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Screening of larvicidal activity of seaweed extracts against the mosquito *Aedes aegypti*

Mafalda Santos Coutinho

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MAFALDA SANTOS COUTINHO

SCREENING OF LARVICIDAL ACTIVITY OF SEAWEED EXTRACTS AGAINST THE MOSQUITO *AEDES AEGYPTI*

Dissertation for the degree of Master in Environmental Contamination and Toxicology, presented to Institute of Biomedical Sciences Abel Salazar of the University of Porto

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Abstract

Mosquito-borne diseases are a big global concern, being responsible for 300 million of annual cases of infection, according to World Health Organization (WHO), and being one of the main causes of deaths worldwide. Some of these diseases were reduced in the last centuries, particularly in the XX century, but many are remerging and also appearing in places that they were not used to. The genera of mosquitos with more medical importance are *Anopheles*, *Aedes* and *Culex*, but this work focus on *Aedes aegypti* (*Ae. Aegypti*).

Most of these diseases do not have a vaccine or treatment, so the best way to fight against them is controlling the vectors – in this case *Ae. aegypti*, and some authors defend that this control is easier when applied in larvae stage. Four main strategies have been used: physical, genetic, chemical and biological control. Chemical control, namely the use of phytochemicals such as seaweed extracts, is receiving more attention lately, once the other methods pose problems like the environmental persistence, hazard in non-target organisms, and resistance by the insects.

Seaweed are a big source of compounds that have a wide range of bioactivities serving several applications, as antibacterials, antifungals, antivirals, antitumorals, among others. In this work, the mosquito larvicidal potential of five organic extracts from two macroalgae species - *Fucus vesiculosus* (*F. vesiculosus*) and *Ulva lactuca* (*U. lactuca*) – were accessed in *Ae. aegypti*. The extracts were obtained with the solvents ethanol, methanol, chloroform, hexane and dichloromethane. Mortality assays, according WHO guidelines, were performed counting the dead larvae after 24 and 48 hours of exposure. Body length and the weight of surviving larvae were also measured to investigate potential gross impacts of the extracts on larval growth/development. From these assays, dichloromethane extract from *F. vesiculosus* was the only one that showed significant differences compared with the control, reaching 58% mortality after 48 hours of exposure. Body size measurements and weighings showed no differences between treatments. Assays with *Artemia salina* (*A. salina*) were also carried out with 24 hours of exposure, to access possible effects of these extracts in non-target aquatic organisms. In the same concentration used in mosquito larvae, no significant differences were observed between the extracts and the control. However, in a higher concentration, three extracts from *F. vesiculosus* showed toxicity, namely ethanol, chloroform and dichloromethane.

The results showed that dichloromethane extract from *F. vesiculosus* could be a useful source of larvicidal compounds to fight mosquitos, the most important disease vectors for transmitting diseases to Humans. The *A. salina* data support that lethality in non-target

species is only attained at higher concentrations of extract when compared with those for the mosquito. Anyway, a wider range of non-target organisms should be assayed. Having the present work as basis, further studies could try to elucidate the mode of action of the extracts effects when inducing lethality, in addition to isolate and characterize the compounds present in the most promising extracts. By other hand, screening tests using combinations of extracts could be useful to pinpoint yet unknown synergy or potentiation effects. At last, our data support that more studies should be done on a wider range of seaweeds, as extracts from different species do present quite different bioactivities.

Key-words: *Aedes aegypti*; Arthropod-borne diseases; larvae; seaweed extracts; vector control;

Resumo

As doenças transmitidas por mosquitos são uma grande preocupação a nível global, sendo responsáveis por 300 milhões de casos anuais de infeções de acordo com a Organização Mundial de Saúde (OMS), sendo por isso uma das principais causas de morte a nível mundial. Algumas destas doenças foram sendo reduzidas em séculos passados, e em particular no século XX, mas muitas estão a reemergir e também a aparecer em sítios onde não costumavam existir. Os géneros de mosquitos com maior importância médica são *Anopheles*, *Aedes* e *Culex*, mas este trabalho foca-se em *Aedes aegypti* (*Ae.aegypti*).

Muitas destas doenças não têm uma vacina específica ou tratamento, por isso a melhor maneira de lutar contra elas é controlando os vetores – neste caso *Ae. Aegypti*, e alguns autores defendem que o controlo é mais fácil e mais eficaz quando aplicado em estados larvares. Quatro principais estratégias têm sido usadas: controlo físico, genético, químico e biológico. O controlo químico nomeadamente o uso de fitoquímicos como extratos de algas têm, ultimamente, recebido mais atenção uma vez que os outros métodos apresentam problemas como a persistência no ambiente, perigo para outros organismos não alvo, e a resistência por parte dos insetos.

As algas são uma grande fonte de compostos, que têm demonstrado ter várias aplicações tais como antibacterianas, antifúngicas, antivirais, antitumorais, entre outras. Neste trabalho, foi avaliada a potencial atividade larvicida de extratos de duas espécies de macroalgas - *Fucus vesiculosus* (*F.vesiculosus*) e *Ulva lactuca* (*U. lactuca*) – com cinco solventes orgânicos: etanol, metanol, clorofórmio, hexano e diclorometano, em larvas de *Ae. aegypti.* Foram feitos ensaios de mortalidade de acordo com as *guidelines* da OMS, contando as larvas mortas após 24 e 48 horas de exposição. O peso e o comprimento das larvas que sobreviveram, após 48 horas, foram também registados para encontrar diferenças entre as várias condições experimentais testadas. Destes ensaios, o extrato de diclorometano de *F. vesiculosus* foi o único que demonstrou diferenças significativas em relação ao controlo, atingindo 58% de mortalidade, após 48 horas de exposição. As medições do comprimento e as pesagens das larvas não mostraram diferenças entre os tratamentos. Ensaios com *Artemia salina* (*A. Salina)* foram também executados com 24 horas de exposição, para avaliar os possíveis efeitos dos extratos em organismos não alvo aquáticos. Na mesma concentração usada nas larvas de mosquito, não foram observadas diferenças significativas entre os extratos e o controlo. No entanto, numa concentração mais elevada, três extratos de *F. vesiculosus* demonstraram toxicidade; nomeadamente etanol, clorofórmio e diclorometano.

Estes resultados demostram que os extratos de algas com solventes orgânicos podem ser uma ferramenta útil no combate aos mosquitos vetores de doenças, e podem ser combinados com outras estratégias de controlo. No entanto, mais estudos são necessários para perceber o potencial de diferentes espécies de algas e diferentes frações, assim como os compostos responsáveis pela atividade larvicida.

Palavras-chave: *Aedes aegypti*; controlo de vetor; doenças transmitidas por artrópodes; extratos de algas; larvas.

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Abbreviations

ACh – Acetylcholine

AChE – Acetylcholinesterase

Bin – Binary toxin

Bs – *Bacillus sphaericus*

Bti – *Bacillus thuringiensis*

CDC – Center for Disease Control and Prevention

CHIKV – Chikungunya virus

CI – Cytoplasmic incompatibility

CSIs – Chitin Synthesis Inhibitors

DDT – Dichlorodiphenyltrichloroethane

DENV – Dengue virus

DMSO – Dimethyl sulfoxide

EAE – Enzyme-assisted extraction

EIP – Extrinsic Incubation Period

EPA – United States Environmental Protection Agency

FAs – Fatty acids

IHMT – Instituto de Higiene e Medicina Tropical

IGRs – Insect Growth Regulators

ILs – Ionic Liquids

JHAs – Juvenile Hormone Analogs

MAE – Microwave-assisted extraction

Mtx – Mosquitocidal toxins

NIH – National Institute of Allergy and Infectious Diseases

OMS – Organização Mundial de Saúde

OPs – Organophosphates

PEF – Pulsed electric field-assisted extraction

PFA – Paraformaldehyde

PSE – Pressurized solvent extraction

RIDL – Release of insects with dominant lethality

SFE – Supercritical fluid extraction

SIT – Sterile insect technique

SLE – Solid-liquid extraction

SP – Sulfated polysaccharide

TPC – Total phloratannin content

UAE – Ultrasonic-assisted extraction

WHO – World Health Organization

ZIKV – Zika virus

Chapter I

Introduction

Introduction

1. Arthropod and mosquito-borne diseases

Many centuries ago, the association between insects and diseases was made, but only in 19th century it included hematophagous arthropods (Molyneux 1993, Braga and Valle 2007). These can be dangerous vectors of many fatal diseases - called arthropod-borne diseases - as malaria, yellow fever, dengue, etc; transmitted through an arthropod bite to humans or animals (Sanchez-Vargas, Travanty et al. 2004, Benelli 2015, Valentina J 2015). The pathogen transmitted could be parasites (helminths) (Harbach 2007), protozoa, bacteria or a virus (arbovirus) (Kalluri, Gilruth et al. 2007, Beugnet and Marié 2009) transmitted to humans by mosquitos, sandflies, triatomine bugs, blackflies, ticks, tsetse flies, mites, snails and lice (World Health Organization – WHO, 2017).

Among all the arthropods and all orders of insects, Diptera order is the most concerning in a medical perspective (Schmidt 2005, Benelli 2015) due to the family Culicidae (Suborder Nematocera), which correspond to mosquitos (Gubler 2009). According to WHO, mosquitos are even the most devastating vectors of human diseases (Gilles, Schetelig et al. 2014), once mosquito-borne diseases are one of main causes of deaths worldwide, estimated by WHO in 300 million of cases of infection per year (Kalluri, Gilruth et al. 2007). Culicidae family are the most important and most distributed vectors responsible for many epidemics (Molyneux 1993, Vimaladevi, Mahesh et al. 2012), causing a great impact on global and public health (Manilal, Sujith et al. 2009, Carvalho, Costa-da-Silva et al. 2014) and also an impact in the economy (Beula, Ravikumar et al. 2011, Gunn 2012). However, the infection cases are often due to mosquitos of the genus *Anopheles*, *Aedes* or *Culex* (figure 1) (Harbach 2007).

Although some of the major outbreaks were in the last century (Lequime and Lambrechts 2014), some mosquito-borne diseases are now emerging globally (Moreira, Iturbe-Ormaetxe et al. 2009) due to several factors, like the raise of human movements (Lequime and Lambrechts 2014) and climate changes, that interfere with conditions that affect the vector reproduction cycles, the prevalence and the intensity of parasitic infections (Molyneux 1993, Carvalho, Costa-da-Silva et al. 2014).

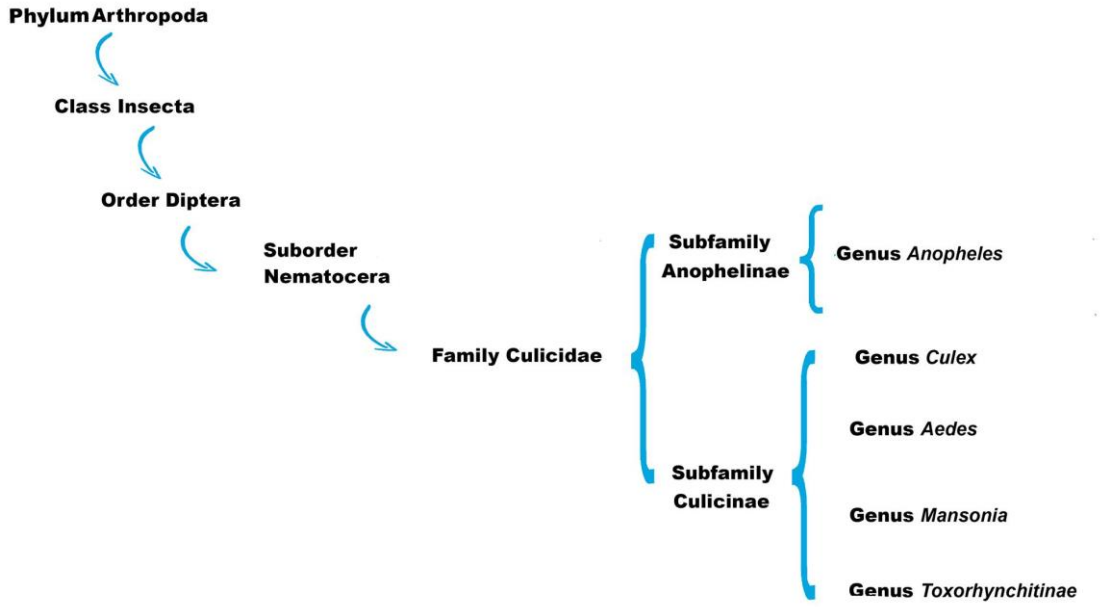

Figure1 – Taxonomic classification (adapted from Neves (2011)).

The three main factors that contribute to mosquitos abundance are: temperature, precipitation, and relative humidity (Githeko, Lindsay et al. 2000, Kalluri, Gilruth et al. 2007). The increasing of only 2 degrees in ambient temperature could double the intensity of some mosquitos, once their rates of development accelerate with the increase of the temperature (Carvalho, Costa-da-Silva et al. 2014). They also digest the blood faster in tropical climate, so it gives opportunity to feed more times and enhance the probability of acquiring and transmitting pathogens (Githeko, Lindsay et al. 2000). That is also the main reason why the majority of mosquitos-borne diseases occur in tropical regions (Ali, Ravikumar et al. 2012), since the ideal temperature for tropical species to develop is between 25 and 28°C (Kalluri, Gilruth et al. 2007); but due to global warming, the increasing of international traveling among other factors, mosquitos are expanding all over the world (Perumalsamy, Jang et al. 2015). The increase of precipitation also increase the number of places where mosquitos could do their egg posture (Githeko, Lindsay et al. 2000). This abundance of mosquitos, together with their longevity, human contact and feeding frequency and the size of the mosquito (because if it is smaller, it feeds more times and enhance the probability of acquiring the pathogen and transmit it), are also factors that influence the incidence of mosquitos-borne diseases (Schneider, Morrison et al. 2004).

