, but this is not a consensus value. In Chlorophyta have been described about 131 species and 31 genera. There are about 140 species and 60 genera in

Phaeophyta. Rhodophyta algae are the most diverse among marine algae in Chile (480 species /150 genera) (Santelices, 1989).

The principal seaweed utilization is the exploitation and cultivation of red and brown algae for obtaining of colloids. The use of colloids ranging from emulsifier in dairy products, leather, textiles and pharmaceuticals, for the treatment of arthritis, metal poisoning, bone grafting, immobilization of biocatalysts in industrial processes, strengthening health treatment, to leverage beauty, etc. (Khalil *et al.,* 2017). Chile is one of the most important exporting countries of phycocolloids in the world. In fact, algal production in Chile is mainly based upon the exploitation of wild stocks and cultivation on a commercial scale remains restricted to agarophytic *Gracilaria chilensis* (Camus *et al.*, 2018b). Due to the demand of algae carrageenophytic in recent years, the supply of these species also relied on the capture of wild populations of Rhodophyta *Sarcothalia crispata*, *Gigartina skottsbergii* and *Mazzaella laminarioides* (McHugh, 2003). Respect to edibles red algae, nine species of *Porphyra* (Chilean nori) in central Chile appears to be important candidates for the development of nori cultivation (Guillemin *et al.*, 2016). Also, a market has opened in Chile for *Callophyllis variegata* and *Chondracanthus chamissoi* (Buschmann *et al.* 2005; Zúñiga-Jara and Soria-Barreto, 2018; White and Wilson, 2015). Brown algae, *Lessonia trabeculata* and *L. nigrescens* are collected for obtaining of colloid alginate (Dhargalkar and Verlecar, 2009; Peteiro, 2017), and *Macrocystis pyrifera* has been harvested for abalone feeding and *Durvillaea antarctica* is used for human consumption in local markets. Furthermore, the cultivation of *M. pyrifera* has been studied to obtain different products for human consumption and the development of organic fertilizers (Camus *et al.*, 2018a; Vásquez, 2016). Also, from Chlorophyta, *Ulva* species have been used like organic fertilizers

(Buschmann *et al.*, 2005). Seaweed biomass has also been used for the production of biogas through anaerobic digestion (Fasahati *et al.*, 2017; Saratale *et al.*, 2018).

Chilean seaweeds have been studied respect their antimicrobial activity against human, plant and food pathogens, among others. Henríquez *et al.* (1979) evaluated the antibacterial activity of petroleum ether extracts obtained from 33 seaweeds against human pathogen, also Jiménez *et al.* (2011) studied the effects of aqueous and ethanolic extracts obtained from 9 macroalgae against plant bacteria and fungi. Lozano *et al.* (2015) found antiviral activity in the lyophilizate of two red macroalgae *Pyropia columbina* and *Gracilaria chilensis* against infectious salmon anemia virus. Respect to chemical structures from algae with antimicrobial activity isolated from Chilean seaweeds, are important the studies carried out by Cáceres *et al.* (2000), Gallardo *et al.* (2018), Matsuhiro *et al.* (2005), Rovirosa and San Martín (1997) and Rovirosa *et al.* (2013). Bioactive monoterpenes, sulfated galactan, carrageenans, polyhalogenated acetogenins were isolated and tested against pathogens responsible of human diseases. However, a number of researches have studied secondary metabolites from Chilean seaweeds, but have only elucidated chemical structures of bioactive compounds and the biological potential have not been explored or have been studied other activities such as cytotoxic, antioxidant, antifeedant among others (Argandoña, *et al.*, 2002, Barroso *et al.*, 1997; Chandía *et al.*, 2005; Chandía and Matsuhiro, 2008; Chandía *et al.*, 2001; Chandía *et al.*, 2004; Cueto, *et al.*, 1997, Darias *et al.*, 2001, Jerez *et al.*, 1997; Leal *et al.*, 2008; Matsuhiro, 1996; Matsuhiro, 2001; Matsuhiro and Urzua, 1996a and 1996b; Matsuhiro *et al.*, 2007; Matsuhiro *et al.*, 1996; Rovirosa *et al.*, 1999, San Martín *et al.*, 1997; Soto *et al.*, 2003, Vasquez *et al.*, 1998; Zúñiga *et al.*, 2006).

1.2.3. Metabolites isolated from *Ceramium* species

Several metabolites have been isolated from *Ceramium* species. The isethionic acid was isolated from *C. flaccidum* (Barrow *et al.,* 1993). Hydroxylated and methoxylated brominated diphenyl ethers and brominated aromatic compounds also were identified in *C. tenuicorne* (Dahlgren *et al.,* 2015; Enhus *et al.,* 2012; Lindqvist *et al.,* 2017) and *C. rubrum* (Kladi *et al.,* 2005; Pedersén *et al.,* 1974). Trichloroethylene was produce by temperate and subtropical *C. rubrum* (Abrahamsson *et al.,* 1995). The compounds mentioned have in common that they are associated with a stress response so-calle oxidative stress that can be initiated by various forms of stress, mainly connected with inhibited photosynthesis or associated with osmotic challenges such as desiccation, changes in temperature, salinity or light (Barrow *et al.,* 1993; Dring 2005). Marine biota produces a variety of halogen-containing organic compounds that have 1 to 30 carbon atoms. Bromine is the halogen found most often in these marine-derived compounds (Abrahamsson *et al.,* 1995). Some of these compounds are highly toxic, such as certain polybrominated ethers. Bioaccumulation of methoxylated and hydroxylated compounds has been observed in some marine food chains (Kelly *et al*., 2008; Losada *et al.,* 2009; Weijs *et al.,* 2009; Dahlgren *et al.,* 2016), but not in others (Zhang *et al.,* 2010; Barón *et al.,* 2013; Dahlgren *et al.,* 2016). For example, Dahlgren *et al.* (2016) study the bioaccumulation of methoxylated polybrominated diphenyl ethers and hydroxylated polybrominated diphenyl ethers produced by the alga *C. tenuicorne*. The methoxylated compounds increased their concentration as they advanced in the trophic chain, but not the hydroxylated compounds, which decreased towards the upper part of the food web. Debromination from penta and hexa brominated forms in *C. tenuircone* to tetrabrominated forms were observed in fish from the upper part of the food chain.

1.2.4. *Ceramium rubrum* biological activities

Ceramium rubrum (Hudson) (Rhodophyta, Florideophyceae) is a marine alga that occurs in both hemispheres, on the tropical as well as the polar coasts (Boo and Lee, 1994). In Chile *C. rubrum* is an epiphytic organism of the marine alga *Gracilaria chilensis* (Buschmann *et al.*, 2005) an important commercial marine resource. *Ceramium rubrum* deeply penetrates the bark and reaches the marrow tissue resulting in destruction of host cells around the infection (Leonardi *et al.*, 2006; Lam *et al.*, 2008).

In the genus *Ceramium*, especially in *C. rubrum* several authors have emphasized in their antibacterial (Hellio *et al.*, 2001; Bansemir *et al.*, 2006; Salvador *et al.*, 2007; Dubber and Harder, 2008, El Shafay *et al.*, 2016) and antifungal (Salvador *et al.*, 2007). Hellio *et al.* (2001) observed antibacterial activity in dichloromethane extract against several marine bacteria involved in the formation of microlayer in the process of fouling. Bansemir *et al.* (2006) also found strong antibacterial activity in dichloromethane extract from *C. rubrum* against *Pseudomonas anguilliseptica*. Tuney *et al.*, (2007) founded in ethanolic extract a moderate to weak activity against *Candida* sp. and *Enterococcus faecalis*. Dubber and Harder (2008) observed that the hexane and methanol extracts of *C. rubrum* were active and inhibited growth of a variety of marine and fish pathogenic bacteria from the genus *Bacillus* spp., *Pseudoalteromonas* spp., *Vibrio* spp. and *Aeromonas* spp, among others species. *Ceramium rubrum* was strongly effective like to streptomycin activity for some bacteria, due the presence of great quantities of fatty acids as responsible of activity. The chloroform-methanol extract of *C. rubrum* presented effective activity on the bacteria *E. coli* (Bouhlal *et al.*, 2019). El Shafay *et al.*, 2016, studied several extracts obtained from *C. rubrum* against Gram positive and Gram negative bacteria. The diethyl ether, methanol, ethanol and chloroform

extracts have been showed strong activity against *Staphylococcus aureus, Shigella flexneri*, *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* Also, the n-hexane, chloroform and methanol extracts showed moderate antimicrobial activity against assayed Gram (+) and Gram (-) microorganisms by MIC method (Güner and Yavaşoğlu, 2018). In an ecological study, also was demonstrated that *C. rubrum* exhibited some antifungal activity. Although the research did not focus on the chemical analysis, showed that some marine fungi and organisms are affected by defense compounds emitted by this macroalga (Enhus *et al*., 2012; Lam *et al.*, 2008).

1.3. Diseases in Chilean salmon industry

The beginnings of Chilean aquaculture date back to the late nineteenth century. In the late '80s began to take off the salmon industry and since has been so successful that in 2006, Chile became the second largest producer of salmon after Norway, until the collapse caused by the ISA virus in 2010 (Fischer *et al.*, 2017). This crisis brought with it the massive death of fish, the closure of operations and a public, administrative and environmental unrest, which marked a before and after in the industry. The environmental and sanitary regulations, the supervisory capacity, the sanctions and the general sanitary management of diseases in farmed fish were strengthened. Several products used to date have been questioned nationally and internationally, so that many diseases regain importance (Sim-Smith and Diggles, 2019). In freshwater stages of the salmon culture, several diseases can be significant due to economical and sanitary effects. This is the case of the enteritis red mouth (ERM) and Saprolegniasis. The ERM disease is an infection and has as a causative agent *Yersinia ruckeri* (Furones *et al.*, 1993). The transmission occurs primarily horizontal during freshwater phase,

however, is possible to find it during cultivation in ocean. The acute phase of the disease is characterized by high mortality due to the onset of sepsis in younger fish (Rodgers, 1991). In adult individuals, the disease occurs as a chronic infection and its main clinical signs are capillary and venous congestion of the brain and eye glasses. Similarly, hemorrhagic lesions are seen in internal organs, muscles and intestines and the body surface (Troncoso *et al.*, 1994). To treat the disease have been tried with little success some oral bacterins, dipping in a solution of sodium chloride hyperosmotic, increase of ascorbic acid or vitamin E in the diet to improve immune response, exposure to iodophor and various antibacterial such as oxytetracycline, erythromycin, quinolones, tiamulin and sulfonamides (Bullock and Cipriano, 2004). On the other hand, saprolegniasis is caused by the oomycete *Saprolegnia parasitica* is an opportunistic, saprotrophic and necrotrophic pathogen, very virulent and can cause primary infections on salmon in freshwater stages (Neish, 1977; Willoughby and Pickering, 1977), occurring in eggs and fishes. The disease is characterized by an outward appearance similar to cotton, which radiates in a circular, crescent-shaped or whorled (Van West, 2006). *Saprolegnia parasitica* can cause cell necrosis and dermis and epidermis damages, including hyphae can penetrate the muscle and blood vessels of infected fish. Severe cases result in lethargic behavior, loss of equilibrium and commonly death of the fish (Hatai and Hoshiai, 1992). Saprolegnia infection is easily controlled by the application of green malachite, a dye that can be applied alone or in combination with other fungicides (Srivastava, *et al.*, 2004). Control of saprolegniasis had turned to a problem since the malachite green treatment was banned worldwide (Noga, 2010). Formalin also has been used, although it has an acute impact on the aquatic environment (Albert *et al.*, 2013).

1.4. Problem statement and Hypotheses

The control of infectious diseases is essential in the success of salmon aquaculture. The current treatments with synthetic products cause a significant ambient detriment, resistant in the pathogens responsible of diseases and enormous economic losses for salmonid producers. Because of this, alternatives natural's agents are needed to be effective and safe for non-target organisms as well as the environment.

