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"Evaluation of the antioxidant activity and antitumor activity of marine invertebrates extracts"

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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"Every second breath we take comes from the ocean"

Contents

Abbreviations

Abs	Absorbance
Ach	Acetil
AChE	Acetilcolinesterase
AChI	Acetylthiocholine iodide
AD	Alzheimer Disease
AlCl3	Aluminum Chloride
ANOVA	Analysis of variances
BChE	Butyrylcholinesterase
BHT	Butylated hydroxytoluene
B.leachii	*Bursatela leachii*
Ca+	Calcium
CE	Catechin
CO2	Carbon dioxide
CTC	Condenced tannins content
Cu2+	Copper
CuSO4.5H2O	Copper sulfate
C+	Positive Control
C-	Negative Control
DMEM	Dulbecco's modified Eagle's médium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	Dopamine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5-dithiobis[2- nitrobenzoic acid
DW	Dry weigth
EDTA	Ethylenediaminetetraacetic acid
FAMEs	Facty acid methyl esters
Fe2+/3+	Iron
FeCl3	ferric chloride
FBS	Fetal bovine serum
FRAP	Ferric reducing/antioxidant power
GAE	Acid gallic equivalents
GC/MS	Gas chromatogragy/ Mass spectrometry
GSH	Glutathione
GSH-Px	Glutathioneperoxidase
H2O	Water
H2O2	Peroxide hydrogen
H.arguinensis	*Holoturia arguinensis*
HCl	Sulfuric acid
HepG2	Hepatic cancer cells
HIV	Human immunodeficiency virus
IC50	Half maximal inhibitory
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
N9	Neuroglia cells

NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxid
NFTs	neurofibrillary tangles
NO	Nitric Oxide
O2	Oxygen
OD	Optical density
OH	Hydroxil
PV	Pyrocatechol violet solution
ROS	Oxygen reactive species
RSA	Radical Savaging activity
RT	Room temperature
RU	Routine
S17	Mourine stromal (non-cancer)
SH-SY5Y	Neuroblastoma cancer cells
TBHQ	Tert-butylhydroquinone
TCA	Trichloroacetic acid
TFC	Total flavonoids content
TPC	Total phenolic content
TYRO	Tyrosinase
UV	Ultraviolet radiation

Resumo

A saúde Humana é tema de grande importância, merecendo assim uma enorme atenção por parte da comunidade científica. As condições ambientais às quais o Homem se encontra sujeito nos dias de hoje, como por exemplo os agravados níveis de poluição e intensos níveis de radiação UV, são por si só potenciais causadores de inúmeros distúrbios e condições perigosas que podem facilmente evoluir para doenças, muitas delas irreversíveis e até mesmo incuráveis. Em associação aos hábitos de vida pouco saudáveis de uma enorme percentagem da população como o tabaco, má nutrição, vida sedentária e o intenso nível stress a que estamos sujeitos diariamente, todos juntos contribuem de uma forma assustadora para o desenvolvimento e progressão de doenças como cancros, Alzheimer, Parkinson e uma série de outras doenças. Tal facto, gera um stress oxidativo no organismo, que como consequência trará uma série de distúrbios ou até mesmo doenças graves. Visando aumentar a esperança média de vida, fontes naturais de compostos bioativos têm sido intensamente estudadas, de forma a prevenir, retardar e até mesmo curar determinadas doenças. Inúmeros compostos bioactivos foram já isolados de frutas, vegetais, plantas e algas, essencialmente oriundos de organismos pertencentes ao Reino das plantas. Parte destes metabolitos naturais fazem ou fizeram parte de ensaios clínicos, os quais inclusive obtiveram para alguns casos obtiveram resultados bastante positivos levando á produção farmacológica. O Oceano é um dos ecossistemas mais ricos do planeta Terra, albergando uma vasta diversidade de organismos permitindo-nos um espetáculo de cor, texturas, tamanhos e diferentes complexidades. Diariamente são produzidos inúmeros metabolitos, muitos deles por descobrir, os quais apresentam as mais variadas bioatividades e aplicações medicinais. Estudos têm vindo a provar que os compostos naturais produzidos nos Oceanos são de facto uma potencial fonte para o desenvolvimento da indústria Farmacológica. Devido á sua interessante e exuberante fisiologia e adaptações ao meio ambiente os invertebrados marinhos são talvez uma das mais promissoras fontes de compostos bioativos. Muito poucos estudos foram ainda realizados nesta área e apesar de ainda existir muito por descodificar e descobrir, estudos prévios apontam para uma forte possibilidade na utilização de invertebrados marinhos como fonte de compostos bioativos. Contudo á que ter em atenção que o estudo dos organismos como fonte de compostos bioativos é ainda bastante recente, tendo sido iniciado à sensivelmente 60 anos, pelo que ainda necessita de muitas reformulações no que toca á extração de compostos. Atualmente aproximadamente 1500 metabolitos de

origem marinha já foram descritos, dos quais alguns deram inclusive origem a medicamentos como: Ziconitide (Prialt™), Yondelis™ e Halaven™, bastante referenciados para a prevenção e tratamento do cancro. Sendo que os referidos medicamentos são derivados da descoberta de compostos bioativos em esponjas. Assim sendo e considerando o potencial que os invertebrados marinhos representam para a indústria farmacológica, neste trabalho foi estuda uma espécie de lesma do mar (*B.leachii*), a qual é considerada invasora no Mar Mediterrâneo. Com o intuito de avaliar o potencial da mesma como fonte de metabolitos naturais foram realizados diferentes ensaios, todos eles complementares, permitindo a quantificação da atividade antioxidante (Inibição do DPPH, atividade quelante do ferro e cobre, atividade redutora do ferro e a inibição do oxido nítrico), neuroprotectora, avaliando o efeito dos extratos de *B.leachii* na inibição enzimática (acetilcolinesterase, butirilcolinesterase e tirosinase) e ainda o seu efeito *in vitro* para culturas celulares, avaliando a capacidade anti-inflamatória dos extratos (avaliando a viabilidade celular após ser induzida uma resposta inflamatória pelo LPS) e o seu potencial na proteção das células contra o efeito de H_2O_2. Os resultados deste estudo mostram que a *B. leachii* apresenta uma atividade antioxidante bastante relevante. Estes resultados são bastante promissores, pois os compostos antioxidantes têm a capacidade de prevenir o stress oxidativo, que quando descontrolado despoleta a iniciação e desenvolvimento de doenças. Os extratos mostraram ainda uma elevada afinidade para inibir a tirosinase, mostrando-se assim bastante promissores para indústria da cosmética (inibindo a produção de melanina) ou até mesmo para a indústria farmacológica. Uma vez que a tirosinase está implícita na progressão de doenças neurológicas como Parkinson, devido à libertação de compostos tóxicos associados á sua atividade, extratos de B. *leachii* podem portanto representar uma esperança na possibilidade do desenvolvimento de tratamento da doença. Ainda referente à atividade neuroprotectora, o extrato de acetona mostrou uma enorme habilidade na inibição do óxido nítrico, apresentando assim uma potente atividade anti-inflamatória, a qual é essencial para a prevenção de doenças neurológicas. De acordo com os dados obtidos para o seu perfil de ácidos gordos, a espécie é de facto possuidora de uma considerável percentagem de ácidos polinsaturados, especialmente ómega-3, destacando-se o EPA, o que vai de encontro á sua capacidade anti-inflamatória. De uma forma geral a espécie de lesma estudada mostrou ser uma potencial fonte de metabolitos naturais os quais apresentaram as mais diversas atividades biológicas sendo possivelmente capazes de beneficiar a saúde Humana, dada a possibilidade de atuação perante determinadas

doenças. Além disso este estudo demonstrou que a lesma do mar estudada apresenta um teor proteico relevante. Pelo que uma possível alimentação em recursos obtidos destes organismos pode beneficiar em muito o sistema imunitário do Homem. Os dados obtidos nesta tese de certa forma acabam por ir ao encontro da teoria que tem vindo a ser desenvolvida pela comunidade científica: Que os compostos presentes na maioria dos invertebrados marinhos na verdade são metabolitos secundários (oriundos da dieta). Finalmente o facto de se falar de uma espécie invasora, e uma vez que a espécie tem todo este potencial, os dados justificam que no âmbito do controle da sua densidade e abundância para os locais onde é invasora, em vez de simplesmente remover o excesso de biomassa, que seria depois desperdiçado, que seja feito o reencaminhamento devido da biomassa para locais onde possa ser devidamente estuda e aproveitada. Este estudo potencializa a abertura de novas portas e oportunidades para o desenvolvimento da saúde humana. Sendo um dos primeiros estudos realizados, não existem estudos comparáveis pelo que todos os resultados são válidos para descartar ou conduzir a novas oportunidades e teorias.

Abstract

Nowadays diseases are evolving and progressing so quickly, every single day thousands and thousands of people die due to several untreatable conditions. The environmental that we live on is full of dangerous agents such as pollution and radioactivity, which contribute for the emerging of new diseases. Considering the risks that those conditions represent for humans health, the research community is doing a huge effort to prevent and hopefully shut down several diseases. Scientists have been using natural sources as a way to extract natural bioactive compounds, with a range of different bioactivities, which can potently be used by the pharmaceutical industry for the new drug development. Oceans are one of the more diverse ecosystems in the world. Aquatic systems are stuffed with a huge diversity of organisms where a magnificent world of colors, shapes, textures and interesting metabolites can be seen. Marine animals had evolved in a complete different environmental and for that they contain a considerable amount of bioactive compounds due to an accurate chemical defensive system, diet and other adaptations to the extreme conditions of oceans. By its interesting physiology, marine invertebrates namely sponges, tunicates, sea cucumbers and sea hare, are considered as a potential resource of bioactive compounds. In the recent years around 1500 marine natural products have been identified, 45 were tested during preclinical and clinical trials. A few of them have been approved for clinical use: this is the case of Ziconitide (Prialt™) used for the chronic pain; ecteinascidin 743 (Yondelis™) used for the treatment of ovarian cancer soft tissue sarcoma and eribulin mesylate (Halaven™) for the treatment of recurrent breast cancer. Another example is bryostatin, which was originally studied for anticancer activity and led to the development of analogues with a high potential to alleviate the symptoms related with Alzheimer' disease. Considering the background and the urgency of the identification of new bioactive compounds, the main goal of this thesis project is to evaluate the biological activities of extracts from *Bursatella leachii,* which is an invasive species of sea hare in the Mediterranean. For the following extracts it was evaluated the antioxidant and neuroprotective activity using different and complementary assays. As for the antioxidant activity, it was evaluated through the RSA of DPPH, nitric oxide inhibition, metal chelating activity and iron reducing/antioxidant power. For the neuroprotective activity it was quantified the ability to inhibition toome specific enzymes and the anti-inflammatory power of the extracts. This work proved that the *B. leachii* could be considered as a potent source of bioactive compounds with a positive effect in

health. As for the results the extracts exhibit a potent antioxidant activity, a high inhibitory power of tyrosinase and a very high affinity to in vitro inhibit the NO production, in other words a potent anti-inflammatory activity. In a near Future it is probably that *B.leachii* could be used by the food, cosmetic or pharmaceutical industry, as a source of bioactive molecules in favor of human's health and quality. Also the study gives propose to the excess of *B. leachii* biomass found in Mediterranean Sea.

Keywords: Anti-inflammatory activity. Antioxidant activity. *B.leachii*. Enzymatic inhibition. Tyrosinase inhibition .Oxidative stress.

1. Introduction

1.1 Justification of the thesis project

1.1.1 Importance of the Ocean

The Ocean covers around 70% of the Earth surface summing a total of 361 million square meters, spread in 5 principal Oceans (Atlantic, Pacific, Indic, Antarctic and Artic). The creation of life started in the ocean at 3.5 billion years ago, which gathered all the necessary conditions for its creation and development. There is no life independent from the sea, directly or indirectly all the organisms ends up attached to it. Humans especially depend on marine systems for a high number of their practices such as food resources, ways to travel around, business and more recently as a source of important metabolites for the cosmetic and pharmaceutical industries (Swathi, *et al*. 2012).

During the last decades studies on marine organism have been growing. The richness of bioactive compounds found in the ocean has turned sea life into a new and prolific source of metabolites, which can be very efficient to improve human health and life quality. Those compounds present a wide range of biological activities such as anti-tumor, anti-microbial, anti-inflammatory and anti-oxidant. Therefore, marine organisms are considered to be the key for the development and conception of new drugs with medicine applications (Bhatnagar & Kim, 2010 and Swathi, *et al*. 2012).

1.1.2 Bioactive compounds

A bioactive compound is a metabolite, which can or not be synthetized by the organism, such as vitamins, proteins or polyphenols. Those are essential compounds presenting a range of different bioactivities including antitumor, anticoagulant, antifungal, antiviral, anti-flammatory, antimicrobial and antioxidant activities (Bhatnagar & Kim, 2010).

Focusing on the positive effects that natural metabolites could have, some industries such as food, cosmetic and pharmaceutical have been developing innumerable

products based on natural bioactive compounds (Biesalski, *et al*, 2009 and Bhatnagar & Kim, 2010).

Bioactive compounds such as antioxidants, are frequently added to food supplies to provide health benefits outside the basic nutritional values (Biesalski, *et al*, 2009). Food industries have tried to improve the quality and nutrition value of their products, defending the association of ordinary diet supplies with natural compounds contributes to a lower risk in the development of chronic diseases, including cancer and cardiovascular disorders (Biesalski, *et al*, 2009).

Although the studies on bioactive compounds extracted from natural sources, are relatively new, those compounds have been used some time ago. Ancient cultures, namely Chinese, always used natural products in their traditional recipes and medicines to treat or prevent some disorders such as cough, asthma, hypertension, anemia, rheumatic congestion, scarring and burns (Hook, *et al.*, 1997).

1.1. 3 Use of marine invertebrates as a source of bioactive compounds

Marine life represents almost half of the total biodiversity that can be found on Earth, and marine ecosystems are considered as the major source for bioactive compounds (Faulkner, 2002).

As it was stressed before, marine organisms have a high importance in the culture of some countries, especially for gastronomy and traditional medicine: being used for example to treat burns and wounds. Nutritionist defend that some marine animals especially mollusks could be the perfect food supply due to their high protein concentration and low fat content (Kobayashi, *et al*. 1991).

Marine invertebrates produce a high amount of natural products as adaptation to their environmental and as result of their life style (Grkovic, *et al.* 2005 and Suarez-Jimenez, 2012). As a result of living in aquatic system, those organisms have face specific biochemical and physiological constrains such as darkness, predation, exposure to ultra-violet radiation, lack of physical defense (soft body), cold temperatures and high pressurized environment's (Grkovic, *et al.* 2005).

Marine invertebrates are mostly soft bodies animals without any strong shell or robust body, which gives them no protection against predator. In order to survive, they have to develop an efficient chemical defensive system, being responsible for their content in bioactive molecules (Regan, *et al.* 1992; Smith, *et al.* 1992; Grkovic, *et al.* 2005 & Haefner, 2003).

Another mediator for the high production of bioactive compounds, especially antioxidants, is the fact that most marine invertebrates live in the intertidal coast living there, organisms have to survive to extreme and stressful conditions as the frequent and high exposure to ultra-violet rays. The exposure can be lethal increasing the amount of free radicals in their bodies, which can severely damage cells and DNA (Regan, *et al*. 1992 and Smith, *et al.* 1992). To avoid and reduce the damages, marine organisms developed some techniques such as the production of "extra" amounts of bioactive compounds, as antioxidants, which will fight back free radicals (Dykens, *et al.* 1992).

Despite all those reasons, a significant amount of natural bioactive compounds present in marine invertebrates, has a dietary origin (Grokvic, *et al* 2005). The majority of those animals are herbivorous, feeding on cyanobacteria and micro/macro algae, which are endowed with some bioactive compounds exhibiting a variety of bioactivities including anti-flammatory (pseudopterosins, topsentins, scytnemin and manoalide), antiobic (marinone), anticancer (sarcodicyin, bryostatins, eleutherobin, discodermolide), and antifungal (Luesch, *et al*. 2002).

The compounds retained from diet are called secondary metabolites, those are not synthetized by the organism and do not have a role in the principal functions such growth and/or differentiation. However, they are crucial to keep the animal alive being used as chemicals in their chemical defense or to fight undesired compounds inside the body, such as free radicals (Grkovic *et al.* 2005).

Marine biotechnology has spent the last decades studying marine organisms as sources of natural bioactive compounds. So far, around 10. 000 marine natural compounds with powerful bioactivities were already extracted from marine invertebrates including tunicates, sponges, soft colors, sea hares, sea cucumbers and bryozoans (Costa Lotufo, *et al*. 2009 and Samuel, *et al.* 2011). Some of those compounds, present a very particular and intense activity being used in clinical and preclinical assays, leading to the

development of new pharmaceutical medicines as the example of Ziconitide (Proalt™), used to treat chronic pains, which was obtained from a mollusk specie, *Conus magus* (Jha, *et al*. 2004; Harvey, 2008; Swathi, *et al.* 2012 and Ngo, *et al.* 2012).

The Fig. 1 summarizes the increasing/evolution in extractions and isolation of bioactive compounds from marine animals, over last forty years (1965-2006). During this time approximately 18.500 natural substances were isolated from marine animals.

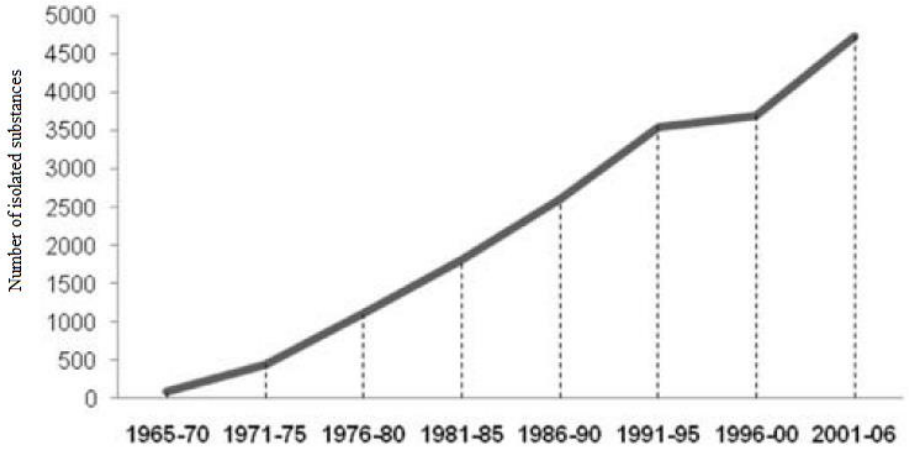

Figure 1. Amount of new marine natural products isolated from 1965 to 2006 (Costa-Lotufo, *et al*, 2009).

Since the sixties marine animals conquered a very important role to the research community, resulting in the isolation of new novel chemical structures including fatty alcochol esters, amino acids, glycosides, terpenoids, alkaloids and proteins, among others. This high diversity of bioactive products was essential for the development and conception of new active products by the pharmaceutical industries (Costa-Lotufo, *et al*, 2009).