1.1. Mosquitos - The particular case of *Aedes aegypti* (Linnaeus, 1762)

Aedes aegypti (*Ae. aegypti*) is primarily originating from Africa (Braga and Valle 2007, Navarro-Silva and Bona 2012), but now it is present almost all over the world between the latitudes 45°N and 35°S (Simard, Nchoutpouen et al. 2005, Braga and Valle 2007), with special incidence in Asia, Africa and Central and South America (Ali, Ravikumar et al. 2012). It was first time described in Americas in 1805 (Eisen and Moore 2013) and there was eliminated in 21 countries between 1948 and 1962. However, it regained territory in 2004 until our days, being more abundant than ever (Schneider, Morrison et al. 2004, Eisen and Moore 2013). This re-emergence could be explained with the exponential population growth, lack of sanitary conditions, the unaware transport of eggs (Schneider, Morrison et al. 2004, Eisen and Moore 2013), and also with the prohibitions of using some chemical insecticides as dichlorodiphenyltrichloroethane (commonly known as DDT) (Conway, Colpitts et al. 2014). *Ae. aegypti* is a diurnal ou a crepuscular habits mosquito that usually only feed on humans indoors (Bush 2001, Kraemer, Sinka et al. 2015).

Mosquitos have an holometabolic development, which means they pass through 4 stages: egg, larva (with four instars – L1, L2, L3 and L4), pupa and finally the adult (Bush 2001, Neves 2011, Navarro-Silva and Bona 2012) in a complete metamorphosis (Schmidt 2005), as we can see in figure 2. The duration of each stage depends on the species, temperature, humidity (Braga and Valle 2007, Navarro-Silva and Bona 2012, Yu, Jantan et al. 2014) and the amount of feeding (Centre for Disease Control and Prevention – CDC, 2017).

They begin the cycle in the water, where the female lays their eggs (Navarro-Silva and Bona 2012) in the inner or the wall off containers or other places where water accumulates (CDC, 2017), about 3 days after the blood feeding (Neves 2011). The eggs are extremely resistant to dry conditions and dissection, remaining viable for months and developing only when the conditions are suitable (Braga and Valle 2007, Navarro-Silva and Bona 2012).

The larval stage is when they feed and growth and so it lasts mainly dependent on the availability of the food, temperature and density of larvae in the breeding site (Navarro-Silva and Bona 2012). The larvae usually stay at water surface and breathe through the siphon, an “air tube” located in the last abdominal segment (Schmidt 2005, Neves 2011). They feed on microorganisms or particulate organic matter (Neves et al., 2011; CDC, 2017). The larvae pass through four instars that will take around 100 to 144 hours after hatching the eggs, depending on the environmental conditions, specially the temperature.

The pupae stage last 3 days when in tropical temperatures, but in more cold regions it could last up to a week before the adult emerges (Braga and Valle 2007). The pupae does not

feed, but it breathes and is very active (Neves 2011). In the case of *Ae.aegypti*, it lasts 10 days from eggs to adults in temperatures around 25°C (Braga and Valle 2007).

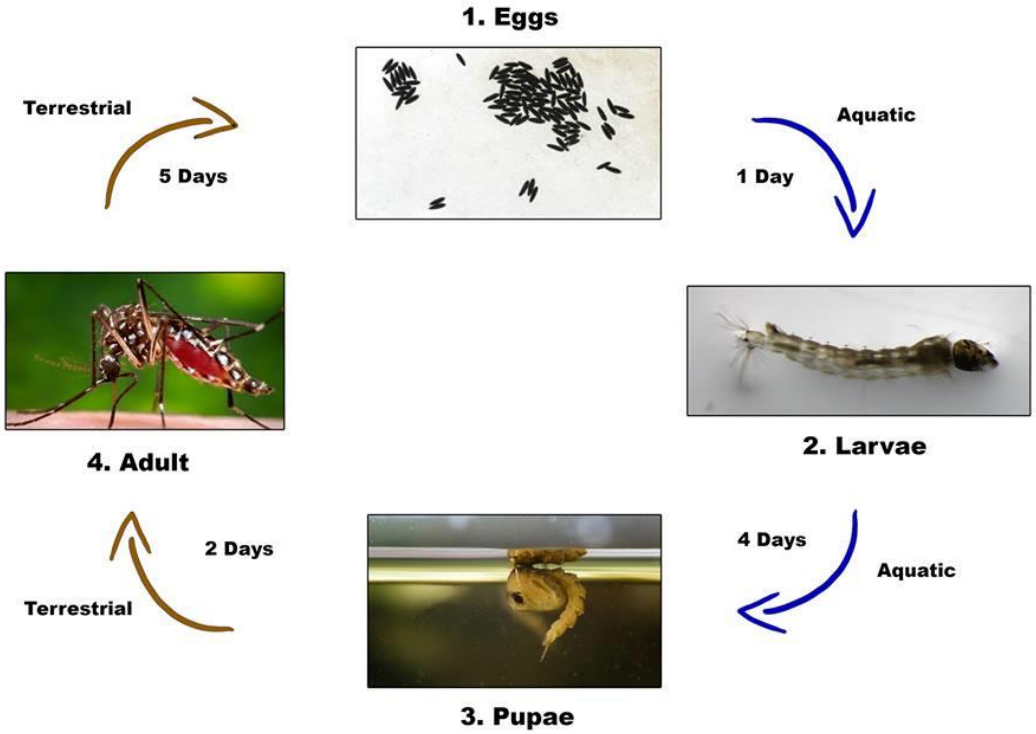

Figure 2 – Life cycle of *Aedes aegypti* (adapted from CDC, 2017).

In what regards the gonotrophic cycle, it has also 4 stages: unfed, blood fed, half-gravid and gravid (Molyneux 1993). This obviously refers to females, which are the responsible for the inoculation of pathogens into vertebrate hosts that leads to the diseases previously mentioned, once they feed on human blood and the males do not. They acquire the virus when they fed on blood of an infected human, and there so becoming the intermediate host or vector. Then the pathogen escapes from digestive tract and must replicate and disseminate before it reaches the salivary glands, waiting for a new blood meal to infect another person (Dubrulle, Mousson et al. 2009, Mousson, Zouache et al. 2012, Carvalho, Costa-da-Silva et al. 2014). The period between the acquisition of the pathogen by the mosquito and its transmission is called Extrinsic Incubation Period (EIP) and it duration depends on the pathogen and temperature (Dubrulle, Mousson et al. 2009). In a single gonotrophic cycle, the female frequently fed several times from different humans, infecting multiple people in a short period (Eisen and Moore 2013). According to Harrington, Edman et al. (2001), females prefer human blood because it confers advantages in energy

synthesis and mosquito fitness, probably due to the biochemical differences between human blood and other vertebrate species.

1.2. Major diseases transmitted by *Aedes aegypti*

Ae. aegypti is the main vector responsible for the transmission of several vector-borne diseases like Dengue, Yellow fever, Chikungunya and Zika, among others (Beula, Ravikumar et al. 2011, Conway, Colpitts et al. 2014), which estimates that causes 50 to 100 million of infections annually (Moreira, Iturbe-Ormaetxe et al. 2009, Wu, Wu et al. 2016). Against Chikungunya there's no vaccine (Moreira, Iturbe-Ormaetxe et al. 2009, Kalimuthu, Lin et al. 2014), for the Yellow fever it's only preventive (Heringer, Johnson et al. 2016) and against Dengue is very recent (discovered in 2015), so it is under tests and is not ready available (WHO, 2017). To prevent Zika infections, the WHO, in 2016, developed one vaccine to be used in an emergence scenario, while several other vaccines are being tested (National Institute of Allergy and Infectious Diseases – NIH, 2017; WHO, 2017). Drug treatments are also not very effective and often expensive, being inaccessible for many populations (Yu, Jantan et al. 2014).

1.2.1. Dengue

Dengue could be caused by 4 serotypes of Dengue Virus (DENV 1-4) (Thongwat and Bunchu 2015). The transmission to humans it's done by a bite of an infected mosquito of the *Aedes* genus, mainly an *Ae. aegypti* female (Mousson, Zouache et al. 2012, Lambrechts, Ferguson et al. 2015).

This disease is a global health concerning from tropical to temperate regions, found in more than 100 countries in Americas, Africa, Southeast Asia and islands from Western Pacific to Eastern Mediterranean (Carvalho, Costa-da-Silva et al. 2014, Thongwat and Bunchu 2015) as we can see in figure 3, being actually the most concerning arbovirus disease in the world (Lequime and Lambrechts 2014). It became more prevalent in the last decades (Lequime and Lambrechts 2014) with up to 390 million dengue fever caser per year (Eisen and Moore 2013, Lees, Knols et al. 2014, Ernst, Walker et al. 2017) (not all symptomatic) and causing about 13 000 deaths annually (Conway, Colpitts et al. 2014, Lequime and Lambrechts 2014). One explanation for these outrageous numbers is given by Eisen and Moore (2013) based on human population susceptibility. The fact that there are 4 serotypes of this virus means that although they have already been infected once, people can be infected again

with a new serotype. That means that having already been infected once with dengue does confers resistance.

In dengue, the EIP, lasts 7 days in tropical regions where the weather is warm and 12 days in regions with a colder temperature (Sanchez-Vargas, Travanty et al. 2004; Ernst, Walker et al. 2017). As previously stated, there's no specific treatment for DENV (Carvalho, Costa-da-Silva et al. 2014), so the programs to prevent and control the disease depend most of the times on vector control (Vimaladevi, Mahesh et al. 2012).

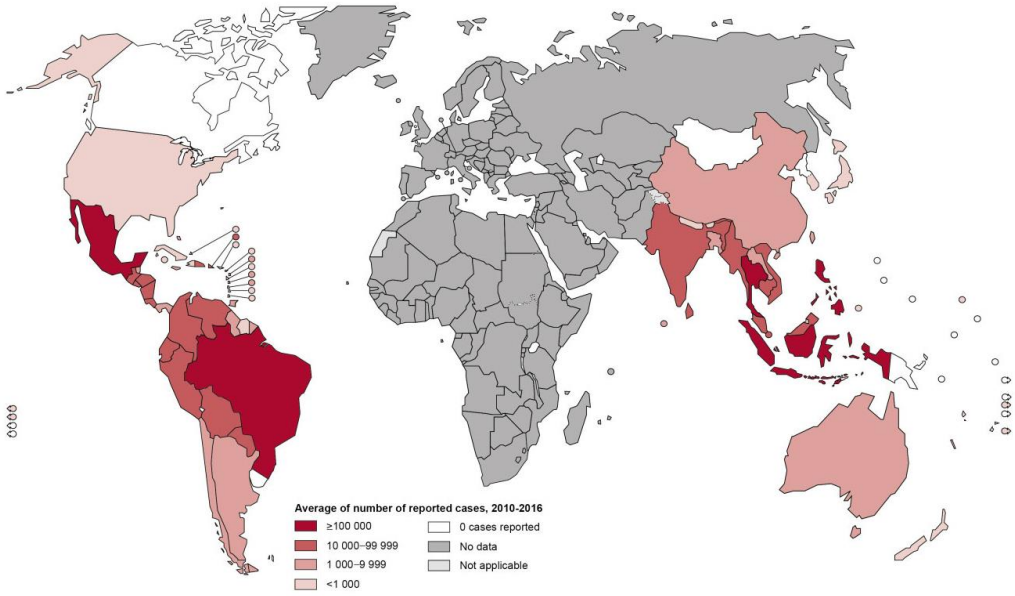

Figure 3 – Global distribution of Dengue in 2016 (WHO, 2017).

1.2.2. Yellow fever

Yellow fever is caused by a RNA virus of the genus *Flavivirus* (Monath and Vasconcelos 2015, Goldani 2017) through a bite of a mosquito of genus *Aedes* or *Haemogogus* (WHO, 2017).

This non-contagious disease is endemic in Africa and South America (Figure 4 and 5) (Monath and Vasconcelos 2015), but most of 90% of cases occur in Africa (Vasconcelos 2003).

Figure 4 – Risk areas of yellow fever in Africa (CDC, 2017).

Figure 5 – Risk areas of yellow fever in South America (CDC, 2017).

It has two types: the jungle yellow fever and the urban yellow fever. Both of types are transmitted to humans through a bite of a mosquito, mainly *Ae. aegypti*. The difference is that in first one the mosquito fed previously on a viremic monkey, while in the second one, it's about inter-human transmission (Monath and Vasconcelos 2015).

According to WHO (2017), there are 130 000 cases each ear only in Africa, responsible for 500 deaths. The majority of cases does not present symptoms, but those who does, have a 50% chance of death (Vasconcelos 2003). The symptoms include fever, chills, headache, myalgia, among others, and they manifests 3-6 days after the mosquito bite (Monath 2001).

