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Analytical and biological studies on the immunomodulatory potential of flavonoids in fish aquaculture

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LIST OF ABBREVIATIONS

WHO	World Health Organization
SGR	Specific Growth Rate
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SOD	Superoxide Dismutase
CAT	Catalase
GSHPx	Glutathione Peroxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
iNOS	Inducible Nitric Oxide Synthase
PUFA	Polyunsaturated Fatty Acid
MDA	Malondialdehyde
PTFE	Polytetrafluoroethylenes
IS	Internal Standard
HPLC	High Performance Liquid Chromatography
HRMS	High-resolution mass spectrometry
MD	Methidathion
LC_{50}	Lethal Concentration
TBARS	Thiobarbituric Acid Reactive Substances
TBA	Thiobarbituric Acid
ALT	Alanine Transaminase
LDH	Lactate Dehydrogenase
TC	Total Cholesterol
H	Hematoxylin
E	Eosin
C	Control group
TC	Toxin Control group
FC	*F. hermonis* control group
SC	*S. marianum* control group
FT	Toxin/ *F. hermonis* treated group
ST	Toxin/ *S. marianum* treated group
H	Hepatocyte
V	Hepatic vein
BD	Bile duct
MMC	Melanomacrophage centres
S	Sinusoid

PL	Primary lamellae
SL	Secondary lamellae
PC	Pillar cell
CC	Chloride cell
PTH	Proliferative tissue hyperplasia
FS	Fusion of the secondary lamellae
BC	Blood congestion
CSL	Curling of secondary lamellae
HCC	Hypertrophy of chloride cells
N	Necrosis
MF	Muscle fiber
LCT	Loose connective tissue

CHAPTER

1

1. INTRODUCTION

Aquaculture (or Aquafarming) is the farming of selected aquatic organisms (such as fish, molluscs, aquatic plants, crustaceans, and algae) by intervention in the rearing process to increase production and private ownership of the stock being cultivated (Martínez Cruz et al., 2012).

The development of Aquaculture and its role as a food source to humanity has environmental and socio-economic limitations, affecting marine habitats and socio-economic scales from local use to worldwide implementation (Martinez-Porchas and Martinez-Cordova, 2012). Global wild fish catches have been for some time at or near the limits of what aquatic habitats can be expected to provide naturally (FAO, 2012). As demand for food increases with growing communities and incomes rise in developing countries (fish is often the lowest-cost animal protein), the global growing food fish supply “gap” has a negative impact on the health and nutrition of the poor populations (Henchion et al., 2017). In this context, Aquaculture (also known as Aqua-farming) has to fulfill that scarcity gap (Duarte et al., 2007).

The aquaculture commercially production has already reached 45 million tons, providing more than 40% of the worldwide food fish supply (FAO, 2016). Aquaculture production, however, is susceptible to increased events of contamination due to intensive production, resulting in severe loss of production (Bondad-Reantaso et al., 2005). Factors such as overcrowding, periodic handling, physical parameters fluctuation, and nutritional malfunctioning contribute to physiological changes in most of the culture's species such as oxidative stress. These interns accelerate the spread of pathogens that might lead to diseases (Cabello, 2006).

On the other hand, industrial chemical contaminants, such as pesticides, insecticides, drugs and their metabolites in Aquaculture, may pose a potential health hazard to aquatic organisms and in some cases in humans (Thompson and Darwish, 2019). These contaminants may bio-accumulate in fish at certain levels that might cause human health problems (for instance, mutagenic and carcinogenic effects).

Industrial chemical (Organic and inorganic) pollutant products are usually present in different amounts in various compartments of the ecosystem according to the anthropogenic inputs, the distribution and fate patterns that occur in the environment (Rhind, 2009). Insecticides, in particular, are considered to be among the most effective environmental contaminants, and their release into the environment is increasing rapidly since the last decades (Carvalho, 2017). Drugs, in particular antibiotics, are frequently used to treat or prevent disease and increase productivity (Brunton et al., 2019). However, they continue to have great attention, in particular, due to the greater understanding of their eco-toxicological importance in different environmental conditions, aquaculture and agricultural activities, and human health (Sabra and Mehana, 2015). Chemical pollutants released in the ecosystem may result from various sources and activities and may enter into the environment by a

wide range of pathways and processes. They can be translocated, dispersed in the environment, and bio-concentrated in aquatic organisms and terrestrial plants, causing increased land degradation, biodiversity loss (Inyinbor et al., 2018). Furthermore, they may enter the food chain, creating health risks for both humans and animals. Terrestrial and aquatic ecosystems are prone to get insecticides from airborne dust, suspended particles, and solutes, and they likely have higher inputs than outputs. Therefore, they can suffer from the accumulation of extremely harmful elements (Stehle and Schulz, 2015).

Among organic pollutants, insecticides are unique because they are manufactured to cause severe injury in target organisms. Because many of their target sites are mostly conserved across many species, they can cause harmful impact to non-target organisms, including fish (Aktar et al., 2009).

One of the most important groups of synthetic insecticides is organochlorine. Because they are extremely persistent, they are bio-accumulative and highly toxic to aquatic organisms. However, most of them have recently been prohibited in many parts of the world (Jayaraj et al., 2016). The next major group is organophosphates. They are less persistent than the organochlorines. However, many are highly toxic to non-target organisms such as aquatic organisms and humans (Fulton et al., 2013).

Although the main mechanism of organophosphate toxicity through the inhibition of acetylcholinesterase enzyme is well known over the past decade, some chronic adverse health effects demonstrate the involvement of organophosphate toxicity in the generation of oxidative stress (Vanova et al., 2018).

In recent years, several veterinary drugs are commonly used in Aquaculture to avoid or prevent oxidative stress outbreaks. Chemotherapy and other veterinary drugs are

administered usually as additives in fish feed or sometimes as baths or vaccination aiming to prevent diseases before they occur, as therapeutics or as growth enhancers (Rico et al., 2013). However, using of veterinary drugs is becoming more restricted recently since they show several side effects either for the environment or health safety. The overdose use of antibiotics has resulted in the development of resistant bacteria strains (Seyfried et al., 2010). Moreover, commercial antidotes are very expensive, and they have a negative aspect that a single vaccine is effective against only one kind of pathogens (Harikrishnan et al., 2011a). Considering the potentially toxic effects of veterinary drug treatments on the environment and humans, including their limited efficacy, disease management should concentrate on undisruptive, preventive, and long-lasting applications. Indeed, disease eruption is commonly associated with fish health, most pathogens taking advantage of stressed fish. As a result, alternative treatments should substantially improve fish immune systems combating pathogen infections (Iguchi et al., 2003). Some of the new proposed solutions are the use of natural products such as plant extracts in Aquaculture (Citarasu, 2010; Lee et al., 2009). Therefore, there is an increasing interest in chemical-free and environmentally friendly feedstuffs. This certainly provokes minimizing the use of chemical products used in Aquaculture and at the same time, support the idea of using natural treatments that might improve the consumption of healthy Aquaculture products.

1.1. THE SIGNIFICANCES AND PURPOSES OF THIS STUDY

The use of veterinary drugs is becoming more restricted since they present numerous side effects for the environment and health safety. Considering the potential harm of veterinary drug treatments on the environment and human health, some of the proposed solutions are the use of plant extracts in Aquaculture, mainly in the form of feed incorporation. Therefore, the goals of this study were to:

1. Measure the cytotoxicity of Methidathion (an organophosphate insecticide) as an oxidative agent model in tissues of exposed fish of Rabbitfish (*Siganus rivulatus*, fish were challenged by Methidathion). Methidathion will serve as a replica of inflammation and the innate immune response to infectious diseases (oxidative agent).

2. Investigate the possible antioxidant efficacy of some local Mediterranean flora (*Silybum marianum* and *Ferula hermonis*) . It is known that the Milk thistle seeds (*S. marianum*) and Zallouh root (*F. hermonis*) may present effects on animal physiology, and can be hypothesized antioxidant properties.

3. Investigate the role of such extracted antioxidants in the protection of *Siganus rivulatus* against the toxicity of Methidathion, through evaluation of fish health status by biochemical assays, pathohistological observations, and statistical analysis.

CHAPTER

2

2. LITERATURE REVIEW

Aquaculture is an essential resource in many Middle-Eastern countries. This is supported by the proximity to the Mediterranean and the Red Sea or large lakes and rivers and a strong tradition of fish production in some Middle-Eastern countries, the success of which induces the implementation in other regions. Water is a scarce resource in Jordan. Therefore, Aquaculture is being conducted in intensive conditions, high fish density and recirculating systems, conditions that pre predispose to stress and disease outbreaks.

Species selected for this study include the Rabbitfish, *Siganus rivulatus* which is a food fish that has considerable market demand in Jordan, Egypt, and other Middle Eastern and Mediterranean countries. It is also greatly valued in the Saudi Arabia markets and has been selected as an essential species in its national mariculture development program (Lichatowich et al., 1984; Bukhari, 2005). In Jordan, *Siganus spp.* holds the highest market price (8-10 US$/kg) of all commercial fish species in

the Gulf of Aqaba. It represent one-third of the national consumption of aquatic products, a total of about 3000 MT (Al-Zibdah et al., 2018). The Siganidae inhabit the Indo-Pacific to the Red Sea and Eastern Mediterranean. Their natural habitat is the seagrass areas close to coral reefs. Although there may be some production of juveniles for on growing, there is a high dependence on the capture of fingerlings from the sea for stocking cages, which may adversely affect natural populations. Exposure to stress and diseases and the resulting losses are among the significant constraints in the Aquaculture investment, and even more so in intensive production. Developing new tools for improving fish health will, therefore, aid in the further development of Aquaculture production in the region.

One of the most promising methods of controlling diseases in Aquaculture is by strengthening the fish's defense mechanisms through prophylactic administration of plant extracts, which is considered a promising alternative to chemotherapy and vaccines because of their broad-spectrum activity, cost-effectiveness, and eco-friendly properties. Medicinal plant extracts can increase disease resistance by enhancing both acquired and adaptive defense mechanisms of fish.

2.1. MEDICINAL PLANT

Plants, which account for a significant component of foodstuffs, have comprised the basis of various traditional medicine systems (Pan et al., 2013). Several plants have been used worldwide for thousands of years to add flavour and conserve food, to treat health disorders and to prevent certain diseases (Singh, 2015). The World Health Organization (WHO) has defined medicinal plants as plants that have properties or components that can be utilized for therapeutic objectives or those that

synthesize metabolites to produce useful drugs (Sofowora et al., 2013). There are about 300,000 species of extant seed plants around the world (Jiao et al., 2011). The number of natural products has now reached over 139,000 (Boopathy and Kathiresan, 2010), and every year, new chemical compounds of vegetal origin are discovered. Several drugs had originated from biologically active plant chemicals, their uses depending on the various aggressive chemicals found in them (George, 2011).

It is well known that the medicinal value of herbs/plants is determined based on the presence of natural active ingredient(s) with drug-like properties (Pan et al., 2013). Now more than 80% of drug substance is either derived directly from natural products or developed from natural compound (Maridass and Britto, 2008). Moreover, about 50% of pharmaceuticals are originated from compounds first identified or isolated from herbs/plants, including organisms, animals, and insects, as active ingredients (Krief et al., 2004). In Aquaculture, the use of natural products for health-promoting and disease control has gain interest, taking advantage of the possible harmful effects of antibiotics and chemicals in Aquacultured species and environment (Na-Phatthalung et al., 2018). Indeed, medicinal plants are used in Aquaculture not just as chemotherapeutics but also as feed additives (Wang et al., 2015), in which they contain a different type of nutrients and chemical compounds (Chang, 2000). They have been used in various forms, either as crude, or extracts or active compounds from the plant, either singly or as a blend of extract compounds, or even as a mixture with other immunostimulants (Van Hai, 2015).

2.1.1. THE POTENTIAL USE OF MEDICINAL PLANT IN AQUACULTURE

Recently plant extract has been exploited in Aquaculture, natural plant products show several biological activities such as antimicrobial, anti-inflammatory, anti-oxidative, anti-parasitic activities, anti-stress and growth-promoting effects, some of which providing benefits for fish health management (Van Hai, 2015; Morales-Covarrubias et al., 2016; Na-Phatthalung et al., 2018). Immunomodulatory activity is one of the predominant properties of medicinal plants (Na-Phatthalung et al., 2018), it has a positive impact on fish immune responses, which enhances a higher degree of disease resistance and stress tolerance (Chakraborty and Hancz 2011; Harikrishnan et al., 2011a).

There is a growing interest in the use of medicinal plants in Aquaculture. Recently, it has become the subject of active scientific research in many countries (Van Hai, 2015). Medical plants can be used in different forms such as crude, extract, or active component. However, crude plants mixture has the benefit of little effort required to obtain and apply it, especially for Aquaculture farmers (Wu et al., 2013). The mode of action of medical plants and their secondary products are assigned to the existence of many active principle components such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Harikrishnan et al., 2011b; Sivaram et al., 2004). Herbs, spices, seaweeds, herbal extracted compounds, traditional medicines, and commercial plant-derived products are used as complementary medicinal plants. The use of such plant taking advantage, because they are not expensive, easy to use, and are active with fewer side effects during the treatment of diseases (Jian and Wu, 2004) and without any impact on the environment and hazardous problems (Citarasu, 2010). Plant secondary metabolites

(such as phenolics, polysaccharides, proteoglycans, and flavonoids) play an essential role in preventing or minimizing the microbes infectious (Citarasu, 2010).