This project will bring together the scientific information necessary to resolve the issues raised, and as a result of it, will provide a package of technology transfer to developing for a pest biological control which may be supplemented by other prevention strategies to assist and support the production of global salmon and decrease the economic losses associated with these infectious diseases and supplement the application of drugs, consolidating salmon and benefiting the region and country.

Because this background, the present study focuses on the new biological activity from *C. rubrum* extract on economically important pathogens of Chilean salmon industry through bioguided assays to characterize chemically the bioactive fractions.

Considering that *Ceramium rubrum* affects the growth and reproduction of several pathogens (i.e., bacteria, fungal, viruses), we hypothesized that *C. rubrum* have amphipathic bioactive metabolites with antibacterial and antifungal activity against *Yersinia ruckeri* and *Saprolegnia parasitica*, pathogens of Chilean salmon industry.

1.4.1. General Objective

To evaluate the antibacterial and antifungal activities, elicited by the red seaweed *C. rubrum* against some pathogens from Chilean salmon industry.

1.4.2. Specific Objectives

i. To evaluate the antibacterial activity elicited by *C. rubrum* against *Yersinia ruckeri*.

ii. To evaluate the antifungal activity elicited by *C. rubrum* against *Saprolegnia parasitica*.

iii. To evaluate the cytotoxic response elicited by *C. rubrum* in brine shrimp, *Artemia franciscana* and zebrafish, *Danio rerio*.

iv. To determine the chemical characteristics of bioactive fractions from *C. rubrum*.

CHAPTER II

"Novel antimicrobial activity of a dichloromethane extract obtained from red seaweed Ceramium rubrum (Hudson) (Rhodophyta, Florideophyceae) against Yersinia ruckeri and Saprolegnia parasitica, agents that cause diseases in salmonids".

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NOVEL ANTIMICROBIAL ACTIVITY OF A DICHLOROMETHANE EXTRACT OBTAINED FROM RED SEAWEED *CERAMIUM RUBRUM* (HUDSON) (RHODOPHYTA, FLORIDEOPHYCEAE) AGAINST *YERSINIA RUCKERI* AND *SAPROLEGNIA PARASITICA*, AGENTS THAT CAUSE DISEASES IN SALMONIDS

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Abstract

Enteric red mouth disease and Saprolegniasis, which are caused by the bacteria *Yersinia ruckeri* and the oomycete *Saprolegnia parasitica*, respectively, are important illnesses that affect salmonid farming. Sanitary problems in farms are addressed by the prevention of disease outbreaks or by the treatment of diseases with chemicals. Environmental and governmental restrictions, toxicity and high treatment costs limit the use of drugs. Marine organisms, such as algae, sponges and corals, have developed an antimicrobial defense strategy based on the production of bioactive metabolites. Among these organisms, seaweeds offer a particularly rich source of potential new drugs. Hence, many pharmacologically active substances have been isolated from seaweeds. In the *Ceramium* genus, *C. rubrum* has been emphasized by several authors for its antimicrobial properties. Based on this background, the present study focused on the antimicrobial activity of a lipophilic extract of *C. rubrum* on *Y. ruckeri* and *S. parasitica*. The alga, collected from the Pacific coast of Chile, underwent an ethanol extraction, and the concentrated extract was partitioned between water and dichloromethane. From the dichloromethane extract, fatty acids, fatty acid esters, one hydrocarbon and phytol were identified by gas chromatography - mass spectrometry analysis (GC/MS). The antimicrobial study showed that the whole extract was more active than the individual components, which suggests a strong synergistic effect among the components. These results may constitute a basis for promising future applied research that could investigate the use of *C. rubrum* seaweed as a source of antimicrobial compounds against fish pathogens.

Keywords: *Ceramium rubrum*, lipophilic extract, antimicrobial activity, *Yersinia ruckeri*, *Saprolegnia parasitica*, salmonids

1. Introduction

Enteric red mouth disease (ERM) and Saprolegniasis, which are caused by the Gram - negative bacteria *Yersinia ruckeri* (Furones, *et al.*, 1993) and the oomycete *Saprolegnia parasitica* (Neish, 1977), respectively, are important illnesses that affect salmonid species and some fish wildlife. ERM disease is controlled with antibiotics, such as quinolones, tiamulines, sulfonamides, oxytetracycline and erythromycin, and antiseptics, such as iodophors (Bullock and Cipriano, 2004). Nevertheless, a decrease in drug efficacy and the development of bacterial resistance have limited their use (Harvey, 2008). A previous study showed that *Saprolegnia* infection could be easily controlled by the application of malachite green, a dye that can be applied alone or in combination with other fungicides (Srivastava, *et al.*, 2004). However, since 2002, the use of malachite green has been banned worldwide due to both carcinogenic and toxicological effects, which has allowed *Saprolegnia* to become a major economic problem in freshwater fish farms (Zaror, *et al.*, 2004). Alternative treatments include bronopol (30 - 100 mg l^{-1}), sodium chloride (30 g l^{-1}), formalin (250 mg l^{-1}), potassium permanganate (10 mg l^{-1}) and hydrogen peroxide (Stueland, *et al.*, 2005a). However, none of these chemicals are more effective than malachite green. In summary, sanitary problems, environmental and governmental restrictions, solubility, toxicity and high cost have limited the use of synthetic antimicrobials (Dixon, 1994); hence, research on new alternatives is necessary.

Many bioactive substances have been isolated from algae. Extracts of seaweeds have been reported to exhibit antibacterial activity (González del Val, *et al.*, 2001; Yi, *et al.*, 2001; Bansemir, *et al.*, 2006; Lakshmi, *et al.*, 2006; Osman, *et al.*, 2010) and antifungal activity (Yi, *et al.*, 2001; Haliki, *et al.*, 2005; Stirk, *et al.*, 2007; Ertürk and Tas, 2011; Stein, *et al.*,

2011). However, scarce literature is available on the antibacterial and antifungal effects of extracts from *Ceramium rubrum* (Hudson) (Rhodophyta, Florideophyceae). *Ceramium rubrum* is a marine alga that is found in both hemispheres on tropical and polar coasts (Boo and Lee, 1994). Hellio *et al.* (2001) observed that the dichloromethane extract of *C. rubrum* that was collected from the French coast elicited antibacterial activity against several marine Gram - positive bacteria. Similar results were reported by Bansemir *et al.* (2006) with samples collected from the German coast. Tuney *et al.* (2006) observed that diethyl ether and ethanol extracts also showed antibacterial properties. Extracts obtained from the German samples using hexane and methanol inhibited the growth of a variety of marine and fish bacteria (Dubber and Harder, 2008). Salvador *et al.* (2007) evaluated the antibacterial effect of fresh and lyophilized extracts and found that *C. rubrum* that was collected from the Mediterranean and Atlantic coasts of Spain was active against Gram - positive and Gram - negative bacteria. There are no reports regarding the antifungal activity of *C. rubrum*.

Seaweeds represent a great source of a variety of natural products (Mayer, *et al.*, 2009), but their veterinary potential in fishes has barely been explored. Therefore, the aim of this study was to investigate the antimicrobial activity of the ethanol and dichloromethane extracts of *C. rubrum* against *Y. ruckeri* and *S. parasitica*, two of the main microbial diseases in Chilean farmed salmonids. The results obtained will form the basis for further studies on marine natural products.

2. Materials and Methods

2.1. Plant collection

The alga *C. rubrum* was collected in the summer of 2010 at the mouth of the Maullín River, which is located to the northwest of the town Maullín (Chile, Los Lagos Region). The collected material was separated and washed with distilled water to remove traces of sand, seaweed and other marine organisms. Finally, the material was kept in plastic bags and frozen at - 20 °C.

2.2. Preparation of the extracts

The alga (20 kg) was extracted using 96 % ethanol 3 times (each extraction period was 24 h). After filtration, the three extracted solutions were pooled, and the solvent was evaporated in a rotary evaporator, which produced the ethanol extract (E). The dichloromethane extract (DCM) was obtained by the liquid - liquid separation of 250 g of the E extract that was suspended in 1000 ml of distilled water and subsequently extracted with three portions of dichloromethane (500 ml). The resulting portions were combined to yielding DCM.

2.3. Gas Chromatography - Mass Spectrometry analysis (GC/MS)

The GC/MS analysis of the DCM was performed through electron impact ionization (70 eV) using a Thermo Finnigan chromatograph (Milan, Italy) that was equipped with a BP - 1 capillary column (30 m 0.22 mm by 0.25 µm; SGE, Victoria, Australia) and used helium as the carrier gas. The GC oven was programmed to ramp from 40 to 300 ºC at 5 ºC min^{-1} and

held for 5 min. The injector and transfer line temperatures were fixed at 250 ºC. The column outlet was inserted directly into the electron ionization source block. The scan range was 40 - 500 Da. Kovats indices (KI) of the DCM compounds were estimated by employing a series of homologous reference compounds. The retention indices were determined relative to an *n* - alkane series by means of linear interpolation. Compounds were identified through a comparison between the KI and the mass spectra with available commercial standards. For non - available standards, spectra were compared with the library mass spectral NIST.

2.4. Antimicrobial tests

2.4.1. Agar diffusion test

Bacterial inhibition was performed using the agar diffusion method (Bauer, *et al.*, 1966). A *Y. ruckeri* pure strain culture was grown on Tryptic Soy Agar (TSA) plates and incubated for 24 h at 26 ± 1 °C. Then, a Tryptic Soy Broth (TSB) suspension was prepared from 5 isolated colonies. The culture tube was incubated for 2 h at 26 ± 1 °C (Furones, *et al.*, 1993), until reaching the turbidity equivalent to 0.5 McFarland standard (1.5×10^8 CFU ml^{-1}). Subsequently, TSA plates were inoculated with the bacterial suspension. Sterilized paper discs (6 mm, Whatman No. 1) were impregnated with 2 µl of a 500 mg ml^{-1} (1 mg $disc^{-1}$) solution of the E, the DCM and the DCM compounds. Finally, the discs were deposited on Petri dishes after solvent evaporation. Additionally, 0.02 % of Tween 80 was gently mixed into the agar after autoclaving the medium at 45 ºC to enhance the solubility of the DCM and DCM compounds (Niu, *et al.*, 2005). All antibiograms were carried out in triplicate. Oxytetracycline discs (30 µg) were used as positive control, and solvents and clean discs

(without solvent) were used as control tests. The plates were incubated for 24 h at 26 ± 1 °C (Furones, *et al.*, 1993), and the inhibition zones were measured taking into consideration the paper disc diameter.

2.4.2. Agar dilution test

The E, DCM and DCM compounds obtained from *C. rubrum* were tested against *S. parasitica* using the agar dilution method. Stock solutions (25,000 µg ml^{-1}) were prepared, and 200 µl of each solution and 19.8 ml of melted Sabouraud agar medium (SA) were pooled into 10 cm plates to achieve the desired concentration (250 µg ml^{-1}). Ten microliters of malachite green solution (500 µg ml^{-1}) and 19.99 ml of melted SA were pooled into positive control plates to achieve the desired concentration (0.25 µg ml^{-1}) (Takada, *et al.*, 2010). Using the same method, we prepared control plates that contained SA alone or SA plus 200 µl of the solvent that was used in the stock solutions. Agar discs (6 mm diameter and 3 mm thick) taken from the margins of actively growing colonies of *S. parasitica* cultures were placed in the middle of the plates. The experiments were carried out in triplicate. All the plates were incubated at 18 ± 1 °C for 5 days in darkness. The fungal growth was measured in cm from the agar disk margin (Macchioni, *et al.*, 1999), and the inhibition of *S. parasitica* was obtained by comparing the fungal colony growth diameter with the controls (only SA) (Meinelt, *et al.*, 2007).