1.2.3. Chikungunya

Chikungunya virus (CHIKV) was first described in Africa in 1952 and it belongs to the genus Alphavirus (Togaviridae family) (Pialoux, Gaüzère et al. 2007, Burt, Rolph et al. 2012, Presti, Lai et al. 2014). It caused major epidemics in the 1950s and 1980s, before it disappeared epidemiologically (Musso and Gubler 2015). However, it re-emerged in 2004 causing outbreaks in the past decade in Indic Ocean islands, India, Southeast Asia and Americas (Figure 6) (Dubrulle, Mousson et al. 2009, Eisen and Moore 2013, Musso and Gubler 2015), and being responsible for 693 000 annual cases of disease only in the Americas (WHO, 2017). It is a disease less common than the others mention above and is occurs essentially during the rainy season when is the pinnacle of vector density (Pialoux, Gaüzère et al. 2007)

Chikungunya's major vector is *Ae. aegypti* mosquito (Sudeep and Parashar 2008, Dubrulle, Mousson et al. 2009, Staples, Breiman et al. 2009). After the inoculation, the symptoms last 2-4 days, and consist in fever, photophobia, skin rash, severe arthralgia, and in the most serious cases it also causes incapacitating joint pain (Pialoux, Gaüzère et al. 2007, Presti, Lai et al. 2014). According to Staples, Breiman et al. (2009) this incubation period is between 3 to 7 days, and it could reach to 12 days.

In spite of the infection confer to infected people a long lasting immunity (Pialoux, Gaüzère et al. 2007), there is still no vaccine, so the treatment goes through non-steroidal anti-inflammatory drugs to relieve the symptoms (Staples, Breiman et al. 2009)

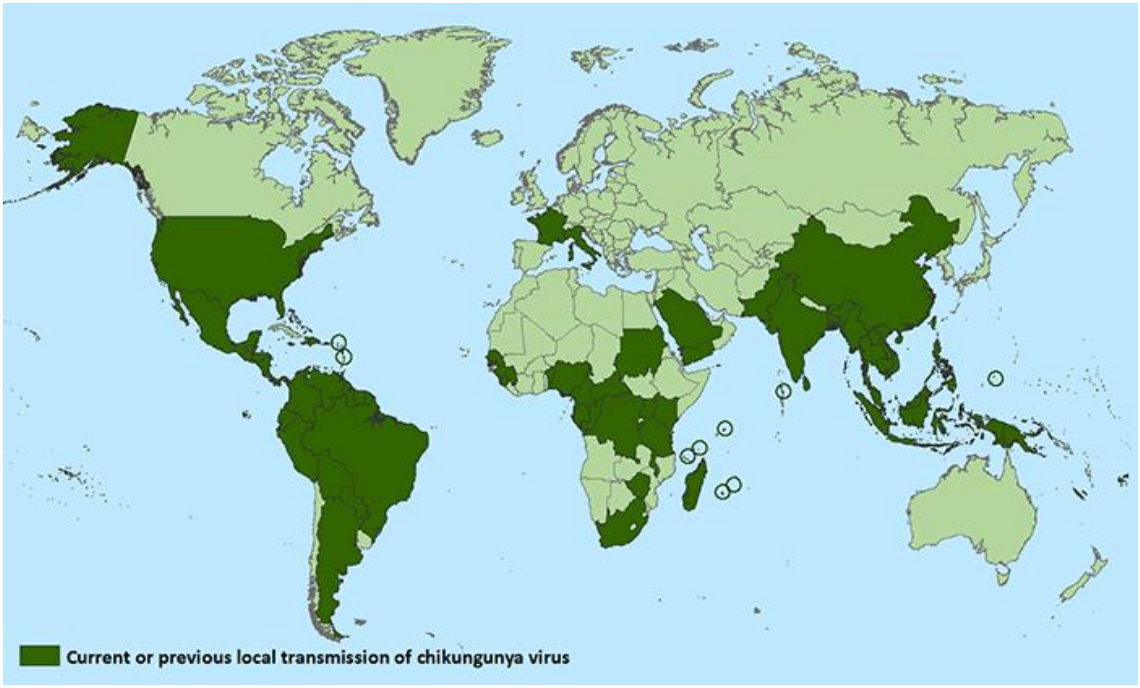

Figure 6 – Global Risk areas of Chikungunya (CDC, 2017).

1.2.4. Zika

Zika virus (ZIKV) is an arbovirus that was first identified in resus monkey (*Macaca mulatta*) in 1947, in Uganda. The vector of the transmission are mosquitos of *Aedes* genus, including *Ae. aegypti* (Ioos, Mallet et al. 2014), but as the yellow fever, it also has two transmission cycles: one where the virus circulates between mosquitos and non-human primates, and a human cycle (Weaver, Costa et al. 2016).

ZIKV was described in many countries in Africa, Asia and Oceania (figure 7) (Ioos, Mallet et al. 2014), but it was introduced in the Northeastern Brazil in 2015, and in February 2016, 30 countries in the Americas already registered the occurrence of the virus, causing the largest zika outbreak ever (Zanluca and dos Santos 2016).

There are no vaccines or specific treatment for ZIKV, whereby the vector control measures and the protection against bites are the best way to fight against the disease (Ioos, Mallet et al. 2014, Zanluca and dos Santos 2016).

Figure 7 – Global map of Zika (CDC, 2017).

2. Vector control

In view of lack of vaccine and/or treatment for the most arbovirus diseases, the best solution is very often to adopt measures to control the vectors (Molyneux 1993), therefore interrupting the disease transmission cycle (Lees, Gilles et al. 2015). The main goal of this control is to reduce the disease incidence and the mortality with not only humanitarian objectives, but also socio-economics (Molyneux 1993, Lacey 2007). Since some diseases affect a big part of population, particularly in African countries, parasitic diseases in humans cause multiple types of financial losses, either by the costs implicated in the diagnosis and treatment, or by preclusion to the people to work (Gunn 2012).

It has been noticed that the aerial insecticides used for killing adult mosquitos are not that efficient, and so the best solution is to attack the larvae and their breeding places (Beula, Ravikumar et al. 2011). Several authors, such Jarić-Perkušić, Hackenberger et al. (2008) and as Poonguzhali and Nisha (2012), affirmed that the latter method is safer, easier and more efficient, once it tackles the most vulnerable stage in mosquito life cycle (Whitten, Shiao et al. 2006). There are four great ways to do this vector control: physical, genetic, chemical and biological (Neves et al., 2011).

2.1. Physical control

This control method comprehends either the destruction of possible breeding places, or other measures that do not involve the use of substances to kill mosquitos.

The reduction or removal of possible breeding sites is an important step to control mosquito populations since it eliminated the source (Floore 2006). Concerning to big breeding environments like swamps or unused pools, they should be drained or destroyed (Pialoux, Gaüzère et al. 2007, Neves 2011) and other standing waters may be changed at least once in a week (Floore 2006). All the containers that could accumulate water after raining as old tires, buckets, etc. should be removed from outdoors since they could offer a possible breeding site (Neves et al., 2011, United States Environmental Protection Agency – EPA, 2017).

A range of traps has been deployed for monitoring *Ae. aegypti* populations by sampling eggs (ovitraps), host-seeking females or gravid mosquitos (Heringer, Johnson et al. 2016). These authors used sticky cards and other agents glue based instead of spatial sprays incorporated with success in the traps. It had as advantages the facility of removing the mosquitos and the fact of being more ecological. Some studies show also that intact screens and air conditioning could also reduce the indoor biting (Eisen and Moore 2013).

2.2. Genetic control

One of the interventions to control adult populations is the release of sterile mosquitos or genetically modified (Heringer, Johnson et al. 2016), commonly called SIT - Sterile Insect Technique (Lees, Knols et al. 2014) and is used since the 1970s (Oliva, Damiens et al. 2014). This method consists in the release of sterile males previously sterilized with one of several ways – chemosterilization, ionizing radiation, cytoplasmic incompatibility, or chromosome translocations (Oliva, Damiens et al. 2014) – causing the population to decline (Lees, Knols et al. 2014). These males will mate with wild virgin females and it will compromise the offspring, in a proved successful way (Bouyer and Lefrançois 2014).

Since males do not feed on blood and therefore do not transmit the disease, they are the base of any genetic control program (Gilles, Schetelig et al. 2014). They are colonized, sterilized, shipped and then released to into the wild population (Lees, Knols et al. 2014). However, there is still no effective method to separate males from females in a large scale,

and this is one of the limitations of this type of control (Gilles, Schetelig et al. 2014, Lees, Knols et al. 2014).

Another method, termed Release of Insects with Dominant Lethality (RIDL), consists in the release of males that carry a dominant-lethal gene. This technique, either reduces flight and induces mortality with age or create mosquitos with enhanced viral resistance (Conway, Colpitts et al. 2014, Oliva, Damiens et al. 2014).

Besides the need of the method to separate females and males, there are other drawbacks on this type of control. It is necessary to have enough knowledge about the target population before starting the program, including distribution features and mating systems (Lees, Knols et al. 2014). For instance, in the case of genetically modified *Ae. aegypti*, the male only inseminated an average of 6.6 females over their lifetime, against the average of 11.5 females inseminated when the males are of the wild-type. However, it is not known yet if this significantly affect the program success (Oliva, Damiens et al. 2014).

2.3. Biological control

Biopesticides derived from nature, like some plants or microorganisms, have increased lately, due to their non-harmful features. The biological control consists often in the use of pathogens, like fungi, parasites, like nematodes, or bacteria (e.g. *Bacillus thuringiensis israelensis* – *Bti* and *Bacillus sphaericus* – *Bs*) (Braga and Valle 2007, Klaassen 2013).

Besides being harmless for humans, they also are often specific to the targets, minimizing the problems of reach non target species, including common domestic animals and plants (Cockerman 1994).

2.3.1. *Bs*

Bs is an aerobic, Gram-positive, spore-forming bacterium (Zahiri, Su et al. 2002, Lacey 2007), commonly found in soil or aquatic habitats and that has mosquitocidal activity (Rungrod, Tjahaja et al. 2009), particularly in the larvae. They can produce different types of toxins that act as larvicides: binary toxin (Bin) produced during vegetative growth and mosquitocidal toxins (Mtx) that are produced during sporulation (Charles, Nielson-LeRoux et al. 1996). They act inducing pore formation and vacuolization (associated with induction of autophagy) in midgut epithelial cells followed by lysis of the cells (Lacey 2007, Opota, Gauthier et al. 2011).

This pesticide is sold in many forms like granules, concentrates, etc. (Lacey 2007). It is quite effective against certain mosquito species, particularly from genus *Anopheles* and *Culex* (Charles, Nielson-LeRoux et al. 1996, Lacey 2007). However, it is not very effective in *Ae. aegypti* (Lacey 2007). Despite this, Rungrod et al. (2008) demonstrated that in spite of mtx1 and mtx2 toxins have a low toxicity in *Ae. aegypti* mosquito larvae, they can have higher larvicidal potential when combined to make a synergistic effect by cloning them using *Escherichia coli*.

There are some cases of resistance to Bs (Charles, Nielson-LeRoux et al. 1996, Lacey 2007), but Zahiri, Su et al. (2002) suggested to do a rotation in the methods or mixtures, to avoid the development of that resistance.

2.3.2. *Bti*

These biopesticides are winning the market step by step and the most common is the use of Bti (Klaassen 2013).

Bacillus thuringiensis (*Bti*) is a Gram-positive, spore forming, aerobic bacterium, naturally occurring in a multitude of habitats (Lacey 2007). *Bacillus thuringiensis israelensis* was the first *Bti* used as a larvicidal agent, because it produces different types of toxins that affect specifically the larvae of certain insects (Klaassen 2013, Ben-Dov 2014) but it is not toxic to humans (EPA, 2017). When ingested, they lead to destruction of midgut epithelium cells by several processes, and the insect die from gut paralysis, feed inhibition, starvation, and septicemia (Casida 2009, Klaassen 2013).

This method of control has proven to be extremely effective in combination with other biological methods (Lacey 2007) and it is applied in the possible larval breeding sites as swamps, lakes, irrigation ditches or home standing water (United States Environmental Protection Agency - EPA, 2017).

According to EPA, there is no recording of development of resistance to *Bti* as a larvicide. This lack of resistance is mainly due to different modes of action and the synergistic effects between the toxins produced by *Bti* (Ben-Dov 2014).

2.3.3. *Wolbachia*

Wolbachia are inherited bacteria of arthropods and were first described in 1924 when found in the gonads of the mosquito *Culex pipiens* (Saridaki and Bourtzis 2010). *Wolbachia* are naturally present in 40% of all terrestrial insect species (Dutra, Rocha et al. 2016), and

therefore in some mosquito species such as *Aedes albopictus* and *C. pipiens*; however is not present in *Ae. aegypti* (Conway, Colpitts et al. 2014). It has been used as a control method that interfere with the longevity instead of interfering with mosquito abundance, by introducing of a life-shortening strain of *Wolbachia pipentis* in *Ae. aegypti* populations (Moreira, Iturbe-Ormaetxe et al. 2009). When in reproductive tissues, *Wolbachia* can induce several reproductive alterations, being the main the cytoplasmic incompatibility (CI) (Saridaki and Bourtzis 2010, Beckmann, Ronau et al. 2017). It also reduces transmission of some arthropod-borne diseases (Moreira, Iturbe-Ormaetxe et al. 2009) like DENV, CHIKV (Conway, Colpitts et al. 2014) and Zika (Dutra, Rocha et al. 2016).

2.3.4. Use of larvae predators

Another biological control consists in the use of larvae and pupae predators (Moreira, Iturbe-Ormaetxe et al. 2009). They could be aquatic insects from the orders Coleoptera, Diptera, Hemiptera and Odonata (Shaalan and Canyon 2009), mosquitofish, like *Gambusia sp*. (Blaustein and Chase 2007, Braga and Valle 2007), or copepods (Moreira, Iturbe-Ormaetxe et al. 2009). Mosquitos' predation occurs mainly during the larval and pupal stages and it depends on several biological and physical factors. A better knowledge about the relation between predator-prey can increase the effectiveness of this control strategy (Shaalan and Canyon 2009).