Milk thistle extracts (*Silybum marianum*) such as silymarin, can help in the treatment of disorders of the spleen, liver, and gall bladder (Wahsha and Al-Jassabi, 2009). It was primarily used as a therapeutic agent in liver disorders, including hepatitis, alcoholic liver diseases, and cirrhosis and was found useful for toxin-induced liver toxicity (Katiyar, 2005). Indeed, silymarin is a well-tolerated and effective antidote in hepatotoxicity produced by several toxins, including phalloides, ethanol and psychotropic drugs (Fraschini et al., 2002). Moreover, it acts as a free radical scavenger, with other liver specific properties that make it a unique hepatoprotective agent (Fraschini et al., 2002). Silymarin can offer strong protection against sub lethal doses of the cyanobacterial toxin, Microcystin, in mice (Wahsha et al., 2010), and also showed to prevent the peroxidation of lipid after the exposure to the hepatotoxin carbon tetrachloride and microcystin (Singh et al., 2002; Lakshmana et al., 2004; Dixit et al., 2007).

Different activities were also reported for *Ferula hermonis* including antibacterial, anti-fungal and insecticidal activities (Al-Jafari et al., 2011); anti-inflammatory (Geroushi et al., 2011) and cytotoxicity (Auzi et al., 2008; Elouzi et al., 2008). The antimicrobial capacity of the essential oil of some *Ferula sp.* were reported, in this contest studies were carried out on *Ferula gummosa* (Eftekhar et al., 2004), *Ferula narthex* (Kar and Jain, 1971) and *Ferula lycia* (Kose et al., 2010) as well as the gum resin of *Ferula gumosa* (Vaziri, 1975). This also together with the isolated constituents of *Ferula persica* root (Shahverdi et al., 2005), *Ferula communis* rhizomes (Al-Yahya et al., 1998) and *Ferula kuhistanica* fruit (Tamemoto et al.,

2001). However, still work is needed on the antimicrobial and antioxidant activities of *Ferula hermonis* (Hilan et al., 2007; Abourashed et al., 2011).

The immuno-stimulating activity of herbal components has been most widely studied in mice, chickens, and human cell lines. In Aquaculture, medicinal plants were recently used as chemotherapeutics and feed additives. Moreover, the antioxidants (immune stimulants) have the properties of growth promoting ability, a tonic to improve the immune system, antimicrobial capability, and stimulating appetite and anti-stress characteristics (Chang, 2000; Citarasu, 2010). The methods of treating microbial diseases in fish are problematic, neither useful nor cost efficient, because a large amount of chemotherapeutic agents is needed and then discharged into the environment, this poses a risk to animals and human health. Drugs like antibiotics accumulate in the fish muscles and may cause a negative effect health-wise (Cabello, 2006; Romero Ormazábal et al., 2012), moreover, their dispersion in the environment at low concentration may induce antibiotic-resistant bacteria (Monteiro et al., 2018).

2.1.2. MEDICINAL PLANT AS APPETITE STIMULATORS AND GROWTH PROMOTERS

Several studies have reported that medicinal plants can be used to stimulate appetite and as growth promoters when they are administered to cultured fish (Pavaraj et al., 2011; Takaoka et al., 2011; Harikrishnan et al., 2012). Ordinarily, the process onset with the enhancement of digestive enzymes, and then increased growth and survival rate of fish (Harikrishnan et al., 2012; Awad et al., 2012). Plant extracts have been shown to enhance digestibility and availability of nutrients giving an increase in feed conversion and promoting to higher protein synthesis (Nya and

Austin, 2009; Citarasu, 2010; Talpur et al., 2013b). On this topic, Mahdavi et al. (2013) proved that the use of *Aloe vera* extract by (0.1, 0.5 and 2.5%) was efficient to stimulate appetite and promote weight gain for common carp where a considerable increment in weight gain and specific growth rate (SGR) was observed after 8 weeks of feeding. In addition, Santoso et al. (2013) reported that incorporated diet with 1% of ethanolic katuk extract (*Sauropus androgynous*) could stimulate appetite, growth and improved food utilization in grouper *Ephinephelus coioides*. Moreover, rainbow trout recorded a significant increase in growth performance, especially weight gain, SGR and digestive enzymes after two months feeding with a diet supplemented with 1% and 2% of lupine, mango and nettle (Awad et al., 2012).

Francis et al. (2002) reported an increase in weight gain of common carp using saponin (extracted from Quillaja) as a growth stimulator at a level of 150 mg/kg diet for eight weeks. Furthermore, Methanolic extracts of some herb such as *Solanum trilobatum*, *Andrographis paniculata*, and *Psoralea corylifolia* can improve the survival and SGR of black tiger prawns (Citarasu et al., 2003). Moreover, Shalaby et al. (2006) reported an increase in feed intake, SGR and weight gain of Nile tilapia after fed for 12 weeks on a diet containing 10, 20, 30 and 40 g/kg of garlic. Other studies recorded an increase in growth performance of Nile tilapia after administration of a diet incorporated with 15% and 45% of wet date for 3 months (Gaber et al., 2014), 10 and 20 g/kg of garlic for 2 months (Aly et al., 2008) and 0.5% of oregano extract for 10 weeks (Ahmad et al., 2009). According to Lin et al. (2006) using a blend of some herbs and plant materials can affect enzyme activity, nutrients digestibility and stimulated the diet to pass speedily out of the digestive tract of white leg prawns. Moreover, Ji et al., (2007) observed a significant weight gain and total unsaturated fatty acid content of Olive flounder (*Paralichthys olivaceus*) after feeding

with a mixture of plant extract; *Crataegi fructus*, *Artemisia capillaries*, *Massamedicata fermentata*, and *Cnidium officiale* (2:2:1:1). The antimicrobial and anti-stress properties of some herbal products can notably increase the survival rate of black tiger prawn larvae (Citarasu et al., 2002).

On the other hand, it is worth to point out that growth-promoting effect of a medicinal plant depend on the dosage used in diets (Awad and Awaad, 2017). For example, canola extracts more than 300 g/kg of food can cause depression of feed intake, and growth performance whereas the lower doses (100, 200 g/kg) increased the growth rate of Asian seabass (Ngo et al., 2016). Furthermore, Chatzifotis et al. (2008) indicated a reduction in seabream growth when fed diet supplemented with caffeine at a concentration higher than 1 g/kg. Moreover, no change in weight gain and specific growth rate of seabream fed with diet contain cottonseed up to 16% for eight weeks (Sun et al., 2015). Also, *Argyrosomus regius* fed diet contain different amounts of carob seed germ meal (75, 150, and 225 g/kg food diet), exhibit an increase in weight gain, feed intake and feed efficiency ratio just at 75 g/kg concentration (Couto et al., 2016).

2.1.3. MEDICINAL PLANTS AS AN IMMUNOSTIMULANT

The immune system is a set of biological mechanisms that protects living organisms from invading pathogens; it assorts into innate (non-specific) and adaptive (specific) immune systems. The immune responses in fish are intermediated by a variety of cells and secreted soluble mediators, which deed in synergistic form for perfect protection (Secombes and Wang, 2012). The innate immune system of vertebrate acts as the first line of defense against invading pathogens (Narnaware et al., 1994). It is responses to infectious pathogens specified by the evolutionary lineage and

genetic makeup; it has been modified with time due to environmental factors and pathogenic associations (Janeway and Medzhitov, 1998; Du Pasquier, 2001, 2004; Alvarez-Pellitero, 2008). Mainly the innate immune system components are macrophages, monocytes, granulocytes, and humoral elements, together with lysozyme or complement system (Secombes and Fletcher, 1992; Magnadottir, 2006). In fish and shellfish, the innate immune system made up of neutrophil activation, production of peroxidase and oxidative radicals, jointly with the initiation of other inflammatory factors (Ellis, 1977; Ainsworth et al., 1991). The innate immune system starts the mechanism of action of the immune system in responding to a pathogen by pathogen recognition receptors that discover and react to the pathogen-associated molecular patterns (Palm and Medzhitov, 2009). Also, the pathogen recognition receptors detect hazard-associated particle patterns, which are endogenous particles released by damaged or stressed host cells (Secombes and Wang, 2012). An adaptive immunity or specific immunity is qualified to effectively identify particular pathogens and make immunological memory (Harikrishnan et al., 2011c). The responses involve a complex network of cells, genes, protein, and cytokines that promote the host to respond to antibodies and antigens (Uribe et al., 2011).

Fish will have two choices, it will survive if they successfully fight against the infection of the pathogen or die if they may not succeed in preventing the spread of the disease. Therefore, survival or death are mainly depended on the status of the immune system to hostilities the initial infection or the spread of the pathogens (Barman et al., 2013). Immunostimulants are naturally occurring substances that enhance the defense mechanisms of the immune system (innate and adaptive), and as a consequence make the animal able to overcome with diseases (Galindo-

Villegas and Hosokawa, 2004). The biological activities properties of medicinal plants rise interest and encourage their use as immunostimulants in Aquaculture. Numerous studies have reported the amelioration in immunological parameters in several fish species after administration of medicinal plants or extracts like phagocytic activity, respiratory burst activity, nitrogen oxide, myeloperoxidase content, complement activity, lysozyme activity, total protein (globulin and albumin) and antiprotease activity (Yuan et al., 2007; Wu et al., 2010; Talpur and Ikhwanuddin, 2012; Wu et al., 2013; Talpur, 2014). However, disease outbreaks in commercial fisheries may be adjusted by strengthening of innate immunity using natural immunostimulants (Robertsen et al., 1990; Sakai et al., 1991; Anderson, 1992; Siwicki et al., 1994). For best results, it is necessary to retain that the utilization of medicinal plant immunostimulants should be carried before a disease outbreak (Galindo-Villegas and Hosokawa, 2004).

Phenolics, polysaccharides, proteoglycans, and flavonoids are secondary plants metabolites; they play a considerable function in prohibition or controlling infectious microbes (Citarasu, 2010). In addition to that, secondary plant metabolites have the potential to overcome the generation of oxygen anions and scavenge free radicals (Harikrishnan et al., 2011a). In one shrimp culture of *Picrorhiza kurroa* has been efficiently used as an anti-stress compound (Citarasu et al., 1998), likewise, *Ocimum sanctum* has a positive immunostimulatory effect, it improved the antibody response and disease impedance in *Oreochromis mossambicus* against *Aeromonas hydrophila* infection (Logambal and Michael, 2000).

Garlic one of the most used medicinal plants in the Mediterranean countries and throughout human history as a consequence of its biological activities such as antimicrobial, anticancer, pro-circulatory effects, hepatoprotective, and

immunostimulant (Amagase et al., 2001). The immunomodulatory potential of garlic encourages it is used as a food supplement in rainbow trout (Nya and Austin, 2009), hybrid tilapia (Ndong and Fall, 2011), Asian seabass (Talpur and Ikhwanuddin, 2012), and Caspian roach (Ghehdarijani et al., 2016). The studies revealed an increase in cellular innate immune parameters (phagocytic activity and respiratory burst) and humoral immune parameters (total protein, lysozyme, antiprotease, and bactericidal activities) along with enhanced resistance against pathogenic bacteria.

Immunological parameters were monitored (lysozyme activity, respiratory burst activity, alternative complement activity, and phagocytic activity) in Nile tilapia (*Oreochromis niloticus*) after fed with diets containing different doses of mistletoe (*Viscum album* coloratum) for 80 days, the results displayed an increase in immunological parameters activity. Then after that period, fish were exposed to a bacterium *Aeromonas hydrophila*, treated fish had about 42% more survivability than the control group (Park and Choi, 2012).

Common onion (*Allium cepa*) is one of the most widely used vegetable overall in the world, rich in trace elements, flavonoids, vitamins, and sulphur compounds (Breu, 1996). Onion recognized as a medicinal plant due to its antioxidant, antibacterial and anticancer activities (Ramos et al., 2006b; Jeong et al., 2009), besides their ability to rise catabolism of lipids (Kumari and Augusti, 2007). Akrami et al. (2015) showed noticeable enhancement of innate immune parameters of beluga fish (respiratory burst, lysozyme, total protein, globulin, and immunoglobulin) after eight weeks oral administration of 0.5 and 1% of onion bulbs.

Another study showed the effect of monkey head mushroom (*Hericium erinaceum*) on the immunological system in olive flounder (*Paralichthys olivaceus*), the results revealed (30 – 45%) reduction in the mortality of the infected olive flounder

(*Paralichthys olivaceus*) with scutociliate *Philasterides dicentrarchi* when the fish fed with monkey head mushroom (*Hericium erinaceum*) enriched diet (0.1 and 1% respectively) compared to control group which had a mortality of 90%. The treated groups represented an increase in the Lysozyme activity and burst respiratory activity of olive flounder, indicating that enhancement of the immunological system leads to a better protection of olive flounder against *Philasterides dicentrarchi* (Harikrishnan et al., 2011b). Also Kim and Lee, (2008) showed that the use of kelp (*Ecklonia cava*) as dietary supplement enhances the non-specific immune response (increased phagocytes, respiratory burst, serum lysozyme, and myeloperoxidase activities) in juvenile olive flounder (*P. olivaceus*), as a consequence to the high antioxidant and polyphenolic content in *Ecklonia cava*.