2.4.3. Broth dilution test

Minimal inhibitory concentration (MIC) values for *Y. ruckeri* and *S. parasitica* were determined by the broth dilution method. In the antibacterial assays, a two - fold serial dilution of DCM was prepared. Then, 800 µl of TSB, 100 µl of inocula compatible with 0.5 McFarland standard and 100 µl of each DCM dilution were added to an assay tube to achieve concentrations between 1 and 2048 µg ml^{-1}. Oxytetracycline (0.5 - 1 µg ml^{-1}) was used as the positive control. All the mixtures were made in triplicate and incubated for 24 h at 26 ± 1 ºC. Once the incubation time elapsed, the absorbance at 600 nm was monitored (Secades and Guijarro, 1999). The bacterial inhibition was calculated per the following equation: % growth inhibition = 100 - [absorbance of the treatment tube / (absorbance of the growth control tube – the medium control tube)] × 100 (Leippe, *et al.*, 1994). To determine the minimum bactericidal concentration (MBC), a loopful of each assay tube that showed growth inhibition was transferred into a Petri dish that only contained TSA (Sreenivasan, *et al.*, 2010).

For the antifungal assays, *Saprolegnia* agar pieces excised from colonized SA plates were incubated in 500 ml of Sabouraud broth (SB) at 21 ± 1 ºC for two days. Subsequently, the grown mycelia mass was washed twice with sterile distilled water, transferred to an Erlenmeyer flask that contained 500 ml of sterile tap water and incubated at 21 ± 1 ºC for 24 h. The spore suspensions were filtered through sterilized gauze into another flask (Stueland, *et al.*, 2005b), and the cysts were counted using a Neubauer chamber. The cyst suspension was adjusted to 5×10^5 spore's ml^{-1} and transferred to the challenge tubes within 3 h of counting (Ali, 2005). To determine the MIC value, a two - fold serial dilution of DCM was prepared, and 800 µl of SB, 100 µl of inocula and 100 µl of each dilution of DCM were added to an assay tube to achieve the desired concentrations between 250 and 2000 µg ml^{-1}.

Malachite green (0.1 - 0.5 µg ml^{-1}) was used as the positive control (Takada, *et al.*, 2010). All the mixtures were made in triplicate and incubated for 5 days at 15 ± 1 ºC (Hatai and Hoshiai, 1992). When the incubation time elapsed, the absorbance at 550 nm was monitored (Astuti, 2006). The fungicidal inhibition was calculated per Leippe *et al.* (1994), and the minimum fungicidal concentration (MFC) was calculated per Sreenivasan (2010).

3. Results

3.1. GC/MS analysis

Twenty kilograms of *C. rubrum* yielded 250 g of the E extract. Subsequently, the E was partitioned to obtain 75 g of the DCM. GC/MS analysis of the DCM resulted in the identification of 14 compounds *(*one alkane, one ketone, fatty acids, fatty acid esters and one diterpene). Phytol (44.7 %) and palmitic acid (25.8 %) were the major components (Table 1). Seven of the compounds, representing 86.4 % of the DCM, were tested in the antimicrobial assays.

Table 1: Compounds identified by GC/MS analysis in the DCM obtained from *C. rubrum*.

Compound	Rt[1]	Molecular ion	Mean % ± SD[2]	KI (Exp)[3]
Hexadecane	26.44	226	4.8 ± 1.9	1694
Myristic acid	27.72	228	4.1 ± 0.5	1756
Ethyl myristate	28.09	256	1.2 ± 0.5	1763
Hexahydrofarnesyl acetone	29.13	268	0.6 ± 0.1	1823
Hexadecenoic acid, Z – 11	31.28	254	4.5 ± 1.3	1932
Palmitic acid	31.96	256	25.8 ± 4.9	1967
Ethyl palmitate	32.06	284	6.7 ± 3.2	1972
Phytol	34.38	296	43.2 ± 1.5	2097
Oleic acid	34.77	282	2.5 ± 0.7	2120
Stearic acid	35.19	284	1.3 ± 0.3	2144
Ethyl linoleate	37.07	308	0.6 ± 0.4	2150
Ethyl linolenate	37.14	306	0.1 ± 0.2	2165
DHA methyl ester	39.48	342	1.4 ± 0.1	2260
EPA methyl ester	39.80	316	1.6 ± 0.4	2274

[1]Rt: Retention time. [2]SD: Standard deviation. [3]KI (Exp): Kovats Index (Experimental)

3.2. Antimicrobial activity

The E, the DCM and the compounds identified in the DCM inhibited the growth of *Y. ruckeri* and *S. parasitica*, but these were less active than the positive controls ($p < 0.04$, Conover - Inman test). The highest antibacterial inhibition was achieved with the DCM (14.7 mm), whereas the highest antifungal inhibition was achieved with the DCM and with stearic acid (> 17.6 %). None of the DCM compounds inhibited bacterial growth more than the DCM; however, the DCM and stearic acid had similar antifungal inhibition (Table 2). The MIC

values of the DCM resulted in 507.7 µg ml^{-1} and > 2048 µg ml^{-1} for *Y. ruckeri* and *S. parasitica*, respectively.

Table 2: Antimicrobial activity of the investigated extracts and the DCM compounds against *Y. ruckeri* and *S. parasitica*.

Extract/Compound	*Yersinia ruckeri*[1] (mm of inhibition)		*Saprolegnia parasitica*[2] (% of inhibition)
	TSA	TSA + Tween 80	SA
E extract	8.7 ± 0.6[b]*	8.1 ± 0.2[c]	1.7 ± 0.2[f]
DCM extract	-	14.7 ± 0.6[b]	17.6 ± 0.2[b]
Ethyl myristate	nt	7.3 ± 0.6[d]	1.7 ± 0.0[f]
Ethyl palmitate	nt	8.0 ± 0.0[c]	10.8 ± 0.1[c]
Myristic acid	nt	8.0 ± 0.0[c]	2.9 ± 0.2[e]
Palmitic acid	nt	8.0 ± 0.0[c]	2.5 ± 0.2[e]
Oleic acid	nt	7.2 ± 0.3[d]	1.0 ± 0.1[g]
Stearic acid	nt	7.0 ± 0.0[d]	17.9 ± 0.1[b]
Phytol	nt	8.0 ± 0.0[c]	5.0 ± 0.0[d]
Oxytetracycline (30 µg)	28.3 ± 0.6[a]	27.9 ± 0.5[a]	nt
Malachite green (0.25 µg ml^{-1})	nt	nt	44.2 ± 0.1[a]

Concentration tested: [1]1 mg $disc^{-1}$. [2]250 µg ml^{-1} per plate. (-) not active. (nt) not tested. (*) The means that are followed by the same letter do not differ significantly at $p < 0.05$ (Conover - Inman test).

Four DCM compound mixtures were tested: reconstituted sample, the main compounds, fatty acids and fatty acid esters. None of these mixtures were more active than the DCM. There is a direct relation between the antibacterial activity and the percentage of the compounds of the DCM tested, which suggests that the minor compounds could play an important role in

the synergistic effect. In the antifungal assay, the highest activities were achieved when the mixture tested included stearic acid (Table 3).

Table 3: Antimicrobial activity of the DCM compound mixtures against *Y. ruckeri* and *S. parasitica*.

Compound	RS	MC	FA	FAE	IND
Hexadecane					
Myristic acid	•		•		•
Ethyl myristate	•			•	•
Hexahydrofarnesyl acetone					
Hexadecenoic acid, Z – 11					
Palmitic acid	•	•	•		•
Ethyl palmitate	•			•	•
Phytol	•	•			•
Oleic acid	•		•		•
Ethyl linoleate					
Ethyl linolenate					
Stearic acid	•		•		•
Methyl DHA					
Methyl EPA					
% of DCM	86	71	34	8	-
[1]Antibacterial activity (mm)	9.0 ± 0.5^{a}*	8.5 ± 0.2^{a}	7.0 ± 0.1^{b}	7.0 ± 0.1^{b}	$< 8.0 \pm 0.0$
[2]Antifungal activity (%)	15.3 ± 0.1^{b}	11.2 ± 0.2^{c}	18.2 ± 0.5^{a}	10.9 ± 0.2^{c}	$< 17.9 \pm 0.1$

Concentration tested: [1]1 mg $disc^{-1}$. [2]250 µg ml^{-1} per plate. RS: reconstituted sample; FA: fatty acid; FAE: fatty acid esters; MC: main compounds; IND: single compounds (commercial standards). (*) The means that are followed by the same letter do not differ significantly at $p < 0.05$ (Conover - Inman test).

4. Discussion

A variety of organisms are capable of synthesizing substances that have antibacterial and antifungal activity. These properties have been observed in extracts obtained from several algae (González del Val, *et al.*, 2001; Yi, *et al.*, 2001; Lakshmi, *et al.*, 2006; Osman, *et al.*, 2010). Nevertheless, previous studies evaluating this characteristic in *C. rubrum* are scarce. The results from the present study indicate that the antimicrobial ability of *C. rubrum* is primarily driven by the lipophilic extract. This agrees with the results reported by Hellio *et al.* (2001), Bansemir *et al.* (2006) and Dubber and Harder (2008), who observed that lipophilic extracts (obtained using dichloromethane and hexane) inhibited the growth of a variety of marine bacteria. Whereas Bansemir *et al.* (2006) did not find inhibitory effects against *Y. ruckeri,* Dubber and Harder (2008) reported a growth inhibition between 50 and 90 % on the same bacteria. The hexane extract of *C. rubrum* exhibited antibacterial activity at 10,600 μg ml^{-1}. In our research, the DCM of *C. rubrum* presented 100 % bacterial inhibition on *Y. ruckeri* at approximately 500 μg ml^{-1}.

There are no reports regarding the antifungal activity of *C. rubrum*. The present study is the first study that has reported the antifungal activity of this alga against *S. parasitica.* The maximum activity for DCM and for stearic acid (> 17.6 % of inhibition) occurred at 250 μg ml^{-1}. These results are consistent with other studies of antifungal inhibition in Rhodophyta seaweeds. Indeed, the ethyl acetate and chloroform extracts of *Solieria robusta* inhibited the growth of several fruit spoiling fungi (Khanzada, *et al.*, 2007). The results reported by Stein *et al.* (2011) indicated that hexane and chloroform extracts obtained from several *Laurencia* species possess antifungal properties.

The present results agree with those reported in other Rhodophyta algae studies. Bansemir *et al.* (2004) observed that the dichloromethane extract of *Laurencia chondrioides* (2 mg disc^{-1}) was active on *Pseudomonas angulliseptica* (Gram - negative) with an inhibition of 15.0 ± 7.3 mm, but it was not active on *Y. ruckeri.* In another study, the dichloromethane extracts (2 mg disc^{-1}) obtained from *Asparagopsis armata*, *Falkenbergia rufolanosa* and *Gracilaria cornea* were active on *Y. ruckeri* with inhibitions of 21.3 ± 1.7, 13.3 ± 1.9 and 9.0 ± 2.6 mm, respectively, whereas *C. rubrum* extracts were not active (Bansemir, *et al.*, 2006). Karabay - Yavasoglu *et al.* (2007) found that an inhibition of 15 mm was obtained using a chloroform extract from *Jania rubens* (4 mg disc^{-1}) on *Enterobacter cloacae* (Gram - negative). It is difficult to assess the antimicrobial activity against *S. parasitica* because there are no reports about the fungal inhibition induced by algae extracts. Several studies have shown that medicinal plants, chitosan, humic substances and compounds isolated from bacteria have antifungal activity against *S. parasitica*. The active compound Oridamycin A, which was isolated from *Streptomyces* sp., exhibited an anti - *S. parasitica* activity with a MIC value of 0.3 µg ml^{-1} (Takada, *et al.*, 2010). Udomkusonsri *et al.* (2007) reported that crude extracts from Thai plants produced a fungal inhibition growth between 11 and 100 % at 250 µg ml^{-1}. Meinelt *et al.* (2007) observed that humic substances from non - eutrophicated freshwater ecosystems produced a 40 % inhibition of *S. parasitica* growth at 250 µg ml^{-1}.