2.4. Chemical control

One of the main vector control strategies continues to be the use of chemicals. It includes several classes of chemicals pesticides such as organochlorine, organophosphates, carbamates and pyrethroids (Braga and Valle 2007, Poonguzhali and Nisha 2012).

The mode of action of insecticides depend on the molecular target, and in what regards to the major classes of insecticides could be: the inhibition of acetylcholinesterase (AChE); activation or inhibition of sodium channels, activation of nicotinic acetylcholine receptors; inhibition of GABA receptor-gated chloride channels; activation of glutamate-gated chloride channels; activation of octopamine receptors; inhibition of mitochondrial complex I or activation of ryanodine receptors (Klaassen 2013).

Besides being effective, with time these compounds typically trigger a big problem, namely the development of resistance in the target population. This is a serious setback once it favors the emergence or re-emergence of certain diseases (Carvalho, Costa-da-Silva et al.

2014). Also, among the consequences of using synthetic insecticides is the environmental pollution, once some of them are very persistent. Another negative issue is the chemicals toxicity for humans (Ali, Ravikumar et al. 2013, Heringer, Johnson et al. 2016), since the insecticides are often non selective and toxic to mammals (Klaassen 2013).

2.4.1. Organochlorine insecticides

Organochlorine insecticides do not have high acute toxicity when compared to organophosphates (Cockerman 1994), but in a long term exposure could cause problems due to their high lipophilicity and they could persist in the environment (Waker 2006). Thereat, in the past 30 years there have been an effort to forbid their use in the most of countries, but some of them are being reintroduced because of their efficacy to control mosquito vectors of some diseases (Klaassen 2013).

These insecticides are usually divided in three groups: the hexachlorocyclohexanes, the cyclodienes, and the most known, which is DDT and their derivate compounds (Waker 2006, Klaassen 2013).

DDT group is probably one of most known insecticides. It was synthesized for the first time in 1874, but only 65 years later was discovered its activity as insecticide by Paul Mueller (Cockerman 1994), and it was used to control malaria since 1945 with great efficacy (Timbrell 2002). The insecticides from DDT-type are neurotoxic by opening persistently the sodium channels, and inhibit the GABA receptor-gated chloride channels (Cockerman 1994). However, it was a devastating impact in other non-target species, and is extremely persistent in the environment (Cockerman 1994).

2.4.2. Organophosphates insecticides

Organophosphates (OPs) are less stable and persistent than organochlorine compounds (Cockerman 1994, Waker 2006). However, they are in many cases more toxic to mammals (Timbrell 2002) and yet represent half of the used insecticides nowadays (Klaassen 2013).

There are neurotoxic once they affect insect nervous system (EPA, 2017). They act by inhibiting AChE (Waker 2006), leading to accumulation of acetylcholine (Ach) and hyper stimulating the nerves, resulting in the death of the insect (Cockerman 1994, Timbrell 2002).

Temephos was one of the most used organophosphate insecticides and is the most used against *Ae. aegypti* (Melo-Santos, Varjal-Melo et al. 2010). It is very effective against mosquito larvae and relatively cheap (Grisales, Poupardin et al. 2013), and is applied in standing and drinking water anywhere that could represent a larval breeding site. However, since the end of the year 2016, it is illegal in USA to sell products containing temephos, once is consider a pollutant (EPA, 2017). Its decrease in use also have to do with the development of resistance by some mosquito species. These resistance has already been reported in many Latin American countries (Grisales, Poupardin et al. 2013), maybe due to the changes in the target site of the insecticide or due to increase of the insecticide metabolism (Diniz, de Melo-Santos et al. 2015).

2.4.3. Carbamates insecticides

These types of insecticides derive from carbamic acid, and has the same mode of action as the organophosphates, namely, the inhibition of AChE leads to Ach accumulation at the synapses resulting in overstimulation of Ach receptors (Waker 2006, Klaassen 2013, Martin-Reina 2017).

They have several formulations from solid (granules) to liquids, but the first ones are the most toxic (Waker 2006). They usually are not persistent, but they could raise problems from acute toxicity when ingested (Klaassen 2013).

2.4.4. Pyrethroids

Pyrethrins are originally insecticides extracted from the flower *Chrisanthenum cinerariefolium*. However, natural pyrethrins are highly decomposed by light, so it started to be synthesized analogs, named pyrethroids (Klaassen 2013) and commonly used as spray (Waker 2006). This ones are usually most stable than the natural pyrethrins (Cockerman 1994, Waker 2006). They are recommended by WHO to combat mosquitos, once is very effective, quick to act, non-persistent in the environment due to its biodegradability (Waker 2006), shows a low toxicity to mammals (Ngoagouni, Kamgang et al. 2016), and ultimately seem to not induce insect resistance (Klaassen 2013).

Pyrethroids act similar to DDT, being a neurotoxic insecticide (Waker 2006), interacting with the voltage sensitive sodium channel. They are very quickly metabolized resulting in hyperexcitation of the sodium channels (Klaassen 2013). However, there have been reported an increasing in the resistance to these insecticides, decreasing drastically its

effectiveness (Ranson, N'Guessan et al. 2011). The resistance in *Ae. aegypti* is a global problem, but it differs according to the area and the mosquito stage. The adult mosquitos usually show more resistance in Caribbean (especially in Cayman Islands (Harris, Rajatileka et al. 2010), Mexico and South America, and lower in Asia, while larvae show higher resistance in Asia, and lower in the Caribbean (Smith, Kasai et al. 2016). In order to solve this problem, more studies are needed to understand de mechanism of resistance, namely studying the genes involved in it development (Liu, Xu et al. 2006).

2.4.5. Insect growth regulators (IGRs)

Insect growth regulators (IGRs) are a class of compounds that disrupt the insect development, both embryonic and post-embryonic development (Mondal and Parween 2000). According with their mode of action IGRs are divided in three types: chitin synthesis inhibitors (CSIs), juvenile hormone analogs and mimics (JHAs) (The Fruit Research & Extension Center, 2017), and ecdysone agonists (Belinato, Martins et al. 2009). The most known IGRs are pyriproxyfen, methoprene, diflubenzuron, cyromazine and novaluron (Shaalan, Canyon et al. 2005, Lau, Chen et al. 2015).

CSIs were discovered in 1970s and as the name suggests, inhibit the synthesis of chitin, leading to disturbances in the molt and cuticle abnormalities (Belinato, Martins et al. 2009, Farnesi, Brito et al. 2012). They are usually as larvicides, and the larvae develop until molting, but then cannot ecdyse, not being able to get out of the cuticle (Mulla 1995, Tunaz and Uygun 2004). According to the results of Belinato, Martins et al. (2009), even the surviving adults live less and have problems in their reproduction potential.

JHAs are more effective in eggs, last larval instars or beginning of pupa. When applied to eggs, it disrupt the embryogenesis; in larvae result in the development of supernumerary instars, and in pupa result in an abnormal pupation (Tunaz and Uygun 2004). Finally, ecdysone agonist are substances that act at the ecdysone binding site disrupting the molting (Casida 2009).

They are generally selective and non-harmful for non-target species including humans (Tunaz and Uygun 2004). Nevertheless, some substances could affect crustaceans or other species that have the same molting hormones that mosquitos (Mulla 1995, Tunaz and Uygun 2004). This was demonstrated recently by Truong, Gonnerman et al. (2016), in a study with zebrafish, which showed that the IGR pyriproxyfen could be toxic to this species, leading to changes in morphology and behavior.

2.4.6. Phytochemicals

Phytochemicals are secondary metabolites produced by plants that can act as natural insecticides as a mechanism to defend on herbivorous insects (Ghosh, Chowdhury et al. 2012). They are great alternatives to synthetic products due to their quick biodegradation, low costs (Beula, Ravikumar et al. 2011, Guedes, Carvalho et al. 2014), less harmful nature to non-target organisms (Shaalan, Canyon et al. 2005), and multiple target-sites that reduce the resistance potential (Beula, Ravikumar et al. 2011, Poonguzhali and Nisha 2012, Perumalsamy, Jang et al. 2015). Nowadays, the phytochemicals, represent 1% of world's pesticide market (Ghosh, Chowdhury et al. 2012). About 334 species of plants from the 2000 known as source of secondary metabolites, have known mosquitocidal activities (Ghosh, Chowdhury et al. 2012, Sharma, Kumar et al. 2015). They are usually neurotoxic but also can have growth inhibiting effects (Shaalan, Canyon et al. 2005).

The bioactivity of the metabolites is different depending on many factors as plant species, plant part (leaves, roots, seeds, flowers and bark), solvent use in the extraction and mosquito species (Shaalan, Canyon et al. 2005, Kamaraj, Rahuman et al. 2008).

Mosquito larvae from genera *Aedes*, *Anopheles* and *Culex* are susceptible to a great part of phytochemicals; however, *Aedes* larvae are the most used in laboratory screening since it is usually the most resistant to extracts. Information published about effects in non-target species is scarce, but the little available shows a very low degree of toxicity in non-target species, and it also did not show evidence of development of resistance (Shaalan, Canyon et al. 2005).

In spite of the promising potential, there are some failures to fill in what regards to use of phytochemicals as insecticides chemical characterization of the compounds extracted from the plants (Benelli 2015) and the development of standardized protocols to extraction and assays with these compounds (Shaalan, Canyon et al. 2005).

3. Seaweeds as a source of compounds

Seaweeds have been used in many areas such feeding, medicine (Manilal, Sujith et al. 2009, Kalimuthu, Lin et al. 2014, Yu, Jantan et al. 2014), fertilizer, source of medical drugs, as raw material in the industrial production of agar, alginate, and carrageenan (Manilal, Sujith et al. 2009, Yu, Jantan et al. 2014) and as a source of bioethanol to produce renewable energy (Wi, Kim et al. 2009).

They have the ability to produce secondary metabolites such as terpenes, alkaloids, lectins, halogenated compounds, among others with known antibacterial, anti-fungicidal, antiviral, antitumoral (Guedes et al., 2014), antimicrofouling and antiprotozoan activities (Pérez, Falqué et al. 2016). Larvicidal activity has been less evaluated. Although, some secondary metabolites such as polyhalogenated monoterpenes, saturated fatty acids and alkaloids, exhibited larvicidal properties against the mosquitos (Alarif, Abou-Elnaga et al. 2010, Poonguzhali and Nisha 2012, Yu, Jantan et al. 2014); those compounds can also be used in combination with other insecticides (Bianco, Pires et al. 2013). However, many studies still needed to understand some features of their effects and fates, like degradation and environmental persistent rates, effect in non-target organisms and the possibility of developing resistance in target populations (Yu, Jantan et al. 2014). Regarding to its mosquitocidal properties, is also important to understand their mechanism of action, which still unknown. Nevertheless, in the review of Yu, Jantan et al. (2014), the authors reported that seaweed extracts and isolated compounds exhibit significant inhibition effects on cholinergic system, which could be the answer to the mechanistic problem. Morphological aberrations (e.g. deformation of anal papillae, deformation of larvae, prepupa that has not have succeed to come out of the larval exoskeleton), changes in the swimming behavior, abnormal growth and development, decrease of life span and fecundity problems are some of the toxic effects induced by seaweed and their compounds.

Ultimately, it is important to stress here that the biological activities of certain seaweed and their chemical composition depend on the species, physiological aspects, pollution, season and environmental factors (Pérez, Falqué et al. 2016).

3.1. *Ulva lactuca* (Linnaeus, 1753)

U. lactuca (Phylum Chlorophyta, Class Ulvophyceae, Order Ulvales, Family Ulvaceae) (Silva et al., 2013; Algaebase, 2017)**,** also known as “sea lettuce”, is an edible green algae with the margin ruffled, translucent membrane, and formed by two layers of cells (figure 8) (Suganya and Renganathan 2012, Thanh, Quach et al. 2016).

It is distributed worldwide, commonly found from tropical to polar climates, living in a wide range of different environments, varying the strains in each region (Bruhn, Dahl et al. 2011, Thanh, Quach et al. 2016). *Ulva* species are usually from saline waters, but they have a big proliferating potential, and could be founded also in freshwater (Silva, Vieira et al. 2013).

Among the three main divisions of seaweed: green seaweed (Chlorophyta), brown seaweed (Phaeophyta) and red seaweed (Rhodophyta), green seaweeds are the one less explored, even though they are easier to collect (Silva, Vieira et al. 2013, Thanh, Quach et al. 2016).

However, in what regards to *U. lactuca*, extracts from this seaweed had proven to have several bioactivities such as anticancer, antimicrobial, antibacterial, anticoagulant, preservative, antioxidant, antifungal, anti-inflammatory anticoagulant, antiproliferative and antiviral, among others (Alang, Kaur et al. 2009, Thanh, Quach et al. 2016). Also, recent studies had revealed a potential of the oil extracted from the seaweed to be a possible source for biodiesel production (Suganya and Renganathan 2012) or a source for bioenergy, once the biomass converted in gas (Bruhn et al., 2011).

In terms of chemical composition, this species is characterized by a high content in polysaccharides (54% of the dry weight) (Yaich, Garna et al. 2011). Regarding to secondary metabolites, they have a wide range of compounds, be the main terpenes, polyphenols and steroids as in the general green algae. However, the specific chemical composition depends largely on geographical distribution and seasons, among other environmental factors. These compounds have proven to be good to human health when the seaweed is consumed as food. For instance, sterols were reported to have the capacity to reduce blood cholesterol and reduce the fat deposition in heart and liver. Besides, Polyunsaturated Fatty Acids (PUFAs) have good nutritional values, and this seaweed also have high levels of protein and dietary fibers (Silva, Vieira et al. 2013).