Some of those products are already in the market and others are being used in clinical/preclinical trials as the example of Zicotine (Prialt™) used for chornic pains; Citarabina™ an anticancer agent; eceinascidin 743 (Yondelis™) to treat ovarian cancer and soft tissue sarcoma; eribulin mesylate (Halaven™) fighting breast cancer. All of them extracted from marine animals: *Conus magus, Cryptotheca Crypta, Ecteinascidia turbinate* and *Halichondria spp*., respectively. There are also some compounds being studied aiming to treat and prevent neurodegenerative diseases as it was for the

bryostatins, which were extracted from *Bugula neritina* originally studied for anticancer activities but ends up leading to the conception of analogues, which can be potential used to alleviate and related Alzheimer' symptoms (Jha, *et al*. 2004; Harvey, 2008 and Swathi et al. 2012 and Ngo et al. 2012).

The Table 1 summarizes the new sources of bioactive compounds extracted from marine organisms.

Table 1. Marine natural compounds with several biological activities

Phylum	Organisms	Bioactive compound	Drug Class	References
Chordata	*Aplidium albicans*	Aplidine/ Aplidine®	Anti-Cancer	Costa-Lotufo, *et al.*, 2009
	Squalus acanthias	Neovastat®	Anti-cancer	Costa-Lotufo, *et al*., 2009
	Ecteinascidia turbinata	trabectidin/ Yondelis®	Anti-cancer & Anti-inflammatory	D`Incalci, *et al.*, 2003
	Aplidium albicans	Aplidin	Anti-cancer	Haefner, 2003
	Aplidium sp	Ascidiathiazones A	Anti-inflammatory	Pearce, *et al*., 2007
	Styela plicata	Plicatamide	Antibacterial	Tincu, *et al*., 2003
Cnidaria	*Pseudopterogorgia elisabethae*	Elisapterosin B	Antituberculosis	Rodriguez, *et al*., 2000
	Clavularia sp.	Stolonidiol	Neuroprotective	Yabe, *et al*., 2000
	Junceella fragilis	Frajunolides B and C	Anti-inflammatory	Shen, *et al*., 2007
	Aurelia aurita	Aurelin	Antibacterial	Ovchinnikova, *et al*., 2006
	Arcophyton glaucum	Sarcophines	Anti-inflammatory & Anti-cancer	Sawant, *et al*., 2006
	Lobophytum durum	Durumolides A-C	Anti-inflammatory	Cheng, *et al*.,2008
	Subergorgia suberosa	pseudopetrocina-E	Anti-cancer	Wang, *et al*.,2002
Echinodermata	*Actinopyga lecanora*	Holothurin B	Antifungal	Kumar, *et al*., 2007
Mollusca	*Dolabella auricularia*	Dolastantines	Anti-cancer	Costa-Lotufo, *et al.*, 2009
	Jorunna funebris	Zalypsis®	Anti-cancer	Costa-Lotufo, *et al.*, 2009
	Conus magus	Ziconotide	Chronic pain	Haefner, 2003
	Dolabella auricularia	Cemadotin	Anti-cancer	Jordan, *et al.*, 1998
	Conus sp.	Conantokin G	Neuroprotective	Adams, *et al*., 2000
	Dolabella auricularia	Dolabellanin B2	Antibacterial	Iijima, *et al*., 2003

	Bursatella leachii	bursatellanina-P	Anti-inflammatory (HIV)	Rajaganapathi, *et al*. ,2002
	Cryptothetia crypta	Ara-A, Ara-C	Antiviral & Anti-leucemic	Pomponi, 1999
	Luffariella variabilis	Manoalide	Anti-inflammatory	Soriente, *et al*., 1999
	Halichondria okadai	Halicondrina B	Anti-cancer	Simmons, *et al*., 2005
	Dysidea avara	Avarol	Anti-inflammatory (HIV)	Müller, *et al*., 1985
Porifera	*Oceanapia sp*	Acetylenic acid	Anti-bacterial	Matsunaga, *et al*., 2000
	Psammaplysilla purpurea	Mololipids	Anti-inflammatory (HIV)	Ross, *et al*., 2000
	Dysidea sp.	Cavernolide	Anti-inflammatory	Posadas, *et al*., 2000
	Aaptos aaptos	Alkaloid	Antibacterial	Jang, *et al*., 2007
	Lendenfeldia sp.	Deidrofurodendin	Anti-inflammatory (HIV)	Chill, *et al*., 2004

All the organisms belongs to *Animalia Kingdom*, ®: Drugs synthetized based on compounds extracted from marine organisms.

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1.1.4 Sea hares as potent source of bioactive compounds extraction

In the recent days, potent bioactivities were reported from sea hares, containing substances such as toxins, antitumor agents, antibacterial factor, anti-HIV compounds and other chemical defensive substances (Iijima, *et al*. 2003 and Wang, *et al*. 2013).Those are mainly low molecular weight compounds derived from algae (Wang, et al, 2013). Some high molecular weight compounds were isolated from *Aplysia kurodai*, *A. juliana*, and *Dolabella auricularia* (aplysianins, julianins and dolabellanins, respectively), presenting cytotoxic, antimicrobial, antifungal and anticancer activities. Scientist believe that sea hares may produce water-soluble biopolymers and glycoproteins from different sizes, presenting several biological activities (Yamazaki, 1993 and Barsby, 2006).

The purple ink released by sea hares when disturbed, has been the subject of study to many recent works, resulting in the reporting of several interesting bioactivities. In the search for natural bioactive compounds from sea hares, early in 1970, Pettit, *et al*. isolated a new and extreme potent anticancer compound called dollastatin 10, a small lipophilic polypeptide, from *Dollabela auricularia* (Madden, *et al*, 2000 and Simmons, *et al*, 2005) The discovery of this compound was very important leading the conception of three synthetic derivatives: Cemadotina (LU-103793), Soblidotina (TZT-1027), Sintadotina (ILX-651), which are being used in clinical assays related to cancer (Yamada, *et al*. 2002 and Yamazaki, 2003).

At the begging it was thought, that the compounds are produced by the sea hares. Later, some studies have proved that dolastatins were in fact synthetized by cyanobacterias: *Symploca hydnoides* and *Lyngbya majuscule* which are part of *Dolabella auricularia* diet. This fact quickly contributes for the appearance of theories defending that sea hares can sequester several compounds from their diet (Pettit, *et al*. 1998).

Currently natural bioactive compounds isolated from sea hares are one of the most promissory sources for cancer prevention, as anticancer agents, being used in clinical trials as the example of the ILX651, Dolastatin-10 and Cemadotin, which are microtubule interfering agents (Yan, 2004; Simmons *et al*., 2005). During the last 30 years, compounds with anticancer activities including Aplyrorine A, dolastatins 10 and malyngamides (O, P, S) have been isolated from different species of sea hares species (Suntornchashwej, *et al*., 2005).

Regarding to *B. leachii*, until today a very little is known about the bioactive and potentials of the organisms as source of natural compounds. Although studies from the purple ink of these organisms detected the presence of very unusual metabolites (Gopichand & Schmitz 1980 and Appleton, *et al*. 2002), which leads to the discovery of an anti-HIV activity and isolation of the respective compound (bursatellin-P) (Rajaganapathi, *et al*. 2002).

So far, only two bioactive compounds were isolated from the species, the bursatellin-P, a protein with a molecular weight of 60 kDa, and malyngamide S, that exhibited a range of antimicrobial, anti-inflammatory and cytotoxic activities (Appleton, *et al*. 2002). To B. leachii those compounds are considered to be secondary metabolites presenting an algae origin (diet) which are synthetized by *Lyngbya majuscule*, supporting the idea that sea hares have the ability to store in their tissue metabolites from their prey (Spinella, *et al*. 1997; Rajaganapathi & Kathiresan 2005; Capper, *et al*. 2005 and Suntornchashwej, et al, 2005). A study to better understand the pathway of the diet compounds inside sea hares was done, which concluded that in the case of *B.leachii*, the compounds are addressed to the chemical defense, being present in the external organs in higher concentrations comparing to the internal ones (Capper, *et al*. 2005)

Assuming the potentials and the lack of knowledge on the subject, this work aims to bring new information regarding to possible bioactivities and bioactive compounds from *B. leachii.*

1.1.5 Oxidative stress and antioxidant activity

Physiologically oxidative processes, such as aerobic respiration, are responsible for the energy production supporting, which allows all the basic functions of cells. However, during the generation of energy some unstable compounds (free radicals) are released inside the organism, which can trigger some disorder or disease progression (Pietta, 2000; Park *et al.* 2011).

Free radicals are unstable molecules such as superoxide anions (O_2^{-}), peroxide and hydroxyl ($^{\cdot}OH$), with one or more unpaired electrons that can easily react with oxygen, creating unstable oxygen forms (Andreo *et al*., 2006). These highly reactive

molecules have the ability to react with polyunsaturated fatty acids in cellular membranes, nucleotides in DNA, lipids (causing lipid peroxidation, decreasing the membrane fluidity and increasing the permeability to Ca^{+}, which could cause necrosis or apposite and in critical sulfhydryl bonds in protein, leading to DNA deterioration, protein, and lipids (Andreo *et al*., 2006). Considering the effects of those compounds, it is important to control (neutralize) them, otherwise severe negative effects in health, with irreversible damages, could be achieved (Andreo *et al*., 2006).

Normally, the production of free radicals and other highly reactive forms happens as consequence of essential biochemical processes. Mitochondria are believed to consume over 90% of the cellular oxygen during energy producing, and are also considered as one of the major sources in the production of undesired compounds like free radicals and other oxidant forms (Abele & Pintarulo, 2004).

Mitochondria is not the only source of free radicals. Microsomal systems of the endoplasmic reticulum, various enzymatic oxidase reactions and also some exogenously reactions such as tobacco' smoke, high intensity of ultraviolet radiation, air pollutants, exposures to solvents, drugs abuse and pesticides are also responsible for the synthesis of free radicals (Abele & Pintarulo, 2004).

Besides free radicals there are also non-free radical molecules like peroxide hydrogen (H_2O_2), that are synthetized by a reducing of O_2^{-} or by superoxide dismutase in the mitochondrial electron chain. If H_2O_2 is not neutralize, it will freely diffuse in mitochondria and cellular membranes causing serious damage. Moreover, H_2O_2 can also be transformed trough the Fenton reaction in a very dangerous and highly aggressive oxygen from, OH^{-}, which is the major promoter of cellular damages. This happens because it will change the amine groups or amino acids into carbonyls in the presence of redox cycling cations (Fe^{2+} and Cu^{2+}) (Halliwell & Gutteridge, 1985).

The human body is constantly suffering from free radical exposure, which without the proper control can be severely engaged, suffering from lipid peroxidation and destruction of structural proteins, enzymes, carbohydrates and nucleic acids (DNA). Organisms have developed some ways to counteract the excessive level of reactive oxygen specie (ROS) like the production of antioxidants (Andreo, *et al*., 2006). However,

in some situations an unbalance between the production of free radicals and the presence of antioxidants occurs, which is called oxidative stress (Petta, 2000).

Oxidative stress is characterized by a high concentration of intra and extracellular free radicals, causing a disturbance in the redox balance leading to the evolution of sickness and chronic diseases. Triggering oxidative stress is showing several pathophysiological states including ischemia-reperfusion injury, hypoxia and iron overload, which can be caused by a high exposure to ultra-violet radiation, pollution and intoxication (Staniek and Nohl, 1999 & Abele and Pintarulo, 2004). Furthermore oxidative stress is a very dangerous condition because it gathers the necessary conditions for the development of illness such as diabetes, cancers, heart problems and earlier aging processes.

To avoid the excess of free radicals and oxidative stress, the organisms synthetize antioxidants which are natural compounds with the power to neutralize and reduce the effects of undesired metabolites. Antioxidants can or not be produced by the organisms and are usually small molecules such as enzymes, glutathione, ferritin, phenolic compounds, carotene, uric acid, bilirubin, several metalloenzymes including peroxidase (selenium), catalase (iron), superoxide dismutase (copper, zinc, manganese), proteins (ceruloplasmin) and also vitamins such as tocopherol (vitamin E) and ascorbic acid (vitamin c) (Machiln, 1997 and Zengin, et al., 2010).

Natural antioxidants can be classified as primary (molecules capable to scavenge free radical, such as phenolic compounds) and secondary (compounds able to reduce the initiation of free radical reactions, exhibiting metal chelating activity as an example of the nitric acid and the ethylenediaminetetracetic acid (Gordon, 1990; Andreo *et al*. 2006).

Once oxidative stress is achieved, the organisms can suffer irreversible damages including early aging process, apoptosis, alteration of the redox potential and progression of chronic diseases such as cancer, neurodegenerative, arthritis, cardiovascular problems and diabetes (Fig. 2) (Halliwell et al., 1992 and Pietta, 2000).

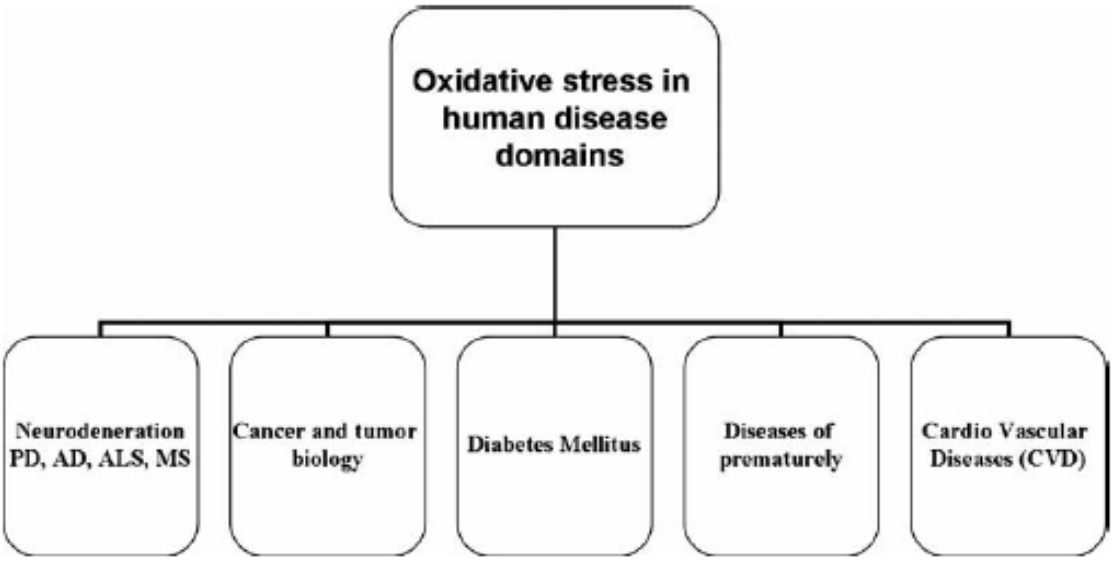

Figure 2. Human diseases where oxidative stress plays a direct or indirect role (Uttara, et al, 2009).

Previous studies have shown that antioxidants when administrated at small quantities can help in the prevention of the consequences mention above, showing a high potential for therapies of diseases caused by the oxidative stress (Noguchi e Niki, 2000; Andreo, *et al*., 2006 and Zengin, *et al*. 2010). Thus, there is a growing interest in the study of natural antioxidant sources as a way to create supplements to be an intake of antioxidants in foods or drugs.

1.1.6 Neuroprotective activity

The underlying causes of neurodegeneration are not fully understood. Environmental and genetic predisposition are considered to be the principal causes, but oxidative stress also plays an important role (Uttara, *et al*, 2009). The brain contains a high level of fatty acids, which are very susceptible to free radical attacks, and therefore it is very sensitive to oxidative damage. Moreover, brain has low amounts of antioxidant molecules, which facilitate the development of oxidative stress (Floyd & Camey, 1992).

There are evidences that oxidative stress is one of the main causes for the onset of several neurological disease. The accumulation of oxygen reactive species (ROS) in the brain generates high concentrations of H_2O_2, nitric oxide (NO) and OH^-, which are toxic

to brain cells causing deterioration and apoptosis leading to the development of neurodegenerative diseases such as Alzheimer (AD) and Parkinson (PD). Neuroprotective activity has an extremely vital significance to brain, working as a defensive mechanism system (Uttara, *et al.* 2009).

Once oxidative stress has an important role as a mediator for the development of neurodegenerative diseases, antioxidants will play a very important part as neuroprotective agents. Since a high amount of free radicals is present in AD, it is vital to the organism has a significant amount of antioxidants fighting the negative effects of free radicals. Recent studies and observations have been clarifying some aspects related with AD, proving that an absence of antioxidants, such as Vitamin E, the development of the disease or also other neurodegenerative pathological disorders including PD is leading (Frank & Gupta, 2005). Besides neutralizing the effects of free radicals, the presence of antioxidants in the brain is really important, acting as chelating compounds to the ion metals accumulated in brain with the development of neurodegenerative disorders. This accumulation could lead to serious damages reacting with oxygen reactive species generating H_2O_2, NO and some other ones, which will severely engage brain (Weinreb *et al.* 2011).

Free radicals and the interaction between metal ions with the surrounding cells could trigger an inflammatory response in the brain. The result of the interaction of ROS with neuronal cells often leads to inflammation in brain, therefore it can easily be deteriorated, compromising the neurons and neurotransmission. A severe inflammatory response could be lethal, causing unfixable damages. Indeed the main promoters of neurodegenerative diseases are states of inflammation in the brain, which evolves to diseases such as AD or PD (Stuchbury & Much, 2005).

1.1.6.1 Alzheimer disease (AD)

AD, the more common responsible for dementia in humans, is a neurodegenerative disease characterized by a progressive loss of memory, task performance, speech, learning capabilities and recognition of people, places and objects (Roos & Poirier, 2004). AD is the result of deterioration in the brain cells, especially the pyramidal neurons in the hippocampus, entorhinal cortex, cholinergic neurons in the median forebrain and entirhinal cortex (Sirnonian & Coyle, 1996).

Although the complete etiology of the diseases is still unknown, there are three recognized hallmarks of this condition. All of them are responsible for the loss of the neuronal functioning: accumulation of neurofibrillary tangles associated to the tau protein; accumulation of insoluble plaques formed from the amyloid-β peptides (Aβ) and loss of neurons (Juneja, 2006).

In a general way, AD is characterized by the aggregation of beta amyloid fibrils on brain being responsible for the neurotransmission loss (Berg *et al.* 1993). The lack of signal compromises daily functioning causing alteration into memories, behavior disorders and affecting motor skills (Scarpini, *et al.* 2003; Souza, 2011).

The deposition of extracellular aggregates of P-amyloid peptides (AP) is one of the neuropathologic hallmarks of AD, and so the disease is known for the deposition of amyloid plaques. This accumulation can be very dangerous, because metal ions such as Cu^{2+}, Zn^{2+} and Fe^{3+} get retained, which can react with oxygen reactive species, creating toxic compounds, leading to cell brain damages or death (Behl, *et al*, 1992).

Supporting the affirmation mentioned above, AP has shown to be toxic in vitro neurons, therefore it is suggested the hypothesis that in Alzheimer, the neuronal deterioration could be provoked by the toxic effect of AP (Yankner, *et al*, 1989). It is important to stress, the concentration and toxicity of AP can be significantly increased in the presence of oxidative stress (Behl, *et al*, 1992). On the other hand, the aggregation of P-amyloid peptides can be inhibited by antioxidants, which allow to scientists believe that high presence of free radicals (oxidative stress), is the principal responsible for the formation of amyloid plaques (Sirnonian & Coyle, 1996).

Another pathologic hallmark in AD is the polymerization of tau, one of the main constituent of intracellular neurofibrillary tangles (NFTs). NFTs are aggregations of tau protein commonly known as a primary maker of AD. NFTs are also related with oxidative stress (Sirnonian & Coyle, 1996).