Dubber and Harder (2008) hypothesized that the antibacterial effect of *C. rubrum* extracts is due to the presence of significant quantities of unidentified fatty acids. Hence, this is the first report about active metabolites from the lipophilic extract of *C. rubrum*. In this study, 7 of the 14 identified metabolites were tested on the pathogens. These metabolites included fatty acids, fatty acid methyl esters and an acyclic diterpene alcohol. Stearic acid was the most

active compound against *S. parasitica*, but none of the identified compounds were more active than the dichloromethane fraction against *Y. ruckeri*. Per Bansemir *et al.* (2004), the antimicrobial activity of the extract could be a synergistic effect of its constituents because the pure compounds only showed a weak effect (the exception was the stearic acid activity against *S. parasitica*).

Many antibacterial metabolites have been isolated from aqueous or very polar extracts of red algae (Takahashi, *et al.*, 2002; Vairappan, 2003; Xu, *et al.*, 2003). Despite numerous studies that show the biological activity of polar compounds, it is not uncommon to find antimicrobial activity based on lipophilic extracts obtained from algae. It seems that the use of lipophilic compounds in defense is an effective adaptation that ensures the presence of the active compound in the aquatic system if possible. In addition, water - insoluble compounds are also advantageous in that they diffuse slowly into the seawater, which ensures that the potency of the metabolite will remain intact for some period (Bryan, *et al.*, 1997). Several antimicrobial metabolites, including diterpenes, bromophenols, sesquiterpenoids and halogenated metabolites, share two aspects: their small molecular size and their lipophilic character. These physicochemical attributes are suggested to be of critical significance for metabolites to function in natural defense (Harder, *et al.*, 2004).

Other than the stearic acid activity against *S. parasitica*, none of the DCM compound mixtures were more active than the DCM. The findings that none of the DCM compounds were responsible for the antibacterial activity and that the reconstituted sample (86 % of the DCM) was less active than the DCM indicates that a synergistic effect may be occurring. Although there are no reports of synergism among algae metabolites, this phenomenon is observed in plants. Sökmen *et al.* (2004) observed synergism between the antimicrobial

compounds obtained from the essential oil of *Achillea biebersteini.* The essential oil was fractioned by column chromatography, and 9 extracts were obtained and tested. The authors observed that the activity was mainly observed in the extracts that contained eucalyptol and camphor, followed by those containing borneol and piperitone. In addition, Carpinella *et al.* (2005) observed an antifungal synergistic effect between scopoletin, vanillin, 4 - hydroxyl - 3 - methoxycinnamaldehyde and pinoresinol that were isolated from *Melia azedarach* fruits. Although the major components of a natural sample generally represent its biological features, they are not necessarily responsible for the greatest activities (Lis - Balchin, *et al.*, 1998). Various minor components may contribute to the biological activities (Patharakorn, *et al.*, 2010). Chairgulprasert *et al.* (2008) indicated that minor components of volatile oils may produce an antimicrobial effect when combined with other minor active components. Thus, it is possible that 2 - decen - 1 - ol, a minor component of the essential oil of *Elettariopsis curtisii*, may enhance the essential oil's antimicrobial activity. In the DCM of *C. rubrum*, minority compounds (14 % of DCM) or their mixtures could be responsible for activity.

The present results could indicate the occurrence of synergy between the DCM components because i) weak antibacterial activity was observed when the DCM compounds were evaluated separately, ii) the reconstituted sample (86 %) had weak antibacterial activity, and iii) there was a trend toward increased antibacterial activity as more of the DCM compounds were included in the tested sample.

The possible mechanisms of the antimicrobial activity of fatty acids can explain the synergy between the DCM components. The antibacterial activity of fatty acids depends on their structure. Although there is disagreement in the literature, some general considerations are

possible. The free carboxylic group is the most important structural requisite for antibacterial activity because it allows an optimal insertion into cells that have hydrogen - bond - acceptor groups in the membrane. The corresponding esters have little or no activity (Zheng, *et al.*, 2005). Other structural factors that modulate the antimicrobial activity of fatty acids are the length and degree of unsaturation of the carbon chain and the stereochemistry of the unsaturation.

Due to the amphipathic character of fatty acids, the microorganism's membrane is the target of fatty acids. After fatty acid - induced damage occurs, other molecules can penetrate the membrane and affect different metabolic processes (Desbois and Smith, 2010).

Ceramium rubrum is an interesting candidate to investigate for active metabolites against *Y. ruckeri* and *S. parasitica*. In summary, the growth of the *Y. ruckeri* and *S. parasitica* pathogens was inhibited to varying degrees by extracts from *C. rubrum*. Because of the natural origin of the extracts or pure compounds from *C. rubrum*, they have a lower hazard potential than drugs and are a potential alternative to prevent and control outbreaks of salmonid diseases.

5. Conclusions

The present study determined that *C. rubrum* contains metabolites that possess antibacterial and antifungal activity against fish pathogens. The lipophilic extract was shown to be active against *Y. ruckeri* and *S. parasitica*; thus, the results suggest the use of *C. rubrum* as a source of antimicrobial compounds.

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CHAPTER III

"Antibacterial activity of ethyl acetate extract and isolated compounds from red algae Ceramium rubrum (Hudson) (Rhodophyta: Florideophyceae) against Yersinia ruckeri, responsible of the enteric red mouth disease, leaded by bioautographic assay"

ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACT AND ISOLATED COMPOUNDS FROM RED ALGAE *CERAMIUM RUBRUM* (HUDSON) (RHODOPHYTA: FLORIDEOPHYCEAE) AGAINST YERSINIA RUCKERI, RESPONSIBLE OF THE ENTERIC RED MOUTH DISEASE, LEADED BY BIOAUTOGRAPHIC ASSAYS

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Abstract

Yersinia ruckeri is a bacterium responsible of the enteric red mouth disease that affects to salmon species and some wildlife fishes. This illness has been addressed, principally, using antibiotics, though, the develop of antibiotic resistance, environmental and governmental restrictions, and toxicity problems have limited their use. Thus, alternatives to cope with this disease are necessaries. Seaweeds are bountiful reservoirs of bioactive compounds which exhibit substantial antibacterial activity against a broad range of bacteria. However, literature about the antibacterial effects of *Ceramium rubrum* is scarce. Previously, we reported the antibacterial effect of the dichloromethane extract, and some of its compounds of *C. rubrum* against *Y. ruckeri*. This time, we evaluated the antibacterial activity of 9 compounds obtained from ethyl acetate extract against *Y. ruckeri*, leaded by direct bioautographic assays. The ethyl acetate extract was obtained by liquid-liquid extraction from an ethanolic liquor and partitioned by column chromatography. The bioautographic assay pointed 15 antibacterial spots, which 9 were isolated by pTLC. The phytochemical screening performed to the compounds was positive to phenolic compounds only, and their IR spectrums showed characteristic vibrations for aliphatic, aromatic, carbonyl and hydroxyl groups. Minimal inhibitory concentrations, determined by microdilution broth tests, ranged from < 0.053 to 54.075 µg/mL. Therefore, *C. rubrum* possesses strong antibacterial activities, showing a great potential as source of antibacterial agents to deal with *Y. ruckeri* strains.

Keywords: *Ceramium rubrum*, *Yersinia ruckeri*, ethyl acetate extract, bioautography, antibacterial activity, phenolic compounds.

1. Introduction

Enteric red mouth (ERM) disease, which is caused by the Gram-negative bacteria, *Yersinia ruckeri* (Furones *et al.*, 1993), is an important illness that affects to salmon species and some wildlife fishes. ERM disease has been addressed taking prevention measures and by treating with antibiotics, such as, quinolones, tiamuline, sulfonamides, oxytetracycline and erythromycin; and antiseptics such as iodophors (Bullock and Cipriano, 2004). However, the decrease of the antibiotic efficacy and the continuous appearance of antibiotic-resistant bacterial strains have limited their use (Bhowmick *et al.* 2020; Harvey, 2008). These matters and others, such as, environmental and governmental restrictions, solubility and toxicity problems and high cost of treatments have limited the use of antibiotics (Dixon, 1994). Therefore, alternatives to cope with this disease are necessaries and marine natural products are increasingly receiving attention in this quest (Bhowmick *et al.* 2020; Mayer, *et al.*, 2020).

Aquatic sources harboring myriad life forms that are bountiful reservoirs of bioactive compounds exhibit in immense potential can no longer be neglected (Bhowmick *et al.* 2020). Antibacterial compounds have been isolated from several aquatic sources, such as, seaweeds, sponges and corals (Donia and Hamann, 2003). Algae exist in almost all kinds of ecosystems around the world. Their ability to survive in extreme conditions and under competitive environments facilitates their production of a heterogeneous group of bioactive compounds via complex metabolic pathways that are largely different from those of terrestrial organisms (Gorelova *et al.*, 2019; Sasso *et al.*, 2013). The metabolites of algae represented various classes of natural products, including terpenes, polysaccharides, alkaloids, and steroids (Rengasamy *et al.*, 2020; Mayer, *et al.*, 2020) and those bioactive compounds exhibit substantial antibacterial activity against a broad range of bacteria, including multidrug-

resistant strains (Bhowmick *et al.* 2020). Particularly, antibacterial activity has been widely studied in red algae (Rhodophyta) (González del Val, *et al.*, 2001; Yi, *et al.*, 2001; Lakshmi, *et al.*, 2006; Osman, *et al.*, 2010), However, literature about the antibacterial effects of the red algae, *Ceramium rubrum* (Hudson) (Rhodophyta: Florideophyceae) is scarce. *Ceramium rubrum* is a marine red alga that is found in both hemispheres on tropical and polar coasts (Boo and Lee, 1994), and some authors have emphasized their antibacterial properties. As far as we know, Kesternich *et al.* (1997), reported for the first time, the antibacterial activity of five compounds isolated from liposoluble extract of *C. rubrum* against Gram-negative bacteria. Hellio *et al.* (2001) demonstrated that the dichloromethane extract of *C. rubrum* collected in France showed antibacterial properties against several marine Gram-positive bacteria. Similar results were reported by Bansemir *et al.* (2006) with samples collected from Germany coast. Hexane and methanol extracts obtained from other German samples inhibited the growth of a variety of marine and fish bacteria (Dubber and Harder, 2008). Tuney *et al.* (2006) informed that diethyl ether and ethanol extracts showed antibacterial activities. Salvador *et al.* (2007) reported that the fresh and lyophilized extracts of *C. rubrum* collected from Mediterranean and Atlantic coasts of Spain were active against Gram-positive and Gram-negative bacteria. A few years ago, El Shafay *et al.* (2016) evaluated the antibacterial activity of diethyl ether, methanol, ethanol and chloroform extracts from *C. rubrum* and three brown algae (Phaeophyta), collected in Hurghada coastal along the Red sea (Egypt), against ten multidrug resistant (MDR) clinical isolates of Gram-positive and Gram-negative bacteria. Data revealed that *C. rubrum* diethyl ether extract showed antibacterial activity against nearly all tested species and all the other extracts showed activity against at least three bacterial strain. Previously, we reported the antibacterial effect of the dichloromethane extract, fatty acids, fatty acid esters, one hydrocarbon and phytol obtained

from *C. rubrum* collected from the pacific coast of Chile, against *Y. ruckeri*, one of the main microbial diseases in Chilean farmed salmonids. The antimicrobial study showed that the whole extract was more active than the individual components, which suggests a strong synergistic effect among the components (Cortés *et al.*, 2014). This time, we evaluated the antibacterial activity of ethyl acetate extract and 9 compounds isolated from *C. rubrum* against *Y. ruckeri*, leaded by Thin layer chromatography - Direct bioautography (TLC-DB).

2. Materials and Methods

2.1 Plant Material

Ceramium rubrum algae was collected at the Maullín River mouth (41°36’ S, 73°38’ W), located to the northwest of Maullín town (Chile, Los Lagos Region) in summer 2010. The material was placed in plastic bags and kept cool on ice. In the laboratory, the alga was washed with distilled water to remove surface salts, sand and epiphytes.

2.2 Ethyl acetate *C. rubrum* extract obtention

Fresh and milled *C. rubrum* algae (10 kg) were macerated in ethanol (96%) at room temperature for 24 h. The suspension obtained was filtered through a frit funnel and the resulting ethanolic extract was evaporated under reduced pressure using a rotary evaporator yielding a dark green ethanolic extract (115 g). Then it was suspended in 1L of distilled water and it was partitioned by liquid–liquid extraction using 10 portions of 500 mL of ethyl acetate. Portions were pooled and filtered through a frit funnel. The resulting extract was dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure on rotary evaporator, yielding a yellow-brown ethyl acetate extract (EAE, 2.2 g).