It is important to highlight ulvan, which is a major sulfated polysaccharide (SP) water-soluble found in the cells of the green algae, being 8-29% of the seaweed dry weight (Silva, Vieira et al. 2013, Thanh, Quach et al. 2016). It is mainly composed by sulfated rhamnose, glucuronic acid, iduronic acid or xylose and low content in galactose, glucose and protein (Yaich, Garna et al. 2011, Silva, Vieira et al. 2013). This SP have been reported to have several biological activities such as cytotoxic against several cancers, anticoagulant, antifungal and antioxidant (Thanh, Quach et al. 2016).

Regarding to phytochemicals, a study by Beula, Ravikumar et al. (2011) in *U. lactuca* report to have found alkaloids, flavonoids, saponins and sugars as constituents of this seaweed. Other applications of extracts from *U. lactuca* include insecticidal. In a study by Abbassy, Marzouk et al. (2014), organic solvents and petroleum extracts from this species showed larvicidal activity against *Culex pipiens* and *Spodoptera littoralis* larvae, inhibition of pupation and adult emergence. However, in spite of the vast literature about seaweed with insecticidal properties, there is a lack of studies of these effects with this particular species of algae.

Due to its capacity to accumulate minerals and above all, heavy metals (manganese, lead, copper and cadmium), this seaweed could also be used to bioremediation in polluted waters (Yaich, Garna et al. 2011, Silva, Vieira et al. 2013).

Figure 8 – *Ulva lactuca* (Algaebase, 2017).

3.2. *Fucus vesiculosus* (Linnaeus, 1753)

F.vesiculosus (Phylum Ochrophyta, Class Phaeophyceae, Order Fucales, Family Fucaceae) (Algaebase, 2017) is also known as bladderwrack for its floating bladders (figure 9) (Mata, Blazquez et al. 2008) and is a brown seaweed (Zaragozá, López et al. 2008). It is edible, being a source of magnesium, protein and vitamin A (Algaebase, 2017).

It is commonly distributed north Atlantic as the Baltic Sea, Norway Sweden, Britain, Ireland, the Atlantic coast of France, Spain and Morocco, Madeira, the Azores, Portugal, the North Sea coast of Denmark, Germany, the Netherlands and Belgium and the eastern shores of United States and Canada (Alang et al., 2009; Biotic, 2017; Encyclopedia of life, 2017), as we can see in the map in figure 10.

Figure 9 – *F. vesiculosus* (Algaebase, 2017).

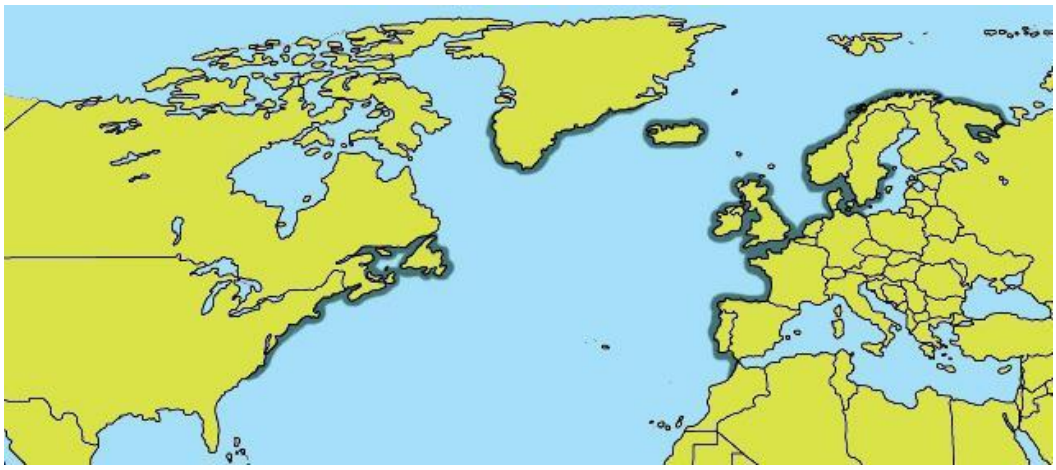

Figure 10 – World distribution of *F.vesiculosus* (Fucus vesiculosus, 2017)

This species is rich in phlorotannins (only present in brown seaweeds), and it also contains fucoxanthin (the carotenoid responsible for the color of the seaweed) (Zaragozá, López et al. 2008, Li, Wijesekara et al. 2011, Wang, Jónsdóttir et al. 2012), laminaran and fucoidan (Rioux, Turgeon et al. 2007).

Phlorotannins are a defense against herbivory since sometimes herbivory induced the production of these polyphenols (Jormalainen and Honkanen 2004). They have a great antioxidant potential against free radical mediated oxidation damage (Li, Wijesekara et al. 2011), and the total phloratannin content (TPC) is correlated with these antioxidant properties (Wang, Jónsdóttir et al. 2012). They also have other activities like enzyme inhibitory effect (which in the case of butyl cholinesterase could be a door open to treat Alzheimer's disease), bactericidal, anticancer, antiallergic and Anti-HIV activity, and radioprotective effect (Li, Wijesekara et al. 2011).
Fucoidan is known mostly by their use in medicine, having several biological activities such as antitumor, antivirus, anticoagulant, anti-inflammatory, antioxidant and gastric protective effects (Li, Lu et al., 2008, Morya, Kim et al., 2012, Wijesinghe and Jeon 2012, Senthilkumar, Manivasagan et al. 2013). It is composed of fucose, uronic acids, galactose, xylose and sulfated fucose (Rioux, Turgeon et al. 2007). Laminaran is a small glucan that is capable of modulating immune response, and also have antitumor and apoptosis activities (Rioux, Turgeon et al. 2010).

There is a lack of studies of insecticidal properties from *F. vesiculosus* extracts, and even in other bioactivities, the information is scarce. However, many papers are available in what

regards of the effects of the major compounds of this seaweed isolated, being known many properties of them.

As *U. lactuca*, also *F. vesiculosus* has a great absorbent capacity of heavy metals, and could be used in bioremediation in contaminant waters (Mata, Blazquez et al. 2008).

3.3. Extraction methods

There are several methods to perform seaweed solid-liquid extraction (SLE). Yet, the extraction efficiency depends not only on them, but also on the nature of phytochemicals, sample particle size and the solvent used (Do, Angkawijaya et al. 2014). Due to the differences in the extraction methods, the qualitative and quantitative composition of extracts from the same plant could also be different, which obviously will influence their bioactivity (De Monte, Carradori et al. 2014). Traditional SLE techniques include Soxhlet extraction, hydro distillation, maceration, infusion, percolation, etc. However, since the 20th century, more efficient and selective methods have been developed, namely Microwave-Assisted Extraction (MAE), Ultrasonic-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), Enzyme-Assisted Extraction (EAE), Pressurized Solvent Extraction (PSE), Pulsed Electric Field-Assisted Extraction (PEF), and extraction with switchable solvents and Ionic Liquids (ILs) (Do, Angkawijaya et al. 2014, Grosso, Valentão et al. 2015, Dhanani, Shah et al. 2017). These alternative methods have some advantages when compared to traditional ones: MAE, UAE and PEF are faster, produce high yields and use less solvent (De Monte, Carradori et al. 2014); SFE, PSE, PEF and EAE are eco-friendly (Grosso, Valentão et al. 2015, Ospina, Castro-Vargas et al. 2017), once organic solvents are often extremely harmful to the environment (Kadam, Tiwari et al. 2013). ILs improve the selectivity of the method by interacting with specific polar and non-polar compounds, and could be applied to other methods like MAE or UAE, among other features. However, they also have drawbacks as high energy consumption and above all, the monetary cost evolved in all the equipment required (De Monte, Carradori et al. 2014).

4. *Artemia* genus in toxicology

Artemia spp., commonly named as brine shrimp, is a genus of small animals from the subphylum Crustacea, class Branchiopoda, order Anostraca (Dvorak 2012, Libralato, Prato et al. 2016). It was first described in 1755 by Schlösser (Mendes 2014), and play an important role in the food chain (Kanwar 2007), being an important primary consumer and being economical important for its use in aquaculture (Libralato, Prato et al. 2016). The

genus has seven described bisexual species *A. salina, A. monica, A. urmiana, A. franciscana, A. persimilis, A. sinica, A. tibetiana,* and a parthenogenic species, *A. parthenogenetica* (Nunes, Carvalho et al. 2006, Dvorak 2012, Mendes 2014). *Artemia* is widely distributed in the world once its adaptability to extreme conditions, being present in all the continents except for Antarctica (Dvorak 2012). While in Europe, Africa and Asia, both bisexual and parthenogenic strains can be found, in the Americas only the first ones exist (Nunes, Carvalho et al. 2006). Typical habitats of *Artemia* spp. are salt lakes and the base of its diet is bacteria, protozoa and algae (Dvorak 2012).

The reproduction strategies on *Artemia* vary by environmental conditions, switching between ovoviviparity to oviparity (Varó, Amat et al. 2006). When the conditions are propitious, embryos develop normally in the body of the females and "born" as nauplii (first stage of brine shrimp life cycle) (Nunes, Carvalho et al. 2006, Kanwar 2007). However, when the conditions are stressful, they female can spawn cysts, which are resistant embryos that can survive to desiccation or other extreme conditions as anoxic or low temperature, through years, due to a chorion that protects it. When hydrated, the cysts proceed their normal development, rising the larvae (nauplii) (Varó, Amat et al. 2006). The nauplii have about 0.4 mm of length and its body consists of a head with a naupliar eye and two pairs of antennae, and a short thorax (Dvorak 2012).

Depending on salinity, temperature, food availability and the features of each species, the life span of *Artemia* spp. varies between 2 and 4 months (Libralato, Prato et al. 2016), but the average of the time life cycle is 1½ months, since not always the environmental conditions are optimal. Light, pH and oxygen may also interfere in the survival (Kanwar 2007).

In figure 11, we can see the different stages of *A. salina* life cycle. Adult individuals have ± 1 cm in length (Kanwar 2007), and depending on the diets and environmental conditions like salinity they can be white, orange red, pink, blue or green. The body has three eye naked distinguishable parts: head, thorax and abdomen (Dvorak 2012, Mendes 2014).

Figure 11 – Different stages of life cycle of *Artemia salina* (Zootecnica domestica, 2017).

Due to its ease of culture and manipulation, short life cycle, wide geographic distribution, commercial availability of its cysts, and cost-effectiveness of performed tests, *Artemia* spp. is one of the most used species for laboratory toxicity testing (Rao, Kavitha et al. 2007, Kalčíková, Zagorc-Končan et al. 2012, Libralato, Prato et al. 2016). Also, its ecology and biology is well known, it has a small body size turning easy to perform test in microplates, and have a high adaptability to test conditions (Dvorak 2012, Libralato, Prato et al. 2016). Its use is also growing in the last decades, since they have been an effort to substituting the use of laboratory animals in toxicological tests due to the suffering and the high costs, according to the "3Rs principle" (replace the experiments which use animals as models, reduce the number of animals used in tests, and the refine of methodologies) (Parra, Yhebra et al. 2001, Kanwar 2007). Sorgeloos, Remiche-Van Der Wielen et al. (1978) affirmed that it is one of the most suitable organisms for toxicity tests on microscopical invertebrates. More recently, Dvorak (2012) claimed that *Artemia* testing is assuredly an alternative to pre-screening chemical toxicity using mammals.

Artemia is an organism used since 80 years ago (Dvorak 2012) in toxicity assessment of many contaminants, such as metals, pharmaceuticals, insecticides, organic solvents (Nunes, Carvalho et al. 2006, Kalčíková, Zagorc-Končan et al. 2012), toxins, plant extracts (Parra, Yhebra et al. 2001, Kanwar 2007), or other biologically active compounds (Ferraz Filha, Lombardi et al. 2012, Bucker, Falcao-Bucker et al. 2013). Lhullier, Horta et al. (2006) also highlighted how the evaluation of lethality in a less complex organism as *Artemia* could be used to quickly access the potential of phytochemicals. Acute endpoints that could be studied besides the lethality are the impacts in hatching, growth and swimming, and biomarkers. Long-term chronic tests could study the effects on growth, reproduction and survival from larval to adult stage (Libralato, Prato et al. 2016). All the stages of the life cycle

could be used to perform different toxicity tests, but the most used is the period of 24-48 hours after hatching (Kanwar 2007).

Despite all these advantages, some publications refer downsides when using *Artemia*. Its absence in most of marine ecosystems and the low solubility of some chemical substances in saline water are some of the drawbacks. But the main criticism is its low sensibility to chemical exposure, once they are much more resistant in comparison with several other organisms under the same test conditions, and so extrapolation and correlation to what may occur with other animals may not be so representative as we could think (Nunes, Carvalho et al. 2006, Dvorak 2012). However, and contradicting this idea, some studies have proven that there is a correlation between the *Artemia* bioassays and toxicological effects in other organisms (or cells). Parra, Yhebra et al. (2001) found a good correlation between *Artemia* and mice in a bioassay with 20 plant extracts; and as this paper, many others correlated results with plant extracts. Carballo, Hernández-Inda et al. (2002) compared the cytotoxicity of marine products extracts in *Artemia* nauplii with 2 human cell lines. Also in a publication from Ferraz Filha, Lombardi et al. (2012), a good correlation with trypanocidal, antitumor and pesticidal activities was mentioned. All these examples and the above advantages allow to conclude that brine shrimp is indeed one of the suitable models for toxicity testing.

Chapter II

Objective

Objective

The objective of this work was to test mosquito larvicidal potential of five different solvent extracts of two seaweeds: *Fucus vesiculosus* and *Ulva lactuca*. To accomplish this purpose, were carried out assays with *Aedes aegypti* mosquito larvae in late 3rd or early 4th instar, evaluating several parameters, such as mortality, mobility, weigh and length after 24 (only mortality) and 48 hours of exposure. *Artemia salina* (brine shrimp) was used as a biological model for estimating the acute-toxicity in non-target organisms. *A. salina* were exposed to the same extracts from and the mortality assessed after 24 hours.