Neurodegenerative diseases as other illnesses are strongly related with life style, the exposure to free radicals and other toxic compounds varies from person to person, as well as the diet. A healthy life style together with a diet rich in antioxidants can indeed be the very first step for the prevention of diseases such as Alzheimer (Santos, 2009).

1.1.6.2 Parkinson disease (PD)

PD is a chronic neurodegenerative disease characterized by the loss of dopaminergic neurons, due to the sensibility of those to oxidation. Dopaminergic such as dopamine transistors and receptors, are related to dopamine, which is a neurotransmitter in the vertebrates. These neurons are more vulnerable, they are exposed to toxic compounds including free radicals and others oxygen reactive species, which are produced as a result of dopamine pathway and so oxidative stress can be induced (Olanow, & Arendash, 1994).

Dopamine is a chemical substance released by the nerve cells during neurotransmission. This neurotransmitter is an amine formed by the removal of a carboxyl group from L-DOPA molecule, in the presence of tyrosine. Besides neurotransmission, dopamine is also responsible for the regulation of hormones doing motor control (Elsworth, & Roth, 1997).

Dysfunctions in the dopamine system are related with the onset of some neurodegenerative diseases such as PD, known from the motor impairment, rigidity of the body, movement slowing and tremors, caused by the deterioration of dopamine-secreting neurons in the midbrain area (substantia nigra). This dis-regulation can also cause attention deficits, schizophrenia, hyperactivity disorders and restless legs syndrome, which are all associated to a decrease in the dopamine levels due to the degradation of the dopamine secreting cells as a result of the toxic compounds released during it production (Elsworth, & Roth, 1997).

Patients suffering from PD usually exhibit low dopamine level in the brain; the usual treatment for this disease is the administration of L-DOPA, the metabolic precursor for the systemization of dopamine. However this treatment is not able to recover or undo the damaged cells, but it allows the healthy cells to continue producing dopamine at higher concentrations in order to compensate for the death ones (Elsworth, & Roth, 1997).

Dopamine is synthetized in the presence of tyrosine, by the tyrosine hydroxylase, which release H_2O_2 as a result of the metabolism. H_2O_2 can be very prejudicial for the organism, because nigral neuromelanin has the ability to bind metals like iron in H_2O_2. In fact, H_2O_2 can react with metals, reducing them to highly reactive form, which lead to oxidative stress and destruction of the surrounding cells (Sirnonian & Coyle, 1996).

Prevention of the presence of H_2O_2 is crucial in PD, because patients that suffers from this condition usually presents high concentration of metals, and the H_2O_2 combined with metals increase the oxidative stress trough the production of OH^- via the Fenton reaction (Riederer, *et al*, 1989).

1.1.6.3 The role of cholinergic systems in neurodegenerative diseases

During the last decades, some substantial progress has been made in the search for new treatment as ways to prevent neurodegenerative diseases (Scarpini, *et al*, 2003).

Respectively to Alzheimer a massive effort has been done in the search for prevention ways. So far, the cholinergic hypothesis seems to be the best approach to avoid the progression of this condition. This hypothesis states that AD and other neurodegenerative disease are due to a deficit of cholinergic transmitters, which are crucial for neurotransmission. Therefore the most common way to accomplish AD treatment is the inhibition of the enzymes (cholinesterase) responsible for the hydrolysis of the neutransmitters in order to increase their levels and restore the brain activity (Scarpini, *et al*, 2003).

Acetylcholine (ACh) is a neurotransmitter responsible for the transmission of the nervous impulse, for muscle contractions, and is also involved in memory and learning capabilities (Houghton, et al, 2006). Previous studies revealed that Alzheimer patients exhibited low levels of Ach, which will consequently decrease neurotransmission, causing an inability to transmit the neurological pulse (Souza, 2011).

Considering the previous fact, the most common way to increase ACh levels in the brain is through the inhibition of the enzymes responsible for the degradation of ACh in the synaptic cleft, the Achetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Both, AChE and BChE, are involved in the breakdown of acetylcholine in the synaptic cleft, breaking it into choline and acetate trough hydrolysis (Giacobini, *et al.* 2004) (Fig. 3).

In AD patients, the inhibition of the activity of those enzymes is extremely important and this inhibition is carry out using some medications: donepezil, rivastigmine and galantamine (Filho, *et al.*, 2006 and Souza, 2011). This inhibition is not only essential

for AD but also to another neurological disorders such as senile dementia, ataxia, myasthenia gravis and PD (Filho et al., 2006 and Pulok; et al. 2007).

Comparatively to AChE, the role of BChE is not completely known, but there are evidences that it can replace AChE when its activity decreases. Moreover, recent studies have shown that high BChE levels, contribute for the maturation of senile plaques, which are a signal of AD (Wiebusch, *et al*. 1999).

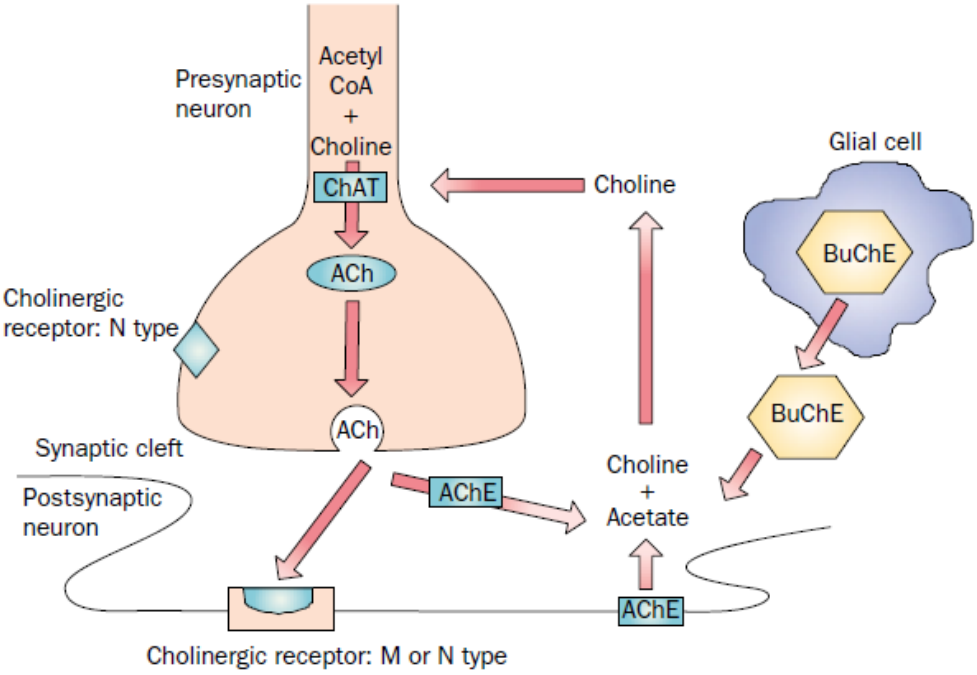

Figure 3. Representation of the importance that AChE and BChE has, as a regulator of acetylcholine levels, breaking it into choline and acetate.

1.1.6.4 Tyrosinase as a trigger for neurodegenerative diseases

Tyrosinase is a copper-containing metalloprotein enzyme, also known as monphenol monooxygenase, commonly distributed in animals and plants. This enzyme is vital for melanin biosynthesis and a few polyphenolic compounds related with skin and hair determining color (Hasegawa, 2010).

Humans have been searching and attempting to develop products able to artificially change the skin color, either through whitening or darkening. Products with this ability are very important to balance and fix a pigmentation disorder that comes with

aging processes. Besides that, products with ability to whitening the skin tone are intensively used in countries from Asia and Africa (Silva, 1998).

The use of skin lightening/darkening products has been increasing in the last years, and it is done in a beauty context, or as a result of skin color disorders namely vitiligo (Shevlin, 1974 and Benech, 2002).

For that reason during the last decades, cosmetic industries have developed pharmaceutical or cosmetic products to be used against skin disorders, namely hyperpigmentation or hypopigmentation. An example of such compound is hydroquinone, which inhibits the activity of tyrosinase and consequently the synthesis of melanin (Lee & Choi, 1997 and Zhai & Maibach, 2001).

Besides melanin production, tyrosinase acts an essential enzyme in the synthesis of dopamine (DOPA), which is a very important neurotransmitter being responsible for movement control. The synthesis of DOPA occurs in the brain, through the tyrosinase activity, a process that converts tyrosine into L-DOPA, which later is descarboxylated resulting in dopamine. Although this is an essential process, during dopamine production some neurotoxic compounds and other oxygen reactive species (ROS) are released (Fig. 4), causing severe brain damage that can evolve to PD (Raper, 1928; Kang, *et al*. 1993; Olivares, *et al*. 2001 and Hasegawa, 2010).

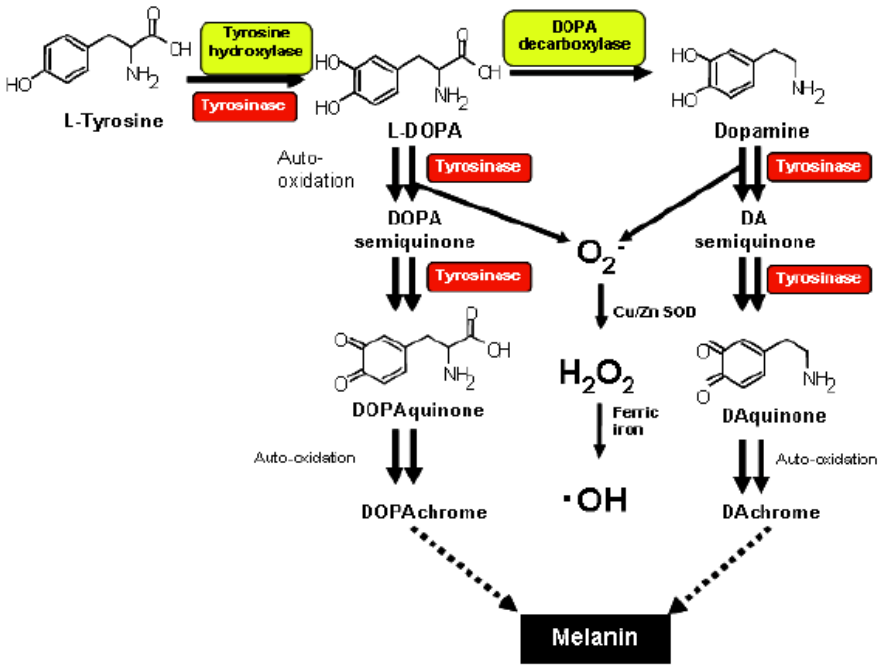

Figure 4. Process of melanin and dopamine production through the tyrosine activity, which produces oxygen reactive species during the enzymatic activation (Hasegawa, 2010).

As it can be observed in Fig. 4, tyrosinase activity and consequently dopamine production releases ROS to the organisms (H_2O_2 and $^{\cdot}OH$), which can covalently be incorporated into lipids, proteins and nucleic acids triggering illness. Considering that, the overproduction of tyrosinase must be stopped: the tyrosinase inhibitors are the best promising sources in the prevention of PD (Stokes, *et al*. 1996 and Hasegawa, 2010).

A few studies on the search for these inhibitors were developed obtaining an interesting correlation between the concentration of some compounds (phenols, flavonoids and tannins) with tyrosinase activity. Suggesting the compounds rich on phenols, flavonoids or tannins exhibit higher levels of tyrosinase inhibition (Maeda & Fukuda, 1991 Lee and Choi, 1998).

1.2 Study species, *Bursatella leachii*

The sea hare *Bursatella leachii* (Blainville, 1817) is a mollusk, belonging to the Ophistobranchia order, subclass Gastropoda, family Aplysiidae (Hickman, *et al*. 2006). This marine invertebrate is a soft body medium/large size organisms without a strong physical defense, although it hides a small shell buried inside. All the individuals exhibit a brownish color with some bright blue spots that camouflage the animal perfectly among the seaweeds and sea grasses (Fig. 5). Covering the body there are present numerous long, branching fleshy papillae that give them a very strange appearance (Rupert & Fox, 1988).

B. leachii is widely distributed around the world, presenting a global distribution in the shallow water from tropical environmental, through the Indio-Pacific, Atlantic Ocena and recently it stated to colonize the Mediterranean Sea (Palige, 1988 and Zenetos, *et al*. 2004).

Previous studies had shown that the Mediterranean Sea is quite diverse respectively to marine mollusks, being identified around 400 opisthobranchs species (Cattaneo-Vietti & Thompson 1989), counting with 21 alien species, namely *B.leachii*

(Zenetos *et al.* 2003; 2004 and 2005). In fact, *B.leachii* was found for the first time in Palestine (O'Donoghue and White, 1940) being then reported in Turkey, Malta, Israel, Sicily, Tunisia, Italy, Solvenia, Greece, Lebanon, Sardinia and in the Southern of Spain (Swennen, 1961; Bebbington, 1970; Eales, 1970; Piani, 1980; Enzenross and Enzenross, 2001; Fasulo et al., 1984; Jaklin and Vio, 1989; Koutsoubas, 1992 and González-Wangüemert, *et al*. 2014). Considering this pattern of distribution, the dispersal that is observed in the Mediterranean suggested that we are assisting to a Lessepsian specie, meaning that this *B. leachii* is performing a migration through the Suez Canal (González-Wangüemer *et al*. 2014).

Alien species represent to be a worldwide threat for the resident communities, economy and human health, therefore it is essential to control them. Also, invasive species contribute for the declining of the native population causing a strong competition and stress (Ricciardi, 2004). Actually the impact and effects of invasive species is considered to be one of the biggest causes for habitat and biodiversity degradation (Breithaupt, 2003).

Considering the threat that alien species represent for the environmental, there is a huge effort to reduce and irradiate the present of those species. Usually the solution implies a massive killing or by controlling their reproduction.

Little is known about the biology and other characteristics from *B. leachii*. This species is commonly known as ragged sea hare, and it is an hermaphrodite organism with an accurate chemical defensive system, expelling a purple ink when it feels threatened, which release noxious and unpalatable compounds protecting the animal from the predators (Kaplan 1988 and Palige, 1988). This species is feeding on micro/macro algae, some cyanobacteria colonies, specifically *Lyngbya majuscula* and also benthic detritus, grazing the surface layers of the mud (Fenical, *et al*, 1979; Palige, 1988 and Kamiya, *et al*. 2011).

Wu (1980) and Clarke (2004) report a possible dietary preference for *Enteromorpha* over cyanobacteria in the Pacific populations, but such preference appears not to be universal. Ragged sea hares are known to consume *Lyngbya majuscule*/ *Microcoleus lyngbyaceus*, a cyanobacterial species abundant in shallow marine systems (Fenical, *et al*, 1979; Palige, 1988 and Kamiya, *et a*l 2011). Capper et al., (2005) suggested that sea hares are likely to derive a dietary benefit from sequestering toxic

metabolites as the lyngbyatoxin-a from *Lyngbya majuscule,* into the digestive gland and in body secretions.

As it was mentioned, *B. leachii* is an alien species in the Meditearean Sea, therefore considering that during the invasive species control a lot of biomass is wasted, it could be useful if we prove that *B. leachii* is a potential source of natural bioactive compounds. On this sense, the industry could use this species to extract natural bioactive compounds and identify new bioactive molecules to use as model for some drugs.

Figure 5. Spcimen of *B.leachii* (Photo by González-Wangüemert).

2. Objectives

Considering the potential that marine animals represent as natural sources of new potent bioactive molecules for the pharmaceutical industries, the main goal of this Msc thesis is to evaluate several biological activities, including antioxidant and anti-inflammatory, for *B.leachii* extracts.

Moreover, in order to understand better the nature of the molecules responsible for those activities it was performed a chemical characterization including the determination of total phenol (TPC), flavonoids (TFC) and tannins content (CTC) and quantification of liposoluble pigments (β-carotene, Lycopene, Chlorophyll am

Chlorophyll b). Besides that it was also determined the moisture and ash content of the samples and it was traced the fatty acid profile (GC/MS).

As marine invertebrates had proved to be a potential sources for the pharmaceutical industries, this Msc Thesis is focusing on determining and quantifying some biological activities that *B. leachii* could present. Also, this work has as goal, find propose and utility for the excess of biomass that *B. leachii* represents as an invasive species.

3. Material and Methods

3.1 Sampling area and sample collection

The individuals of *B. leachii* used in this study were caught in Mar Menor (Figure 6), Spain. Mar Menor is one of the largest Mediterranean coastal lagoons (135 Km^2), located on the southeastern coast of Spain (37º389 N, 0º429 W) (Fig. 6) (Arévalo, 1988). Mar Menor is a hypesaline lagoon (39-47psu), isolated from the Mediterranean Sea by a 22 km long and 0.1 to 1.5 km width sandy bar known as "La Manga del Mar Menor" (Pérez-Ruzafa, 2005). The area is characterized as shallow waters, with a medium deep of 4 m achieving a maximum of 7 m, with water temperature ranging from 10ºC to 31ºC in winter and summer, respectively. The central area is covered by a dense community of the algae *Caulerpa prolifer*a or *Cymodocea nodosa* sea grasses patches (González-Wangüemert, *et al*., 2009; 2014).

Figure 6. Study area, Mar Menor

Fifty adult individuals from *B. leachii* were sampled by snorkelling from the shallow benthic habitats, in December of 2012 by Dr. Mercedes González-Wangüemert. Individuals were cleaned from contaminants, frozen dried, reduced to powder and stored at 0ºC.

3.2 Preparation of the extracts

Through a simple extraction 4g of dried biomass of *B.leachii* was added, in a proportion of 1:40 (w/v), to 5 solvents of different polarities index (PI), namely dichloromethane (PI=3.1), ethyl acetate (PI=4.4), acetone (PI=5.1), methanol (PI=5.1) and water (PI=10.1). The mixture was left to extract over the night at room temperature (RT) and then homogenized using the IKA Ultra Turrax (2 min), vortexed (1 min) and finally centrifuged (5000rpm, 10 min, RT, Megafuge 16R Centrifuge, Thermo Scientific). The upper layer (supernatant) was pooled in a schott, and 40 ml of solvent was put into a falcon in order to repeat the centrifugation, the process was repeated for other 4 times. After it, samples were filtered (Whatman no.4) and further evaporated using a rotary evaporator (IKA R10 Digital S93 with water bath IKA HB10 Digital S93), with 60°C for acetone and methanol and 50ºC for ethyl acetate and dichloromethane, rotation was set at 90 rtpm. All the extracts were resuspended in DMSO and stored at 4ºC until use in the assays.

3.3 Chemical characterization

3.3.1 Phytochemicals

3.3.1 a) Total phenolic content (TPC)

Phenolic compounds are a group of bioactive metabolites able to neutralize free radicals due to their hydroxyl groups. In this sense, the phenolic contents might be directly related with the antioxidant activity of an extract and its potential against the cellular oxidation (Tosun *et al*., 2009). During this work it was determined the amount of phenolic compounds present in the extracts using Folin-Ciocalte (F-C) colorimetric method (Julkunen-Tiitto 1985), according to the protocol established by Velopglu *et al*. (1998).