In general, plant extracts have the potential to enhance the phagocytic activity in different fish species (Logambal et al., 2000; Chakrabarti and Rao, 2006; Gopalakannan and Arul, 2006). Phagocytic activity is a primitive defense mechanism, in which phagocytes produce toxic oxygen forms during a process called respiratory burst (Neumann et al., 2001). Several studies revealed an increase in phagocytic activity resulted from the administrations of plant extracts, for example the phagocytic activity increased when kelp grouper were fed *Lactuca indica* extract supplemented diets (Harikrishnan et al., 2011c), Chinese sucker (*Myxocyprinus asiaticus*) were fed *Herba epimedii* extract enriched diet (Zhang et al., 2009), and olive flounder were fed *Prunella vulgaris* extract enriched foods (Harikrishnan et al., 2011b).

2.1.4. MEDICINAL PLANTS AS FISH ANTI-PATHOGENIC

Several studies have highlighted a wide range of bioactivities proved by natural products from plants, fungus, and algae, the antimicrobial properties of medicinal plants and their active by-product compounds were inspected by many researchers (Tagboto and Townson, 2001; Zheng et al., 2007; Zahir et al., 2009). Numerous studies showed that plant extracts exhibited high antibacterial activity, besides their ability to used to treat specific diseases caused by viruses, parasites, and fungi (Hai, 2015).

Antibacterial properties of phytochemicals with potential applications in Aquaculture systems represented the most studied bioactivities (Reverter et al., 2014). Castro et al. (2008) examine 31 methanolic extracts of Brazilian plants against pathogenic fish bacteria, the authors found that all the examined methanolic extracts exhibited antibacterial activities (agar diffusion assay) against pathogenic bacteria such as *Streptococcus agalactiae*, *Flavobacterium columnare*, and *Aeromonas hydrophila*, being *Flavobacterium columnare* the most susceptible microorganism to most of the tested extracts. Wei and Musa (2008) studied the susceptibility (minimum inhibitory concentration) of *Staphylococcus aureus* and *Streptococcus agalactiae*, *Citrobacter freundii*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* and 18 isolates of *Edwarsiella tarda* to garlic aqueous extract (500, 250, 125, 62.5 mg/ml), and found that all garlic extracts were efficient against the tested pathogenic bacteria.

Chitmanat et al. (2005) found that Indian almond can be used as an alternative antibacterial remedy for tilapia ectoparasites and the bacterial pathogen *Aeromonas hydrophila*. Abutbul et al. (2004) used *Rosmarinus officinalis* to treat *Streptococcus* infection in tilapia (*Oreochromis sp.*). Shangliang et al. (1990) studied the potential

use of herbal extract of five Chinese plants (*Stellaria aquatica*, *Impatiens biflora*, *Oenothera biennis*, *Artemisia vulgaris*, and *Lonicera japonica*) against thirteen bacterial fish pathogens, his results revealing that *Aeromonas salmonicida* and *Edwardsiella ictaluri,* were the most susceptible to these extracts. Cinnamon was found to have the potential to use as an antibacterial activity inimical to *Aeromonas hydrophila* infection in Nile tilapia (Ahmad et al., 2011). *Ocimum sanctum* poses immunostimulatory effects, and it was also strengthening the antibody response and disease resistance in *Oreochromis mossambicus* against *Aeromonas hydrophila* infection (Logambal et al., 2000).

Ginger roots are generally used as a spice or drink (Afzal et al., 2001). They are associated with different biological activities such as antibacterial (Jagetia et al., 2003), anti-inflammatory (Chrubasik et al., 2005; Grzanna et al., 2005), antifungal (Agarwal et al., 2001), antiviral (Denyer et al., 1994), anti-tumour properties (Nagasawa et al., 2002), addition to immune-modulatory agent for many fish species (Nya and Austin, 2009; Immanuel et al., 2009; Talpur et al., 2013a). Ginger showed positive influence in the defense mechanism of Asian seabass (Talpur et al., 2013a) and rainbow trout (Nya and Austin, 2009) against pathogenic bacteria; *Vibrio harveyi* and *Aeromonas hydrophila*, respectively. The mechanism of action comprising the activation of the cellular immune response (phagocytosis and respiratory burst) and humoral immune response (lysozyme, bactericidal, antiprotease, and total protein) was monitored in fish with the effective dose of 1% ginger.

Indian major carp (*Labeo rohita*) fed diets incorporated with (0.2%) Indian ginseng (*Achyrantes aspera*) and (0.5%) prickly chaff flower (*Whitania somnifera*) showed a reduction in mortality up to 41% and 49% respectively when it was challenged against *Aeromonas hydrophila* compared to control groups (Vasudeva-Rao et al.,

2006; Sharma et al., 2010). In a similar study, tilapia (*Oreochromis mossambicus*) intraperitoneally injected with water extracts of *Solanum trilobatum* (400 mg/Kg) and *Toona sinensis* (8 mg/kg), the results revealed that, after challenged against *Aeromonas hydrophila,* 27% and 57% respectively in reduction of mortality compared to control groups (Divyagnasweri et al., 2007; Wu et al., 2010).

2.1.5. CLINICAL EFFECTS AND APPLICATIONS OF MEDICINAL PLANTS IN AQUATIC ORGANISMS

One of the most promising methods of controlling diseases in Aquaculture is by strengthening the defense mechanism of fish through prophylactic administration of antioxidants (immuno-stimulants), it is considered as a promising alternative to chemotherapy and vaccines because of their broad-spectrum activity, cost-effectiveness and eco-friendly disease preventative measure. The immuno-stimulants are effective means of increasing immuno-competency and disease resistance by enhancing both specific and non-specific defense mechanisms of fish and shellfish and other animals. Several immuno-stimulants have been developed and found to be effective in fish and shellfish, including chemical agents, bacterial components, polysaccharides, and animal or plant extracts. Numerous reports have demonstrated that *Polygonum multiflorum* extract exhibits a variety of pharmacological effects, such as antioxidative action (Chiu et al., 2002) and free-radical scavenging effects (Chen et al., 1999). Several plants or their by-products contain phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine, and polypeptide compounds, many of which are effective alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds (Harikrishnan et al., 2011a). The immuno-stimulating activity of herbal components mostly has been widely studied in mice,

chickens, or human cell lines. In Aquaculture, medicinal plants were recently used as chemotherapeutics and feed additives.

Moreover, the antioxidants (immune stimulants) have the properties of growth promoting ability, a tonic to improve the immune system, antimicrobial capability, and stimulating appetite and anti-stress characteristics (Chang, 2000; Citarasu, 2010). The methods of treating microbial diseases in fish are problematic, neither practical nor cost-efficient, because a large amount of chemotherapeutic agents is needed and then discharged into the environment, poses a risk to animals and human health in addition to stimulating the development of resistant bacteria. A possible method to confront these problems might be to use the bioencapsulation technique. Bioencapsulation with live feed is a suitable approach to convey extracts into the hosts such as *Artemia* (Immanuel et al., 2004). *Artemia* enriched with butanol extract from *Withania somnifera* reduced *Vibrio parahaemolyticus* and *Vibrio damsela* infection in prawns (Praseetha, 2005) or with the combination of five herbs increased the growth and survival of black tiger prawns (Citarasu *et al.,* 2002).

Several studies have proved that herbal plants can be used as promising antibiotics that after challenging with pathogens, the survival rates of infected fish priority fed various immunostimulants, vaccines, and probiotics, increased. After a challenge with *Aeromonas hydrophila*, the best survival rate was observed in fish treated with herb and boron. The methanolic extracts of three ayurvedic herbals via *Solanum trilobatum, Andrographis paniculata,* and *Psoralea corylifolia* showed the protection of *Penaeus sp*. Against nine pathogens such as *Bacillus subtilis, Proteus vulgaris, Salmonella typhi, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Vibrio sp., Staphylococcus aureus,* and *Aeromonas hydrophila.* Butanolic extract of *Withania somnifera* through *Artemia* enriched diet successfully

controlled *Vibrio parahaemolyticus* and *Vibrio damsela* infection in prawns (Praseetha, 2005). *Psidiumguajava* could control disease caused by *Aeromonas hydrophila* in Nile tilapia. They also showed that green tea, cinnamon, and American ginseng improved the resistance of Nile tilapia against *Aeromonas hydrophila* infection dietary intake of tulsi (*Ocimum sanctum*) (Logambal et al., 2000) as well as in Belly Tilapia (Wahsha and Al-Zibdah, 2014). It was also reported that heartleaf and moonseed leaf extracts protected *Oreochromis mossambicus* against *Aeromonas hydrophila* (Sudhakaran et al., 2006). Both herbs enhanced the survival rate after a challenge with *Aeromonas hydrophila* (Ardó *et al.,* 2008). Nile tilapia *Oreochromis niloticus* fed a mixture of *Astragalus,* and *Lonicera* extracts supplementation diets enhanced the respiratory burst, phagocytic activity of blood phagocytes and increased plasma lysozyme activity, and increased survival rate against *Aeromonas hydrophila*. *Azadirachta indica* (Neem) plant extract at a concentration of 150 mg/l in vivo was reported as another possible choice of antibiotics for treating bacterial infection *Citrobacter freundii* in *Oreochromis mossambicus* (Thanigaivel, 2015). In common carp (*Cyprinus carpio)* and large yellow croaker *Pseudosciaena crocea* fed a ration containing a mixture of *Astragalus membranaceus* (root and stem), *Polygonatum multiflorum*, *Isatis tinctoria*, and *Glycyrrhiza glabra* containing a mixture diets with 0.5 and 1% observed that significantly increased phagocytosis, respiratory burst activity and total protein level (Yuan et al., 2007). Phagocytosis by white blood cells and lysozyme activity in the serum of crucian carp were both increased by feeding four different herbs. Namely, *Rheum officinale, Andrographis paniculata* and *Lonicera japonica* (Chen et al., 2003). A supplementation of *Ocimum sanctum* and *Withania somnifera* improved the immune system and reduced mortality in greasy grouper juveniles during *Vibrio*

harveyi infections (Sivaram et al., 2004). Herbal extracts enhanced the immune responses of grouper *Epinephalus tauvina* against the pathogen *Vibrio harveyi*. The dietary supplementation of *Lactuca indica* extract increased disease resistance in kelp grouper against *Streptococcus iniae* infection (Harikrishnan et al., 2011c). Dietary *Angelica sinensis* polysaccharide enhanced some cellular immune parameters and disease resistance against *Edwardsiella tarda* in *Epinephelus malabaricus* (Wang et al., 2011). Recently, a bacteriolytic activity and leucocyte function was improved by mixtures of Chinese herbs in shrimp (*Penaeus chine*nsis) and tilapia (Chansue et al., 2000). In addition, *Penaeus indicus* juveniles fed with seaweed extracts were protected from *Vibrio parahaemolyticus* (Immanuel et al., 2004). Diets with five herbal extracts decreased the *Vibrio harveyi* load in black tiger prawn after bath challenging with these bacteria. Guava eliminated luminous bacteria from black tiger prawns more effectively than did oxytetracycline (Direkbusarakom, 2004). Brown seaweeds were also used as alternatives to antibiotics to control common diseases in black tiger prawns (Immanuel et al., 2010; Vaseeharan et al., 2011). The traditional Chinese medicine formulation of four herbs was used as a prophylactic approach for disease control and replaced the use of antibiotics for treating enteritis in grass carp (Choi et al., 2014).

2.2. ENVIRONMENTAL STRESS IN AQUACULTURE

Aquaculture activities are exposed to a considerable number of biological and environmental factors such as changes in feed, climatic variables, handling, regrouping, therapeutic and prophylactic activities, various stressors, and so forth.

The ability of the cultivated organisms to compete against these factors is vital for the maintenance of their productivity.

This section is about the interactions between Aquaculture and its ecosystem in terms of environmental induce oxidative stress and how these interactions can be managed in the best interests of environmental sustainability.

2.2.1. OXIDATIVE STRESS

Animals in their life are subjected to a high number of biological and environmental factors like modification in feed and rearing practices, climatic changes, therapeutic and prophylactic activities, and various stressors. The ability of an animal to withstand these factors is critical for the maintenance of their health and productivity (Rahal et al., 2014). Several research suggest that diet act as a powerful tool for the control of some chronic diseases (Kumar et al., 2010, 2011). Diet enriched with medical plants have been pointed out to exert a protective role in overcoming a variety of diseases, such as cardiovascular disease and cancer (Cox et al., 2000; Ahmad et al., 2006; Mahima et al., 2012). The chemical compounds thought to award protection offered by medical plants are the antioxidants (Eastwood, 1999; Rahal et al., 2013).

Several articles focus on the potential role of oxidative stress in the generation of chronic pathological conditions and even aging in general. Consequently, it has been proposed that oxidative damage and Reactive Oxygen and Nitrogen Species (ROS, RNS) play an important role in the initiation or development of numerous disorders (Ames et al., 1995; Hoeschen, 1997). In farm animals, oxidative stress may be implicated in various pathological conditions, in addition to the terms that are

relevant for animal production and the general well-being of the individuals (Lykkesfeldt and Svendsen, 2007). Furthermore, Antioxidant therapy provides a potentially essential and cheap alternative solutions for the treatment of diseases related to oxidative stress, although its use remains controversial (Lykkesfeldt and Svendsen, 2007).