Chapter III

Material and methods

Materials and methods

1. Extract preparation

The protocol for seaweed extraction was adapted from (Guedes, Carvalho et al. 2014). Dried seaweeds *U. lactuca* and *F. vesiculosus* were bought from Algaplus – Produção e Comercialização de Algas e Seus Derivados Lda. Seaweeds were crushed with a kitchen blender (Moulinex® 1,2,3 A327R1) in order to obtain the smallest pieces possible to enhance the extraction efficacy. Fifty grams of crushed seaweed were suspended in 100 mL (*F. vesiculosus*) or 300 mL (*U. lactuca*) of solvent (ethanol, methanol, chloroform, dichloromethane or hexane) in a glass bottle. According to Pérez, Falqué et al. (2016), these solvents are among the best to extract bioactive compounds. Regarding to *U. lactuca*, once it is lighter, a bigger volume was necessary to reach the 50 g, and to make the suspension possible. This suspension was macerated with continuous agitation for 72 hours in a stirring plate. After this time, the suspension was filtered in a vacuum pump, and new solvent was added to the seaweed material for new maceration. The filtered was stored at 4ºC in amber glass bottles. This process was repeated 3 times (3 x 72 hours). In the end the filtrates were dried in the fume hood chamber in glass Petri dishes at room temperature. Once dried, each extract was weighed to calculate extraction percentage and then stored at 4ºC. The percentage of extraction was calculated with the following formula according to Beula, Ravikumar et al. (2011):

% of extraction = (weight of the dry extract / weight of the seaweed material) x 100.

When needed, all extracts were diluted in dimethyl sulfoxide (DMSO) in a concentration of 25 000 µg/mL, followed by several cycles of vortex and sonicator bath to dissolve better. Extracts aliquots of this stock solution were stored at -20°C.

2. *Aedes aegypti* larvae assays

2.1. Mortality assays

The *Ae. aegypti* larvae were provided by the IHMT (Instituto de Higiene e Medicina Tropical, Lisboa) and the assays were performed according to WHO Guidelines WHO, 2005) for *mosquito in vivo* testing. We used 25 late 3rd instar/ 4th early instar larvae for cup (disposable plastic cups), each one with 99 mL dechlorinated water plus 1 mL of test solution (stock

solution diluted in a concentration of 10 000 µg/mL). Two replicates were made for each experimental condition. Nine extracts were tested: *F. vesiculosus* ethanol, methanol, dichloromethane, chloroform and hexane extract, and all the same extracts from *U. lactuca* except for the hexane, once the yield was not sufficient to use in the assays. All the extracts were tested at 100 µg/mL. Three controls were used: dechlorinated water, water with 1% DMSO, and temephos at 3 µg/mL (the latter being an organophosphate larvicide that served as positive control). The larvae were fed only before the beginning of the experiment with dried flakes for tropical ornamental fish, in accordance with the practices at IHMT. The mosquitos were maintained in a system with water at 26 °C ± 2 and with a photoperiod of 12 hours light and 12 hours dark (figure 12). Dead larvae were registered at 24 and 48 hours of exposure. The number of death larvae was obtained making the subtraction between the initial number and the counted alive larvae, once the dead ones start to fade and sometimes turn to completely transparent make it impossible to count. The ones that did not fade were considered dead if they stayed at the bottom of the cup and do not respond to touch or water movement. Also, was tried to observe if the larvae exhibit an abnormal movement or problems in its mobility or swimming performance. The mortality percentage was calculated dividing the number of dead larvae by the total number of larvae, and then multiplicated by 100 to obtain the percentage. In the cases that mortality in DMSO control was between 5 and 20%, mortality values were corrected with recourse to the Abbott's formula (Abbott 1987):

$$\%\ \text{Mortality} = \frac{\%\ \text{of survival in untreated control} - \%\ \text{of survival in treated sample}}{\%\ \text{of survival in untreated control}} \times 100$$

After each assay, three alive larvae were collected from each cup and fixed with PFA (paraformaldehyde) at 4% for 24 hours, and then placed in ethanol 70%, either for being studied under stereomicroscopy and for future histopathological analysis. The rest of living larvae were frieze in liquid nitrogen to study enzymatic activity posteriorly. These samples were not processed yet, but the procedures will allow us to understand the alterations produced by the extracts. All the procedure was repeated 4 times in different days.

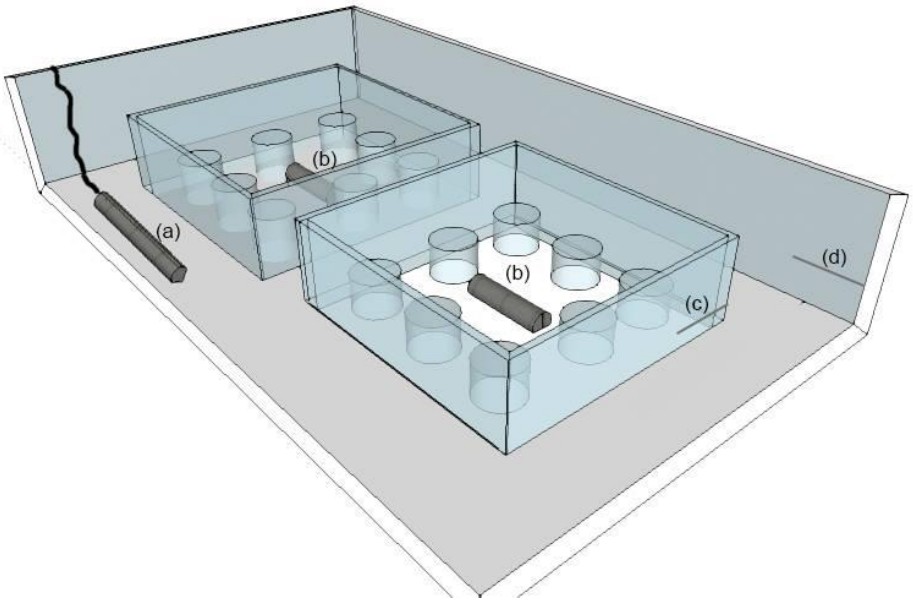

Figure 12 – 3D representation of one of the two boxes of the system for larvae bioassays. (a) Thermostat to keep the water temperature stable; (b) iron bars were put in the small boxes to avoid the fluctuation; (c) and (d) indicate the water level used to conduct the temperature of the small boxes and the exterior box, respectively. Each box contained 8 cups for putting the mosquito larvae.

2.2. Larvae body length

Every larva collected after the mortality assays was observed under a stereomicroscope (Olympus SZX10, Japan) and photographed with a digital camera (DP21, Olympus, Japan). The pictures were processed in an image analysis software (ImageJ, version 1.47, NIH, Bethesda, Maryland, USA), being the length measured with the segmented line option. The software was calibrated using as reference a photographed scale of 1 mm (figure 13). The data were used to look for differences between treatments in regard of the larvae body size.

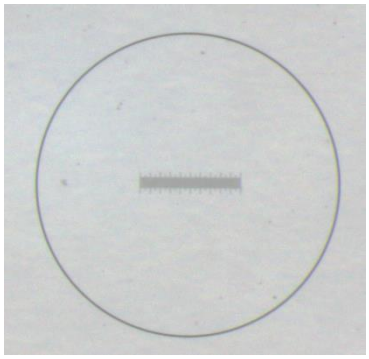

Figure 13 – Scale used to calibrate the Image J in order to measure the larvae length.

2.3. Larva mass

The same larvae used for length evaluation were weighed. For each replicate, three larvae per condition were weighed together and the average mass of the larvae was determined for the treatment. Final results are expressed as the mean of four independent experiments.

3. *Artemia salina* mortality assays

A. salina toxicity could be used to evaluate acute toxicity of certain compounds in non-target organisms (Guedes, Carvalho et al. 2014). The protocol followed was the "Artemia Reference Centre-test (ARC-test)", developed by Vanhaecke and Persoone (1984). About 175 mg of dry cysts (Ocean Nutrition™ Lot no. ON13280) were weight and left to hydrate in a small flask for about 5 hours in artificial salty water (the same water was used in all the experiment, i.e., 35 g/L of Tropic Marin PRO-REEF Sea Salt, stored at 4°C). Then the cysts were placed in a conical flask with 600 mL of saline water with strong aeration by an air pump, in a water bath at 26 °C and a light period of 14 hours light and 10 hours dark, in a system represented in figure 14. They were left to hatch for 36 hours (to reach the state between the instar II and III).

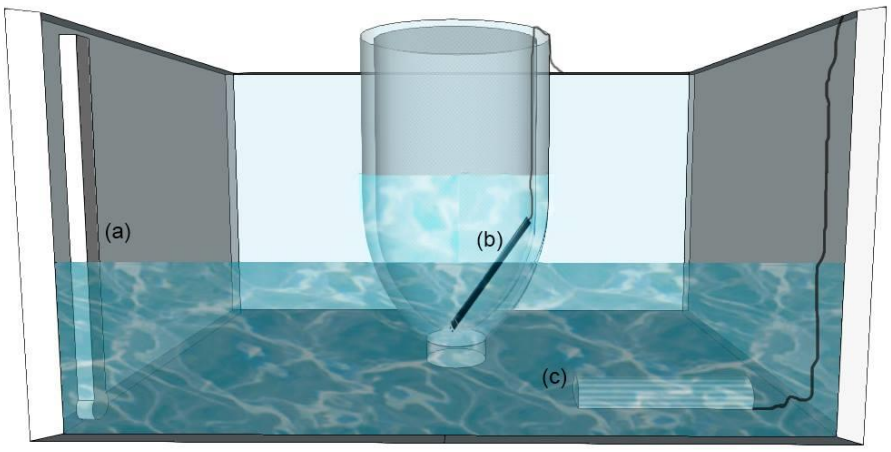

Figure 14 – 3D representation of the system used to hatch the *Artemia* cysts. (a) Represent the thermometer used to control water temperature; (b) is the aeration probe inside the bottle where the cysts are hatched; (c) is the thermostat used to keep the water temperature stable.

The assays of seaweed extracts toxicity were carried out in 24 well-microplates. In each well, 10-20 free-swimming nauplii were transferred in 1 mL of water with a micropipette,

being the same in all wells. The final volume of each well was 2 mL. The quantity of the compound/extract to add was calculated in function of this volume and the concentration to be tested.

The same extracts tested in *Ae. aegypti* larvae were used, plus the extract of *U. lactuca.* prepared with hexane. Since the brine shrimp is known for its tolerance to compounds, a higher concentration than that used in the mosquito larvae was tested initially (500 µg/mL). Extracts that showed toxicity were thereafter tested in the same concentration as for the mosquito larvae (100 µg/mL). Temephos was also tested in the same concentration as in the larvae assays.

Three kinds of control groups were used: saline water, DMSO at 1% (solvent control), and growing concentrations of potassium dichromate (6, 12, 5, 25, 40, 50, and 75 mg/L). The last compound was used as a positive control, to evaluate the current sensibility of the *Artemia* nauplii, once its LC_{50} could be calculated easily through the probit analysis method.

After compound or extract exposure, microplates were left in an incubator (Ehret, Germany) at 25°C, for 24 hours, in the dark. After this time, dead individuals were counted and registered. Then 400 µL of Bouin's fluid were added to each well to kill all the nauplii, and all the individuals were counted in each well in order to calculate the mortality percentage.

All the conditions were tested at least three times, in three different days (n = 3). Two extracts from *F. vesiculosus* were tested more than three times: Chloroform at 500 µg/mL – n=4; and Ethanol in the same concentration – n=5. All the procedure was done using a stereomicroscope (Zoom 2000, Leica, Germany).

4. Statistical analysis

Statistical analysis was done with the software GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA), and the results were expressed as mean ± standard deviation (SD), from 3 to 5 independent experiments depending on the situation. Significant differences ($p \leq 0.05$) were evaluated either using one-way or two-way ANOVA, with multiple post-hoc comparisons between the extracts and the DMSO control being made either by Tukey's or Dunnett's test, as appropriate. The ANOVA assumptions of normality and homogeneity of variances were confirmed by the Shapiro–Wilk's and Levene's test, respectively.

Chapter IV

Results and discussion

Results and discussion

1. Extraction yield from seaweed extracts

In spite of the main limitations of classical methods such as time consumption, lower yield and use of great amount of organic solvents, we used them because very few equipment is needed, they are cheaper than other methods, and, as first approach, they fit the objective; that was to screen larvicidal activity of the extract and not to extract specific compounds. Thus, to perform the extraction, the seaweeds were crushed into small pieces and agitated with one of five solvents (ethanol, methanol, chloroform, hexane and dichloromethane) for 72 hours for 3 times. The protocol followed was adapted from Guedes, Carvalho et al. (2014). he results of extraction yields are presented in Table 1.

Table 1 - Extaction yield (%) from *U. lactuca* and *F. vesiculosus* extracts using 5 different solvents.

Solvent	***Ulva lactuca***	***Fucus vesiculosus***
Ethanol	6,17	4,50
Methanol	13,65	5,72
Chloroform	1,98	4,22
Hexane	0,04	4,23
Dichloromethane	11,41	2,33

Methanol was in both seaweeds the solvent with higher percentage of yield. In *F. vesiculosus* the extraction yields were quite similar in all solvents used, while in *U. lactuca* the percentages were very different depending on the solvent. Hexane extract in *U. lactuca* had very low yield, as opposed to what happened with methanol and dichloromethane. There are several factors that could explain the reduced extraction yield for hexane, but surely that one of them was the inefficiency of the agitation. When carried out, it was observed that the seaweed deposited on the bottom of the bottle, and the magnet stir bar was not able to mix the suspension. Even when strong manual shaking was tried, the deposit formed in the instant that the bottle was put over the magnetic stir plate. With an inefficient agitation the cell walls are not properly disrupted, not allowing the solvent to fully enter into the natural matrix to extract the compounds (Grosso, Valentão et al. 2015).