2.3 Ethyl acetate extract separation

The EAE (2 g) was fractioned by column chromatography (3.5 × 45 cm) with 100 g of silica gel 60 (70-230 Mesh, 0.063-0.200 mm particle size) as a stationary phase (Pavia, *et al.*, 2005), using the following elution gradient: hexane (HE), HE-EtOAc step gradient, EtOAc, EtOAc-acetone step gradient, acetone, and MeOH. Fractions were collected each 20 ml. The resulting fractions were evaporated under reduced pressure and were analyzed using thin layer chromatography on silica gel 60 F_{254} pre-coated plates. The plates were examined under UV light at 254 and 365 nm. Similar fractions were combined (Fried and Sherma, 1999), concentrated in a rotary evaporator up to 2 mL in volume, and then were transferred to amber vials and dried under N_2 flow to obtain 20 fractions (EAE-1 up to EAE-20). The fractions were subject to a TLC-DB assay to screening the bioactive compounds.

2.4 Thin layer chromatography-Direct bioautography (TLC-DB)

Yersinia ruckeri clinical strains were grown on Tryptic Soy Broth (TSB) (Sigma-Aldrich, US) medium and incubated at 26 °C for 8 h (Furones, *et al.*, 1993) with agitation (220 rpm). Each EAE fraction (5 mg) were seeded onto 10 x 10 cm thin layer chromatography (TLC) aluminum plates coated with silica gel (Merck, 60 F_{254}). The plates were developed using the following elution gradients: EAE-1 – 3: HE:EtOAc 95:5; EAE-4 – 10: HE:EtOAc 72:25; EAE-11 – 14: HE:EtOAc 65:35; EAE-15 and EAE-16: HE:Acetone 45:55; EAE-17 and EAE-18: HE:Acetone 35:65; EAE-19 and EAE-20: HE:Acetone 20:80. After, the TLC plates were sprayed with a *Y. ruckeri* at 0.5 McFarland standard concentration (1.5 x 10^8 CFU/mL). Oxytetracycline (0.5 µg/mL) was used as positive control. The sprayed plates were placed into Petri dishes (150 × 25 mm) on paper filter moistened with sterile distilled water and

incubated overnight at 26 ºC. After incubation time, a 0.2% aqueous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, US) was sprayed onto the plates (Tiew *et al.*, 2003). The plates were incubated overnight at 26 ºC again. The dehydrogenases of living microorganisms convert tetrazolium salt into a purple formazan. Thus, the inhibition zones appearing as creamy spots against a purple background (Choma and Jesionek, 2015).

2.5 Bioactive compounds isolation

Each fraction containing active compounds was seeded onto 20 x 20 cm preparative glass chromatoplates (pTLC) coated with 1 mm silica gel (Merck, 60 F_{254}). The plates were developed two times using the following elution gradients: EAE-8 – 10: HE:EtOAc 72:25; EAE-11 – 14: HE:EtOAc 65:35; EAE-15: HE:Acetone 45:55; EAE-17 and EAE-18: HE:Acetone 35:65. The selected bands were removed from the chromatoplates and the adsorbed compounds were extracted from the silica gel powder using CH_2Cl_2 - MeOH (7:3). Subsequently, the suspensions were vacuum filtered, and the filtrates were dried with anhydrous Na_2SO_4. Finally, each sample was evaporated to dryness using a rotary evaporator, yielding 9 bioactive compounds.

2.6 Chemical characterization

2.6.1 Phytochemical screening in EAE fractions by thin layer chromatography (TLC).

For the phytochemical screening, each fraction of EAE containing the bioactive compounds was analyzed by TLC on silica gel 60 F_{254} pre-coated plates (10 cm x 10 cm) and were developed using the following elution gradients: EAE-1 – 3: HE:EtOAc 95:5; EAE-4 – 10: HE:EtOAc 72:25; EAE-11 – 14: HE:EtOAc 65:35; EAE-15 and EAE-16: HE:Acetone 45:55;

EAE-17 and EAE-18: HE:Acetone 35:65; EAE-19 and EAE-20: HE:Acetone 20:80. Classification of the bioactive compounds present in EAE fractions were done by spraying the chromatoplates with the following spray reagent: For terpenoids – *p*-anisaldehide/sulfuric acid reagent (Wagner *et al.*, 1984); alkaloids - Dragendorff reagent (DRG) (Wagner *et al.*, 1984); amino acids and biogenic amines – ninhydrin reagent (NIH) (Wagner *et al.*, 1984); phenolic compounds: Folin-Ciocalteu (FC) reagent, potassium hydroxide reagent (KOH), and lead acetate reagent (LA) (Aslan *et al.*, 2019); carbohydrates – Molisch reagent; and steroids – phosphoric acid reagent (Merck, 1972).

2.6.2 Infrared spectroscopy analysis

Bioactive compounds from EAE were analyzed by attenuated total reflectance fourier transform infrared spectroscopy (ATR)-FT-IR. The IR spectra were recorded on a Cary 630 FTIR spectrometer (Agilent Technologies, Inc., CA, USA) equipped with a liquid transmission, ZnSe beam splitter, and DTGS detector was used to analyze fluid samples. Aliquots of each samples (3 µL) were placed on the zinc selenide (ZnSe) crystal, and IR spectra were collected in the absorbance mode by using a DialPath transmission technology in the portable MIR selected at 30 µm fixed path length created by an optical head that rotates into position, sandwiching the sample between two IR transparent ZnSe windows. In-between measurements, the crystal was carefully cleaned with ethanol and dried with tissue. The samples were scanned at room temperature. The spectrum was collected in the frequency range of 4000-650 cm^{-1} using a 4 cm^{-1} resolution, and 64 interferograms were co-added to improve the signal to-noise ratio. Subsequently, the spectra were corrected against the background spectrum of air. Infrared spectra of background and samples were observed using Agilent MicroLab PC software.

2.7 Minimum inhibitory concentration (MIC) determination

The minimal inhibitory concentration (MIC) determination assays were performed as established by the Clinical and Laboratory Standards Institute (CLSI, 2005) guidelines. The two-fold standard micro broth dilution method was performed in 96-well plates (Falcon, Becton Dickinson). Briefly, *Y. ruckeri* clinical strain was incubated overnight in 3 mL of MH broth at 26 °C with shaking at 220 rpm. The bacterial cultures were then diluted in tris-buffered solution (TBS) to McFarland 0.5 (1.5 x 10^8 CFU/mL). To each well, 188 µL of MH broth, 10 µL of the sample (0.2 to 108.0 µg/mL), and 2 µL of a bacterial suspension were added to McFarland 0.5 to achieve a final volume of 200 µL. In addition, some wells were used as solvent and sterility controls. The plates were incubated at 26 °C for 24 h. Then, 10 µl of MTT aqueous solution (5 mg/ml) was added and the plates were incubated again (Pieters, *et al.*, 1990). After that, the optical density was measured at 600 nm (OD_{600}) (Secades and Guijarro, 1999) in an Epoch microplate reader (BioTek Instruments, Inc., VT, USA). The MIC of Oxytetracycline (0.5-1 µg/ml), used as positive control, was determined by the same method. All results are expressed as µg/mL, and experiments were performed in triplicate in three individual experiments. The bacterial inhibition was calculated according Leippe, *et al.* (1994), and the MIC was defined as the lowest concentration of a sample for which no growth was observed.

3. Results

A yellow-brown resin was isolated using from the ethanolic extract of the red algae *C. rubrum* using ethyl acetate. The resin, denominated ethyl acetate extract (EAE, 2.2 g, 0.022 % in fresh matter), was chromatographically portioned yielded 20 fractions: EAE-1 (14 mg),

2 (128 mg), 3 (64 mg), 4 (59 mg), 5 (238 mg), 6 (59 mg), 7 (40 mg), 8 (15 mg), 9 (29 mg), 10 (61 mg), 11 (42 mg), 12 (15 mg), 13 (33 mg), 14 (132 mg), 15 (13 mg), 16 (150 mg), 17 (173 mg), 18 (85 mg), 19 (83 mg), 20 (488 mg). Each fraction was subject to a TLC-DB assay to screening the bioactive compounds.

3.1. Direct-bioautographic assays

The direct-bioautographic assay proved that nine EAE fractions could effectively inhibit the growth of *Y. ruckeri*. Among those, EAE-8, 9 and 10 showed three strong inhibition zones in each of those fractions, suggesting that more than one compound from each fraction possessed an antimicrobial effect. Interestingly, EAE-11, 12, 14, 15, 17 and 18 showed promising antibacterial effects. The remaining 11 fractions did not present any detectable inhibition zone, thus precluding any antibacterial activity against *Y. ruckeri*. Following the results of TLC-DB assay, 9 bioactive compounds (BC) were isolated by pTLC. The amount of the bioactive compound from *C. rubrum* is summarized in Table 1. Bioactive compounds were analyzed by a phytochemical screening and by attenuated total reflectance Fourier transform infrared spectroscopy (ATR)-FT-IR.

3.2 Phytochemical screening of the bioactive compound

The results of phytochemical screening performed to bioactive compounds is outlined in Table 1. The qualitative tests performed to all bioactive compounds were negative to ninhydrin reagent, *p*-anisaldehide/sulfuric acid reagent, Dragendorff reagent, Molisch reagent, and phosphoric acid reagent, ruling out the presence of amino acids and biogenic amines, terpenoids, alkaloids, (Wagner *et al.*, 1984), carbohydrates and steroids (Merck, 1972). On the contrary, the screening was positive to Folin-Ciocalteu, potassium hydroxide,

and lead acetate reagents, showing that all bioactive compounds isolated from ethyl acetate extract of *C. rubrum* corresponding to phenolic compound, a large family of natural products, including phenolic acids, flavonoids, coumarins, tannins, among others (Croteau *et al.*, 2015).

Table 1. Amounts and analysis of phytochemicals in bioactive compounds (BC) isolated from EAE extract of *C. rubrum*.

Fraction	Amount (mg)	Tp	Am	Ak	PC			Ch	St
					KOH	LA	FC		
BC-1	2.8	-	-	-	+	+	+	-	-
BC-2	3.5	-	-	-	+	+	+	-	-
BC-3	1.2	-	-	-	+	+	+	-	-
BC-4	3.0	-	-	-	+	+	+	-	-
BC-5	3.0	-	-	-	-	+	+	-	-
BC-6	4.0	-	-	-	+	+	+	-	-
BC-7	4.7	-	-	-	+	+	+	-	-
BC-8	17.7	-	-	-	+	+	+	-	-
BC-9	3.8	-	-	-	+	+	+	-	-

Tp: Terpenes, Am: Amino acids and biogenic amines, Ak: Alkaloids, PC; Phenolic compounds, Ch: Carbohydrates, St: Steroids, (+): Presence, (-): Absence.

3.3 Characterization by FT-IR

The IR analysis performed to the bioactive compounds could identify the nature of bonding and types of functional groups in the samples. The IR spectra were similar among the bioactive compounds and revealed seven significant vibration signals. A very strong C-H valence vibration frequencies ranging from 2975 to 2840 cm^{-1}, and a medium-strong valence at 1446 – 1460 cm^{-1}, both corresponded to the aliphatic part of the molecules; a characteristic

strong C–O stretching vibration withing 1705 – 1730 cm^{-1} assigned to carbonyl group; C–C(=O)–O asymmetric stretching and C–C–O asymmetric stretching bands observed withing 1260 – 1275 cm^{-1}, and 1077 cm^{-1}, respectively, probably corresponding to ester group vibrations; and two medium vibrations ranging from 1618 to 1543 cm^{-1} characteristic of an aromatic ring. Particularly, the IR spectrum of BC-4 to BC-9, presented a broad medium-weak O-H stretching vibration withing 3100 – 3600 cm^{-1}, and both, C-OH deformation and stretching vibrations ranging from 1366 – 1376 cm^{-1} and 1173 cm^{-1}, respectively. Those characteristic band come from the phenol group vibrations, and were absent in BC-1, 2 and 3 spectrums, suggesting that those compounds lack of any phenol group.