Any stimulant, whatever it concerns the social, the physiological, or the physical, it is understood by the body as challenging, threatening, or demanding, can be classified as a stressor. The existence of a stressor results in the activation of neurohormonal regulatory mechanisms of the body, leads to maintain the homeostasis (Dimitrios et al., 2003). The total physiological effect of these factors and the adaptation capacity of the body govern the alteration in growth, development, productivity, and health status of the animals (Hoult et al., 1994; Lundgren et al., 2013). Thus any variations in homeostasis result in increasing production of free radicals, much more the detoxifying capacity of the local tissues (Trachootham et al., 2008). These free radicals then react with other biomolecules within cells created oxidative damage to proteins, membranes, and genes (Rahal et al., 2014).

2.2.2. OXIDATIVE STRESS AND ANTIOXIDANTS

Normal biochemical reactions, exposure to the environment, and elevated levels of dietary xenobiotics result in the production of unstable compounds known as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Bagchi and Puri, 1998). ROS and RNS are the mediator for the oxidative stress in several pathophysiological conditions (Kim and Byzova, 2014). Cellular components are changed under oxidative stress conditions, give rise to various disease states (Nimse and Pal, 2015). The body can be effectively neutralize oxidative stress by

promoting cellular defenses in the form of antioxidants (Sies, 1997; Pal and Nimse, 2006). Specific compounds act as in vivo antioxidants by elevating the levels of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) increases the level of endogenous antioxidants (Thomas and Kalyanaraman, 1997). Moreover, dietary antioxidants, those that can reduce the deleterious effects of ROS (Demmig-Adams and Adams, 2002), have been proposed to be useful for health promotion (Piskounova et al., 2015).

2.2.3. GENERATION OF FREE RADICALS

To understand the mode of action of antioxidants, it is essential to learn about the generation of free radicals and their damaging reactions (Nimse and Pal, 2015). The generation of ROS starts with quick uptake of oxygen, activation of NADPH oxidase, and the production of the superoxide anion radical ($O_2^{\cdot-}$), then $O_2^{\cdot-}$ rapidly converted to H_2O_2 by SOD. The reactive species can also be produced by the myeloperoxidase halide H_2O_2 system, which is present in the neutrophil cytoplasmic granules. In case of existence of the chloride ion, which is prevalent, H_2O_2 is converted to hypochlorous (HOCl), a strong oxidant and antimicrobial agent (Babior, 1999). In addition, the ROS can also be generated from $O_2^{\cdot-}$ and H_2O_2 through 'respiratory burst' by Fenton and/or Haber–Weiss reactions (Knight, 1999). On the other hand, enzyme nitric oxide synthase generates reactive nitrogen species (RNS), such as nitric oxide ($NO^{\cdot}$) from arginine. An inducible nitric oxide synthase (iNOS) has the ability to generating continuously a large amount of $NO^{\cdot}$, which act as a $O_2^{\cdot-}$ quencher. Also $NO^{\cdot}$ and $O_2^{\cdot-}$ can react together to produce peroxynitrite (ONOO), a potent oxidant, thus, each can modulate the effects of others. Even though neither

$NO^{\cdot}$ nor $O_2^{\cdot-}$ is a strong oxidant, peroxynitrite is a powerful and versatile oxidant that acts to attack a wide range of biological targets (Zhu et al., 1992). The other sources that can generate free radicals included cyclooxygenation, lipooxygenation, lipid peroxidation, metabolism of xenobiotics, and ultraviolet radiation (Shahidi and Zhong, 2010).

2.2.4. DAMAGING REACTIONS OF FREE RADICALS

Oxidants are compounds that have the ability of oxidizing target molecules, which happen by one of three actions: abstraction of a hydrogen atom, the concept of an electron or the addition of oxygen. However, oxidants can also be endogenous act as signaling molecules that manage the major cascades, such as apoptosis and inflammation (Lykkesfeldt and Svendsen, 2007). The outcome of uncontrolled oxidative stress in cells, tissues, and organ injury due to oxidative damage (Nimse and Pal, 2015). It has been recognized that high levels of free radicals or ROS can inflict direct damage to the significant class of biomolecules, mainly polyunsaturated fatty acid (PUFA) of cell membranes (Ayala at al., 2014). The oxidative damage of PUFA, well known as lipid peroxidation, is exceptionally destructive, since it proceeds as a self-perpetuating chain reaction (Park et al., 2009).

Mainly hydroxyl radical ($HO\cdot$) and hydroperoxyl ($HO\cdot_2$) are most predominant ROS thoroughly affect lipids. The hydroxyl radical ($HO\cdot$) is a small, highly mobile, soluble in water, and chemically most reactive form of activated oxygen (Ayala et al., 2014). This molecule has short life spam generated from the metabolism of O_2 in cells and under a variety of stress conditions. Surprisingly a cell can produce around 50 hydroxyl radicals every second, which can be neutralized or attack biomolecules (Lane, 2002). As a consequence of Hydroxyl radicals generation, oxidative damage

to cells may take place since they unspecifically attack biomolecules, far from its site of production less than a few nanometres (Halliwell and Gutteridge, 1984).

The oxidative degradation of lipids (generally known as Lipid peroxidation) is a chain reaction that takes place when oxidants like free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially PUFA that require hydrogen abstraction from carbon, with oxygen addition resulting in lipid peroxyl radicals and hydroperoxides (Yin et al., 2011). In general, the lipid peroxidation process includes three steps: initiation, propagation, and termination (Kanner et al., 1987; Girotti, 1998; Yin et al., 2011). In the first step initiation, free radicals such as hydroxyl radical attack PUFA and abstract hydrogen forming the carbon-centered lipid radical. In the second step, the propagation, lipid radical speedily reacts with oxygen generating a lipid peroxy radicals, which also abstracts hydrogen from another lipid molecule producing a new lipid peroxy radicals and lipid hydroperoxide (Figure 1). In the third step termination reaction, when free radicals react with a nonradical, it regularly produces a new free radical, and this is why the process is often called a chain reaction mechanism. The free radical reaction ends when two radicals react to form a nonradical molecule. This occurs exclusively when the level of free radicals is sufficient to be a high probability of collision of two free radicals. However, living organisms have various molecules that speed up the termination by neutralizing free radicals and, accordingly, protecting the integrity of the cell membrane. (Yin et al., 2011; Sfriso et al., 2019).

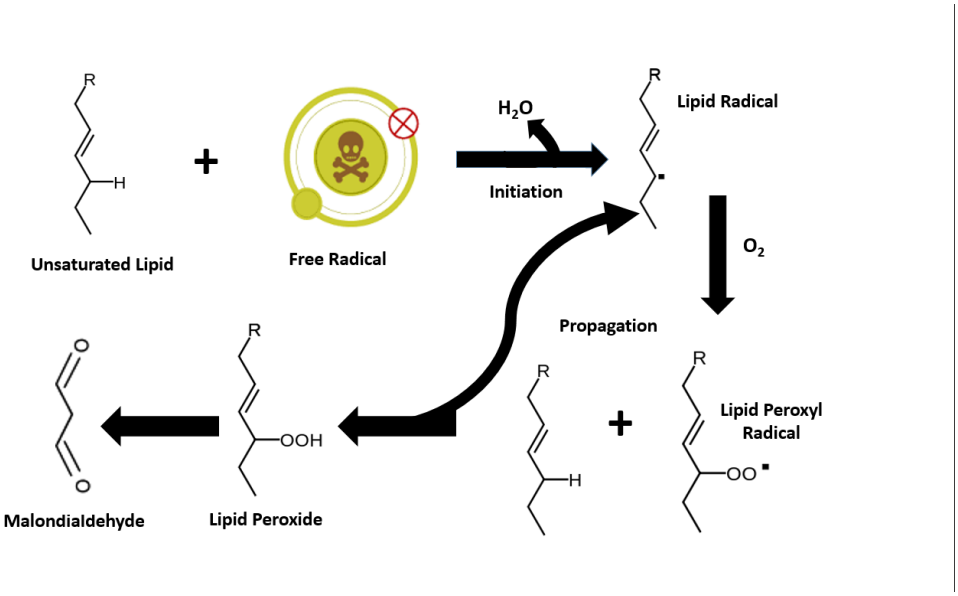


Figure 1: Lipid peroxidation process

Lipid hydroperoxides all the time break down to aldehydes, many of these aldehydes are biologically active compounds, which can spread out from the original site of the attack and spread the attack to the other parts of the cell (Devasgayam et al., 2003). Among several markers of oxidative stress, malondialdehyde (MDA) believe to be the most critical markers (Singh et al., 2011). Malondialdehyde (MDA) is a three-carbon compound generated from peroxidation of polyunsaturated fatty acids, mainly arachidonic acid, it is one of the end products of membrane lipid peroxidation (Fig. 1) (Rahal et al., 2014). The continued break down of peroxides to produce aldehydes ultimately results in loss of membrane integrity by modulation of its fluidity, which eventually leads to the inactivation of membrane-bound proteins (Ayala et al., 2015).

2.2.5. MECHANISMS OF DEFENSE AGAINST OXIDATIVE STRESS

Aerobic organisms have acclimatized to overcome oxidative stress. The defense mechanisms can be categorized into at least three levels given to their function of quenching oxidants, repairing/removing oxidative damage or encapsulating non-

repairable damage (Fig. 2) (Lykkesfeldt and Svendsen, 2007). The first line of defense against oxidants is so-called antioxidant network, in which antioxidants can donate electrons to oxidant, resulting in quenching their reactivity and making them harmless to cellular macromolecules (Lykkesfeldt et al., 2003). A second and highly significant level is the ability to find and repair or remove oxidized and damaged molecules. The last one, in case the oxidative damage overrides the repairing and lifting capacity, the organism is equipped with one final weapon, controlled cell suicide, or apoptosis (Payne et al., 1995).

The antioxidant network can be classified into two primary groups, namely the enzymatic antioxidants and non-enzymatic antioxidants regulate the free radical reactions. Enzymatic antioxidants such as CAT, GSHPx, and SOD are significant in the prevention of lipid peroxidation and protect the structure and function of cell membranes (Koruk et al., 2004). SOD's present in the cytosol and mitochondria, catalyzes the dismutation of two molecules of $O_2^{\cdot-}$ into oxygen and H_2O_2 (Gough and Cotter, 2011), while catalase present in the peroxisome directly remove H_2O_2 and convert it to water and oxygen (Lykkesfeldt and Svendsen, 2007). The GSH peroxidase converts the H_2O_2 leaking from the electron transport chain into water (Cabiscol et al., 2000; Arthur, 2000).

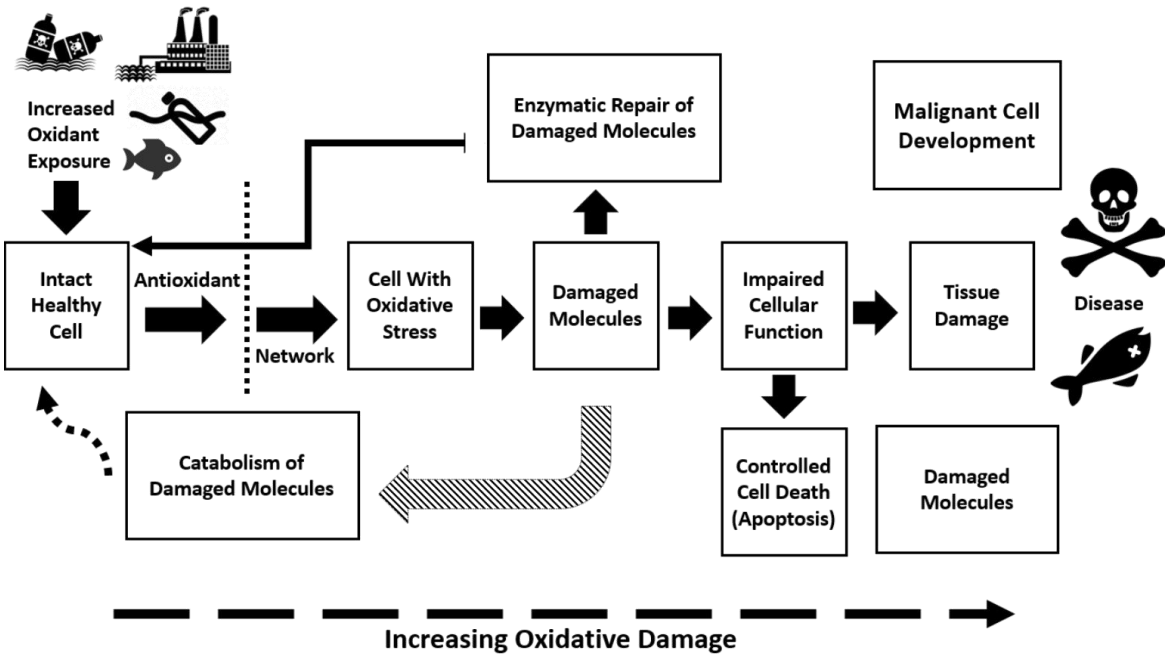


Figure 2: Schematic outline of cellular defenses against oxidative stress-mediated cellular damage. The expansion of oxidative stress is initially faced by the antioxidant network. Injured molecules are then repaired or catabolized. Programmed cell suicide can be started if furthermore oxidative damage gives rise to impaired cellular function. When these signaling cascades are broken, or the oxidative damage surpasses the capability of the defense mechanisms, uncontrolled cell death, tissue injuries, and malignant cell development can proceed into disease. Modified after Lykkesfeldt and Svendsen, (2007).