The total extraction yields and the type of compounds extracted depend also strongly on the polarity of the solvents (Sultana, Anwar et al. 2009). Non-polar (or apolar) solvents are used to extract lipophilic substances, such as alkaloids, fatty acids (FAs), flavonoids and terpenoids. Contrariwise, to extract hydrophilic substances, polar solvents should be used, to obtain, for instance, flavonols, lectins, flavones, polyphenols, tannins and saponins (Sultana, Anwar et al. 2009, De Monte, Carradori et al. 2014, Do, Angkawijaya et al. 2014). According to several authors, such as Do, Angkawijaya et al. (2014) and Ospina, Castro-Vargas et al. (2017), the polarity also influences the extraction yield, increasing with the polarity of the solvent, and decreasing with the decrease of the solvent polarity (Wang, Jónsdóttir et al. 2012). Because hexane and chloroform are apolar, dichloromethane are less polar, and ethanol and methanol are polar (De Monte, Carradori et al. 2014), it would be expected that ethanol and methanol would show the higher yields when compared to chloroform and hexane. Our results are consistent with the expectation. Additionally, most soluble compounds of the seaweeds are high polar (Wang, Jonsdottir et al. 2009).

It is also due to the polarity, that many papers refer the aqueous mixtures of solvents as more suitable to extract compounds (Sultana, Anwar et al. 2009, Do, Angkawijaya et al. 2014), once water has high polarity (Dhanani, Shah et al. 2017). Do, Angkawijaya et al. (2014) obtained extracts from *Limnophila aromatica*, an aquatic plant found in southeast Asia, with methanol, ethanol and aqueous mixtures of both. They concluded that the yield percentage was higher in the mixtures than in the used of the solvent alone. Dhanani, Shah et al. (2017) tested the extraction from the terrestrial plant, *Withania somnifera,* with pure solvents and aqueous mixtures, using different methods. The mixtures showed always higher yield regardless of the extraction method. Sultana, Anwar et al. (2009) also presented results that showed that extracts from aqueous mixtures of ethanol and methanol from plant materials exhibited better antioxidant activities and higher phenolic contents in the two methods of extraction tested, when compared with the solvents alone. Beula, Ravikumar et al. (2011), Ali, Ravikumar et al. (2013) and Bianco, Pires et al. (2013) also reported the enhance of extract yield when aqueous mixtures were used.

It is difficult to compare the values of extraction yields with existent literature, once the few works that used the same species, used different extraction methods, solvent mixtures and some of publications do not even present the extraction yield. Guedes and Cutler (2014) considered the yield too low when below 0.1%, which is the case of the hexane extract obtained here from *U. lactuca*. Ali, Ravikumar et al. (2013) obtained a yield of 5.32% in an ethanolic extract from *U. lactuca*, which is a little less than we did. Still in *U. lactuca*, our results were better than those of Tan, O'Sullivan et al. (2012), in the methanol, ethanol, chloroform, and mainly in dichloromethane extract, in which we reach 11.41% of yield,

against 0.01% of the cited authors. On the other hand, they obtained 0.18% from the hexane extract, and we only got 0.04% of yield. We also had higher yields in the chloroform extract than Alang, Kaur et al. (2009). Even in the ethanol:water extract, these authors obtained a lower yield than we did from pure ethanolic extract. Regarding to *F. vesiculosus*, most publications were based in aqueous mixtures and so they ca not be compared. Anyway, the yields obtained are way higher than our results. Wang, Jónsdóttir et al. (2012) reported a yield of 23% when using aqueous ethanolic 1:1 extract. Also in pure ethanol, Rioux, Turgeon et al. (2007) obtained an yield of 18.3%, which is around 4 times higher than our results.

Besides the polarity, it is also possible that the use of the water with the solvent turns easier to extract compounds that are soluble in water and/or the solvent (Do, Angkawijaya et al. 2014). So, despite we obtained the extracts, in the future, and to try to optimize the protocol and improve the extraction yield, the use of the solvents in mixture with water instead, of the absolute solvent should be tested. Also, once most of publications that report seaweed extraction use them as a powder, therefore a more potent blender is advisable, to better macerate the seaweed material, and ultimately making the agitation easier and likely more efficient.

2. *Aedes aegypti* larvae assays

2.1. Mortality assays

The mortality assays with *Ae. aegypti* mosquito larvae were carried out following the WHO Guidelines for laboratory and field testing of mosquito larvicides (World Health Organization. Dept. of Communicable Disease Prevention and Scheme 2005). Mortality percentage of *Ae. aegypti* larvae after 24 and 48 hours of exposure to the extracts are represented in figure 15.

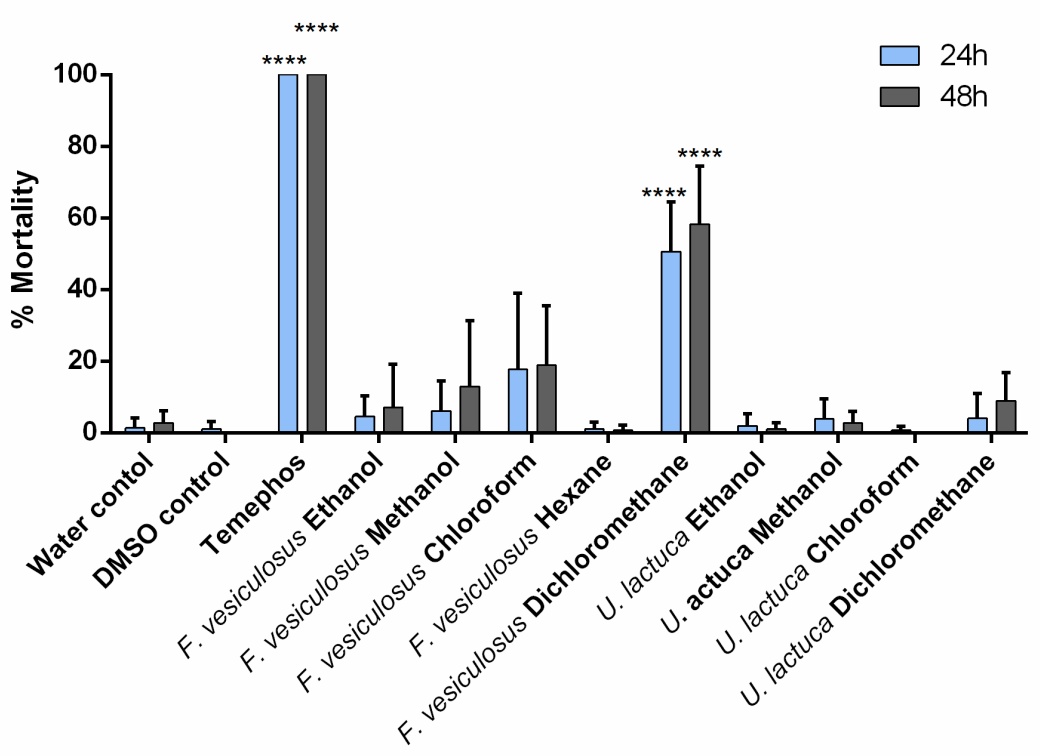

Figure 15 – Percentage of mortality in *Aedes aegypti* larvae after 24 hours (blue bars) and 48 hours (grey bars) of exposure to the seaweed extracts. Results are expressed as mean ± standard deviation (SD) of four independent experiments. Significant differences (**** $p \leq 0.0001$) were calculated by two-way ANOVA, followed by Dunnett's test, comparing the experimental conditions with negative control (DMSO 1%).

Both negative controls (water and DMSO 1%) showed low mortality, once it was below 20%, as recommended by the guidelines. After 48 hours, the average mortality for water and DMSO 1% controls was of 2.79% and 0%, respectively; without significant differences between them. Dimethyl sulfoxide was used at 1%, according to the above cited WHO recommendations, to evaluate the effect of the solvent on the larvae mortality. Positive control (temephos, at 3 µg/mL) was chosen to induce larvae death, and showed 100% mortality since the 24 hours of exposure. The concentration of temephos was chosen with basis in literature, to assure the larval death. It should be stressed that the values of LC_{90} in the literature are very distinct for this larvicide, depending also on the species tested and if it is a resistant strain (Biber, Dueñas et al. 2006, Floore 2006).

Significant differences were not found between the majority of the extracts and the solvent control, except for the *F. vesiculosus* dichloromethane extract. This extract showed to be very toxic to larvae, reaching 58% of mortality after 48 hours of exposure. In the larvae that survived, an abnormal behavior was observed. Instead of staying still at the surface of the water for breathing, the larvae were restless, swimming continuously up and down. There

is a lack of studies about how toxicity in mosquito larvae affect the swimming behavior, since the majority of the papers that study behavioral aspects usually focus in other aspects like oviposition or adult emergence. Moreover, the papers that study the effect of a toxic compound in mosquito larvae swimming, report an increasing in resting and a deceleration in swimming velocity (Tomé, Pascini et al. 2014, Marriel, Tomé et al. 2016), as opposed to observed in these assays. Regarding the time exposure, in all the conditions it was not found significant differences between 24 and 48 hours of exposure. This leads to the hypothesis that the mode of action of the extracts may be quick, once the only one who showed toxicity, only exhibit a difference of 7.8% between 24 to 48 hours.

U. lactuca extracts showed to be less harmful that *F. vesiculosus* ones. This is in agreement with the results of Manilal, Thajuddin et al. (2011). These authors tested the larvicidal activity of methanolic extracts from 20 different seaweeds from Chlorophyta (green seaweeds), Phaeophyta (brown seaweeds), and Rhodophyta (red seaweeds), in two species of mosquito: *C. quinquefasciatus* and *Ae. aegypti*. In that study it was concluded that brown algae show more larvicidal potential than the green algae. Guedes, Carvalho et al. (2014) also tested dichloromethane, methanol and ethanol extracts from *U. lactuca,* but the extracts did not show larvicidal activity against *Ae. aegypti*. In what regards mosquito larvicidal effects of extracts from *F. vesiculosus*, no studies were found. Due to this caveat, we can only analyze the effects of extracts from *Ulva* genus in other mosquito species, or the potential of other seaweeds against *Ae. aegypti* mosquito larvae, to evaluate on the one hand the potential of *Ulva* and, on the other hand, the possibility of using seaweed extracts against *Ae. aegypti* larvae. Anyway, despite there are several publications on this subject using other seaweeds, their results cannot be entirely extrapolated and compared to ours. Poonguzhali and Nisha (2012) tested methanol, acetone and benzene extract from *Ulva fasciata* against *Culex* larva. The extract did not show high toxicity, contrary of other previous studies. According to Manilal, Thajuddin et al. (2011), that studied the effects of extracts from 20 different seaweeds against *C. quinquefasciatus* and *Ae. aegypti* larvae, the first species was the most sensitive. Dichloromethane/methanol (2:1) extracts of *Canistrocarpus cervicornis*, *Hypnea musciformis* and *Chaetomorpha antennina* showed that more than half of the larvae of *Ae. aegypti* died at concentrations of 300 µg/mL (Bianco, Pires et al. 2013). In the same study, in a concentration of 50 µg/mL, extracts from *Laurencia dendroidea* showed a larvicidal activity of more than 91%.

Other example using seaweed extracts against 4th instar *Ae. aegypti* larvae was described by Ali, Ravikumar et al. (2012). Aqueous extracts (3:1) of *Syringodium isoetifolium*, *Cymodocea serrulata* and *Halophila beccarii* were tested. *S. isoetifolium* was the one which showed maximum larvicidal activity extract with minimum concentration of the extract with

LC_{50} values of 0.0604 µg/mL. A paper recently published (Adaikala Raj, Jayaraman et al. 2017) tested hexane, chloroform, ethyl acetate, acetone and methanol extracts of *Halimeda macroloba*, *Caulerpa racemosa* and *U. lactuca*, against *Ae. aegypti* larvae. The results showed that hexane, chloroform, ethyl acetate, acetone and methanol extracts of *H. macroloba, C. racemosa* and *U. lactuca* showed larvicidal activity against *Ae. aegypti*, but in all the three seaweeds, the ethyl acetate was the extract that produced higher mortality, reaching 90% at 1000 µg/mL in *C. racemosa*. Ahmad, Yu et al. (2016) tested methanolic extracts of 15 seaweeds in *Ae. aegypti* and *A. albopictus. Bryopsis pennata* was the only sample that exhibited larvicidal activity against both mosquito species, having LC_{50} values lower than 200 µg/mL; *Padina australis* and *Sargassum binderi* had larvicidal activity, with LC_{50} values comprised from 200 to 500 µg/mL, and the other seaweed extracts had LC_{50} values of more than 500 µg/mL, having therefore less potential. Yu, Wong et al. (2015) also used methanolic extracts from *B. pennata*, *S. binderi* and *P. australis* to access the larvicidal activity in *Ae. aegypti*, but with liquid–liquid partitions of hexane, chloroform and aqueous. Chloroform partition of *B. pennata* had the highest larvicidal potential (LC_{50} = 82.55 mg/mL), followed by the methanol extract from the same seaweed (LC_{50} = 160.07 mg/mL) and the chloroform partition of *S. binderi* extract (LC_{50} = 192.43 mg/mL).