Briefly, 5 µL of extract at the 10 mg/ml concentration were mixed in a 96 well-plates with 100 µL of the Folin reagent (1/10 in distilled water, v/v) during 5min at RT. Then, 100 µL of sodium carbonate (Na_2CO3, 75 g/L in water) were added, and incubated for 90 min, RT at the dark. The absorbance was measured at 725 nm using a microplate reader (BioTek Synergy 4 plate reader). Results were expressed as gallic acid equivalents (GAE) per gram of sample (dry weight) through the use of a calibration curve of gallic

acid standard solutions for the following concentrations (0, 0.00375, 0.0075, 0.015, 0.03, 0.125, 0.25, 0.5, 0.75, 1 mg/ml)

3.3.1 b) Total flavonoid content (TFC)

Flavonoids are a group of low molecular secondary metabolites, belonging to the polyphenols class, usually present in high amounts on plants and other vegetal organisms. These compounds, which are very popular for the wine and tea production, can be synthetized following several routes as the example of the acetylcholine A. Flavonoids have a range of biological activities including: anti- inflammatory, hormonal control, anti-tumor, anti-alergic and antioxidant (Harborne & Williams, 1992).

This technique is based in the measurement of the absorbance of the complex formed between the aluminum reagent and flavonoid, forming a yellowish compound.The total flavonoids content was carried according to the protocol develop by Zou, et al (2011), adapted to 96-well microplates. Aliquots (30 µL) of extracts, at the concentration of 10 mg/ml were mixed with 180 µL of distilled water and 10 µL of 50% $NaNO_2$, for 6min of incubation at RT. Then 20 µL of 10% of AlCl3 (in methanol) were added to each well, after 6 min 60 µL of 4% NaOH were added and the plate further incubated for 15 min. The absorbance was measured in the microplate reader at 510 nm. Results were expressed as milligrams of rutin equivalent per gram of sample (mg RE/g, DW), by constructing a standard curve of rutin using different concentrations (0, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 mg/ml).

3.3.1 c) Condensed tannin content (CTC)

Tannins are water-soluble polymeric phenols with the ability to form protein complexes (Hagerman and Butler, 1981 and Arnold & Targett, 2002) synthetized by plants and algae, with a very particular and important role, protecting then against herbivores or pathogens. Previous studies reported that tannins are important to health, when ingested, as for example in vegetables, as an intake of antioxidants (Hagerman and Bulter, 1981 and Arnold and Targett, 2002).

The CTC was done according to the method developed by Zou, et al (2011) with slight modifications. In a 96-well pate, 10 µl of the extracts, with a concentration of 10 mg/ml, were mixed with 200 µl of 1% DCAMA w/v (in methanol) and 100 µl of 37% hydrochloric acid (HCL). After 15 min, the absorbance was read at 640 nm using the microplate reader. The values were expressed as milligrams of catechin equivalents per g samples (mg CE/g), obtained by the use of a catechin calibration curve with the follow concentrations of 0, 0.0039, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.

3.3.2 Liposoluble pigments

The analysis of liposoluble pigments (β-carotene, lycopene, chlorophyll a and chlorophyll b) was done according to the method described by Yamashita (1992) and Barros *et al.*, (2010). Dried biomass from *B. leachii,* 150 mg, was mixed with 10 ml of an acetone-hexane solution (4:6 v/v), and vortexed during 1 min. Then the extracts were filtered filtered through Whatman No. 4 filter paper, diluted to ½ and the absorbance of the filtered solution was read at 453, 505, 645 and 663 nm.

The content of liposoluble pigments was calculated using the values of the absorbances at the different concentrations according to the follow formulas:

β-carotene (mg/100 mL) = 0.216×A663 −1.220×A645 −0.304×A505 + 0.452×A453;
Lycopene (mg/100 mL) =−0.0458×A663 + 0.204×A645 −0.304×A505 + 0.452 ×A453;
Chlorophyll a (mg/100 mL) = 0.999×A663 −0.0989×A645;

Chlorophyll b (mg/100 mL) =−0.328×A663 + 1.77×A645

The results were expressed in mg/100 g of dry weight (considering the ½ dilution).

3.3.3 Moisture and ash content

Fresh sea hare fragments were weight and then dried in oven for 96h at 52ºC, until the pieces were completely dried. The ash content was determined using a protocol described by Gressler et al. (2010), where the ash content is calculated according to weight difference before and after 5 hours of incineration in the muffle furnace at 550°C.

3.3.4 Fatty acid profile (GC/MS)

Fatty acids were extracted and converted to the corresponding fatty acid methyl ester (FAME) by a direct transesterification method with acetyl chloride/methanol followed by direct extraction into hexane according to Lepage and Roy (1984) with some modifications. Briefly 0.1 mg of *B. leachii* extracts biomass were treated with 1.5 ml of derivatization solution (methanol/acetyl chloride, 20:1, v/v). Cell disruption was further accomplished with an UltraThurrax homogenizer in 3 cycles of 30 s each. After the addition of hexane (1 ml), the mixture was heated for 1 hour at 100ºC. The vials were then cooled in an ice bath, and 1 ml of distilled water was added. For fast phase separation, samples were centrifuged and the hexane phase removed and dried with anhydrous sodium sulphate. Methyl esters were analysed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System, 5973 Inert Mass Selective Detector) equipped with a DB5-MS capillary column (25 m x 0.25 mm internal diameter, 0.25 μm film thickness, Agilent Tech). The temperature program was 60ºC (1 min), 30ºC/ min to 120ºC, 5ºC/min to 250ºC, and 20ºC/min to 300ºC (2 min). Injection temperature was 300ºC. For identification and quantification of the fatty acid methyl esters, total ion mode was used. Due to differences in the response factors, separate calibration curves were performed in triplicate for each FAME. In the case where no standard was available, the response factor of the most similar FAME, in terms of structure, was used.

3.4 Determining of anti-oxidant activity

Anti-oxidant activity of *B. leachii* extracts (methanol, acetone, ethyl acetate, water and dichloromethane) were analyzed by different complementary assays including: free radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, metal chelating activity (iron and copper), iron reducing power and nitric oxide inhibition.

3.4.1. Free radical scavenging activity on DPPH radical

The assay measures the inhibitory activity of the extracts, based on the decreasing of the absorbance, at 517 nm, according to a reduction of the DPPH in the presence of antioxidant compounds. This is a colorimetric assay, where a change of color can be notice, when the DPPH is reduced, transforming the initial purple into a pale yellow. During the DPPH assay, Antioxidants react with DPPH•, a stable free, which is reduced to the DPPH-H with a consequent discoloration indicates an antioxidant ability of the extract in terms of hydrogen donating. The scavenging reaction between (DPPH•) and an antioxidant (H-A) can be written as is shown Fig. 7.

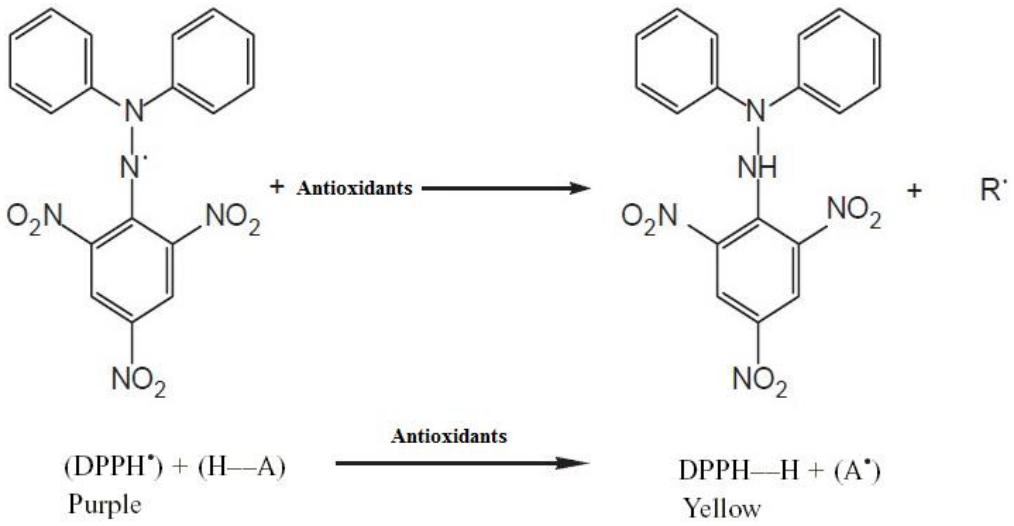

Figure 7. Scavenging reaction between (DPPH•) and an antioxidant (H-A). Adapted from Mosquera, *et al*. 2007.

Free radical scavenging activity on DPPH was performed following the method described by Moreno et al. (2006). Briefly, in a 96 well fat bottom plates, 22 µl of the extracts at different concentrations (1, 5 and 10 mg/ml) were mixed with 200 µl of daily prepared DPPH solution (120 µM in methanol), left for a 30 min incubation period in the dark. Negative and positive controls were also prepared, by using DMSO and butylated hydroxytoluene (BHT, 1 mg/mL, a synthetic antioxidant), respectively, instead of sample. Due to the color of the extract, color controls were prepared, by replacing DPPH for methanol. The optical density was measured at 517 nm using a spectrophotometric microplate reader (Synergy 4, Biotek instruments, USA).

The results from this assay were expressed as a percentage of DPPH inhibition, which is the percentage of antioxidant activity. The calculation of the antioxidant activity was done according to the negative control using the following formula (1):

$$\%\ antioxidant\ activity = 100 - \left[\frac{(OD_{517} - \overline{OD}_{colour\ control}) \cdot 100}{\overline{OD}_{negative\ control}}\right] \quad (1)$$

Where OD_{517} consists in the absorbance of the sample; OD represents the color controls and $OD_{negative\ control}$ represents the mean absorbance of the negative control.

3.4.2. Chelating activity

The term chelate, from the Greek "chele" for "claw", describes the potential of a metabolite to aggregate itself with metal ions such as calcium, lead, mercury, cadmium, copper, aluminum and iron. This very useful ability is extremely important for medicine allowing the extraction of dangerous metals from the organism. Chelating agents as the EDTA are often realized into the bloodstream with the aim to grad undesired minerals and metals, removing them from the system, delivering them to the kidneys, which excrete them in the urine. Furthermore, once a chelate binds to those minerals, they get unable to react in undesirable ways.

Nowadays, chelation therapy is often used as a complementary or alternative treatment for the reduction of atherosclerosis, oxidative stress or tumor growth.

3.4.2. a) Iron chelating activity

Iron chelating activity was evaluated through the ability of the extracts to inhibit the formation of Fe^{2+}-ferrozine complex in the present of antioxidants, according to the method described by Megias *et al*. (2009). Antioxidants will work as chelate compounds, binding to iron, on this way, there is no iron available for the formation of Fe^{2+}-ferrozine.

The extracts, 30 μL at the concentrations of 1, 5, 10 mg/mL, were mixed with 200 ml of distilled water and 30 μL of Iron(II)-chloride ($FeCl_2$, 0.1 mg/mL in distilled water) in 96-well plates for 30 min at RT, in the dark. Then, 12.5 μL of ferrozine (40 mM in distilled water) were added to each well and 10 min later the absorbances were read at 562 nm using a microplate reader (BioTek Synergy 4 plate reader). Negative and positive controls were prepared replacing the extracts for distilled water and ethylenediaminetetraacetic acid (EDTA, 1 mg/mL in distilled water), respectively. Color controls were prepared by replacing $FeCl_2$ and ferrozine by distilled water. The chelating activities were then calculated using the formula (1) indicated on the section of the Free radical scavenging activity on DPPH radical.

3.4.2. a) Copper chelating activity

Copper chelating activity was determined according to Megías et al. (2009), where 30 μL of extracts, at the concentrations of 1, 5 and10 mg/ml, were mixed in a 96-well plates with 200 μL of sodium acetate buffer (50 mM, pH 6.5), 100 μL of copper sulfate ($CuSO_4.5H_2O$, 50 μg/mL, w/v), and 6 μL of pyrocatechol violet solution (PV, 40 mM in buffer), left for an incubation period of 5 min at RT. Negative and positive controls were prepared replacing the extracts for distilled water and ethylenediaminetetraacetic acid (EDTA, 1 mg/mL in distilled water), respectively. Color controls were prepared by replacing copper sulfate and PV by sodium acetate buffer. The optical density was then measured with the use of a microplate reader (BioTek Synergy 4 plate reader) at 632 nm and the chelating activities calculated the same as for the iron assay.

3.4.3 Iron reducing power

Compounds with reduction potential are essential and useful for medicine, allowing the transformation of some undesired metabolites, which sometimes can not be chelated due to the unstable form, into other less dangerous forms being afterwards eradicated. Based on the iron reducing power, compounds with a reductive potential will react with potassium ferricyanide (Fe^{3+}) forming potassium ferrocyanide (Fe^{2+}), which afterwards reacts with ferric chloride resulting on ferric ferrous complex's (absorbed at 700 nm) (Jayanthi, P. & Lalitha, P., 2011), has it can be seen in the Fig. 8.

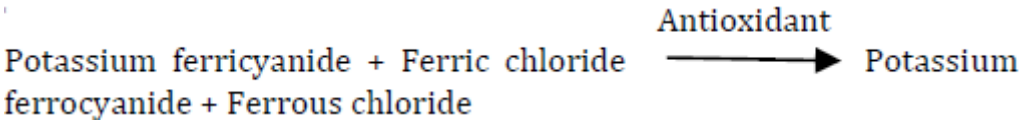

Figure 8. Iron reducing power assay concept.

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu (1986), and modified by Megías et al (2009). Samples (50 µl at the concentrations of 1, 5 and 10 mg/ml), distilled water (50 µl) and 1 % potassium ferricyanide (50 µl) were mixed and incubated in at 50 ºC for 20 min. Then, 50 µl of 10 % trichloroacetic acid (w/v) and ferric chloride solution (0.1 %, w/v) were added, and the absorbances were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as a positive control at the concentration of 1 mg/m.

3.4.4 Nitric oxide (NO) scavenging activity

NO is a free radical involved in many physiological and pathological processes, known as a bioproduct in several organisms including bacteria, plants, fungi and animal cells. This is a very dangerous and highly reactive molecule, which can easily interact with cells compromising the good functioning. Because of its reactivity, this unstable molecule can indeed be the major responsible of some illnesses. NO is usually associated to oxidative stress situations and inflammatory responses (Kelm, et al, 1997 and Yan & Rao, 1997).

In the Nitric oxide scavenging assay, the ability of the extracts to inhibit NO activity is quantified based on the concept that sodium nitroprusside generates NO, which

reacts with oxygen producing nitrite ions that can be quantified by colorimetry using Griess reagent, which in the presence of nitrite ions forms a pink color (Marcocci, *et al.* 1994).

Once sulphanilic acid is added, nitrites form a diazonium salt; when the azo dye agent (N-alpha-naphthyl-ethylenediamine) is added, shows a pink color (Fig. 9).

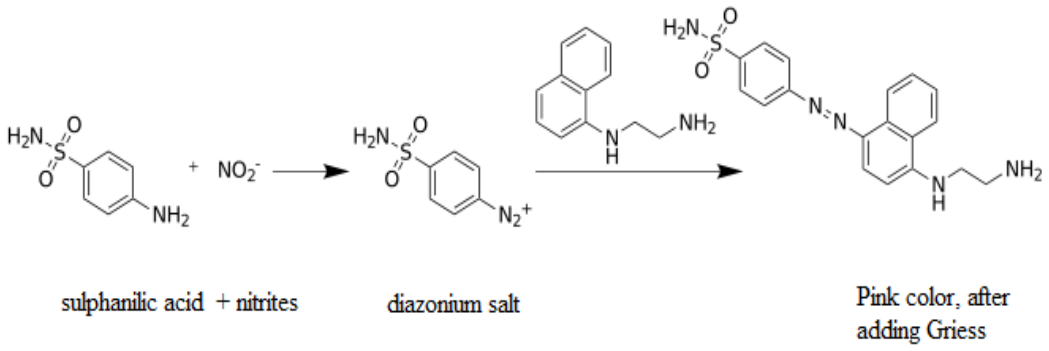

Figure 9. Final step in the NO assay.

The NO scavenging activity was evaluated according to Ho et al (2010). The extracts (50 µl at the concentrations of 1, 5 and 10 mg/ml) were mixed in 96 well plates with 50 µl of 10 mM sodium nitroprusside in phosphate buffer (PBS) and incubated in the light for 90 min at RT. Then, 50 µl of Griess reagent (1% of sulphanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO3 were added and absorbances were read at 546 nm. Ascorbic acid was used as the positive control at the concentration of 1 mg/ml. The results were expressed as the inhibitory rate (%) for the synthesis of nitrites, consequently NO inhibition.

3.5 Determining of neuroprotective activity

Neuroprotection is essential to the organisms allowing the maintenance of a good neuronal functioning. The neuroprotective activity of *B. leachii* extracts was analyzed using different assays including: inhibitory activity of enzymes (AChE, BChE, Tyrosinase), protective effect against H_2O_2-induced injury in neuronal cells and anti-inflammatory activity on LPS-stimulated microglia cells.

3.5.1 Inhibitory activity of enzymes

3.5.1 a) AChE and BChE inhibitory activity

The AChE and BChE inhibitory activity was determined according to Orhan et al (2007). Briefly 140 µl of phosphate buffer (0,1 mM, PH 8) were mixed in a 96-well plates with 20 µl of the extracts, at the concentrations of 1, 5 and 10 mg/ml for BChE and 125, 250, 500 and 100 µg/ml for AChE, with 20 µl or AChE or BChE for an incubation period of 37ºC for 15 min. Then 10 µl of acetylthiocholine iodide (AChI) or butyrylthiocholine chloride (BChI) were added to 20 µl of 5,5-dithiobis[2- nitrobenzoic acid] (DTNB). A negative and positive controls were also prepared, replacing the extract for DMSO and galanthamine at 1 mg/ml, respectively. Due to the color of the extract, color controls were prepared using only the buffer solution and BChE or AChE. Finally the absorbance was measured at 412 nm in the microplate reader and the calculations of the enzyme activity were done according to the formula (1). Results were expressed as the inhibitory activity of BChE or AChE.

3.5.1 b) Tyrosinase inhibitory activity

The tyrosinase activity was quantified according a protocol described by Nerya *et al* (2003). In 96-well plates was mixed 70 µl of extracts, at the concentrations of 125, 250, 500 and 100 µg/ml, with 30 µl of tyrosinase (diluted 1x in 0,1mM, phosphate buffer, PH 6.8), followed by an incubation period of 5 min at RT. 110 µl of L-tyrosine (1mM) was then added to the wells and incubated for 45 min. Negative and positive controls were made, replacing the extracts by phosphate buffer and arbutin, respectively. Due to the color of the extracts, color controls were also prepared, where 70 µl of extracts were mixed with 140 µl of phosphate buffer. Finally, absorbances were read at 492 nm, in the microplate reader and the calculations were done according to the formula (1). Results express the inhibitory activity of tyrosinase.

3.5.2 Neuroprotective activity using *in vitro* cellular models

3.5.2. a) Cell culture and determination of cellular viability

The human neuroblastoma cell line (SH-SY5Y cells) was maintained in Dulbecco's modified eagle medium (DMEM) with 4500 mg/mL of glucose, 10% heat inactivated FBS, l-glutamine (2 mM), penicillin (50 U/mL) and streptomicin (50 µg/mL), and were grown in an incubator at 37◦C, 5.1% CO2 in humidified atmosphere. To evaluate the effect of the extracts on the viability of SH-SY5Y cells, they were seeded in 96-well plates at a density of 2 × 104 cell/well and incubated for 24 h. Then, 100 µl of the acetone, methanol and water extracts at the concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL were added and incubated for 24 h. Cell viability was determined by the MTT assay (Mosmann, 1983).