3.4 MIC determination

The MICs of EAE and bioactive compounds were evaluated in *Y. ruckeri* clinical isolated strains. The MIC values are summarized in Table 2. The antimicrobial study showed that the whole extract was less active than the isolated components, which suggests a strong antagonistic effect among the components. All bioactive compounds acted as antibacterial agents. Among all those, BC-1, 2, 3, 5 and 6 were the most active compound with MIC values < 0.053 µg/mL. It was followed by BC-9, BC-8 BC-4 and BC-7, which produced values of 0.106, 0.845, 3.38 and 54.07 µg/mL, respectively, the latter being the less active.

Table 2. MICs of ethyl acetate extract (EAE), and isolated compounds from *C. rubrum* on *Y. ruckeri* strain.

Samples	MIC (µg/mL)
EAE	432.6
BC-1	< 0.053
BC-2	< 0.053
BC-3	< 0.053
BC-4	3.380
BC-5	< 0.053
BC-6	< 0.053
BC-7	54.075
BC-8	0.845
BC-9	0.106

4. Discussion

The primary goal of this study was focus on the search of new and natural antibiotic agents against *Y. ruckeri*, causing the Enteric red mouth disease that affects to salmon species and some wildlife fishes. The strategy was to obtain potential antibacterial extracts and compounds from *C. rubrum* following a direct bioautography assay. Generally, direct bioautography is used to perform an antimicrobial screening by absorbing chemicals onto the surface of thin layer chromatographic plates and placing them directly in contact with a medium inoculated with microorganisms (Horváth *et al.*, 1993). Microorganism cultures can grow directly on the TLC plate; thereby, clear zones detected corresponding to microbial growing inhibition (Wedge and Nagle, 2000). Comparing with common antimicrobial screening methods, TLC-DB is more suitable for evaluating complex extracts and facilitates a quick, economic and easy evaluation (Horváth *et al.*, 1993) and it has been performed to

find antibacterial agents against animal, plant and human pathogens (Nostro *et al.*, 2000; Chomnawang *et al.*, 2005; Saravanakumar *et al.*, 2008). The present study was the first to investigate the antibacterial activity of compounds ethyl acetate extract obtained from *C. rubrum* against *Y. ruckeri* using TLC-DB.

In a developed TLC plates, containing the EAE fractions from *C. rubrum*, a thin layer of agar inoculated with *Y. ruckeri* clinical isolated strain was placed on the TLC plate. After incubation, the identification of 15 spots that produced bacterial inhibition using MTT solution regeant was quickly and easily, such as was described by Chomnawang (2005). The spots were observed in 9 of the 20 EAE fractions. However, according with the phytochemical screening and IR analysis, the agents responsible for inhibition spots in EAE-9 and 10 were the same agents in EAE-8, reducing the potential bioactive compounds (BC) from 15 to 9. Thus, 9 bioactive compounds (BC) were isolated using pTLC, BC-1 to BC-9. Moreover, the antimicrobial study of those compounds indicated that MIC values ranging from < 0.053 to 54.075 µg/mL. Although, most compounds showed MIC values lower than 3.380 µg/mL. Additionally, the ethyl acetate extract produced a MIC value of 432.6 mg/mL. As indicated by Duarte *et al.* (2007); Wang *et al.* (2008), and Oliveira *et al.* (2013) plant extracts can be classified as antibacterial agents in accordance with their MIC values; MIC < 500 µg/mL indicate strong inhibitors, MIC between 600 and 1,500 µg/mL indicate moderate inhibitors, and MIC > 1,600 µg/mL indicate weak inhibitors. Therefore, the ethyl acetate extract and bioactive compounds from red algae *C. rubrum* can be classified as strong growth inhibitors of *Y. ruckeri*.

Based on the phytochemical screening, the bioactive compounds isolated here, corresponding to phenolic compounds, a large family of natural products. Phenolic compounds are generally

characterized as aromatic metabolites that possess, or formerly possessed, one or more "acidic" hydroxyl groups attached to the aromatic arene ring, including phenolic acids, flavonoids, coumarins, tannins, among others. Most, but not all, plant phenolics are derived from the phenylpropanoid and phenylpropanoid acetate pathways (Croteau *et al.*, 2015). The flavonoids constitute an enormous class of phenolic natural products. Among their subclasses are the anthocyanins, proanthocyanidins or condensed tannins, and isoflavonoids. On the other hand, the coumarins belong to a widespread family of metabolites called the benzopyranones. Additionally, it includes linear furanocoumarins, angular furanocoumarins, pyranocoumarins, and pyrone substituted coumarins. Hydrolyzed tannins, a group of mostly polymeric substances, are typically copolymers of carbohydrates and the shikimate derived gallic and ellagic acids (Croteau *et al.*, 2015). Nevertheless, the identity of the bioactive compounds isolated here, may not coincide with some phenolic compounds for many reasons. Concordant with the portioning methods developed in this study, the water-soluble compounds could not be present in the ethyl acetate extract. Then, water-soluble metabolites, such as, hydrolyzed tannins, proanthocyanidins, saponins, catechins, glycosides, and others similar, should be ruled out. Moreover, per the IR analysis, BC-1 to BC-3 did not possess the hydroxyl group. Thereby, those can be neither simple phenols nor phenolic acids. Many natural phenolic compounds have at least one hydroxyl group combined as an ether or ester. Ethers or esters are less soluble in water than parent phenols (Robinson, 1991). It agrees with the ester group vibrations observed in IR spectrums of BC.

Furthermore, the phytochemical screening was positive to Folin-Ciocalteu, KOH, and lead acetate reagents, indicating the presence of phenolic compound. However, lead acetate reagent is frequently used for flavonoids identification (Vimalkumar *et al.*, 2014), and KOH

reagent is commonly used to identify coumarins, which react through Bonträger reaction (Wagner *et al.*, 1984). Also, vibrations observed in IR spectrums of BC were like vibrations produced by functional group from this kind of secondary metabolites (Sharma and Janmeda, 2017). Then, even the bioactive compounds corresponding to phenolic compounds, those compounds may be flavonoids or coumarins.

Our results are not so far from other bioactive metabolites studies in Rhodophyta. Red algae are the largest group containing bioactive compounds. Polysaccharides, lipids and polyphenols, steroids, sterols glycosides, flavonoids, tannins, saponins, alkaloids, terpenoids, antheraquinones and cardiac glycosides have been reported in red algae (Aziz *et al.*, 2020, Cotas *et al.*, 2020). Despite this, constituent profile may differ from species. For example, Seetharaman *et al.* (2016) reported the phytochemical screening of the antibacterial methanol extract from red alga *Kappaphyccus alvarezii*, proving the presence of alkaloids, saponin, phenols, terpenoids, coumarins, protein, carbohydrates, flavonoids, and tannins. But steroids, glycosides and anthraquinone were absent.

5. Conclusions

The search for new functional ingredients from marine natural sources is one of the challenges in science and pharmacology, which basically try to answer the demand for new drugs for human and animal health, and the possibilities for extracts and compounds derived from algae to become natural ingredients to treat diseases in salmon industry should be discussed. The present study determined that the ethyl acetate extract and its constituents were shown to be strong antibacterial agents against Gram negative bacteria, *Y. ruckeri*, responsible for the Enteric red mouth disease that affects to salmon species and some wildlife

fishes. Thus, this results together with others, suggest that *C. rubrum* possesses significant antibacterial activity, showing a great potential as source of antibacterial agents, and in future extracts from *C. rubrum* may pave the way for designing the new drugs for controlling bacterial diseases.

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CHAPTER IV

"Toxicological effects of dchloromethane and ethyl acetate extracts obtained from Ceramium rubrum, using indicators organisms Artemia franciscana and Danio rerio"

(Unpublished data)

TOXICOLOGICAL EFFECTS OF DCHLOROMETHANE AND ETHYL ACETATE EXTRACTS OBTAINED FROM *CERAMIUM RUBRUM*, USING INDICATORS ORGANISMS *ARTEMIA FRANCISCANA* AND *DANIO RERIO*

(UNPUBLISHED DATA)

1. Introduction

An important aspect in the search of antimicrobial metabolites is the toxicity of the obtained products. In these sense, the brine shrimp *Artemia franciscana* and zebrafish *Danio rerio* were a powerful tool in pharmacology for drug discovery (Domínguez-Martín *et al.*, 2020; Zon and Peterson, 2005, Rubinstein, 2006). Due to these organisms exhibits a short development time, high fecundity rates and a cheap maintenance, the lab handling is very simple (Iturriaga-Vásquez *et al.*, 2012; Sorgeloos *et al.*, 1986). Other characteristic such as the rapid absorption of chemical substances and the genome fully sequenced, making the brine shrimp and zebrafish suitables to toxicological assays (Goldsmith, 2004, Kaufman *et al.*, 2009: Vanhaecke *et al.*, 2000).

In this chapter, unpublished data are presented about toxicological tests performed using the indicator organisms *A. franciscana* and *D. rerio* are presented. The results would form the basis for further studies on marine natural products.

2. Materials and Methods

2.1. Brine shrimp toxicity

The *A. franciscana* nauplii stage was used as test organism. The cysts were incubated in a cylinder container with artificial seawater up to density of 5 g L^{-1} of cysts, for 48 h at controlled temperature (28ºC), salinity of 33 ppt (pH 8.0), constant illumination (2,000 lux)

and aeration (2 mg L^{-1}) (Sorgeloos *et al.*, 1986). Two-fold serial dilutions of DCM extract and EAE extract were prepared using 1:1 ethanol-dimethyl sulfoxide (DMSO) and ethanol as adequate solvents, respectively. The *A. franciscana* acute toxicity assay was performed per the method described by Meyer *et al.* (1982). Ten individuals were transferred to an assay tube (8 mL) containing 4.95 mL freshly prepared artificial seawater plus 50 µL of each dilution of DCM and EAE extracts, achieving the desired concentrations between 31.25 - 1,000 mg mL^{-1}. Three control groups were established, one with artificial seawater and the carrier solvent (50 µL), another control group was being maintained only with artificial seawater, and the other group carried out with a solution of sodium lauryl sulfate (98%). A serial dilution (1 to 20 µg mL^{-1}) was performed from this (Prefectura Naval Argentina, 1998). All lethality experiments were carried out with 5 replicates/each concentration at 25°C and the mortality was recorded at 24 h of exposure. Percent mortalities were used to estimate the lethal concentration required to produce a decrease of 50% survival (LC_{50}) using prbit regression (Throne *et al.*, 1995). Finally, doses-response curves were elaborated.

2.2. Zebrafish toxicity

2.2.1. Larvae production

The set-up involved two aquaria, one of this of 80 L and another of 20 L of capacity. A breeding stock of mature zebrafish *Danio rerio* (~6 months-old) was maintained in the big aquarium. To maintain a suitable temperature range (26 ± 1°C), heaters were installed and the oxygen was supplied through pumps installed in the aquaria. Dry flaky food was supplied manually about 2 times a day to the fishes. Besides, live *A. franciscana* nauplii were administered once a day to promote breeding (Adu and Thomsen, 2011). In the small aquarium, the reproduction occurred. Two mature males and one mature female

(distinguished by the bulgy abdomen) were placed. Due to adults' prey on fertilized eggs (Spence *et al.*, 2008), a spawn-trap was necessary to separate the eggs from their parents. This consists in two layers of glass spheres (1 cm diameter), enough to keep off adults to fertilized eggs. The fishes were maintained in the small aquarium for 24h. If the female's abdomen continues bulging, the adults were maintained for another period of 24h. Once gametes were released, the adults were transferred to the big aquarium. The hatching of eggs occurred between 24 - 72h post-fertilization.