With the above examples we infer that seaweed extracts are a promising tool to combat mosquitos. In respect to our results in particular, they are primary screening assays, which "only" show the possible potential of the tested extracts from *F. vesiculosus* when applied alone. More studies will be needed to determine the LC_{50} levels, like in the above examples.

2.2. Larvae measurements and weighings

After 48 hours of exposure, live larvae were collected and the length was measured. The results obtained are represented in figure 16 as body lenght expressed in millimeters.

The body size was similar in all the conditions with no significant differences when compared with the negative control. This means that regardless the toxicity, it does not affect larvae growth, at least in length. With Temephos it was not possible to measure the larvae length, once they all died after 24 hours of exposure and shrunk. There is a great deficiency in studies including larvae body length measurements. Most of published papers that evolve mosquito measurements are mainly focused in wing length or the body size of adult mosquitos (e.g. Lyimo, Takken et al. (1992); Strickman and Kittayapong (2003) or Armbruster and Hutchinson (2002)).

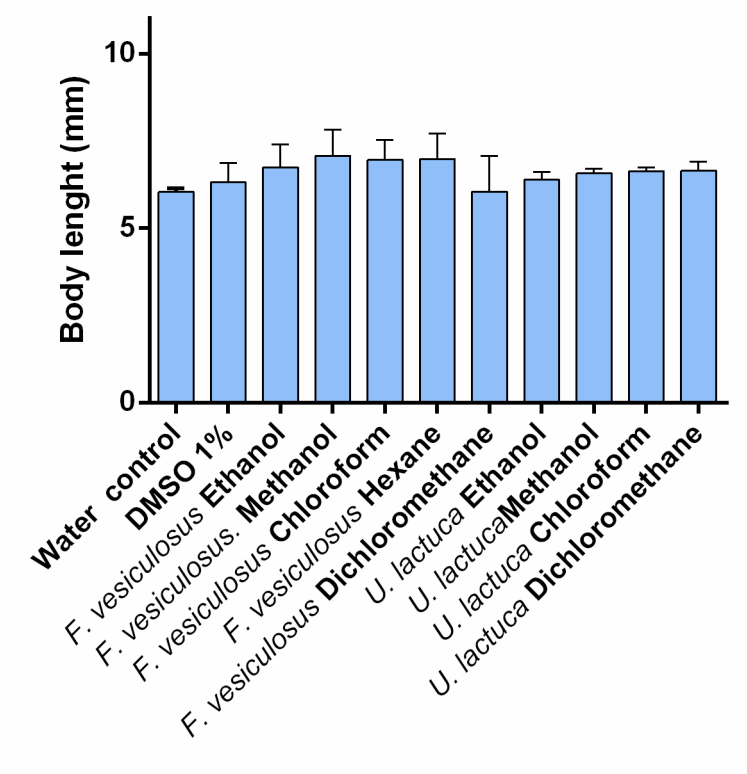

Figure 16 – Total length of the larval body, from the different treatments, given in millimeters (mm). Results are expressed as mean ± standard deviation (SD) of four independent experiments.

The results of larvae mass are represented in figure 17.

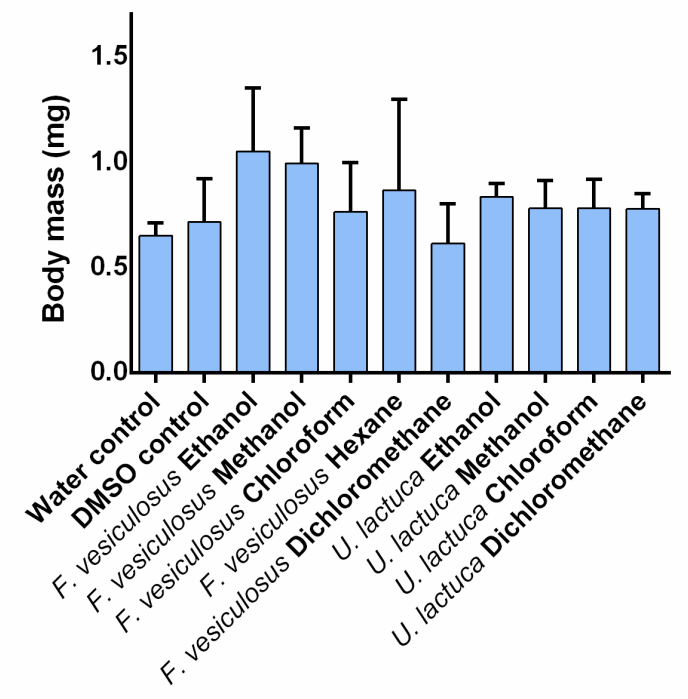

Figure 17 – Mass of the larvae from the different treatments, given in milligrams (mg). Results are expressed as mean ± standard deviation (SD) of four independent experiments.

No significant differences existed between the water and the DMSO control, meaning that the latter is not toxic and is safe to use herein. There were also no significant differences among the different conditions.

3. *Artemia salina* lethality assays

A. salina is widely used as a test organism for the assessment of acute toxicity of substances, and also as a non-target model organism, in several areas as ecology, ecotoxicology, aquaculture, among others (Nunes, Carvalho et al. 2006) (Guedes, Carvalho et al. 2014) .

Once *A. salina* is known to less sensitive when compared to some other organism models used in ecotoxicology testing (Nunes, Carvalho et al. 2006), a higher concentration that the one used in mosquito larvae were tested (500 µg/mL). The percentage of mortality after 24 hours of exposure represented in figure 18.

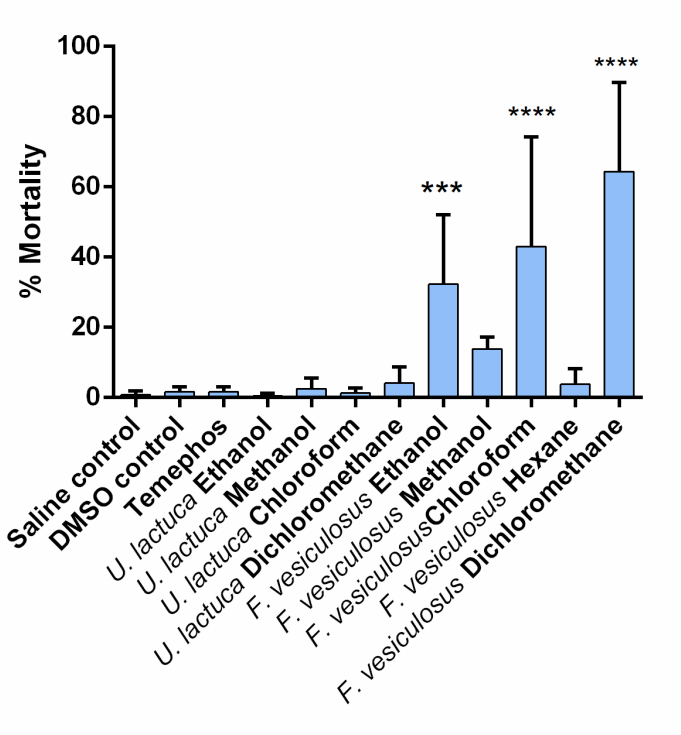

Figure 18 - Percentage of mortality of *Artemia salina* exposed to 500 µg/mL of the extracts. Results are expressed as mean ± standard deviation (SD) of at least three independent experiments. Significant differences (*** $p \leq 0.001$; **** $p \leq 0.0001$) between extracts and DMSO control were tested by One-way ANOVA, followed by Dunnett's multiple comparisons test.

To match the amount of DMSO present to reach the 500 µg/mL concentration, solvent control was readjusted to 2% DMSO, which showed to be nontoxic to nauplii and did not exhibit significant differences when compared to saline control. Three extracts (ethanol, chloroform and dichloromethane) from *Fucus vesiculosus* showed significant increase when compared to DMSO control, inducing 32%, 43% and 64% of mortality, respectively. All the other extracts showed very low or non-existent toxicity, with no significant differences comparing to solvent control. Only those extracts which showed some toxicity in the 500 µg/mL concentration were tested in the same concentration used in mosquito larvae (100 µg/mL) (figure 15). These results are expressed in figure 19.

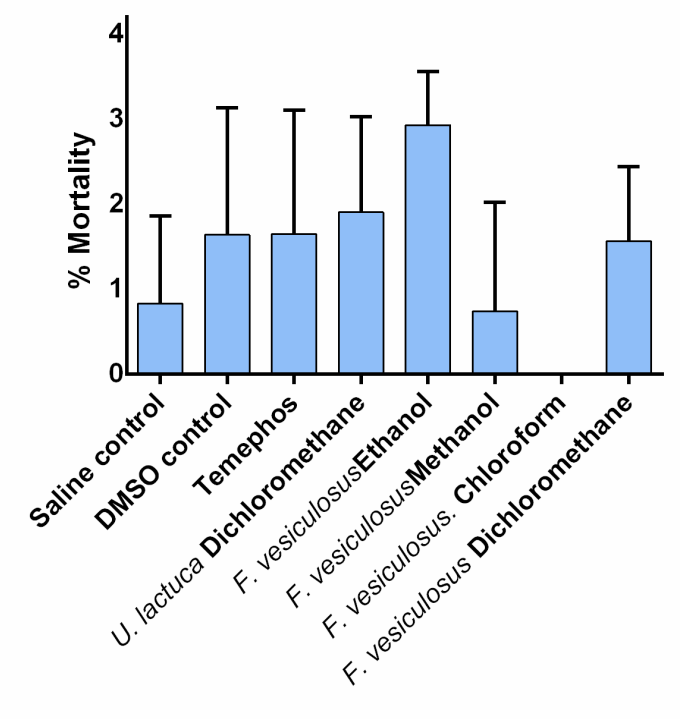

Figure 19 - Percentage of mortality of *Artemia salina* exposed to 100 µg/mL of the extracts. Results are expressed as mean ± standard deviation (SD) of at least three independent experiments.

When exposed at this concentration, the extracts exhibit a very low toxicity to *A. salina*, not showing significant differences in comparison with the control.Several studies already tested innumerable seaweed extracts and fractions against *A. salina*. Guedes, Carvalho et al. (2014), tested the same extracts mentioned in the topic 2.1. in brine shrimp. Only the chloroform and hexane fractions of *Hypnea musciformis* showed mortality, but with low toxicity. Manilal, Sujith et al. (2009) demonstrated that dichloromethane/ethanol 1:1 extracts from 13 seaweeds exhibit 100% mortality when in a concentration of 400 µg/mL, which is a lower concentration that the higher that we used. In our work, ethanol, chloroform and dichloromethane extracts at 500 µg/mL showed toxicity in *A. salina*, but with lower toxicity

than that observed in the work of Guedes, Carvalho et al. (2014). However, since the seaweed species are different, we cannot infer that the higher toxicity is due to the potential of the algae species or the differences in the solvent. Ara, Sultana et al. (1999) tested ethanolic extracts from 22 different seaweed species to access their toxicity in brine shrimp. More species from brown algae showed cytotoxicity when compared with green and red algae. LC_{50} values were in all the seaweeds ≥ 500 µg/mL, and in 16 of them, the LC_{50} was above 1000 µg/mL, which regards on the different species, which show that *A. salina* is way more resistant than mosquito larvae. *U. lactuca* extract deserves more attention, once is a species common with our work. In Ara, Sultana et al. (1999) study, ethanolic extracts from this green seaweed showed no toxicity at 100 µg/mL, which is consistent with our results. However, the authors showed an increase of the toxicity at 500 µg/mL, and this did not occur in our study. We can suppose that differences between studies can are attributed to technicalities between experiments, warning for the need of replication, namely under various conditions, to gain more robust knowledge on *U. lactuca* extracts.

Chapter V

Conclusions and future perspectives

Conclusions and future perspectives

The aim of this study was to screen the larvicidal activity of ethanol, methanol, chloroform, hexane and dichloromethane extracts from two different seaweed species found in Portugal – *F. vesiculosus* and *U. lactuca* – against the mosquito *Ae. aegypti*. These extracts were also tested in a well-established model organism in toxicity assessment – *A. salina* – to investigate possible effects of these extracts in non-target aquatic organisms.

The publications about seaweed extracts and their possible uses have been growing. However, there are distinct protocols of extraction, and there is a lack of information on the compounds extracted in each method. Herein, the extracts yields varied, depending on the solvent and on the seaweed used. So, in line with literature, our data also backs the need to standardize efficient extract protocols that could be suitable for every algae species, and to carry out more in-depth investigations in order to understand better the compounds to be extracted from each species and with different solvents.

The mortality assays with mosquito larvae showed that dichloromethane extract from *F. vesiculosus* have a high larvicidal potential. However, more studies are needed to access LC_{50} dosage and its mode of action. Also, it is needed to be tested in other models in order to ensure its non-hazard nature to other non-target species. Anyway, the current data show that the extract is well worth exploring as to the characterization of the exact compounds it contains, so to see which one(s) are the responsible for the larvicidal effect. As to the other extracts, that did not show significant mortality levels, more specific tests can be performed to look for possible sub-lethal cellular (functional or structural) injuries, once the body length and mass were not altered when exposed to the extracts, when compared with the control.

There is no much information in literature about the larvicidal potential of *Fucus* species against mosquitos, but our data support that extracts from other species from this genus are worth investigating to access for such potential, not only in the genus *Aedes*, but also in other genera with public health implications.

It should also be considered the untested possibility of combinatory effects of two or more extracts, to analyze if they have synergistic or potentiating effects. Another perspective that could be explored is the combination of the extracts (or of compounds extracted from them) with conventional used insecticides, with the same purpose in mind.

Chapter VI

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