The non-enzymatic antioxidants can be devised into two groups, the synthetic antioxidants, and natural antioxidants. In general, some plants have various importance due to the numerous active compounds presented in theirs. Antioxidants are one of the essential constituents of the plant structure, which shown to have a strong capacity to inhibit ROS generation and to scavenge free radicals (Citarasu, 2010). Plants act as a potent source of antioxidants (Ghasemzadeh et al., 2010), for example, carotenoids are powerful antioxidants produced mostly by plants be endowed with scavenging potential of free radicals. They protect cells against oxidative damage, posse's immune stimulant function, and anti-inflammatory action

(Craig, 1999). Fish cannot synthesize carotenoids in their bodies, so they must get these compounds from their diets (Christiansen et al., 1995). Carotenoids are classified as a non-enzymatic group of the cell's antioxidant network and act through two mechanisms to protect cells against oxidative damage; quenching of ROS and scavenging of free radicals (Wang et al., 2006). According to Christiansen et al. (1995) and Page et al. (2005) who revealed a relationship between the concentration of carotenoids in fish diets and antioxidants status in liver and muscle of Atlantic salmon and rainbow trout.

Flavonoids considered a large group of natural polyphenolic compounds that are present commonly in plants (Mohiseni, 2017). Until now, around 6000, chemical compounds were isolated and identified as flavonoids (Ghasemzadeh and Ghasemzadeh, 2011). These bioactive compounds can be extracted from several vegetables, nuts, seeds, grains, and herbs (Kandaswami and Middleton, 1994). Flavonoids are considered as a primary antioxidant due to their numerous antioxidant activities, they act as free radical acceptor and chain breaker (Rice et al., 1997; Škerget et al., 2005; Balasundram et al., 2006). Today, phenolics and flavonoids are known as great antioxidants, in which various report have focus attention on their higher efficiency compared with well-known antioxidants, vitamin C, E, and carotenoids (Ghasemzadeh and Ghasemzadeh, 2011; Syahidah et al., 2015; Mohiseni et al., 2017).

2.3. INSECTICIDES AND AQUACULTURE

Chemical insecticides are well known as an economical approach to controlling pests; at the same time, such chemicals are incredibly harmful to other species in

the environment. Presently there is growing interest worldwide over the use of insecticides that result in environmental pollution and toxicity risk to cultivated aquatic organisms.

Responses to insecticides by aquatic organisms are varied depending on the chemical type, exposure period, water properties, and the type of cultivated marine organisms.

This section covers the interactions between Aquaculture and insecticides in terms of eco-toxicological effects and the environmental fate of insecticides in the environment.

2.3.1. INSECTICIDE TOXICITY IN FISH

The use of industrial chemicals with insecticidal characteristics has led to considerable increases in agricultural yields through pest well control (Popp et al., 2013). Insecticides are pollutants of sole interest because the majority of their role of toxic effects for insect pests overhang with those of non-target organisms. Therefore, the regulation of insecticides must balance the advantages of pest management with both environmental and human health issues (Fulton et al., 2014).

Insecticides can reach water bodies as a result of spray flux, runoff, or wastewater misapplication. Due to conservation across many ways (e.g., neurological targets), aquatic organisms such as fish, can suffer adverse effects from insecticide exposure that are similar to outcomes in target organisms (insects) (Jayaraj et al., 2016). If concentrations are sufficient, fish kills may result. At a low concentration level, invertebrates that are more sensitive may be affected, therefore, reducing the food

availability for fish consumption (Pimental, 2005). Pesticides are reported to kill 6–14 million fish yearly in the United States (Pimental, 1992). Moreover, in addition to mortality, insecticide exposure may produce sublethal effects in fish. Insecticides have been shown to reduce fish growth, performance, reproduction, and immune capacity. The nature of these outcomes differs extensively based on many factors such as the type of insecticide, the species and as well as the age of the exposed fish (Sabra and Mehana, 2015).

Methidathion is an insecticide that belongs to the non-systemic organophosphorous. This chemical is used to control a wide range of insects in many vegetables and fruits. It is particularly helpful against scale insects. It works by inhibiting specific enzyme action sites in the target insects. It is commercially available in emulsifiable concentrate, wettable powder, and ultra-low volume liquid formulations (Kamrin, 1997).

2.3.2. TOXICOLOGICAL EFFECTS

Acute toxicity: Methidathion is extremely poisonous via the oral route, with reported critical oral LD_{50} values of 25 to 54 µg/g in the rat, and 18 to 25 µg /g in the mouse (Gallo, and Lawryk, 1991; Kidd and James, 1991). Other studies reported oral LD_{50} values to include 25 µg /g in guinea pigs, 80 µg /g in rabbits, and 200 µg /g in dogs. Furthermore, It is highly toxic via the dermal route; Wagner, (1989) reported that the LD_{50} values of 85 to 94 µg /g in the rats. Methidathion is only a mild skin irritant in rabbits and is nonirritating to the eyes. Through the inhalation route, it may be slightly poisonous; Kidd and James, (1991) demonstrated that 4-hour inhalation LC_{50} of 3.6 µg /mL in rats. Its toxic effects due to acute Methidathion exposures are similar

to those induced by different organophosphates and may include vomiting, nausea, cramps, salivation, diarrhea, headache, muscle twitching, dizziness, difficulty in breathing, blurred vision, and tightness in the chest. Severe high exposure may cause serious breathing difficulties, including the insensibility of the respiratory muscles (Gallo, and Lawryk, 1991).

Organ toxicity: Target organs in animal researches include the neurotic system, liver, gall bladder, and ovaries (Kamrin, 1997).

2.3.3. ECOLOGICAL EFFECTS ON AQUATIC ORGANISMS

The compound is extremely harmful to both vertebrate and invertebrate marine organisms. In this context, the reported LC_{50} values of the mixture are 10 to 14 mg/l in rainbow trout, and 2 to 9 mg/l in bluegill sunfish (Mayer and Ellersieck, 1986; Kidd and James, 1991). Experiments on lobsters showed that the combination of Methidathion and another organophosphate insecticide, Phosphamidon, was more harmful than either compound individually or than would be expected if the toxicities were added together (Kidd and James, 1991). Researchers with bluegill sunfish demonstrate that there is only a small potential that the compound would bio-accumulate in fish tissues (Smith, 1993). The maximum concentration of the residues of the pesticide after one month of exposure to low levels in the water (0.05 mg/l) was 1.0 mg/kg in the edible tissue, 3.9 mg/kg in non-edible mass and 2.4 mg/kg in whole fish. These levels show a low bio-concentration factor of 46 for the entire fish. After two weeks in water without Methidathion, the concentration in entire

fish fell by nearly 80% (Gallo and Lawryk, 1993). Methidathion is slightly toxic to bees (Sanchez-Bayo and Goka, 2016).

2.3.4. ENVIRONMENTAL FATE IN SOIL AND GROUNDWATER

Methidathion is of moderate persistence in the soil ecosystem; reported field half-lives are 5 to 23 days, with a symbolic value of about seven days (Wauchope et al., 1992). The breakdown of the compound in soil happens through the activity of soil microorganisms (Gauthier et al., 1988). Under basic situations, Methidathion is quickly destroyed by chemical action (U. S. National Library of Medicine, 1995). Soils poorly bind Methidathion and its breakdown products and therefore may be transportable (U.S. Environmental Protection Agency, 1988; Wauchope et al., 1992). However, they have not been reported in any groundwater reservoir yet. This is apparently due to the quick half-life of the Methidathion and its degradates.

Regarding the breakdown of Methidathion in water: No data are currently available.

CHAPTER

3

3. MATERIALS AND METHODS

3.1 PLANT SAMPLES

Validated and identified Zallouh roots (*Ferula hermonis*) was purchased from Teeba Investment for Developed Food Processing Co., Jordan (Voucher No. 6253501437132). Fresh Milk thistle (*Silybum marianum*) seeds have been harvested from fresh plants (at the same vegetative phase and healthy morphological appearance) from local areas (Al-Sareh and Huson), North Jordan according to the methods recommended by Benton (2001). Samples were packed in ice bags and transported to the laboratory in Aqaba (South Jordan). Plant species (Milk thistle) was classified according to Webb et al. (1988) as *Silybum marianum*.

At the laboratory*, F. hermonis* roots and *S. marianum* seeds were gently washed with tap water, rinsed with distilled water to remove moisture without causing appreciable thermal decomposition; all plant samples were oven-dried overnight at 50 °C (Benton, 2001; Chumroenphat et al., 2011 and Sukrasno, 2014). Dried plant tissues were ground into a fine powder (< 5 µm) with an agate mill and then stored for further analysis.

3.2. PLANT METABOLITES IDENTIFICATION

3.2.1. PLANT SAMPLE PREPARATION

The metabolite extraction procedure was based on the protocol described by De Vos et al. (2007) with minor modifications. Homogenized samples of air-dried rhizomes and roots of *F. hermonis* and seeds of *S. marianum* were analyzed after being grinded using a ball mill (MM 400, Retsch, Verder Scientific, Haan, Germany). The mill was equipped with two polytetrafluoroethylenes (PTFE) vessels and grinder balls (10 min with a vibration frequency of 30 Hz) to achieve a final particle fineness of ~5 µm. For each plant, three replicated samples were analyzed to check the repeatability and method precision. Moreover, we analyzed three blank samples to avoid any possible contamination driving from sample handling. An internal standard (IS), namely, phenyl-$^{13}C_6$ salicylic acid, was added to the root and seed before extraction to correct for variation in extraction and detection of all mass signals over the samples. The homogenized powder of roots/rhizomes and seeds was weighed (50.0 mg ± 0.5) into Eppendorf tubes of 2 ml and then extracted for 30min in an ultrasonic bath with 1.5 ml of methanol/water (75:25, v:v). Samples then were centrifuged for 20 min at 14,000 rpm, and the supernatant was collected and filtered simultaneously with PTFE syringe filters (Ø 25 mm, 0.2 mm) which were previously activated with 1 ml of both methanol and ultrapure water. The obtained solution was directly introduced in the HPLC-HRMS system .

3.2.2. INSTRUMENTAL ANALYSIS

The analytical method used in this study was in accordance to that described by Rizzato et al. (2017) and Scalabrin et al. (2015). Analyses were carried out on an UltiMate 3000 (Dionex), coupled to an ESI-LTQ Orbitrap XL (Thermo Fisher

Scientific, Waltham, USA). The chromatographic separation was accomplished on a SB-Aq Narrow Bore RR 2.1 X 150 mm, 3.5 µm column (Agilent Technologies, Wilmington, USA), two eluents were used: eluent A (0.01% formic acid in ultrapure water) and eluent B (0.01% formic acid in acetonitrile). Initially and during the first 5-min, the chromatographic separation was operated in an isocratic phase at 100% of eluent A. This was followed by a 40-min gradient until 100% of eluent B, then the elution continued using the same eluent for 15-min in isocratic mode, finally last 15-min step back to the initial eluent proportions. The eluent flow rate was 200 µl/min and the sample-injected volume was 5 µl. The ESI source was operated in both negative and positive mode, the capillary temperature was set at 275 ˚C and the vaporization temperature at 300 ˚C; the sheath, auxiliary and sweep gas flow rate were set at 35, 5 and 0 µl/min respectively. The analyses were operated in full scan mode, at a resolving power of 60,000, with a mass range between 90 and 1500 m/z. Data-dependent acquisitions were performed, in order to obtain a complete fragmentation pattern of the molecules.

3.2.3. DATA PROCESSING

To extract relevant information from raw chromatographic data, they were processed by two dedicated software with best processing parameters; MetAlign 3.0 (Lommen and Kools, 2012) for spectral alignment and baseline correction, and MSClust (Tikunov, et al., 2012), to obtain clustered mass signals (i.e., reconstructed metabolites). The list of masses resulting from MetAlign software was processed by MS excel, to be ready for MSCLust processing. Molecular formulas were determined using XcaliburTM software (Thermo Scientific Inc.). In addition, a metabolite

identification protocol was applied on the basis of the most probable molecular formula assigned considering the monoisotopic mass and the fragmentation pattern; mass spectra were compared with available online libraries (Metlin, HMDB, Dictionary of Natural Products, and LIPID MAPS Structure Database) and literature data. The identification level was assigned, according to Sumner et al. (2007).

3.3. FISH SAMPLES

Identified fish samples (*Siganus rivulatus*), with the same age (6 months) and average body weight (75 ± 15 g) were collected from the Aquaculture unit available at the Marine Science Station in Aqaba during summer 2017 (Fig. 3).

Figure 3: ***Siganus rivulatus.***

3.4. TOXIN MODEL

Methidathion (MD) was used in this study as a model of an oxidative agent. The stock toxin solution was purchased from Veterinary and Agricultural Products Manufacturing Co. VAPCO, Jordan.