The cell viability was evaluated through the MTT method, which is a colorimetric assay based on the precipitation of formazn, with a characteristic purple color when dissolved in DMSO that is a reductive product of tetrazolium due the mitochondrial activity. This mitochondrial activity and precipitation of formazan is then direct associated with the cell viability (Fischer, *et al*. 2002).

Briefly, 2 h prior to the end of the incubation period, 20 µL of MTT (5 mg/mL in PBS) were added to each well and incubated further at 37ºC. Then, 150 µL of DMSO waswere added to each well in order to dissolve the formazan cristals and absorbance was measured at 590 nm (Biotek Synergy 4). Samples allowing cell viability higher than 80% were selected and used to assess their protective effect against H2O2 induced oxidative stress on SH-SY5Y cells.

3.5.2 b) Protective effect against H_2O_2 induced oxidative stress on SH-SY5Y cells

SH-SY5Y cells were seeded at a density of 2×10^4 cells/well in 96-well culture plates, left to attach overnight, treated with different concentrations of the extracts in culture medium and incubated for 24 h. Then, H_2O_2 (100 µM) was added for 30 min and cell viability was determined by the MTT assay (Mosmann 1983) as described on the previous section. The stock solution of H_2O_2 was prepared on phosphate-buffered saline (PBS, pH 7.4) and diluted with DMEM without FBS immediately before use.

3.5.2 c) Anti-inflammatory activity on LPS-stimulated microglia cells

Murine microglial cells (N9 cell line) were cultured in Dulbecco's modified eagle medium (DMEM) in the same conditions described on section 3.6.1 for neuronal cells. To evaluate the effect of the extracts on the viability of N9 cells, the extracts were applied at concentrations ranging from 3.125 to 100 μg/mL, and incubated for 24 h. Cell viability was determined by the MTT assay (Mosmann 1983). Samples allowing cell viability higher than 80% were selected and used to assess their anti-inflammatory activity, through the study if their inhibition of NO production on LPS-stimulated microglia cells.

N9 cells were seeded at density of 1 x 10^6 cells/well in24-well plates and incubated for 24h. Then, cells were treated with 200 μl of different extracts (hexane, dichloromethane, ethyl acetate, and methanol) at various concentrations 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL in serum- and phenol-free culture medium, for 24 h, with 200 μL of LPS (10 μg/mL). Control cells were treated with DMSO at the highest concentration used in test wells (0.5%) with and without a lipopolysaccharide (LPS). NO production in cell culture medium was measured spectrophotometrically using the Griess reaction (Miranda *et al*. 2001). Stock Griess solution was prepared with equal amounts of a solution of 1% (w/v) sulphanilamide + 0.1% of N-(1-Naphthyl)-ethylenediamine dihydrochloride (NED) and 2.5% (v/v) phosphoric acid. After the incubation period with the extracts and/or LPS, 100 μl of the culture supernatants were mixed with the 100 μl of Griess reagent in 96-well microplates. Upon 20 min of incubation at RT, in the dark, absorbance was measured at 540 nm on a microplate reader (Biotek Synergy 4). Nitrite concentration was determined using the calibration curve prepared with several known concentrations (1.7, 3.1, 6.2, 12.5, 25, 50 and 100 μM) of sodium nitrite as standard. Results were expressed as Inhibition of NO synthesis (%).

3.6 Isolation and identification of bioactive compounds - Bioguided fractionation

A bioguided fractionation of the most bioactive extract (acetone) was performed in order to isolate and identify the bioactive compound(s). The acetone extract was fractionated through a liquid-liquid extraction. The dried extract was dissolved in water, placed in a fractioning ballon, and mized with 150 ml of *n*-hexane, being vigorously shaken. After settling down, 2 distinct phases were observed: hexane (lower lawyer) and

water (upper lawyer). The hexane-fraction was collected and the process was repeated again twist. The hexane fractions were pooled, and the extraction procedure was repeated with dichloromethane, ethyl acetate and *n*-butanol. Five fractions were obtained: hexane, dichloromethane, ethyl acetate, *n*-butanol and water, which were evapotated, resuspended in appropriate solvent and tested for the most significant biological activities, tyrosinase inhibitory activity.

3.7. Statistical analysis

Except for ash content, moisture content and fatty acid profile, for all the assays it was always done 6 replicates and the results were expressed as mean ± standard error. Respectively to the color controls instead of 6 it was done 3 replicates. Regarding to the ash content and fatty acid profile it was used 4 replicates and for the moisture content it was used 3 replicates. In order to check if there were some significant differences between the concentrations of the same extract per each assay it was done an analysis of variance (ANOVA) according to Ducan ($P<0.05$). Using GraphPad Prism V 5.0 program it were calculated the half maximal inhibitory concentration (IC_{50}) were calculated by sigmoidal fitting of the data

4. Results

4.1 Extraction and chemical characterization of the extracts

4.1.1 Total content of phenolics (TPC), flavonoids (TFC) and tannins (TTC).

The chemical characterization of *B. leachii* extracts was made in terms of total phenolic compounds, flavonoids and tannins (Table 2).

Table 2. Total content phenolic (TPC, mg GAE/g extract, DW), flavonoid (TFC mg RE/ g extract, DW) and tannins (TTC, mg CE/g extract, DW) of *B.leachii* organic extracts.

***E*xtracts**	**TPC**	**TFC**	**TTC**
Acetone	40.9 ± 0.2^{a}	228.6 ± 9.0^{a}	23.59 ± 0.7^{a}
Methanol	7.5 ± 0.1^{b}	171.2 ± 9.6^{b}	2.83 ± 0.1^{b}
Ethyl acetate	3.2 ± 0.1^{c}	32.6 ± 8.4^{c}	1.53 ± 0.2^{b}
Dichloromethane	1.6 ± 0.3^{c}	50.8 ± 8.4^{c}	2.80 ± 0.2^{b}

Results are expressed as mean ± SEM (n = 6). In the same column, values followed by the same letter are not significantly different at $P < 0.05$ according to the Duncan's multiple range test.

As it can be seen on Table 3, acetone extracts had the highest levels of TPC (40.99 mg GAE/g extract, DW), TFC (228.66 mg RE/ g extract, DW) and TTC (23.59 mg CE/g extract, DW), followed by the methanol extract, which 7.58 mg GAE/g extract, DW, 171.28 66 mg RE/ g extract, DW and 2.83 59 mg CE/g extract, DW for TPC, TFC and TTC, respectively.

The ethyl acetate and dichloromethane extracts had the lowest content for all the tested phenolic groups, and no significant differences were observed between them.

It is also possible to see in the Table 2 that Flavonoids were the most abundant group of compounds and that between methanol, ethyl acetate and dichloromethane extracts did not present significant differences between them regarding to the tannins content.

4.1.2 Nutritional profile, proximate composition and pigments

The proximate composition of *B. leachii* is summarized on Table 3.

Table 3. Moisture (g/100g of wet weight), ash (g/100g DW), total protein (g/100g DW), lycopene (mg/100mg DW), chlorophyll a (mg/100mg DW) and chlorophyll b (mg/100mg DW) of *B. leachii*.

Macronutrients			**Pigments**		
Moisture	Ash	Total protein	Lycopene	Chlorophyll a	Chlorophyll b
35.5 ± 0.8	10.1 ± 0.9	32.0 ± 1.21	1.0 ± 0.0	2.0 ± 0.1	3.5 ± 0.0

B. leachii had a moisture content of 35.5 %, a low level of ash (10.1%) and 32% of total protein. Regarding to pigments, chlorophyll b was the most abundant pigment with 3.52 mg/100 mg (DW) followed by chlorophyll a and lycopene with 2.04 and 1.04 mg/100 mg (DW) respectively. β-carotene was not detected (data not shown).

4.1.3 Fatty acid profile (GC/MS)

The FAME contents of *B. leachii* are summarized on Table 4.

Table 4. FAME profile of *B. leachii* expressed in percentage.

Name	Structure	Percentage (%)
Myristic acid	**C14:0**	5.4
Pentadecanoic acid	**C15:0**	2.9
Palmitic acid	**C16:0**	5.3
Margaric acid	**C17:0**	3.4
Stearic acid	**C18:0**	11.0
∑ SFA		28.3
Myristoleic acid	**C14:1**	12.6
Palmitoleic acid	**C16:1**	4.8
Ginkgolic acid	**C17:1**	2.3
Oleic acid	**C18:1 Cis**	2.9
Elaidic acid	**C18:1 Trans**	12.4
∑ MUFA		35.1
α-Linolenic acid (ALA)	**C18:3n-3**	7.9
Linoleic acid (LA)	**C18:2n- 6**	3.5
Eicosapentaenoic acid (EPA)	**C20:5n-3**	17.5
Eicostrienoic acid	**C20:3n-3**	7.4
∑ PUFA		36.4
∑ n-3		32.9
∑ n-6		3.5

ΣSFA= Total percentage of saturated fatty acids (FAs); ΣMUFA= Total percentage of monounsaturated FAs; ΣPUFA= Total percentage of polyunsaturated FAs. Σn-3= Total percentage of omega-3 PUFAs, Σn-6= Total percentage of omega-6 PUFAs.

B. leachii exhibited a higher percentage of PUFAs than monounsaturated (MUFAs) and saturated fatty acids (SFAs), with 36.46, 35.18 and 28.35 %, respectively. The total percentage of omega-3 PUFAs was higher than omega-6 PUFAs.

Regarding to all the fatty acids determined and detected by the assay, the one with the higher percentage was Eicosapentaenoic acid with 17.53% followed by Elaidic acid and Stearic acid, with 12.46% and 11.06%, respectively.

4.2 Antioxidant activity

4.2.1 Radical Scavenging Activity (RSA) on DPPH•

The extracts were tested for radical scavenging activity (RSA) on DPPH free radical (Fig. 10).

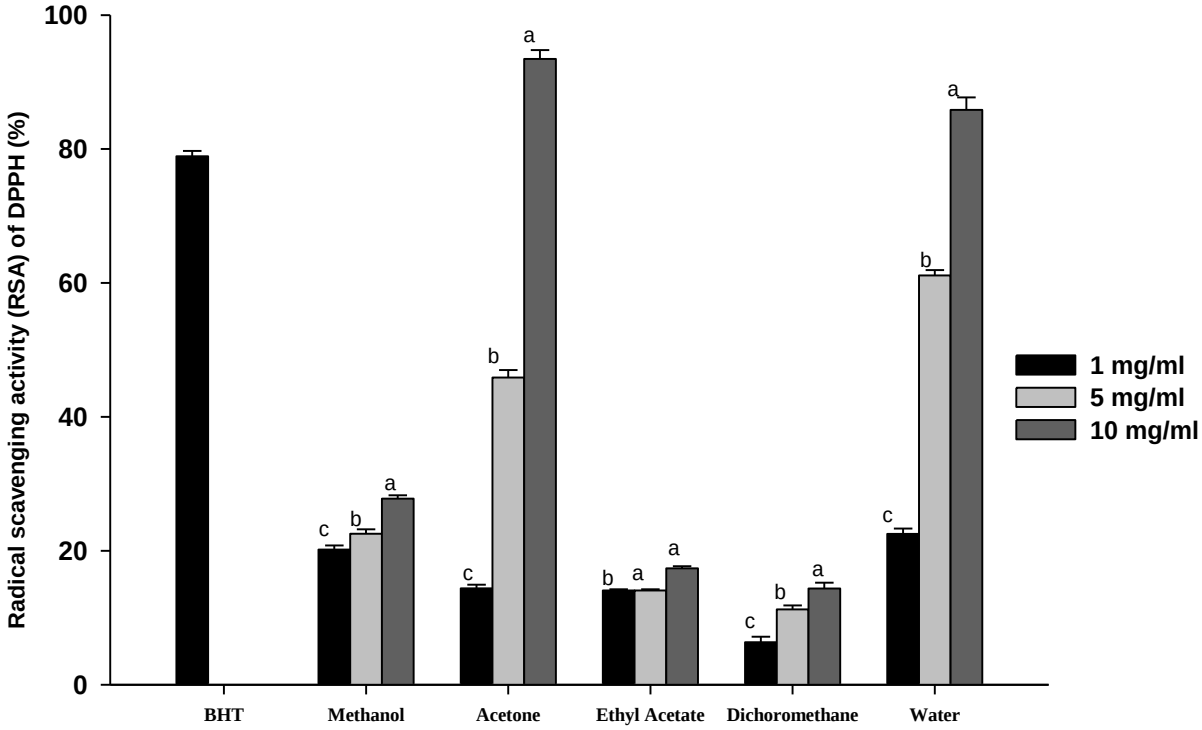

Figure 10. Radical scavenging activity (RSA, %) on DPPH free radicals of organic and water extracts of *B. leachii* and the positive control (BHT). Each value represents mean ± SEM (n = 12). Significant differences between concentrations for the same extracts were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-c).

All *B. leachii* extracts had RSA, which was concentration-dependent (Fig. 10). Acetone had the highest RSA with 93.4, 45.8 and 14.3% at the concentrations of 10, 5 and 1 mg/ml respectively (Fig. 10), and an IC_{50} value of 2.54 mg/ml. The water extract also had a high RSA with values of 85.5, 61.1 and 22.5% for the concentration of 10, 5 and 1 mg/ml respectively (Fig. 10), with and an IC_{50} of 1.67 mg/ml. BHT had an IC_{50} value of 0.07 mg/ml.

4.2.2 Metal chelating activity

The chelating activity for iron and cooper ion metals was determined and results are summarized on Table 5. The extracts had no relevant iron or copper chelation potential, with values always below 40% at the highest concentration tested.

Table 5. Metal chelating activity (%) on iron and cooper ions of organic and water extracts of *B. leachii*.

	Iron chelating activity (%)			**Cooper chelating activity (%)**		
	1 mg/ml	**5 mg/ml**	**10 mg/ml**	**1 mg/ml**	**5 mg/ml**	**10 mg/ml**
Water	9.9 ± 1.56^{b}	16.2 ± 1.8^{b}	20.6 ± 1.8^{a}	28 ± 1.5^{a}	24.2 ± 1.8^{a}	28.8 ± 1.8^{a}
Acetone	13.1 ± 1.56^{b}	29.8 ± 1.8^{a}	32.4 ± 1.8^{a}	28.2 ± 2.2^{b}	$24.3 \pm 3.65\ 1.5^{b}$	35.6 ± 4.1^{a}
Methanol	6.2 ± 2.02^{c}	26.4 ± 2.7^{b}	36.2 ± 0.7^{a}	24 ± 4.7^{b}	21.2 ± 3.3^{b}	30.5 ± 4.7^{a}
Ethyl acetate	4.2 ± 1.30^{b}	3.8 ± 0.6^{b}	10.7 ± 1.97^{a}	14.4 ± 1.6^{b}	13 ± 0.5^{b}	18.6 ± 1.7^{a}
Dichloromethane	-2.9 ± 1.90^{b}	3.4 ± 2.7^{ab}	7.9 ± 2.7^{a}	11.2 ± 3.2^{b}	14.6 ± 3.7^{ab}	20.2 ± 4.3^{a}
EDTA*	92 ± 0.3	nt	nt	81.3 ± 1.2	nt	nt

Each value represents mean ± SEM (*n=12*). Significant differences between concentrations for the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-c). nt: not tested, * positive control

4.2.3 Ferric reducing/antioxidant power (FRAP)

The FRAP assay was used to determine the ability of the extracts to reduce potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). Results are shown on Fig. 11.

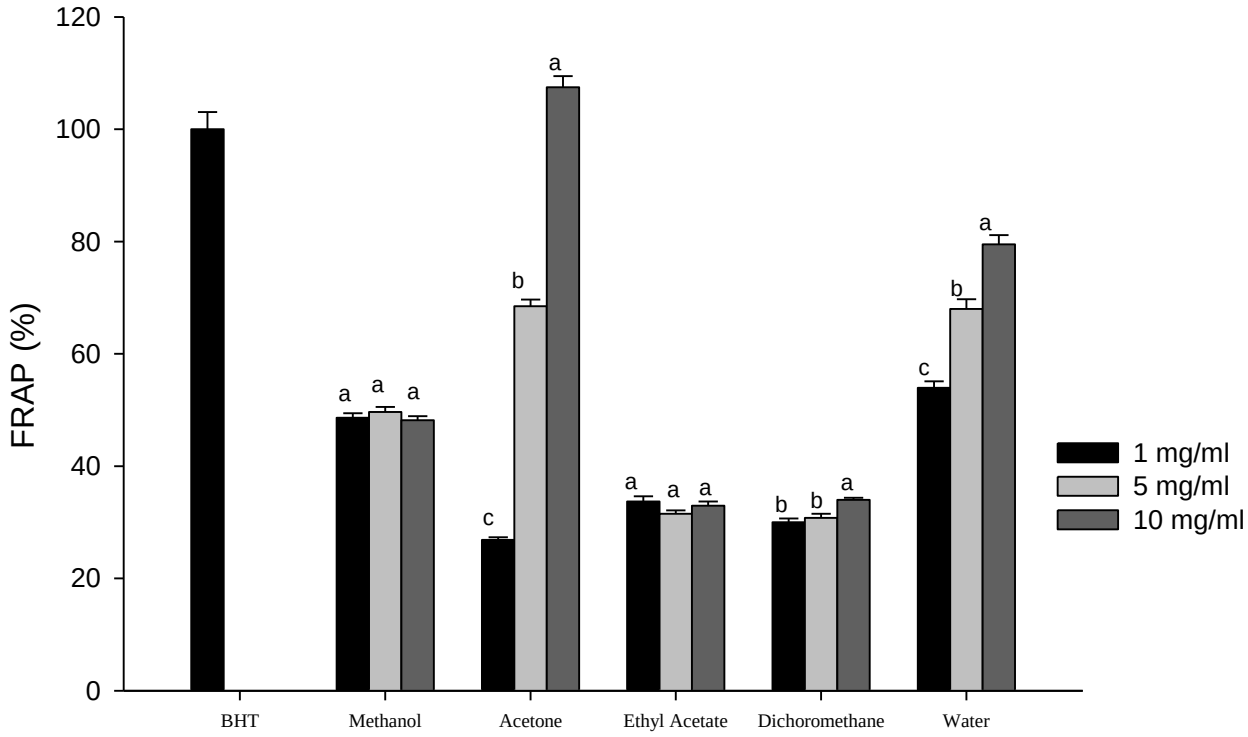

Figure 11. Ferric reducing/antioxidant power (FRAP) of organic and water extracts of *B. leachii* and the positive control (BHT). Each value represents mean ± SEM (n = 12). Significant differences between different concentrations for same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-c).

The acetone and water extracts had the highest reducing power in a dose dependent way (Fig. 11). The acetone extract had FRAP values of 104.4, 68.4 and 26.8% at the concentrations of 10, 5 and 1 mg/ml respectively, and an IC_{50} value of 1.75 mg/ml. The water extract also allowed significant results, with values of 79.4, 67.9 and 53.9% at the concentration of 10, 5 and 1 mg/ml respectively, with and IC_{50} value of 0.8 mg/ml.