2.2.2. LC_{50} determination

The *D. rerio* larvae (< 24h-old) were used as test organism. Two-fold serial dilutions of DCM extract and EAE extract were prepared using distilled water and 1% of ethanol as adequate solvent. The toxicity assay was performed per the method described by Martínez-Jerónimo and Espinosa (2008). Ten individuals were transferred to an assay flask containing 99 mL of freshwater plus 1 mL of each dilution of DCM and EAE extracts, achieving the desired concentrations between 50 – 200 mg mL^{-1} for the DCM assays and 10 – 80 mg mL^{-1} for the EAE assays. Three control groups were established, one with freshwater and the carrier solvent (100 μL), another control group was being maintained only with freshwater, and the other group carried out with a solution of copper solution (as $CuSO_4 \times 2\ H_2O$; copper 0.5 μg mL^{-1}) as positive control (Kammann *et al.*, 2006). A serial dilution (70 to 95 ng mL^{-1}) was performed for this purpose. All lethality experiments were carried out with 5 replicates/each concentration at 26 ± 1°C, photoperiod 16:8h (light-darkness cycles) and 1,000 lux of illumination (environmental lab). Besides, the assay was carried out with certified fishes in Metazoan technologies Lab. The mortality was recorded at 1, 3, 6, 24, 48, 72 and 96h of

exposure. Percent mortalities were used to estimate the LC_{50} using probit regression (Throne *et al.*, 1995), doses-response curves were elaborated.

3. Results

3.1. Brine shrimp toxicity

The toxicity of DCM and EAE extracts obtained from *C. rubrum* were evaluated using the brine shrimp assay. The 50% of mortality were reached at 172.0 mg mL^{-1} of DCM extract and 54.8 mg mL^{-1} of EAE extract, per predictive model. Experimentally, the LC_{50} values were 166.0 mg mL^{-1} and 49.6 mg mL^{-1}, respectively. The theoretic LC_{50} value for sodium lauryl sulphate was 7.6 μg mL^{-1}, while the experimental value was 7.0 μg mL^{-1} (Table 1). Assays performed with the solvents used in the stock solutions not presented toxic effects on *Artemia* nauplii (data not shown). The comparison of the antimicrobial results presented in the previous chapters and the toxicological results are shown in Table 3. The toxic concentration is higher than the antibacterial and antifungal concentration of the extracts and compounds obtained from *C. rubrum*. This is a very important result in the development of bioguided chemical analysis.

Table 1. Toxicological activity at 24h of DCM and EAE extracts obtained from *C. rubrum* on *A. franciscana* nauplii.

Extracts	LC_{50} value (mg mL^{-1})	
	Predicted	**Experimental**
DCM	172.0	166.0
EAE	54.8	49.6
Positive control*	7.6	7.0

(*) Concentration in μg/mL.

3.2. Zebrafish toxicity

The toxicity of DCM and EAE extracts obtained from *C. rubrum* were evaluated using the zebrafish assay. With non-certified fishes, the 50% of mortality were reached at 102.2 mg mL^{-1} of DCM extract and 58.0 mg mL^{-1} of EAE extract, per predictive model. Experimentally, the LC_{50} values were 104.5 mg mL^{-1} and 60.0 mg mL^{-1}, respectively. The theoretic LC_{50} value for copper solution was 85.7 ng mL^{-1}, while the experimental value was 86.3 ng mL^{-1}. With certified fishes, less toxic effects were observed (Table 2). Assays performed with the solvents used in the stock solutions not presented toxic effects on larvae (data not shown). The comparison of the antimicrobial results presented in the previous chapters and the toxicological results are shown in Table 3. The toxic concentration is higher than the antibacterial and antifungal concentration of the extracts and compounds obtained from *C. rubrum*. This is a very important result in the development of bioguided chemical analysis.

Table 2. Toxicological activity at 96h of DCM and EAE extracts obtained from *C. rubrum* on *D. rerio* larvae, using certified and non-certified fishes.

Fishes / Extracts	LC_{50} value (mg mL^{-1})	
	Predicted	**Experimental**
Non-certified fishes		
DCM	102.2	104.5
EAE	58.0	60.0
Positive control*	85.7	86.3
Certified fishes		
DCM	117.3	119.1
EAE	66.4	67.2
Positive control*	88.4	89.3

(*) Concentration in ng/mL.

Table 3. Comparative chart of antimicrobial and toxicological activities of DCM and EAE extracts obtained from *C. rubrum*.

Fraction	Activity	Organism	Parameter	Concentration (µg mL^{-1})
DCM	Antibacterial	*Y. ruckeri*	MIC	507.7
	Antifungal	*S. parasitica*	MIC	>2,048
	Toxicological	*A. franciscana*	LC_{50}	166,000
	Toxicological	*D. rerio*	LC_{50}	104,500
EAE	Antibacterial	*Y. ruckeri*	MIC	432.6
	Antifungal	*S. parasitica*	MIC	>2,048
	Toxicological	*A. franciscana*	LC_{50}	54,800
	Toxicological	*D. rerio*	LC_{50}	60,000
BC-1	Antibacterial	*Y. ruckeri*	MIC	< 0.053
BC-2	Antibacterial	*Y. ruckeri*	MIC	< 0.053

BC-3	Antibacterial	*Y. ruckeri*	MIC	< 0.053
BC-4	Antibacterial	*Y. ruckeri*	MIC	3.380
BC-5	Antibacterial	*Y. ruckeri*	MIC	< 0.053
BC-6	Antibacterial	*Y. ruckeri*	MIC	< 0.053
BC-7	Antibacterial	*Y. ruckeri*	MIC	54.075
BC-8	Antibacterial	*Y. ruckeri*	MIC	0.845
BC-9	Antibacterial	*Y. ruckeri*	MIC	0.106

4. Discussion

One of the main objectives of the search of bioactive compounds is to identify the interactions between this and one or more physiological functions of the organism. This primary objective must be based on researches *in vitro*, later in animal models and finally they must be corroborated in studies of observation in target organisms (human, fishes, other) in clinical trials (Plaza *et al.*, 2008). Because of this, the aim of this study was to evaluate the toxicity of DCM and EAE extracts obtained from *C. rubrum* using brine shrimp and zebrafish larvae. The results presented in this report are the basis for future and promising applied research in the use of natural products.

The principal guideline to follow in the use of natural products is to increase to the maximum the benefit and to reduce to the minimum the risk. Increasing the benefit implies look for a physiological wide effect, assuring that existing bioavailability is going to be kept along all the useful life of the food. On the other hand, to reduce the risk, it is necessary to carry out toxicity studies (Plaza *et al.*, 2008). In this aspect, the toxicity results presented in this report are promising. The toxic concentrations are much higher than the antibacterial and antifungal concentrations of the DCM and EAE extracts obtained from *C. rubrum*, indicating that their use is safe.

Is important to consider that the toxic effects of a metabolite decrease as moves up the food chain. The intermediate organisms can metabolize and inactive compounds and prevent the accumulation of toxic metabolites, reducing the risk of exposure to higher trophic levels (Canton *et al.*, 1975; Correa-Reyes *et al.*, 2007). The results show that the toxicity of the EAE extract decreases as the level in the trophic chain increases, contrary to what happens with the DCM extract, which was more toxic to zebrafish than to brine shrimp. Considering this, high LC_{50} concentrations can be expected in salmon, suggesting the safe use of EAE extract and compounds isolated from it.

Regarding the mechanisms of action of toxic effects on Artemia nauplii and zebrafish embryos, several authors have indicated that the compounds may interact with the surface of the test animal and combine with external proteins (Bellas *et al.*, 2004; Shafshar *et al.*, 2001). Also, can penetrate through a lipoid barrier or modify its solubility entering tissues and inhibiting metabolic processes (Glaberman *et al.*, 2016; Pretti *et al.*, 2013; Taylor *et al.*, 2005). Particularly in fishes, Könemann (1981) developed a structure–activity relationship in which the narcotic potency of many chemicals, expressed as LC_{50}, was related to the presence of nonpolar and polar compounds and their relationship, eliciting differing respiratory-cardiovascular responses and lethal body burdens in fish acute toxicity syndromes (Vaes *et al.*, 1998). In this sense, Fernández-Pumarega *et al.* (2017) found in their study that are important descriptors in the narcosis of both nonpolar and polar compounds the hydrophobicity, dipolarity and polarizability, and hydrogen bond basicity.

Finally, the Artemia nauplii and zebrafish embryo test provide a valuable tool to characterize the effect of a substance in a quantitative as well as in a qualitative manner, although the latter was not developed in this study, it opens an opportunity for new research.

5. Conclusion

It has been shown in this study that under laboratory conditions, DCM and EAE extracts have effects on the development of Artemia nauplii and fish embryos at very high concentractions; concentrations of these substances have been detected in the mg mL^{-1} range, whereas positive controls appear in clearly lower µg mL^{-1} and ng mL^{-1} for Artemia nauplii and zebrafish embryos, respectively, realizing its biological safety for other uses. Summarizing these findings, it concludes that EAE and DCM extracts would not contribute to toxicity and they may not be toxic to other species that are larger or higher in the food chain, such as farmed salmon. However, more information is needed about that to confirm this assumption.

CHAPTER V

Global discussion and General conclusions

GLOBAL DISCUSSION AND GENERAL CONCLUSIONS

Macroalgae, being sessile organisms, have had to develop a series of defense and adaptative mechanisms against predators, pathogens and epilithic algal communit. It is for th is reason that the chemical production of biologically active metabolites it's interesting in these organisms. The bioactivity of sdecondary metabolitres produced by algae has been studied since ancient times, being the Rhodophyta division the most investigated and from which the greatest number of compounds with biological activity have been obtained.

Considering the arguments, this study hypothesized that the red seaweed *Ceramium rubrum* Rhodophyta) have amphipathic bioactive metabolites with antibacterial and antifungal activity against *Y. ruckeri* and *S. parasitica*, pathogens of Chilean salmon industry.

The literature on antibacterial and antifungal activity of *C. rubrum* is scarce, and only one evaluated the bioactivity at the level of crude extracts. The results of this thesis correspond to the first approximation on the chemical identification of active extracts against pathogens of salmon industry. The DCM extract resulted active against *Y. ruckeri* and *S. parasitica* and 7 of the 14 identified metabolites were tested. These metabolites included fatty acids, fatty acid methyl esters and an acyclic diterpene alcohol. On the other had, EAE extract resulted with antibacterial and antifungal activity, however 9 active compounds isolated from them were tested only against *Y. ruckeri*, because because it was more promising. These compounds would be associated to phenolic compounds, possibly flavonoids.

The results of the antimicrobial activity of the DCM extract agree with other investigations. Dubber and Harder (2008) hypothesized that the antibacterial effect of *C. rubrum* extracts was due to the presence of significant quantities of unidentified fatty acids. Bansemir *et al.* (2006) also assume that the active compounds could be, at least partly, lipophilic halogenated

compounds. Similar conclusions were reported by Güner and Yavaşoğlu (2018). They indicated that the strong activity of hexane extract of *C. rubrum*, can be due to the presence of lipophilic antioxidants, such as tocopherol. The fact that DCM extract showed a significant higher antibacterial activity than both pure compounds and reconstituted sample indicates that a synergistic effect may be occurring. In these sense, Bansemir *et al.* (2004) indicated that the antimicrobial activity of the extract could be a synergistic effect of its constituents because the pure compounds only showed a weak effect. In terrestrial plants is a common phenomenon. Lis – Balchin *et al.* (1998), Patharakorn *et al.* (2010) and Chairgulprasert *et al.* (2008) concluded that major components are not necessarily responsible for the biological activity, thus the contribution of minor components in the activity is essential. In our case, minority compounds (14 % of DCM) or their mixtures could be responsible to antibacterial activity of the DCM extract of *C. rubrum*.

On the other hand, Ramkissoon *et al.* (2015) observed in the methanolic extract of *Chaetomorpha crassa* bioactive molecules with antifungal activity on human pathogens, associated to stearic acid and linolenic acid. Mohamed and Saber (2019) evaluated the in vitro antifungal activities of different crude polar (methanol and ethyl acetate) and non-polar (chloroform and petroleum ether) extracts of brown seaweed *Hormophysa cuneiformis* against human and plants pathogens, and found that the chloroform extract exhibited a potential antifungal activity against all tested fungal isolates. GC–MS analysis revealed the presence of saturated, monounsaturated and polyunsaturated fatty acids.