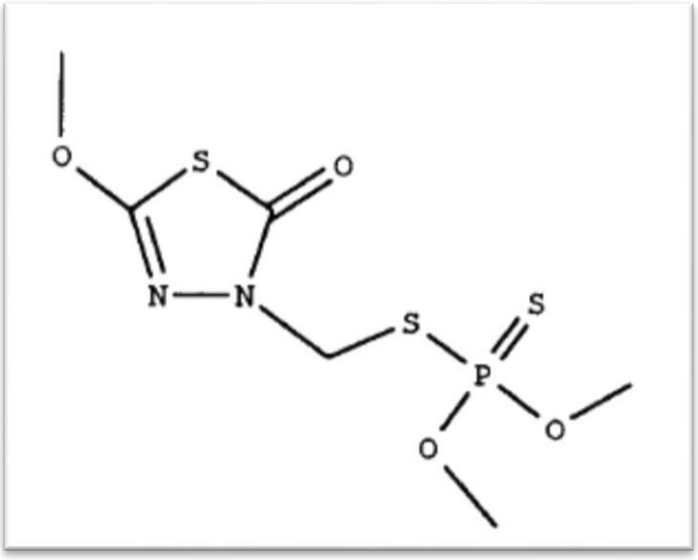


Figure 4: Methidathion. Adopted from Kamrin (2000)

3.5. ANIMALS AND ETHICS

All procedures on care and maintenance of the experimental animals were in accordance with International Guiding Principles for Animal Research (1986). All the present experiments were reviewed and approved by the animal care and ethical committee at Marine Science Station, The University of Jordan and Yarmouk University, Jordan.

3.6. LC_{50} DETERMINATION

In order to establish an adequate dose of Methidathion to investigate toxicity; the toxicity testing was conducted using the Fawell's up and down method (Fawell et al., 1999). The duration of the test was for 24 hours. A stock solution of Methidathion (1.323 M) was prepared in artificial seawater according to Goldman and McCarthy (1978), in order to give specific doses of the toxin. The following concentrations 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, and 0.0157 mg/l, were distributed in separate experimental aquariums (60 Litter/ tank). In each tank, ten fishes with an average weight of 75 g and the average total length of 15 cm were used. All fishes were monitored for 24 hours, and the signs of toxicity such as difficulty in swimming and rubbing against tank objects and weakness were recorded. The experiment was concluded and the death rate was recorded among different fish groups.

3.7. WATER QUALITY

Water quality is is a critical factor that can affecting fish health and performance in any aquaculture systems (Otoo et al., 2019). The Physical (pH, temperature, salinity, dissolved oxygen and oxygen saturation level) and chemical (nitrate, nitrite, and ammonia) water quality parameters of experimental tanks (Fig. 5) were measured according to Strickland and Parsons, 1972.

Figure 5: Experimental Tanks

3.8. FISH BIOASSAY AND TOXICITY CHALLENGE

One hundred eighty (180) *S. rivulatus* fish with the same age (6 months) and average body weight 75 ± 15 g were obtained from the Aquaculture unit at the Marine Science Station, and transferred to the laboratory where the temperature was set at 25 ± 3 °C. Throughout the experiments, artificial seawater with pH value of 8.0 ± 0.2 was used. The fish were allowed to adapt to these conditions for 7 days. Moreover, the fish were fed at a rate of 3% body weight/day with a commercial fish food diet (Jafar Aquatics) during the adaptation period. Experiments were conducted in glass aquaria containing 150 L of test solution. Fishes were randomly divided into 6 groups (30 each, otherwise indicated) as shown below:

Table 1: Fish bioassay and toxicity challenge experimental design.

Group	Description	Action
C	Control group without supplement of crude extract or treatment with toxin	-10 fishes sacrificed after 6 hrs (C6). -10 fishes sacrificed after 12 hrs (C12). -10 fishes sacrificed after 24 hrs (C24).
TC	Toxin Control group treated with Methidathion only by a dose of 7.5µg toxin/L (according to LC_{50} value)	-10 fishes sacrificed after 6 hrs (TC6). - 10 fishes sacrificed after 12 hrs (TC12). - 10 fishes sacrificed after 24 hrs (TC24).
FC	*F. hermonis* Control group supplemented on daily basis with 2.5g F. h/kg* fish body weight for 14 days.	-10 fishes sacrificed after 6 hrs (FC6). - 10 fishes sacrificed after 12 hrs (FC12). - 10 fishes sacrificed after 24 hrs (FC24).
SC	*S. marianum* Control group, supplemented on daily basis with 2.5g S. m/kg* fish body weight for 14 days	-10 fishes sacrificed after 6 hrs (SC6). - 10 fishes sacrificed after 12 hrs (SC12). - 10 fishes sacrificed after 24 hrs (SC24).
FT	Fish supplemented on daily basis with 2.5g F. h/kg* fish body wt for 14 days, and then fishes were treated with Methidathion of 7.5µg/L	-10 fishes sacrificed after 6 hrs (FT6). - 10 fishes sacrificed after 12 hrs (FT12). - 10 fishes sacrificed after 24 hrs (FT24).
ST	Supplemented on daily basis with 2.5g S. m/kg* fish body weight for 14 days, and then fishes were treated with Methidathion of 7.5µg/L	- 10 fishes sacrificed after 6 hrs(ST6). - 10 fishes sacrificed after 12 hrs(ST12). - 10 fishes sacrificed after 24 hrs(ST24).
* The selection of the plant dose was carried out following the standard approaches recommended by Cos et al. (2006). (Data are not presented)		

A blood sample (0.3 ml) was collected immediately after sacrification into a vial without anticoagulant from each fish via cardiac puncture. The serum was separated by using the centrifuge at 3000 X g for 30 min and used for the enzyme assay. The tubes were immediately capped and kept at 4ºC for later use. Moreover, hepatopancreas, gills and muscles of the experimental fish were isolated

immediately after sacrification, washed with phosphate buffer saline (pH 7.2) and stored at -20ºC for further biochemical and histo-pathological tests.

3.9. BIOCHEMICAL ASSAYS

3.9.1. THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) ASSAY

The oxidative degradation of unsaturated fatty acids (known as lipid peroxidation) of the hepatopancreas, gills, and muscles were measured according to the method described by Wahsha et al. (2012a) with some minor modifications. Malonaldehyde (MDA) is the end by-product of lipid peroxidation, MDA reacts with thiobarbituric acid (TBA) to produce a red colored complex (MDA-TBA) which has a maximum of absorbance at 532nm. Fresh sample (0.1g) was homogenized in 5 ml solution of 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid. This mixture was incubated at 95°C for 30 minutes followed by quick ice cooling. Centrifugation of the cold mixture was accomplished at 10,000 X g for 2 minutes, and then the absorbance of the clear supernatant was measured using a spectrophotometer (EMC LAP, EMC-31PC-UV) at 532 nm. Correction for unspecific turbidity was conducted by subtracting the absorbance of the sample at 600 nm. The concentrations of MDA were quantified by the measurement of absorbance and using a molar attenuation coefficient of 0.155 L mol^{-1} cm^{-1}.

3.9.2. BIOCHEMICAL STUDIES

The measurement of the certain serum enzymes has been shown to be of daigonistic importance. This is due to the existence of these enzymes in the serum suggest that cellular or tissue damage has occurred leading to the release of

intracellular components in to the blood (Machetti et al., 1998). The levels of serum Alanine transaminase (ALT), Lactate dehydrogenase (LDH), and total Cholesterol (TC) were measured by one chemistry analyzer (ACCENT 200, Cormay) using Cormay diagnostic kits.

3.10. MICROSCOPY ANALYSIS

According to the procedure described by AL-Haj (2010), fresh tissues (1g), from the hepatopancreas, gills, and muscles were immediately pre-fixed in 10% formalin for 24 hours after sacrifice. Specimens were dehydrated and embedded in wax. Thin sections (6 µm) of the implanted material were stained with Haematoxylin and Eosin stain. The slides were observed by optical microscope (OPTIKA ITALY), using 40X for assessing the histological changes.

3.11. STATISTICAL ANALYSIS

One ANOVA statistical analysis was carried out to determine the significance of the differences between groups, results are presented as averages ± S.D, followed by Student–Newman–Keuls test. Statistical significance was notified when the p-value was equal to or less than 0.05. The statistical analysis was conducted using the Sigma Stat Software version 3.5.

CHAPTER

4

4. RESULTS

4.1. PLANT METABOLITES

4.1.1: *Ferula hermonis*

The total number of compounds detected from the HPLC-HRMS measurements were 132 secondary metabolites (in Figure 6 is reported one typical chromatogram). Of which 26 identified by annotation of the more probable molecular formula by comparison with strucutural information available from literature data and/or online database containing experimental data (level 2), the others were chracterized only for the class to which them belong (level 3) (Sumner et al., 2007).

The compounds are presented in the appendix supplementary material (Table S1) and they are classified as terpenoids (65 compound), flavonoids and polyphenols (18 compound), glycoside (26 compound), essential fatty acid (9 compound), and others (14 compound). The compounds identified in the roots material of *F. hermonis* were mostly represented by terpenoids with a relative abundance of 49 % followed by glycoside and flavonoid, (20 % and 14 % respectively), fatty acids (7 %), and others (10 %) (Figure 7).

4.1.2. *Silybum marianum*

The total number of compounds detected from the chromatogram HPLC-HRMS (Fig. 8) were 126 secondary metabolites, of which 37 identified at level 2 and the others at level 3 (Sumner et al., 2007). The compounds can be classified as belonging to the following classes of metabolites (Appendix supplementary material: Table S2): terpenoids (28 compound), flavonoids and polyphenols (27 compound), glycoside (17 compound), essential fatty acid (11 compound), and others (42 compound). Our results revealed that, the seeds extract of *Silybum marianum* secondary metabolites, were mostly characterized by flavonoids and terpinoids compounds, with approximately equal relative abundance (22% for each), glycoside and fatty acid (14% and 9%, respectively), and other (33%). (see Fig 9). Silymarin, the main constituents of *S. marianum* seed extract, are composed of a mixture of flavonolignans. Our study revealed that the chemical composition of milk thistle besides flavolignan also include other flavonoids such as **(**Naringenin, Eriodictyol, Luteolin, 2,3- Dehydrosilybin, Naringin, 4'-Hydroxy-5,6,7-trimethoxyflavanone, 5,7-Dihydroxy- 8- C- (gamma- methyl- gamma- formylallyl) flavanone 4'- Hydroxy- 5-methoxy- 7- (3- methyl- 2, 3-epoxybutoxy) flavone**)**, terpenoids **(**Loganin, Oleoside dimethyl ester, Darendoside B, Hymenolide, 18-Oxoaustrochaparol acetate, 6alpha-Formyloxygrindelic acid, Celastrol, Ixerisoside N**)**, fatty acid **(**Palmitic acid, alpha-Linolenic acid, Linoleic acid, Helenynolic acid, Ricinoleic acid**)**, N-Acetyldopamine, D-Tryptophan, Nigellicine, and other.

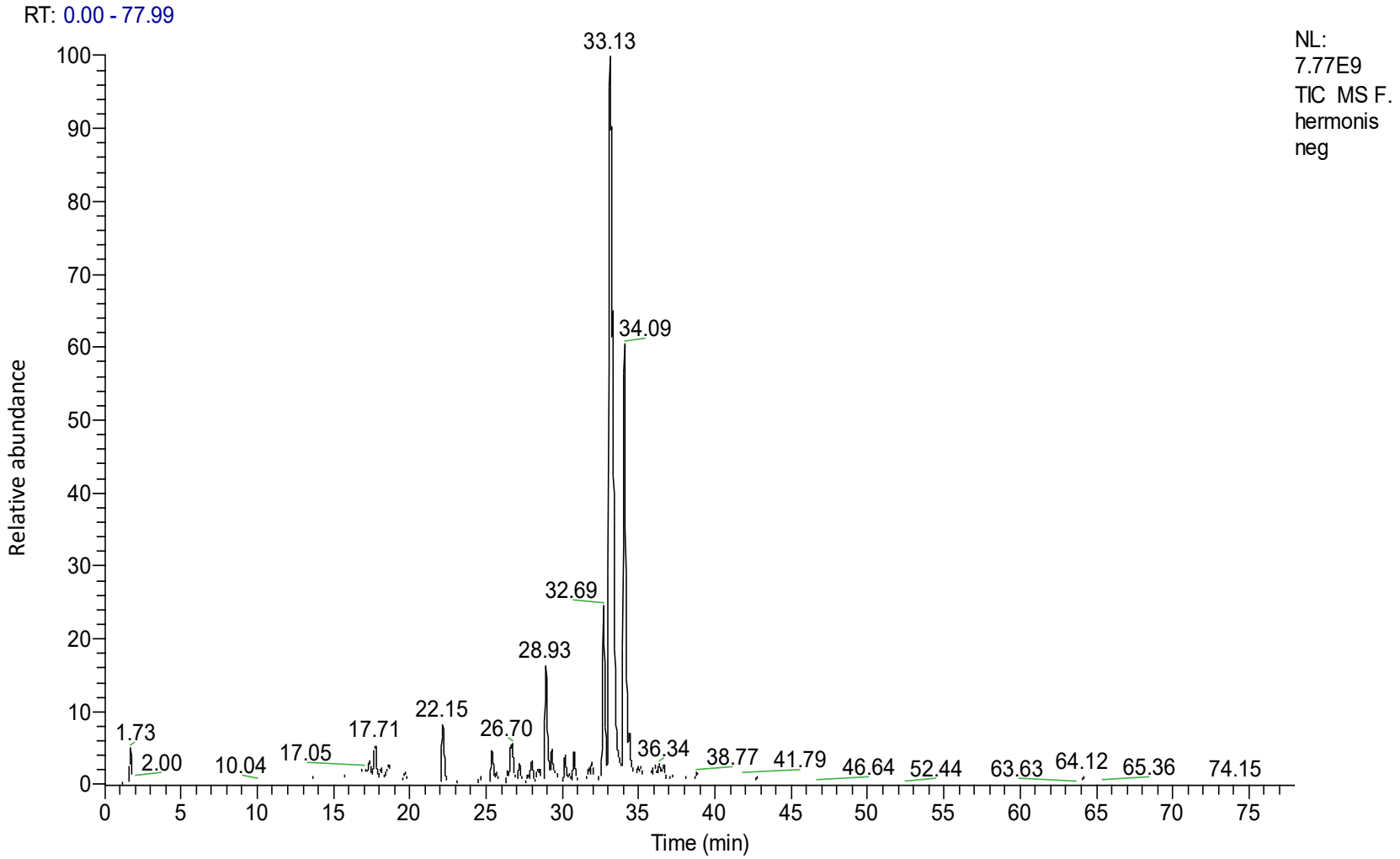


Figure 6: Chromatogram obtained by HPLC-HRMS for the *F. hermonis* roots methanolic extract.