A positive correlation was found between the RSA on DPPH (%) and FRAP values (Fig. 12) only for the water extracts. As for the other extracts the R^2 was below 0.99, and the correlation was not considered.

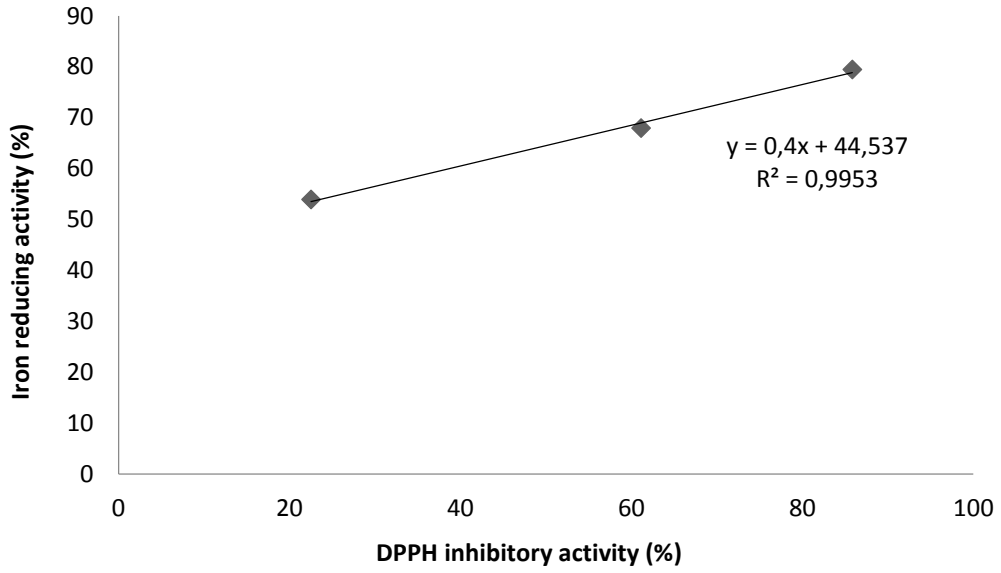

Figure 12. Correlation between the DPPH inhibitory activity (%) and FRAP (%) for *B.leachii* aqueous extracts.

A positive correlation was also found between total phenolics content and the RSA on DPPH and the iron reducing power assay (Fig. 13).

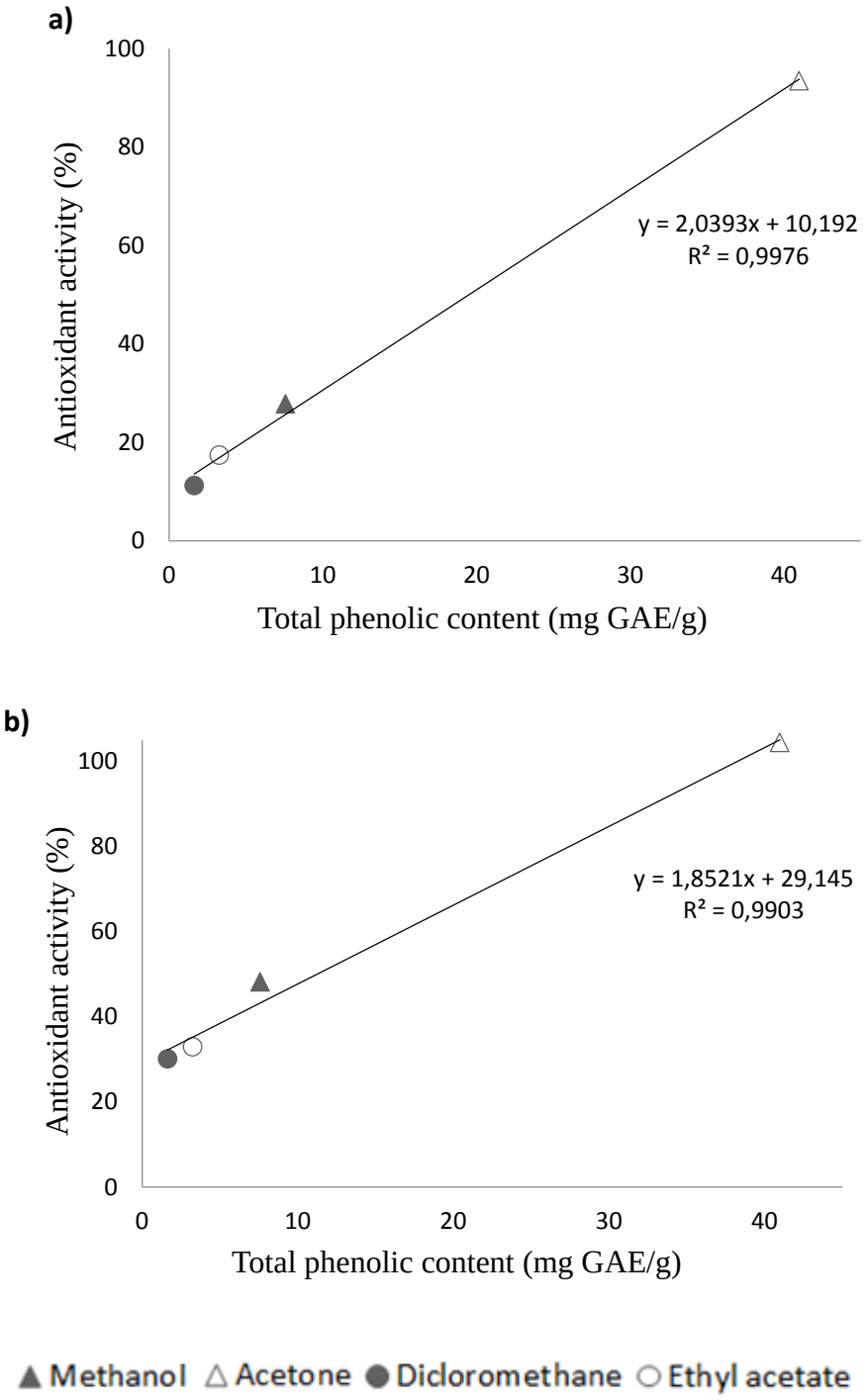

Figure 13. Correlation between the antioxidant activities and phenol compounds for acetone, ethyl acetate, methanol, dichloromethane and water extracts at 10 mg/ml. a) DPPH assay b) Iron reducing power assay

4.2.4 Nitric oxide (NO) inhibition

The ability of *B. leachii* extracts to scavenge NO was tested. Results are summarized on Table 6.

Table 6. NO scavenging activity of organic and aqueous extracts of *B. leachii*.

Samples	**1 mg/ml**	**5 mg/ml**	**10 mg/ml**
Water	31.8 ± 1.2^{b}	37.4 ± 1.5^{a}	39.6 ± 1.2^{a}
Acetone	24.0 ± 2.1^{a}	ni	ni
Methanol	28.2 ± 4.3^{b}	35.0 ± 5.4^{ab}	44.91 ± 2.3^{a}
Ethyl acetate	18.6 ± 1.8^{b}	33.4 ± 2.2^{a}	34.9 ± 3.6^{a}
Dichloromethane	23.7 ± 2.7^{b}	24.0 ± 1.4^{b}	36.6 ± 2.6^{a}
Ascorbic acid*	88.2 ± 2.2	nt	nt

Each value represents mean ± SEM (n = 12). Significant differences between different concentrations of the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-c). * positive control, nt: not tested, ni: no inhibition.

The extracts had low capacity to scavenge NO, with values below 50% at the highest concentration tested (Table 6). As for the acetone extract in contrast to what has been observed so far, the ability to increase NO seems to be higher to lower concentrations (Table 6).

4.3 Neuroprotective activity

4.3.1 Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) inhibitory activity

In this work it was evaluated the capacity of the extracts to inhibit AChE and BChE, and the results are summarized on Tables 7-8. The inhibitory activity (%) was classified as potent (>50%), moderate (30–50%), low (<30%) or null (<5%) (Vinutha, *et al.*, 2007).

Table 7. Inhibitory activity (%) of *B. leachii* extracts and positive control (galanthamine) on AChE.

Samples	**0.125 mg/ml**	**0.250 mg/ml**	**0.5 mg/ml**	**1 mg/ml**
Water	34 ± 2.6^{a}	30 ± 2.1^{a}	31 ± 2.7^{a}	34 ± 4^{a}
Acetone	9.5 ± 4.3^{b}	12 ± 2.8^{b}	12 ± 2.8^{b}	31 ± 3.1^{a}
Methanol	32 ± 3.7^{b}	nt	58 ± 1.3^{a}	59 ± 2.2^{a}
Ethyl acetate	25 ± 2.1^{b}	21 ± 2.6^{b}	23 ± 1.9^{b}	54 ± 0.9^{a}
Dichloromethane	19 ± 2.2^{c}	4 ± 4^{c}	42 ± 12.2^{b}	83 ± 0.8^{a}
Galanthamine*	91.3 ± 0.9	nt	nt	nt

Results are expressed as mean ± SEM (*n*=6). Significant differences between concentrations for the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a - c). * positive control, nt= not tested.

Based on the previous classification the dichloromethane extract had potent AChE inhibition (83%) at the highest concentration tested (1 mg/ml; Table 7), and an IC_{50} value of 0.5 mg/ml. The methanol and the dichloromethane extracts also had potent inhibitions on AChE, with values of 59% and 54%, respectively, at 1 mg/mL (Table 7). The IC_{50} values for those extracts were as follows: methanol: 0.4 mg/ml and ethyl acetate: 1.2 mg/ml.

As can be observed on Table 8 the extracts had a lower capacity to inhibit BChE than AChE.

Table 8. Inhibitory activity (%) of *B. leachii* extracts and positive control (galanthamine) on BChE.

Sanples	**1 mg/ml**	**5 mg/ml**	**10 mg/ml**
Water	23.8 ± 3.2^{a}	17.55 ± 4.8^{a}	19.9 ± 3.3^{a}
Acetone	22.1 ± 2.6^{b}	21.14 ± 2.2^{b}	30.8 ± 3.1^{a}
Methanol	28.7 ± 3.7^{a}	25.35 ± 3.9^{a}	24.6 ± 3.3^{a}
Ethyl acetate	22.1 ± 0.4^{b}	23.84 ± 1.3^{b}	29.8 ± 2.6^{a}
Dichloromethane	24.4 ± 5.4^{a}	22.58 ± 4.6^{a}	27.2 ± 4.0^{a}
Galanthamine	81.3 ± 1.3	nt	nt

Results are expressed as mean ± SEM (*n*=6). Significant differences between concentrations for the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-b). * positive control, nt= not tested.

Except for the acetone extract, with moderate activity (30.8%) at 10 mg/mL, all the extracts had low BChE inhibitory capacity (Table 8).

4.3.2 Tyrosinase (TYRO) inhibitory activity

The ability of *B. leachii* extracts to inhibit the TYRO activity was determined and the results are summarized in the Fig. 14.

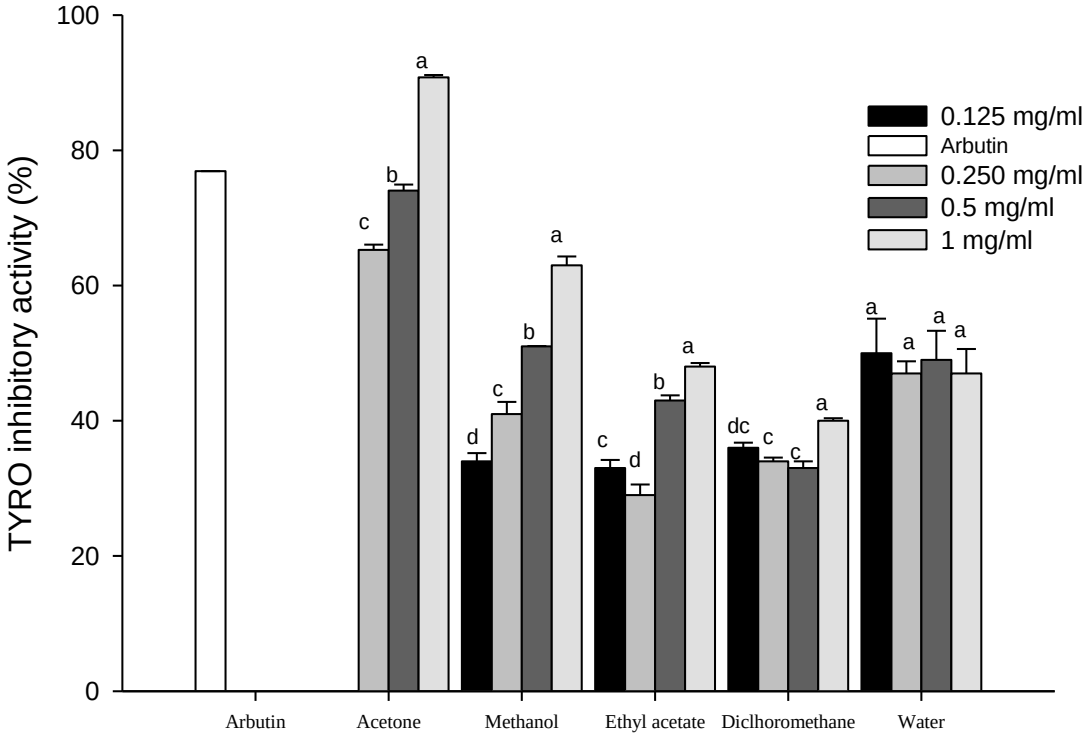

Figure 14. TYRO inhibitory activity (%) of *B.leachii* extracts and for the positive control (arbutin at, 1 mg/ml). The results are expressed as mean ± SEM (*n* =6). Significant differences between concentrations of the same extracts were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a - d).

All the extracts had the capacity to inhibit TYRO, and for the acetone and methanol extracts, there are dependent concentration (Fig. 14).

The maximum inhibitory activity was obtained for the acetone extract, with 90.78% at 1 mg/mL, and an IC_{50} value of 0.073 mg/m. Moreover, acetone inhibitory activity was inclusive higher than the standard used as positive control, arbutin (76.91 %) (Fig. 14). The. Methanol also allowed a potent inhibition at 1 mg/ml (64.3%), and had an IC_{50} value of 0.416 mg/ml. The water extract also exhibit a strong affinity to inhibit the tyrosinase inhibition with an IC_{50} of 0.101 mg/ml.

The acetone extract was further tested for TYRO inhibition, through a bio-guided fractionation by column chromatography eluted with different solvents, namely hexane, dichloromethane + ethyl acetate, N - butanol and water. The obtained fractions were tested at concentrations ranging from 0.125 to 1 mg/ml and the results are depicted on (Fig. 15).

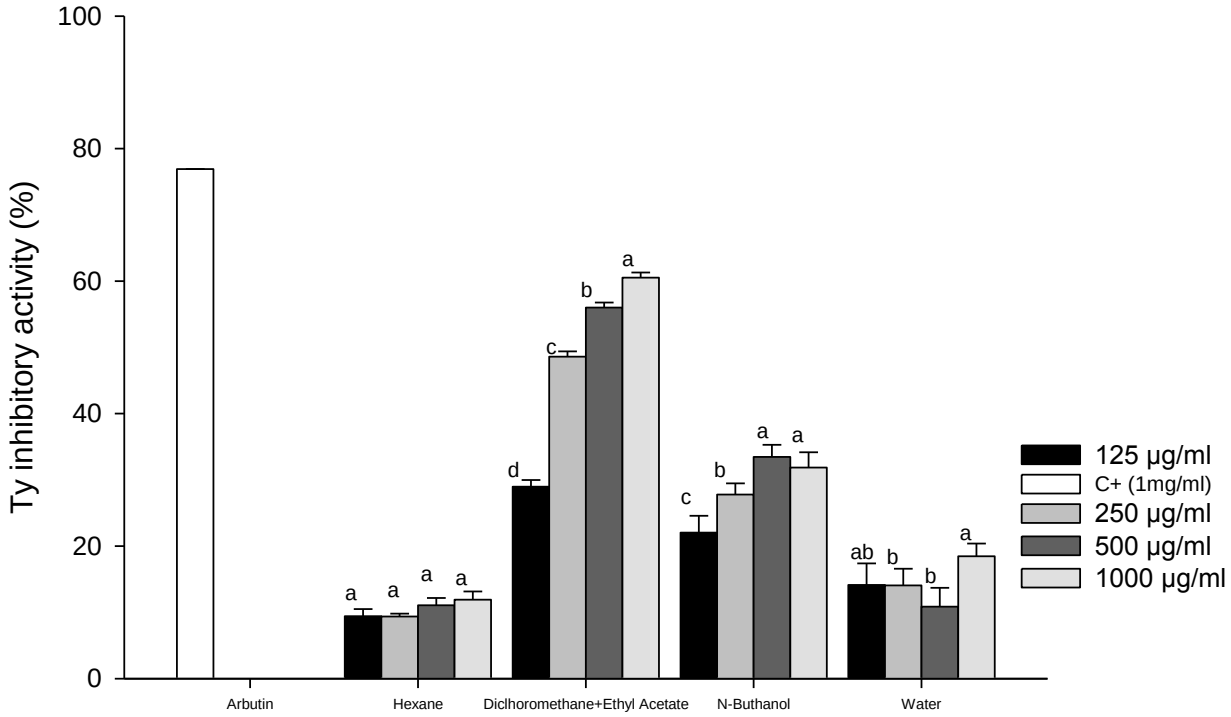

Figure 15. TYRO inhibitory activity (%) of *B.leachii* acetone fractions and for the positive control (arbutin at, 1 mg/ml). The results are expressed as mean ± SEM (*n* =6). Significant differences between concentrations for the same extracts were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a - c).

Regarding to all fractions, the dichloromethane + ethyl acetate fraction presented the higher inhibitory activity, with 62.27% and an IC_{50} of 0.387 mg/ml.

4.3.3 Anti-inflammatory activity on LPS-stimulated microglia cells

Since the acetone, and water extracts allowed the best results in the previous assays, they were further tested for potential neuroprotective potential, namely through the evaluation of their anti-neuroinflammatory activity.

In order to select the non-toxic concentrations of the extracts to be used in the *in vitro* assay for anti-inflammatory activity (i.e. concentrations allowing cell viability values higher than 80%), samples were applied at different concentrations to microglia cells (N9 cell line) for 24h, and cell viability was evaluated by the MTT method (Fig.16). Microglial cells were used in this assay because they are responsible for the defense of the brain, participating in the regulation of many inflammation-related pathological processes in the central nervous system (CNS).