Algal lipids content in seaweed ranges from 0,1 % to 7% (dry weight) approximately, and are composed mainly of phospholipids, glycolipids and non-polar glycerolipids (Kumari, *et al.*, 2013). Phospholipids are characterized by higher contents of fatty acids like oleic,

palmitic, stearic, arachidonic and eicosapentanoic acids. In this study, palmitic acid was the second most important component of the DCM extract reaching 25.8%.

Gas chromatography (GC) is widely used for the analysis of a broad variety of lipid samples, most often used in combination with mass spectrometry (MS). An adequate characterisation and accurate identification of fatty acids remain challenging due to their complexity, incomplete separation dimension resolution often confounded by sample matrix, and sometimes insufficient MS domain differentiation (Waktola *et al*., 2020). To achieve better definition and resolution can be prepared fatty acid esters from fatty acids to quantify them. The preparation of fatty acid esters can be performed either by hydrolysis or methylation (Zainal Abidin and Saha, 2017). However, to obtain accurate quantitative results, it is necessary to address potential procedural difficulties, such as incomplete conversion of the fatty acid into fatty acids esters, formation of artefacts and contamination, the loss of very volatile short-chain compounds, alterations of the original fatty acid profile during esterification (positional and/or geometrical isomers) and subsequent damage of GC column (Rozema *et al.,* 2008; Salimon *et al.,* 2017). Per Salimon *et al.* (2017), no statistically significant differences were found between the concentrations obtained of fatty acid and fatty acids esters by the two compared methods. The methylation method is a suitable alternative for the analysis of a complex mixture of fatty acids.

Direct TLC-Bioautography was used to determine the antibacterial activity of EAE extract. This is an effective planar separation technique that offers a rapid isolation of individual components from complex mixtures associated with the identification of their biological activity (Legerská, *et al.,* 2020). The derivatization step is required for a functional TLC-bioautographic method because the resulting biological reaction product is colorless on a chromatoplate. When a freshly solution of MTT is sprayed on TLC plate, MTT is reduced

with bacterial succinate dehydrogenase to form colored formazan, revealing yellow zones of inhibition on a purple background of live bacteria.

Bioautographic bioassays allowed to determine that 9 of the 20 extracts obtained from EAE resulted active on *Y. ruckeri*. These extracts showed an intermediate polarity and some of the fractions showed activity. Because the polarity of these active extracts, compounds such as flavonoids, coumarins, saponins, phenols, tannins, anthraquinones and other moderately polar compounds could be present (Deyab, *et al.*, 2016; Chakraborty, *et al.*, 2015; Xu, *et al.*, 2015). Colorimetric reactions and the vibrations observed in IR spectrums produced by functional group of secondary metabolites, suggest that the BC of EAE corresponding to phenolic compounds, those compounds may be flavonoids or coumarins. The phytochemical screening was positive to Folin-Ciocalteu, KOH, and lead acetate reagents, indicating the presence of phenolic compound. Lead acetate reagent is frequently used for flavonoids identification (Vimalkumar *et al.*, 2014), and KOH reagent is commonly used to identify coumarins (Wagner *et al.*, 1984). Also, vibrations observed in IR spectrums of BC exhibit characteristic absorption bands of flavonoids at 3600-3100 cm^{-1} (O-H stretching) and 1618 to 1543 cm^{-1} (carbonyl attached with aromatic nucleus) (Mabry *et al.*, 1970) and coumarins at 1750-1700 cm^{-1} (–C=O groups) and 1620-1470 cm^{-1} (–C=C– groups of aromatic rings) (Kuznetsova, 1967). The presence of carbonyl group signal in all BC occur between 1900 and 1600 cm^{-1} in IR spectrum (Smith, 2017). The carbonyl group is probably the most important functional group in organic chemistry and the interaction with substituents may affect carbonyl groups in several different ways. One of the important features of the carbonyl group is the difference in electronegativity between carbon and oxygen, which leads to electron transfer (Wiberg, 1999), and confers distinctive structural and functional properties. For example, thiazolidinones with a carbonyl group in position 2, 4 or 5, have diverse

biological activities such as bactericidal, pesticidal, fungicidal, insecticidal, anticonvulsant, tuberculostatic, antiinflammatory, antithyroidal, potentiation of pentobarbital-induced sleeping time, etc. (Singh *et al.*, 1981).

Our results are agreed with other researches about seaweeds. Seetharaman *et al.* (2016) reported the phytochemical screening of red alga *Kappaphyccus alvarezii* and revealed that the extract contained significant quantity of alkaloids, saponin, phenols, steroids, protein, phytosterols, aminoacids, flavonoids, steroids, tannins and absence of terpenoids, sugars and anthraquinone who are responsible of the activity against clinical isolated bacteria. Ravi *et al.* (2019) studied the *Jania rubens* extracts for treating human and fish microbial pathogens, relating their high antibacterial activity with the presence of steroids, terpenoids, flavonoids, alkaloids, cardiac glycosides, coumarins, quinine, phenols and betacyanin. At this point it is important to consider that the colorimetric tests are only referential and will require complementary analysis to indicate with certainty the presence of a compound. The occurrence of false positives is not uncommon. For example, in the determination of certain compounds it has been indicated that the reagents used in precipitation reactions are activated depending on both the amount of sample evaluated and the concentration of the metabolites present in the sample, yielding often false results (Gunatilaka *et al.*, 1980). Likewise, the reagent of Folin-Ciocalteu is not specific and detects all the phenolic groups that are found in extracts, including proteins, with the possibility of overestimating its result due to the superposition of spectral responses (Naczk and Shahidi, 2004). When Folin-Cioocalteu's reagent is used for the determination of antioxidant activity of polyphenols, a complementary method, for example FRAP, should be used to rule out interference with digestive enzymes, amino acids and sugars, that can give false positives.

Antimicrobial activity of fatty acids depends on their structure (Zheng *et al.*, 2005), specifically the free carboxylic group, that allows an optimal insertion into cells through hydrogen bond that have hydrogen-bond-acceptor groups in the membrane, also depends the length, degree of unsaturation of the carbon chain and the geometry of the unsaturation (Desbois and Smith, 2010). Studies show that long fatty acids (> 14-16 carbon atoms) are highly antifungal (Malina and Shai, 2005). Likewise, optimal antibacterial activity is obtained by testing medium chain fatty acids (C12-C13) and this activity tends to decrease rapidly over C13 (Feldlaufer, et al., 1993, Kabara, 1987, Kubo, et al., 1993, Yoon, et al., 2018), specially against Gram negative bacteria (Umerska *et al.*, 2016), like as *Y. ruckeri.* Introduction of a double bond is necessary to maintain antimicrobial activity once the chain length of the fatty acid exceeds 14 carbons (Feldlaufer, et al., 1993; Kabara, 1987). The formation of an amphipathic structure seems to be required for membrane binding and lytic activity by surface-active action (surfactant) (Giangaspero *et al*., 2001; Kubo *et al.*, 2003). When the concentration of fatty acids reaches a critical concentration, the apolar portion of the molecule, the tail, insert into the microbial membrane to form pores, or destabilize it disrupting the wall curvature and leading to microorganism to death (Huang and Ebersole, 2010; Kubo *et* al., 2003; Lunde *et al.,* 2009; Shai, 2002). The effect of stearic acid on the growth of *S. parasitica* could be related to the amphipathic character of fatty acids. After fatty acid-induced damage to the membrane of microorganisms occurs, other molecules can penetrate and affect different metabolic processes (Desbois and Smith, 2010).

It is very important to emphasize that algae develop in a dynamic ecological environment, so biotic and abiotic factors affect the macroalgae physiology with subsequent variability in the content of metabolites (Kroeker *et al.,* 2020; Lalegerie *et al.,* 2020). For example, the latitudinal variation in phlorotannin contents from brown seaweeds (Ank *et al.,* 2019), the

fatty acids derivatives and polyolefins change in *Lobophora* species when changing their natural habitat, types of substrates and the sea surface temperature and salinity (Gaubert et al., 2019), and the impacts of salinity and UV stress on the metabolic profile in the brown macroalgae *Sargassum cymosum* (Polo *et al.*, 2015). Due to the above, the results presented here represent a "photograph" of the time and place of the sampling. It is interesting to study samples of *C. rubrum* obtained in other latitudes and in another season of the year.

Finally, an important piece of information in any study of antimicrobial activity is the toxicity analysis. A toxicity bioassay basically consists of testing a series of different concentrations of the test sample or a pollutant, using living reference organisms, to obtain a harmful effect. The effects can manifest themselves at different levels, from subcellular structures or enzyme systems, to entire organisms, populations or communities, and will depend on both the chemical properties of the compound and its concentration. Per the duration and frequency of exposure to the toxin -and its relationship with the life cycle of the organism; the tests may be acute or chronic. The main characteristic of the reference organisms is that they are biologically, genetically and physiologically known, that they are easy to grow and that the harmful effects are easily observable, among other qualities. In this study, toxicity tests were performed using two widely described organisms: the brine shrimp *A. franciscana* and zebrafish *D. rerio*. The unpublished data regarding the toxicity of extracts obtained from *C. rubrum*, indicated that EAE extract showed promise for developing bioguided tests against *Y. ruckeri*. It would be expected that the nine compounds isolated from them (BC-1 to BC-9) result toxicologically safe on reference organisms.

Conclusions

Agree with the propose hypothesis "*C. rubrum* have amphipathic bioactive metabolites responsible of antibacterial and antifungal activity against *Y. ruckeri* and *S. parasitica*, pathogens of Chilean salmon industry", the results from this work allow to state that the hypothesis was proven.

The antimicrobial study showed that the whole extract was more active than the individual components, which suggests a strong synergistic effect among them. DCM extract of *C. rubrum* presented 100% bacterial inhibition on *Y. ruckeri* at approximately 500 μg mL^{-1}.

The present work is the first study reporting the antifungal activity of *C rubrum* against *S. parasitica.* The maximum activity of DCM and stearic acid occurred at 250 μg mL^{-1}.

The EAE and its constituents showed a strong antibacterial activity against *Y. ruckeri*, 9 bioactive compunds were isolated from them.

The DCM and EAE extracts did not show toxicity using the brine shrimp, *A. franciscana* and zebrafish, *D. rerio* bioassay.

In summary, this work showed that *C. rubrum* contains metabolites that possess antibacterial and antifungal activity against fish pathogens. These results may constitute a basis for promising future applied research that could investigate the use of *C. rubrum* seaweed as a source of antimicrobial compounds against other fish pathogens. Despite efforts to be mitigated, diseases such as Piscirickettsiosis caused by the bacterium *Piscirickettsia salmonis*, Caligidosis caused by the sea louse *Caligus rogercresseyi*, Bacterial Kidney Disease (BKD) caused by the bacterium *Renibacterium salmoninarum* and Infectious Pancreatic Necrosis (IPN) caused by the virus of the same name, they continue to be great

challenges for the salmon industry in Chile. All of them are persistent diseases that cause fish mortality and increase the use of antibiotics.

Concluding remarks

Until now, most of the antimicrobial activities of bioactive metabolites of marine origin have been observed in *in vitro* models or in small systems *in vivo*. Therefore, an important aspect is scaling to investigations that determine the biological potential in clinical or field studies. The elucidation of chemical structures is also a line of investigation that is pending.

Based on the advancement of metabolic engineering and chemical synthesis, the biosynthesis could provide an environmentally sustainable and cost-effective alternative for mass production of these high-value chemicals in a short period.

Finally, taking into consideration all the investigations made over the last 10 years, along with the advances in technology, the marine environment will play a vital role in the future development and trials of anti-infective drugs. The continuous research and analysis of the potential of natural compounds lead to the discovery and development of a new generation of agents with antimicrobial properties that can effectively control infectious diseases in humans and other animals and plants of great value.

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