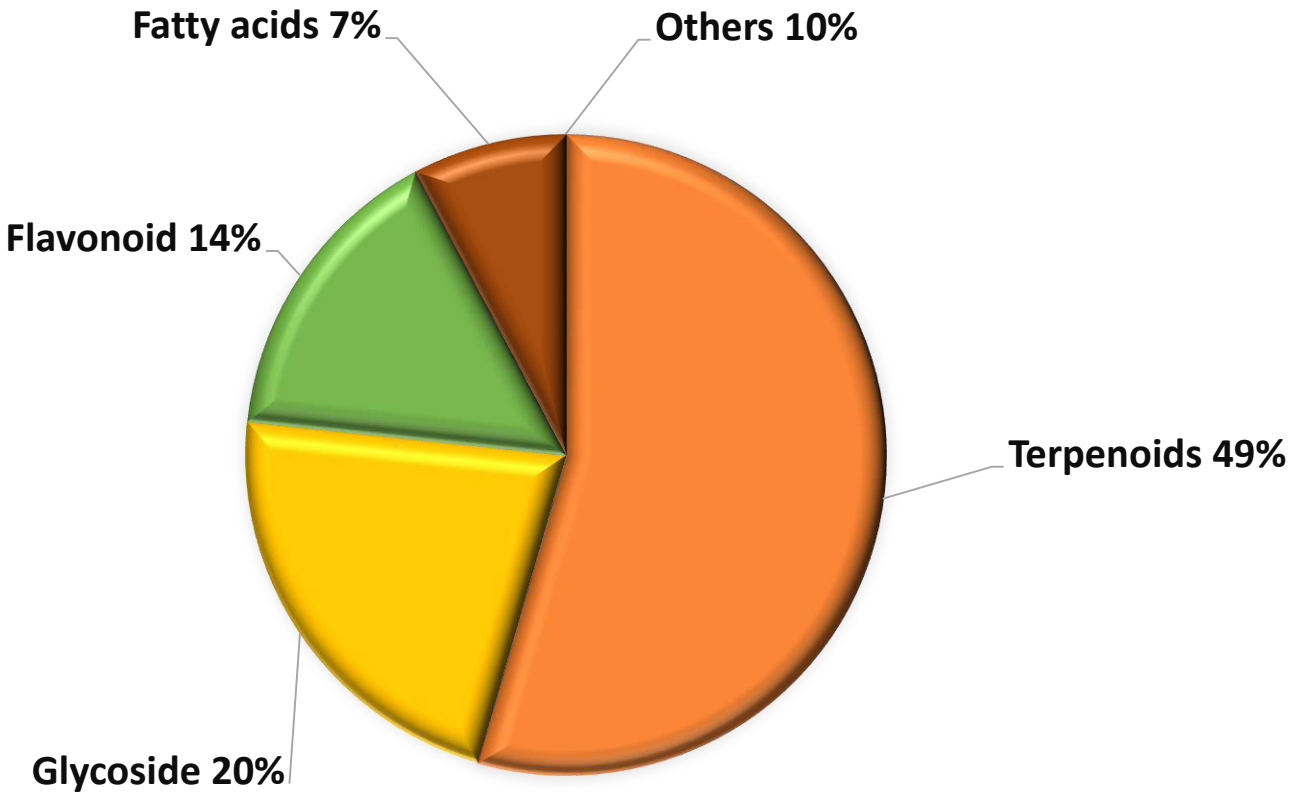


Figure 7: Relative abundance of *F. hermonis* roots metabolites

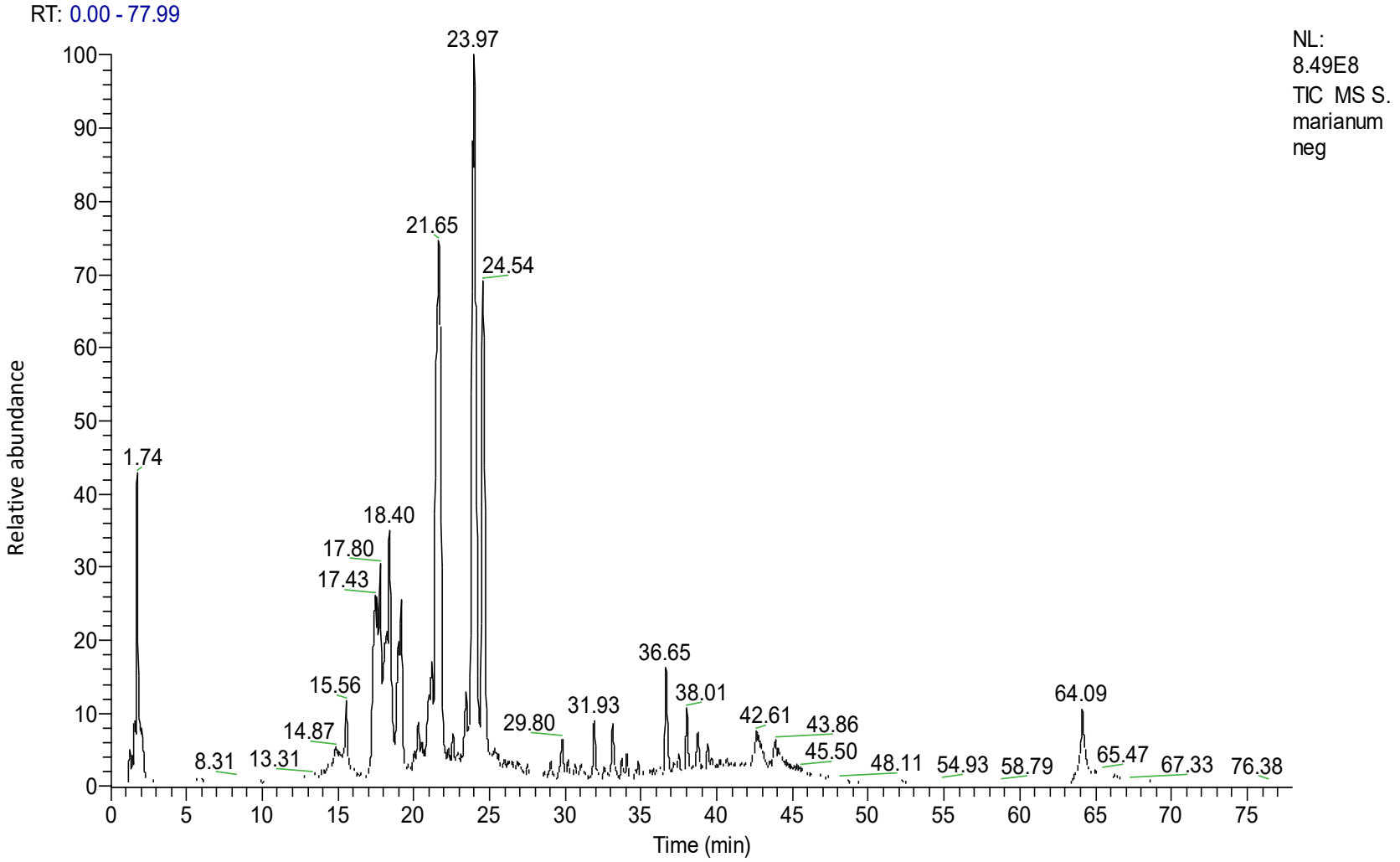


Figure 8: Chromatogram obtained by HPLC-HRMS for the *S. marianum* seeds methanolic extract.

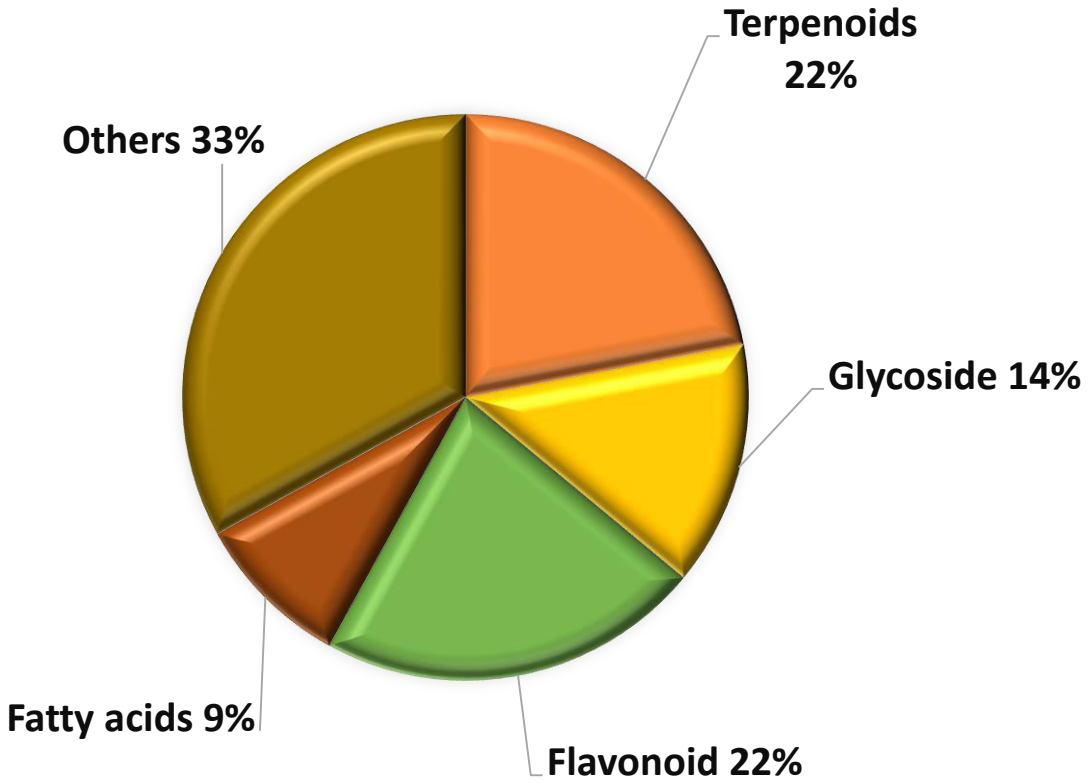


Figure 9: Relative abundance of S. marianum seeds metabolites

Until now, few data are available for *Ferula hermonis* root and *Silybum marianum* seed extracts in the literature, and the sources of data in the literature make evident that additional scientific investigations are necessary. In this study, we have systematically summarized the available biological/pharmacological properties of *Ferula hermonis* root and *Silybum marianum* seed extracts in appendix, annex 1 and 2.

4.2. LETHAL CONCENTRATION DETERMINATION

No fatality correlated with *F. hermonis* and *S. marianum* treatment was witnessed in the experimental fish during the trial period. The performance of fish of the intoxicant group (Methidathion) could be recognized by losing their capacity to move ahead, with a remarkable decline in food intake and consequently to die from exposure to oxidative stress. The estimate LC_{50} of toxin was 7.5µg toxin/L.

4.3. WATER QUALITY

General parameters of seawater characteristics (Salinity, pH, Dissolved oxygen, Oxygen saturation, Temperature, Ammonia, Nitrite, and Nitrate) were analyzed in all seawater samples, and a summary of these analyses is given in Table 2. The water characteristics in the experimental tanks never assume critical values to maintain the functionality of the system; the pH values oscillate from about 8.1 to nearly 8.4, the oxygen concentration ranges between 5.6 to 5.9 mg/L, the saturation was always higher than 88%, the ammonia range between 0.011 to 0.014 mg/L, the nitrite and nitrate concentrations started to increase after 4 and 5 days respectively, the values

increased from 0.22 to 0.24mg/L and from 15.0 to 19.3mg/L, respectively. Other parameters were did not change significantly, the salinity value were 40.4 ppt for the entire experiment, the temperature fluctuated from 27.3 to 27.6 C^{o}.

Table 2: Summary of results of the physical and chemical seawater properties of the experimental tanks.

Days / Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Unit
Salinity	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	40.4	ppt
pH	8.3	8.3	8.2	8.3	8.4	8.3	8.2	8.2	8.2	8.3	8.3	8.1	8.2	8.2	
Oxygen saturation	88	90	92	90	87	89	90	88	88	88	90	90	89	90	%
Temperature	27.5	27.3	27.5	27.5	27.4	27.3	27.4	27.5	27.3	27.6	27.5	27.5	27.3	27.5	C^o
NH_4	0.010	0.010	0.011	0.011	0.011	0.012	0.012	0.012	0.012	0.013	0.013	0.013	0.014	0.014	
NO_2	0.22	0.22	0.22	0.23	0.23	0.23	0.22	0.23	0.23	0.24	0.24	0.24	0.22	0.24	
NO_3	15.0	15.0	15.0	15.0	15.6	15.7	16	16.3	16.3	17	17.9	18	18.6	19.3	
Dissolved oxygen	5.7	5.9	5.9	5.8	5.8	5.9	5.9	5.8	5.6	5.6	5.7	5.8	5.8	5.8	mg/L

4.4. BIOCHEMICAL ASSAYS

4.4.1. MALONALDEHYDE CONCENTRATION THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) ASSAY

As stated earlier, Malonaldehyde (MDA) is the main reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acid (PUFA) constituents of biological membranes. Thus, the concentration of MDA levels in the tissue is generally used as a biomarker (indicator) for cell damage. The MDA concentration was determined by Thiobarbituric Acid reaction, therefore, it is espressed as mol/g of Thiobarbituric Acid Reactive Substances (TBARS).