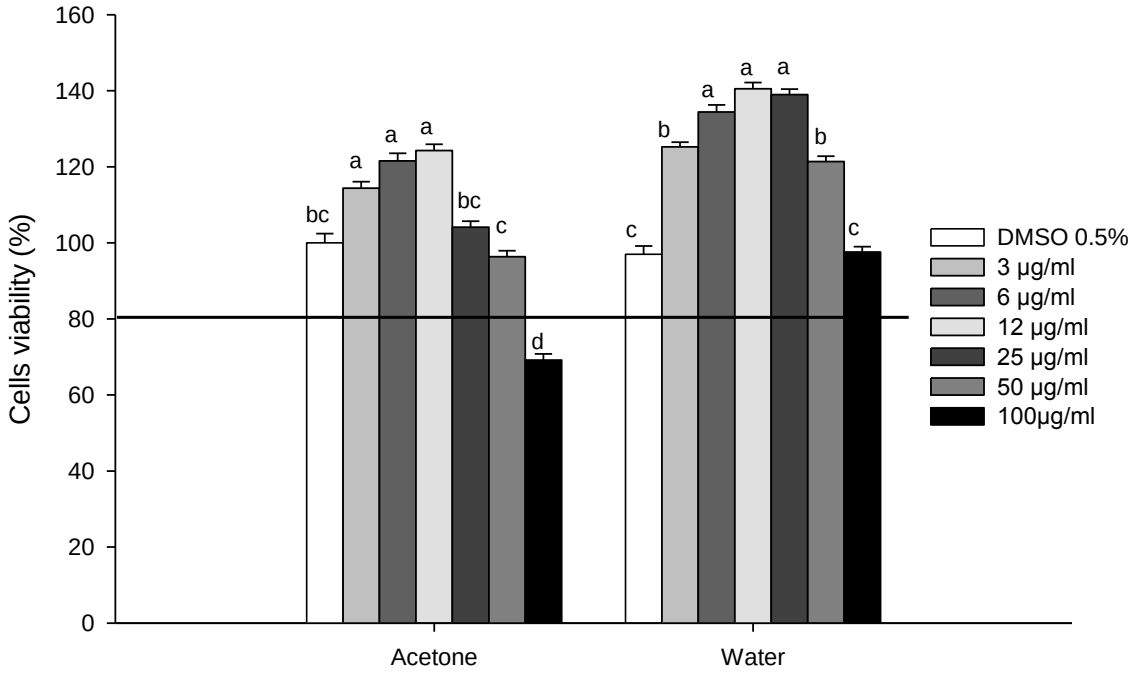

Figure 16. Effect of the application of acetone and water extracts of *B. leachii* on the viability of N9 cell line. Results are expressed as % of viability relative to a control containing DMSO at the highest concentration used in the samples (0.5%, v/v). Solid and errors bars represent the average and SD, respectively (n = 12). Significant differences between control and treated cells are indicated by as follows (a-c), (Duncan's test, $p < 0.05$). The horizontal line represents the minimum percentage possible in order to be considered as non-toxic.

Extracts were not toxic to N9 cells, except for the acetone extract at 100 µg/ml which reduced cell viability to 69% (Figure 14). The concentration considered as non-toxic for the cells, was used in the next step of the assay. N9 cells were treated with LPS at the concentration of 10 µg/ml for 24h, promoting the development of inflammatory response with a NO production of 6.66 µM (Table 9).

Table 9. Nitric oxide (NO) production (%) relative to LPS-stimulated N9 cells incubated with non-toxic concentrations of water and acetone extracts of *B. leachii*.

	NO production (%)							
Sanple	**0 µg/ml**	**3 µg/ml**	**6µg/ml**	**12 µg/ml**	**25 µg/ml**	**50 µg/ml**	**100 µg/ml**	**IC50 (µg/ml)**
Water	100 ± 0.1^{d}	nt	63.6 ± 0.5^{c}	65.0 ± 0.2^{c}	59.6 ± 1.2^{c}	60.5 ± 0.5^{c}	47.7 ± 0.1^{a}	108
Acetone	100 ± 0.1^{e}	56.4 ± 0.3^{d}	50.3 ± 0.4^{d}	57.2 ± 0.5^{c}	22.3 ± 0.2^{b}	7.5 ± 0.1^{a}	nt	5.74
L-NAME	28.8 ± 0.1							

Results are expressed as mean ± SEM (n = 6). Statistical significance in NO production between cells containing DMSO (0.5%, v/v) diluted in culture medium and those treated with the extracts are indicated by different letter (a-d). nt: not tested, L-Name: NG-nitro-L-arginine methyl ester: positive control.

A significant decrease in NO production was observed upon incubation of microglia cells with LPS and the acetone extract at all concentrations. The higher concentrations tested, 25 and 50 µg/ml, were able to reduce the NO production in 88.7 and 97.5%, respectively. Those concentrations were able to reduce NO production in a big percentage than L-NAME (positive control), which inhibit 82.2% (Table 9).

The application of the water extract also allowed a significant reduction of the NO production, concentration of 100 µg/ml, where only 47.7% of NO was produced.

4.3.4 Protective effect against H_2O_2 induced oxidative stress on SH-SY5Y cells

In order to select non-toxic concentrations of the extracts (i.e. that allowed cell viability values higher than 80%), samples were applied to SH-5YSY cells at different concentrations for 24h, and cell viability was evaluated by the MTT assay (Fig. 17).

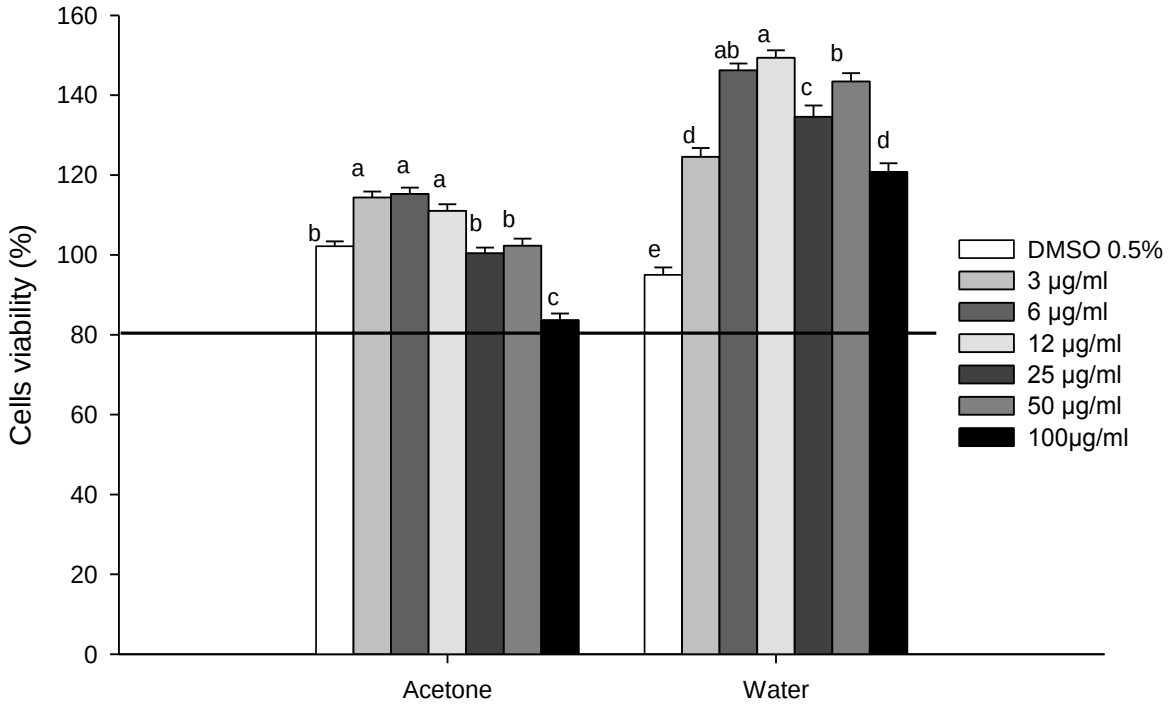

Figure 17. Effect of the application of acetone and water extracts of *B. leachii* on the viability of SH-5YSY cell line. Results are expressed as % of viability relative to a control containing (DMSO; 0.5%, v/v). Solid and errors bars represent the average and SD, respectively (n = 12). Significant differences between control and treated cells are indicated by as follows (a-e), (Duncan's test, $p < 0.05$). The horizontal line represents the minimum percentage possible in order to be considered as non-toxic.

None of the tested concentrations of the extract were cytotoxic for SH-SY5Y cells. Moreover, an increase in cellular viability was observed after treatment with the water (all concentrations), and also with the acetone extract at 3, 6 and 12 µg/mL

The assay requires a concentration of H_2O_2 able to reduce the cell viability in 50%, and so several concentrations of H_2O_2 were previous tested (Table 10).

Table 10. Necessary concentration of H2O2 to inhibit the cell viability in 50%.

H2O2 (mM)	Cell Viability (%)
0,5	106,62 ± 1,68
1	90,19 ± 1,54
2	48,08 ± 2,15
4	47,90 ± 1,7
8	43,00 ± 2,3

Then cells were treated with the non-toxic samples for 24h, exposed to 2mM of H_2O_2 for 30 minutes with concentrations that reduce the SH-SY5Y cells viability to approximately 50%, and cell viability was determined by the MTT assay (Fig. 18).

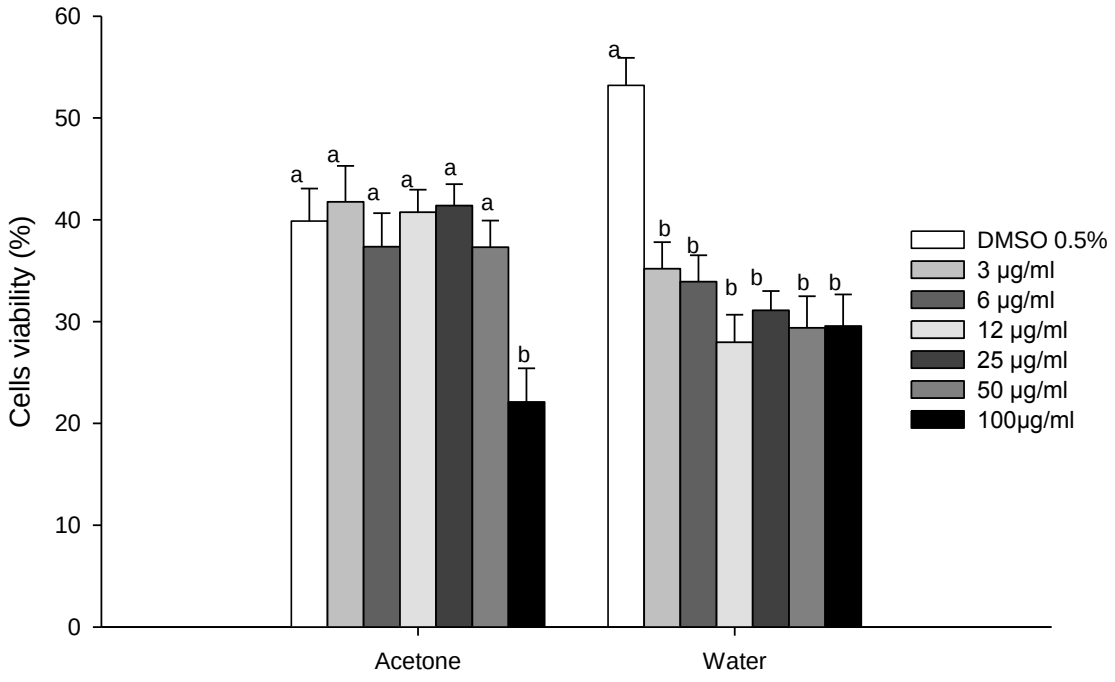

Figure 18. Effect of the application of acetone and water extracts of *B. leachii* on the viability of SH-5YSY cell line, previously treated with H_2O_2. Results are expressed as % of viability relative to a control containing (DMSO; 0.5%, v/v). Solid and errors bars represent the average and SEM, respectively (n = 12). Significant differences between control and treated cells are indicated by as follows (a-b), (Duncan's test, $p < 0.05$).

As can be seen on Fig. 17, the cell viability of cells pre-treated with the extracts was similar or lower to cells treated with H_2O_2 alone, indicating that the extracts were not able to protect cells from the oxidative stress imposed by H_2O_2.

5. Discussion

Marine organisms have attracted the attention of researchers due to their high content of bioactive compounds, with applications in different industries, such as the pharmaceutical (Wang, *et al*. 2013).

Until now a high variety of natural bioactive compounds were isolated from marine organisms (mainly marine invertebrates and algae), including alkaloids, phenols, terpenoids and proteins. Sea hares are considered a promising source of bioactive compounds, due to their strong chemical defenses and ability to sequester metabolites from their diet (Suarez-Jimenez, *et al*. 2012).

In fact, there are a few report of the presence of bioactive compounds in *B. leachii* and only two bioactive compounds were isolated from the species: bursatellin-P and malyngamide S, both with antimicrobial, anti-inflammatory and cytotoxic activities (Appleton, *et al*. 2002). Regarding to *B. leachii* similar to this work there is only been reported an anti-HIV protein, bursatellanin-P (a protein with a molecular weight of 60 kDa), isolated from the purple ink secretion (Rajaganapathi, *et al*. 2002).

Due to the lack of information regarding the biochemical composition and/or biological activities of *B. leachii*, and the fact that it is considered an invasive species in different loccations, namely Mar Menor, this work aimed to evaluate the chemical composition and different bioactivities of the species (Rajaganapathi, *et al*. 2002, Avilla 2006).

Very little is known about the studied species, so far there is only a few papers available on the species or similar organisms. For that reason it was not always possible to compare the data obtained in this work with the work of others authors.

5.1 Chemical characterization of the extracts

5.1.1 Total content of phenolic, flavonoids and tannins.

In this work acetone, methanol, dichloromethane and ethyl acetate extracts were prepared from dried biomass of *B. leachii* and characterized in terms of total contents of phenolics, flavonoids and tannins. Acetone had the highest amount of phenols followed by methanol, ethyl acetate and dichloromethane. To the best of our knowledge this is the first report on the phenolic contents *B. leachii*,

The highest content of phenols in *B. leachii* belong to the acetone extract, with 40.9 mg GAE/g, much higher than the other tested extract, which were all below 8 mg GAE/g. Previous studies reported that phenolic compounds own a potent antioxidants (Zhong, *et al*. 2007), considering this fact and according to the results on the acetone extracts, it is possible that acetone may present a good antioxidant activity, which indeed presented. In this sense, phenolic compounds might be the responsible for the antioxidant activity for the acetone *B. leachii* extract.

As mentioned above, there are no reported studies about the content of phenols for *B. leachii*, although and regarding to marine invertebrates Zhong, et al (2007) reported for methanol extracts of the sea cucumber *Cucumaria frondosa* a total phenolic content of 1.08 mg GAE/g, lower to the TPC determined in this work.

The extracts from *B.leachii* presented a content of flavonoids ranging from 33 mg RE/ (ethyl acetate extract) to 228 mg RE/g (acetone extract), which were three fold higher than the ones reported for ethyl acetate extracts of the sea cucumber *C. frondosa* (Mamelona, *et al* 2007).

Information about the levels of total flavonoids present in marine organisms is scarce and to the best of our knowledge, there is no information regarding the presence of flavonoids in *B. leachii*. Although it is well described for terrestrial plants, as the example of the studied developed by Pourmorad et al (2006), who studied the content of flavonoids for medicinal herbs. Comparing our study with Pourmorad et al (2006), the TFC of acetone and methanol extracts, were higher than the ones reported by Pourmorad et al (2006) for methanol of medicinal plant including *Mellilotus officinalis* (57 mg RE/g) and *Adiantum capillus-veneris* (78 mg RE/g).

It was already reported that Flavonoids exhibit several biological properties such as anti-inflammatory (Ferrfindiz & ALcaraz, 1991) being reported for the inhibition of arachidonic acid, which is responsible for a pro inflammatory activity and Alzheimer (Alcaraz, *et al*. 1988 and Ferridindiz & Alcaraz, 1991). Having in mind that for acetone extract, *B. leachii* presented a TFC of 288 mg RE/g, the results from our work indicates that *B. leachii* could present a good anti-inflammatory activity, also we must not forget that *B. leachii* proved to have higher potential than the previous marine invertebrate studies (*C. frondosa*).

The amount of total tannins was lower than the other studied groups (phenols and flavonoids), and reached the maximum amount for the acetone extracts whit 23.52 mg CE/g and to the best of our knowledge this was first time that they were ever reported for *B. leachii*.

Having in mind that sea hares commonly sequester secondary metabolites from their diets (Carefoot 1987, Faulkner 1992), it is possible that phenols, flavonoids, tannins among other metabolites, could be present in *B. leachii* because of their diet. Indeed most of the compounds present in sea hares have an algal origin (Faulkner, 1992; Pennings & Paul, 1993). As the example of Dollastatin 10, a small lipohilic polypeptide with a potent cytotoxic activity isolated from *Dollabela auricularia*. Dollastin was thought to be produced by the sea hare, but later studies revealed that they were in fact synthetized by the cyanobacterias *Symploca hydnoides* and *Lyngbya majuscule* that were part of *D.auriculata* diet (Madden, *et al*, 2000; Simmons, *et al*, 2005). Moreover, Capper, *et al*. (2005) studied the fate of *Lyngbya majuscule* toxins, which is part of *B. leachii* diet, in their consumer. The author found a very high concentration of lyngbyatoxin-a in the purple ink of *B. leachii*, suggesting that this sea hare developed a mechanism to avoid the toxicity of *Lyngbya majuscule* toxins. The differences between the concentration of the toxins in the digestive gland, ink, and the fecal indicates that *B. leachii* transfer of lyngbyatoxin-a from body tissue to body secretions, as a potential toxin mechanism excretion, giving the high amount of toxins found the purple ink (Capper, *et al*. 2005). This strategy not also makes *B. leachii* avoid the toxic compounds from the diet, but also provides the sea hare with a defense mechanism against predation (Johnson & Willows, 1999).

If we take in consideration the strategy described by Capper, *et al*. (2005) and if we consider the role of tannins in plants, to *B. leachii* it is possible that those compounds could have a similar function as they have in plants, being stored in the purple ink.

Considering this fact and that sea hares have the ability to sequester some compounds from their diet (Madden, *et al*, 2000; Simmons, *et al*, 2005) it is possible that for *B. leachii* the content of phenols, flavonoids and tannins among other compounds have a diet origin.

5.1.2 Nutritional profile

In Asiatic cultures marine invertebrates, such as the spices sea cucumbers *Holothuria nobilis, Apostichopus japonicas* and *Thelenota ananas* are considered edible in containing high of protein levels with a low level of fats (Kobayashi, *et al*. 1991 & Chen, 2003) Moreover, they are used in traditional medicine as for example the species Stichopus chloronotus for wound healing (Fredalina, *et al*. 1999).

In order to evaluate the potential of *B. leachii* as a source of nutritional components, in this work the proximate composition was evaluated and compared with edible marine sea cucumbers, algae and food ingredients (milk and meat) (Table 11); information about other sea hares or close organisms was not found.

Table 11. Proximate composition (%) of B. leachii and other food products.

	Ash	**Moisture**	**Protein**
B.leachii	10%	36%	32%
Stichopus herrmanni *	38%	10%	47%
Thelenota ananas *	25%	15%	55%
Actinopyga mauritiana *	15%	11%	63%
Bohadschia argus *	17%	13%	62%
Enteromorpha spp. **	36%	9%	14%
Meat	-	-	43%
Milk	-	-	26%

* Edible sea cucumber species (Wen, et al. 2010); ** Marine algae species (Aguilera-Morales, et al. 2005); meat and milk (Spolaore, et al. 2006).

The protein content of *B. leachii* was 32% higher than the value reported by Spolaore, *et al*. (2006) for milk (26%) and lower than meat (46%) (Table 11).

The protein content of *B. leachii* (32%) was similar or even higher than the values reported by Spolaore, *et al*. (2006) for milk (26%) and not too far from meat (46%) (Table 22), suggesting that *B. leachii* could be considered as an alternative source of proteins, as for example for animal feeds.

5.1.3 Liposoluble pigments

In this work it was assessed the content of liposoluble pigments (β-carotene, lycopene, chlorophyll a and chlorophyll b in *B. leachii*, by spectrophotometric methods. Results were compared with those reported for medicinal herbs (Table 12).

Table 12. Liposoluble pigments content (β-carotene, Lycopene, chlorophyll a and chlorophyll b) from *B.leachii* and from previous report medicinal herbs. The results are expressed in mg/100 g, represented as mean ± SEM (*n*=3).

	Liposoluble pigments (mg/100 g)			
	β-carotene	Lycopene	chlorophyll a	chlorophyll b
B.leachii	ni	1.04 ± 0.01	2.50 ± 0.02	3.56 ± 0.04
*Castanea sativa**	43.53 ± 0.08	0.05 ± 0.00	1.06 ± 0.00	0.46 ± 0.00
*Centaurea paniculata**	69.25 ± 0.08	-	1.23 ± 0.00	0.35 ± 0.00
*Rubus ulmifolius**	38.77 ± 0.15	0.07 ± 0.00	0.01 ± 0.08	0.59 ± 0.36
*Matricaria recutita**	127.68 ± 0.29	-	170.26 ± 0.00	56.37 ± 0.00
*Trifolium angustifolium**	160.69 ± 1.13	-	4.75 ± 0.00	1.77 0.00

* Medicinal terrestrial herbs studied by Barros, et al. (2010). ni= no inhibition

The results obtained in *B. leachii* are similar to previous studies done on medicinal herbs by Barros et al (2010), who quantified the amount of liposoluble pigments for several medicinal plants (table 23) recommended for the well- functioning of the digestive system, respiratory disorders, hypertension, cholesterol and inflammation (Dawidowicza, *et al*. 2006).

B. leachii did not exhibit β-carotene, but regarding to other pigments, *B. leachi*i presented a concentration of chlorophyll b (3.56 mg/ 100 g) always higher to the medicinal herbs (Table 12), except for *Matricaria recutita* (56.57 mg/ 100 g).

The reasons explaining why *B. leachii* contains those pigments are unknown, but it is strongly possible that they are sequestered from food. It is known, that some marine mollusks are able to acquire and sequester defensive chemical metabolites from their largely algal diets (Fenical, 1996). Most recently, the marine invertebrate *Elysia chlorotica*, impressed the research community with their incredible power to sequester plastids (chloroplasts) by ingestion of its algal food source *Vaucheria litorea*. The organelles were stored in the mollusc's digestive epithelium, where they photosynthesize for months (Rumpho, *et al*. 2008).

5.1.4 Fatty acid profile

The major FAs present were eicosapentaenoic acid (EPA) (C20:5n-3; 17.5%) followed by eicostrienoic acid and stearic acid (Table 4).

The presence of EPA as the most abundant fatty acid is very exciting, which could indicate that *B. leachii* is rich source of omega-3. For that, *B. leachii* could be considered as food supply by the food industry or even as a source for animal feeding. Moreover, the fact that EPA was the most abundant fatty acid, suggests that the study species might own a potent antioxidant and anti-inflammatory activity, considering EPA is known by his anti-inflammatory activity (James, *et al*. 2014).

EPA is an essential fatty acid, which plays an important role in the organisms including eicosanoid metabolism and gene transcription (Harris et al. 2008, Ferraro et al. 2010, Alasalvar et al. 2010), also it increase membrane fluidity preventing cardiac arrhythmias. Furthermore, Harris et al. (2008) and Ferraro et al. (2010) referred to omega-3 PUFAs as benefic compounds in the organism, as nutritional supplements and/or potential sources for drugs development. The author also emphasizes that a rich diet in omega-3 contribute for an improvement of a membrane fluidity and inflammation

prevention (Schmitz & Ecker 2008). So as it can be seen due to the high content of EPA, *B. leachii* could improve human's health.

Furthermore, polyunsaturated fatty acids (PUFAs) were the most abundant (36%) followed by monounsaturated (MUFAs) and saturated fatty acids (SFAs), respectively with 35% and 28% each. Once again we have reasons to believe that the study species owns a potent antioxidant activity, due to the high concentration of PUFAS, considering that lipids and especially unsaturated lipids are good radical scavengers (Plaza et al., 2009). Also PUFAs might also suggest a good affinity to the enzymatic inhibition, considering that they are known for improving cholinergic neurotransmission (Willis, *et al*. 2009).

To the best of our knowledge there is no fatty acid profile reported for sea hares, on the other hand there are a few report on other marine invertebrates as for example sea cucumbers.

In 2011, Aydın et al. reported that MUFAs and SFAs were lower that PUFAs for three species of sea cucumbers (*H. mammata*, *H. polii* and *H. tubulosa*). Our results indeed are in agreement with those, since for *B. leachii* PUFAs were the most abundant fatty acid class, as well.

Although *B. leachii* contains a promisor fatty acid profile, we must not forget that the profile only indicates those are present in *B. leachii* tissue, it does not mean that the extracts would present the same results.

5.2. Antioxidant activity

Oxidative stress is the underlying cause of several diseases, namely cancer, cardiovascular diseases, Alzheimer's and Parkinson's. The use on antioxidants can thus protect the organism from the deleterious effect of free radicals, such as ROS, preventing the onset of several ailments needed (Khan, 2002). To contra balance the effects of oxidative stress, antioxidants are engaged to protect biomolecules and the organisms from the dangerous effects of oxygen reactive species. The interaction between antioxidants and those undesired compounds, neutralize the damaging trough the donation of electron by antioxidants (Kunwar & Priyadarini, 2011).

Acetone and water extracts were the most effective DPPH scavengers, with inhibitory activities of 93.4 and 85.5%, respectively, at the higher concentration tested (10 mg/ml). To the best of our knowledge there is no information about the antioxidant potential of *B. leachii.*

Regarding to sea hares, there is no data published that we know of, and studies on marine invertebrates, with the same aims of this work, are rare. Although regarding to sea cucumbers, with are marine invertebrates as well, there are a few reports on their antioxidant activity using the DPPH assay. As the example of the study developed by by Althunibat, *et al*. (2009), who studied the antioxidant and antiproliferative activities of Malaysian sea cucumber species, *Holothuria leucospilota* and *Stichopus chloronotus*

Althunibat, *et al*. (2009) reported that *Holothuria leucospilota* and *Stichopus chloronotus* presented an IC_{50} of 3.9 mg/ml and 2.1 mg/ml for water extracts. Those values are higher than the IC_{50} obtained for the same kind of extract (water extract) of our study, which was 1.6 mg/ml. In other words, regarding to the same type of extract (water), *B. leachii* presented a higher affinity to scavenge DPPH than *Holothuria leucospilota* and *Stichopus chloronotus.*

On the other hand, studies microalgae are more common as the example of the study developed by Custódio *et al*. (2011), who reported the DPPH scavenger activity on microalgal extracts for *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella minutissima* and *Rhodomonas salina*.

The results obtained with *B. leachii* extracts for the DPPH scavenger activity, for acetone and water extracts, were higher for the higher concentrations (5 and 10 mg/ml)

than those described by Custódio, *et al*. (2012) for methanol and hexane microalgae extracts, where the higher concentration was achieved by the hexane extract of *Nannochloropsis oculata* (70.3%). Although we must considered that we were comparing different solvents, the different activity of extracts, for the same assay, made by different solvents could be explained due to the nature of the solvent used for the extraction. According to the polarity the extraction will work differently, extracting different compounds with different structures. This will cause a differences on the type of extracted compounds, which will have an effect on the activity (Trabelsi, *et al.* 2010).

Working with a different species of the genus *Enteromorpha*, a green macroalgae included in the diet of *B. leachii*, Ganesan, *et al*. (2011) evaluated the antioxidant activity of acetone and water extracts by the DPPH assay. The values obtained by those authors were similar to the ones obtained in this work at the concentration of 1 mg/ml.

In this work, a correlation was observed between the RSA on the DPPH radical and the TPC of the acetone extract (R^2=0.99), which was the extract with the highest contest of phenols (40.9 mg GAE/g). This is in agreement with several reports describing a strong correlation between the antioxidant activity and the phenolic content (Indu & Seenivasan, 2013).

Metal chelating activity is another complementary assay to evaluate the antioxidant activity of the extracts, being very important in the finding of metabolites able to bind themselves to free metal ions in the organism, neutralizing the effect of those. Several metals have been implicated in the development of neurological disorders (Weinreb et al., 2011) being implicated in the development of neuronal disorders (Gaeta & Hider, 2005). In this work the extracts displayed low capacity to chelate metal ions and to the best of our knowledge this was the first attempting of it.

Regarding to FRAP, the highest activity was achieved by the acetone with 104.4% followed by water with 79.4%. Once acetone is medium polar and water high polar, this suggest that the compounds responsible for the activity of the extract might be medium-high polar metabolites. To the best of our knowledge there is no data reported for *B. leachii* and regarding to marine invertebrates, the studies are scarce. In 2009, Wang et al., reported a ferric reduction power for *Porphyra spp.* lower than the results got it for *B.leachii*, at 1 mg/ml, with 0.2 and 0.4 (Abs), respectively.

In our work, it was observed a correlation between the iron reducing/antioxidant power and the ability to scavenge DPPH (Fig 12). As the iron reducing/antioxidant power (%) increase the DPPH inhibitory activity (%) also increase (Fig. 10). The observed correlation it is in agreement with the data reported by Wang, *et al*. (2009), who reported the same correlation for both assays. In this sense, the author suggested that the ability to scavenge DPPH might be related with the iron reducing power due to the presence of reductones, which are responsible for the reducing ability, decomposition of peroxides and radical scavenging (Yildirm, *et al*. 2001).

Taking in account the observed correlation in our study (Fig 12), and what was reported by Wan, et al. (2009), the antioxidant of water extract of our work might be due to the presence of compounds with a high ability to reduce other metabolites, donating hydrogen to free radicals reducing them to a nonreactive specie (Wang, et al. 2008). Also we must not forgot that the extracts with the highest activities for the assay were acetone and water, suggesting that the compounds responsible for the antioxidant activities might be some medium-high polar compounds (according to the polarities of the solvents).

Naturally the antioxidant activity of the same extract and same concentration varies between assays. We have to consider that the extracts are a mixture of several compounds, which are unknown in this case, and that each assay is prepared and developed to detect different kind of compounds. For that reason some of the assays presented a very high antioxidant activity, while others could seem less interesting (Hercberg *et al.*,1998).

5.3 Neuroprotective activity

5.3.1 Enzymatic inhibition

The inhibitory activity against AChE, BChE and TYRO was classified according to Vinutha, et al. (2007) as low (<30%), moderate (30-50%) and potent (>50%).

Regarding to AChE extracts were tested for the concentrations of 0.125, 0.250, 0.5 and 1 mg/ml. The dichloromethane extract had potent AChE inhibitory activity, presenting the highest inhibition (83% at 1 mg/ml), with an IC_{50} of 0.5 mg/ml. Methanol and ethyl acetate were also potent inhibitors.

Despite that fact that the patterns and causes of Alzheimer´s disease are not completely understood, it is clear that low levels of acetylcholine (ACh) are associated to AD. In fact studies have shown that the brains of AD patients contains a low level of Ach, which is the major neurotransmitter in the central nervous system (Filho et al., 2006). In brain, ACh is hydrolysis by AChE, which is considered to be the major enzyme involved in the hydrolysis of ACh and as well AD progression (Filho et al., 2006). Recent studies have proved that AChE inhibitors have the ability to increase the level of ACh, providing a beneficial effect (Zarotsky, *et al.* 2003). Indeed a very well-known medicine, tetrahydroaminoacridine (tacrine), was the very first acetylcholinesterase inhibitor, which evolves into a large-scale clinical trial and commercial launch in USA and part of Europe. Recently it was followed by more recent products such as donepezil, rivastigmnine and metrifonate (McGleenon, *et al*. 1999).

For that reason the search of compounds able to inhibit AChE are extremely important, not only for the prevention of AD, but also as a treatment for other neurological disorders including dementia, Parkinson and ataxia (Pulok, *et al*. 2007).

For the reasons mentioned above, the research community have been focusing their attention the discovery of natural compounds able to inhibit AChE and so benefit human health. Taking in consideration the results from this study, *B. leachii*, most specifically the dichloromethane extract it might be a potent source for the extraction on compounds with a high affinity to inhibit AChE and be used in approach for AD treatment of (Pulok, *et al.*2007 and Natarajan *et al.*, 2009).

As for the other enzyme tested, BChE, the *B. leachii* extracts was not effective in terms of inhibition, presenting a low inhibitory activity. The inhibition of BChE is equally important for the prevention of neurodegenerative diseases as the inhibition if AChE. The degeneration of cholinergic neuros and loss of transmission involved in AD reduces the levels of ACh and in AChE, causing an increase of the BChE activity. Despite the fact that the role of BChE is not completely understood, it known that BChE is able to degradate ACh (Fig. 3). Moreover, recent studies have shown that high BChE levels, contribute for the maturation of senile plaques, which are a signal of AD (Wiebusch, *et al.* 1999).

Rgarding to neurodegenerative diseases there is another enzyme, tyrosinase (TYRO), strongly associated to the prevention of another equally important disease, Parkinson (Berman & Hastings, 1999).

The acetone extract had the highest activity to inhibit TYRO, with an IC_{50} of value of 0.073 mg/ml. The TYRO is involved in the production of dopamine, an essential neurotransmitter. Consequently to the dopamine synthesis, which is requires a TYRO activity, some neurotoxic compounds are releases into the brain, including H_2O_2 and OH among other oxygen reactive species. When overexpressed, tyrosine could severely engaged neurons, which eventually causes apoptotic cell death (Hasegawa, 2010). Considering the negative effects of it, the inhibition of tyrosine is one of the most promisor's pathways to prevent PA.

B. leachii extracts presented a strong affinity to inhibit tyrosinase, never less than 30%, with the highest activity of 93%, at the higher concentration testes (1 mg/ml), with a very low IC_{50} of 0.073 mg/ml.

Flavonoids as mentioned before, are known to contain a potent antioxidant and metal chelating activity. Most recently it had been reported that those compounds are also able to inhibit tyrosinse due to the formation of coopper-flavonoid complexes (Kim, et al. 2006). Therefore we have reasons to believe that the high affinity of the extracts to inhibit tyrosinase it is due to the content of flavonoids. Indeed, the content of flavonoids it is related with the inhibitory activity of tyrosinase, which is as higher as the content of flavonoids.

Once our extracts, especially the acetone extract is rich and flavonoids and consequently contains a high affinity to inhibit tyrosinase, able to reduce 93% of its activity. This suggest that *B. leachii* could be seen as a potential candidate for the extraction of compounds able to successfully tyrosinase and with that benefit human health, either for neurodegenerative diseases or for the cosmetic industry..

5.3.2 Neuroprotective activity using *in vitro* cellular models

When the organism is under an inflammatory response, one of the main mediators is the nitric oxide (NO) (Tuntipopipat *et al.*, 2009). With the aim to test the anti-inflammatory ability of *B. leachii* extracts, it was evaluated the *in vitro* ability of the extracts to reduce the NO levels on microglailcells (N9 cell line). N9 cells are in charge of the immune system of the brain. Those are extremely important to fight an inflammatory response, producing inflammatory cytokines, reactive oxygen and nitrogen species (Tuntipopipat *et al.*, 2009).

The acetone extract had the highest ability to inhibit NO production, with a reduction of 7.5%, at the concentration of 50 µg/ml, when compared with the LPS stimulated cells, and an IC_{50} of 0.57 µg/ml. Moreover, the extract were able to reduce NO in a higher percentage than the positive control, L-NAME, which inhibit 28%. Those results are expressed in a potent anti-inflammatory activity, presented by the acetone extract. To the best of our knowledge there is no data regarding to sea hares in this field. From our results, we can see that *B. leachii* contains a high affinity to inhibit NO, which in other words means that the study species is able to fight an inflammatory response. Considering the high amount of flavonoids that *B. leachii* presented, and having in mind that as mentioned those compounds are known to contain a good anti-inflammatory activity, it is possible that the compounds responsible for the observed anti-inflammatory activity are indeed flavonoids. Moreover, the fatty acid profile presented a high concentration of EPA, which also contains an anti-inflammatory activity. Although the fatty acids profile was not determined for the acetone extract, considering that high anti-inflammatory activity it is possible that EPA is the responsible compound for it. Therefore, the results suggest that *B. leachii* should be considered for the extraction of bioactive with a potent anti-inflammatory activity, for the development of new drugs.

Oxidative stress is a key factor, being responsible for the initiation of several diseases as cancer, Alzheimer, Parkinson, diabetes, cardiovascular problems and so ever. The high amount of free radicals present when the organisms is under oxidative stress can severely engaged the well-functioning of the organisms and as well damage to the cells, especially DNA, which contributes to mutagenesis (Vera-Ramirez *et al.*, 2011).

Oxidative stress is known to prodice H_2O_2 among others toxic oxygen species. As a strong oxidant, H_2O_2 is severe dangerous for the cells leading to oxidative damage causing the disruption of metabolic function, cellular integrity and compromises de DNA (Prasad et al., 1994). An over production of peroxide hydrogen has been reported as a signal of stress. Moreover the excess of H_2O_2 is responsible for the developing of inflammation (Ciccone, *et al*. 2013).

The ability of the extracts to protect the cells against H_2O_2, was performed on human neuroblastoma cells (SH-SY5Y). The extracts presented no ability to protect the cells, since the cell viability did no increase. In fact, the water extract, which was not toxic for the cells, after the treatment with hydrogen peroxide, the cells exhibit a decrease in the cell viability. Somehow the H_2O_2 compromises the cell viability, as it known for the lipid peroxidation and changes in the permeability of the membrane, which put the cells weak (Ciccone, *et al*. 2013).

6. Conclusions and Future perspectives

In summary, the present data suggest that *B. leachi* could be considered a potential new source of metabolites with antioxidant and anti-inflammatory activity. Also with and AChE and TYRO inhibition. The high TYRO inhibition suggests that *B. leachii* could be a source of molecules for the cosmetic industry with bleaching properties. Besides the medicinal potential, the results on the fatty acid profile and proximate composition suggested that *B. leachii* can be used as food and/or feed.

As it was mentioned, *B. leachii* is an alien species in the Meditearean Sea. During the invasive species control a lot of biomass is wasted, because it is useless. As our data prove that *B. leachii* contain a range of potential uses, instead of being wasted, the excess of biomass can now be used for a better propose. On this sense, the industry could use this species to extract natural bioactive compounds and identify new bioactive molecules to use as model for some drugs.

In order to support all the possibilities and to better understand *B. leachii* and all the potential that the species represent, it is necessary proceed to the identification of the bioactive compounds present in the extracts and a purification of the compounds is one of the major priorities of this work.

Several future studies could be developed based on the results obtained in this work., namely the to the identification of the bioactive compounds present in the bioactive extracts, to test the *in vitro* ability of the extracts to inhibit tyrosinase using cellular models, Also and as it was suggested in this thesis, it is possible that the major part of the compounds presented on *B. leachii* responsible for those biological activities have an algae origin. Therefore, studies to understand the pathway of the bioactive compounds presented on *B. leachii* would be very interesting as for example the comparison between *B. leachii* and the organisms the species feed of. In this sense for example it could be developed an assay where the *B. leachii* was feed under controlled conditions, and then make a comparative evaluation of the biochemical profile of both the feed and the different *B. leachii* tissue.

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