Fermonis hermonis **administration experiment:** Control fish (group C) demonstrated normal levels of MDA, and it was 4.2, 12.6, and 2.5 µmol/g in hepatopancreas, gills, and muscles, respectively. A significant rise ($P < 0.05$) was detected in MDA concentrations in the intoxicant group (TC) by 16.4, 24.6 and 3.5 µmol/g in hepatopancreas, gills, and muscles, respectively. Furthermore, fish received antioxidant supplementations showed a significant decrease ($P < 0.05$) in MDA concentrations when compared with TC, only a scarse increase in the gills respect C group was observed. (Table 3).

Table 3: Effect of Methidathion on MDA levels in fish hepatopancreas (H), gills (G) and muscles (M) after bath administration of LC_{50} (7.5 µg toxin/L) with and without F. hermonis. C: Control group without supplement of F. hermonis or treatment with Methidathion; TC: Toxin Control group treated; FC: F. hermonis Control group supplemented on daily basis with 2.5g F.h/kg fish body weight for 14 days; FT: Fish supplemented on daily basis with 2.5 g F.h/kg fish body wt for 14 days, and then fish was treated with LC_{50} 7.5 µg toxin/L. All the values are mean of 3 replicates ± S.D.

MDA concentration (µmol/g)												
Group	**C6**	**C12**	**C24**	**TC6**	**TC12**	**TC24**	**FC6**	**FC12**	**FC24**	**FT6**	**FT12**	**FT24**
H	4.1	4.0	4.2	10.3	13.2	16.4	5.2	4.8	4.5	4.8	5.7	6.0
± S.D	0.1^{yx}	0.1yx	0.1yx	0.4^{xy}	0.5^{xy}	0.7^{xy}	0.4^{yx}	0.8^{yx}	0.9^{yx}	0.5^{xx}	0.4^{xx}	0.2^{xx}
G	12.3	12.4	12.6	18.3	20.4	24.6	14.3	13.7	13.1	17.1	17.3	17.8
± S.D	0.2^{yx}	0.2^{yx}	0.4^{yx}	1.8^{xy}	0.6^{xy}	0.7^{xy}	0.9^{yx}	0.5^{yx}	0.7^{yx}	0.9^{xy}	0.3^{xx}	0.4^{xx}
M	2.6	2.4	2.5	3.3	3.6	3.5	2.4	2.7	2.1	1.3	1.5	1.7
± S.D	0.6^{yx}	0.2^{yx}	0.4^{yx}	0.3^{xy}	0.4^{xy}	0.7^{xy}	0.4^{yx}	0.5^{yx}	0.2^{yx}	0.6^{yx}	0.5^{xx}	0.9^{xx}

The two letter symbols following ± S.D within the same row indicate if there is a significant difference or not when compared to control groups C and TC respectively. x indicates significant difference at $P < 0.05$ and y indicates no significant difference according to ANOVA.

***Silybum marianum* administration experiment**; Fish groups supplemented by antioxidants only, presented regular levels of the MDA with concentration of 5.3, 12.5 and 0.6 µmol/g in hepatopancreas, gills, and muscles, respectively. The group of fishes with Methidathion administration (7.5 µg toxin/L) for 24 hours raised MDA values to 16.4, 24.6 and 3.5 µmol/g in hepatopancreas, gills, and muscles, respectively, which all were significantly different ($P<0.05$) compared to control fish (Table 4). It was found that the pre-treatment with *S. marianum* at 2.5g/Kg fish body weight, significantly inhibits the increase in MDA that was induced by Methidathion LC_{50} (Table 4).

Table 4: Effect of Methidathion on MDA levels in fish hepatopancreas (H), gills (G) and muscles (M) after bath administration of LC_{50} (7.5µg toxin/L) with and without S. marianum. C: Control group without supplement of S. marianum or treatment with Methidathion; TC: Toxin Control group treated; SC: S. marianum Control group supplemented on daily basis with 2.5g S.m/kg fish body weight for 14 days; ST: Fish supplemented on daily basis with 2.5g S.m/kg fish body wt for 14 days, and then fish was treated with LC_{50} 7.5µg toxin/L. All the values are mean of 3 replicates ± S.D.

MDA concentration (µmol/g)												
Group	**C6**	**C12**	**C24**	**TC6**	**TC12**	**TC24**	**SC6**	**SC12**	**SC24**	**ST6**	**ST12**	**ST24**
H	4.1	4.0	4.2	10.3	13.2	16.4	5.1	5.3	5.3	7.2	5.5	5.7
± S.D	0.1^{yx}	0.1^{yx}	0.1^{yx}	0.4^{xy}	0.5^{xy}	0.7^{xy}	0.2^{yx}	0.1^{yx}	0.1^{yx}	0.4^{xx}	0.7^{xx}	0.6^{xx}
G	12.3	12.4	12.6	18.3	20.4	24.6	13.1	12.9	12.5	16.4	12.5	13.1
± S.D	0.2^{yx}	0.2^{yx}	0.4^{yx}	1.8^{xy}	0.6^{xy}	0.7^{xy}	0.2^{yx}	0.1^{yx}	0.1^{yx}	0.4^{xx}	0.7^{xx}	0.2^{xx}
M	2.6	2.4	2.5	3.3	3.6	3.5	0.5	0.5	0.6	2.6	3.3	1.2
± S.D	0.6^{yx}	0.2^{yx}	0.4^{yx}	0.3^{xy}	0.4^{xy}	0.7^{xy}	0.1^{yx}	0.1^{yx}	0.1^{yx}	0.2^{xx}	0.4^{xx}	0.4^{xx}

The two letter symbols following ± S.D within the same row indicate if there is a significant difference or not when compared to control groups C and TC respectively. X indicates significant difference at $P < 0.05$ and y indicates no significant difference according to ANOVA.

4.4.2. BIOCHEMICAL MARKERS

The changes in the levels of blood serum biomarkers, Alanine transaminase (ALT), Lactate dehydrogenase (LDH), and total Cholesterol (TC), within a different periods (T6, T12, and T24) are summarized in (Table 5). There were significant differences with the biomarkers measured over these times in fish treated with Methidathion, as compared to those observed in the control group. The levels of serum biomarkers: ALT and LDH started to show up their significant increased ($P < 0.05$) after Methidathion bath toxification administration and were still high until 24 hours. One exception was the total cholesterol results, in the TC group; levels were significantly lower relative to the control after 24 hours. On the other hand, in FT and ST treated groups, the total cholesterol values were significantly lower ($P < 0.05$) compared to their normal levels after 6, 12 and 24 hours of Methidathion bath toxification as shown in (Table 5).

Table 5: Levels of the blood serum biochemical markers after bath toxification of S. rivulatus fishes (n=3) with Methidathion at LC_{50} (7.5µg toxin/L). Data are presented as means ± SD.

Blood serum biochemical markers (u/l)										
Group	**C**	**TC**	**FC**	**SC**	**TF6**	**TF12**	**TF24**	**TS6**	**TS12**	**TS24**
ALT	11	316	17	36	34	28	204	37	94	23
± S.D	2^{yx}	2^{xy}	1^{yx}	1^{yx}	1^{yx}	1^{yx}	3^{xx}	2^{yx}	4^{yx}	1^{yx}
LDH	338	12643	344	936	1587	1354	1043	1828	1884	1455
± S.D	7^{yx}	8^{xy}	3^{yx}	2^{yx}	3^{xx}	3^{xx}	3^{xx}	3^{xx}	4^{xx}	13^{xx}
TC	625	30	750	726	214	221	196	187	277	253
± S.D	1^{yx}	2^{xy}	3^{yx}	2^{yx}	2^{xx}	3^{xx}	4^{xx}	2^{xx}	2^{xx}	3^{xx}

The two letter symbols following ± S.D within the same row indicate if there is a significant difference or not when compared to control groups C and TC respectively. X indicates significant difference at $P < 0.05$ and y indicates no significant difference according to ANOVA.

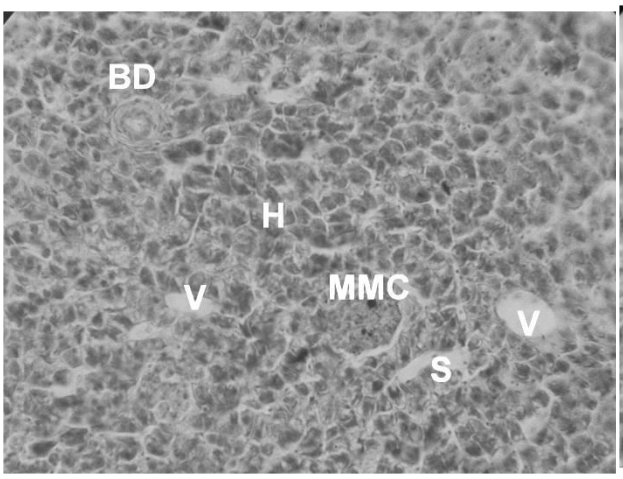
4.5. HISTOLOGY

A total of 754 histological sections were examined under a optical microscope. Representative photomicrograph for hepatopancreas, gills and muscles of all treated groups are presented in Figure10 through 12.

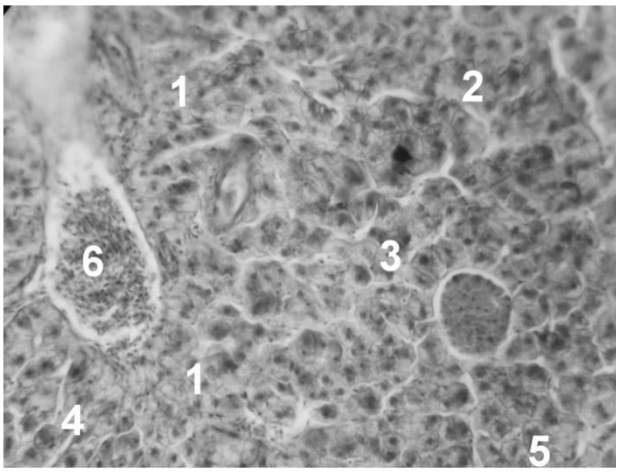
4.5.1. HISTOPATHOLOGICAL CHANGES IN HEPATOPANCREAS

Representative images of hematoxylin (H) and eosin I stained sections of hepatopancreas tissue sections from control group were close to normal in histological appearance (Figure 10 C). However, the construction of the control hepatopancreas of *S. rivulatus* (Fig. 10 C) consists of masses of hepatocytes that are not organized into distinct lobules and interrupted by sinusoids. The hepatocytes were polyhedral in shape with spherical central nuclei. Blood vessels and bile ducts were randomly observed throughout the hepatic parenchyma. Melanomacrophage centres were also seen among hepatic parenchyma, and they were usually located approximately within hepatic arteries or bile ducts. Likewise, none of the fish exposed to antioxidants alone (FC and SC) showed pathological changes. In contrast, the toxin treated fish group (TC) caused moderate damage to hepatopancreas tissue in the form of cellular infiltration, blood congestion and bile stagnation, fibrosis, nuclear hypertrophy, cytoplasmic vacuolization and fatty change, and degeneration of hepatocytes, (Fig. 10 TC). Toxin treated fishes received *F. hermonis* or *S. marianum* supplementation have shown no remarkable changes in the overall structure of hepatopancreas at a dose of 2.5g fish body weight after 6 hours of toxin challenge (Fig. 10 FT6 and ST6). However, after 12 hours, the cytoplasmic vacuoles were noted in both experimental plant extract aquariums (Fig.

10 FT12 and ST12), and some hepatic vein congestion was observed in the case of *S. Marianum* (Fig. 10 ST12). Furthermore, after 24 hours, some lipid-like vacuolization was found (Fig. 10 FT24 and ST24). The degree of damage and deformation appears to be time-dependent.



C



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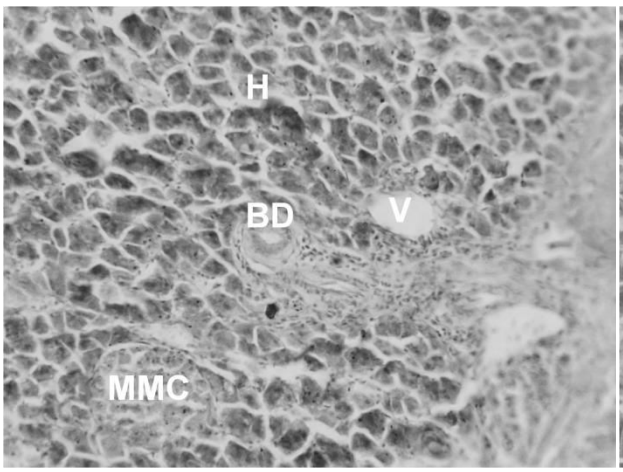
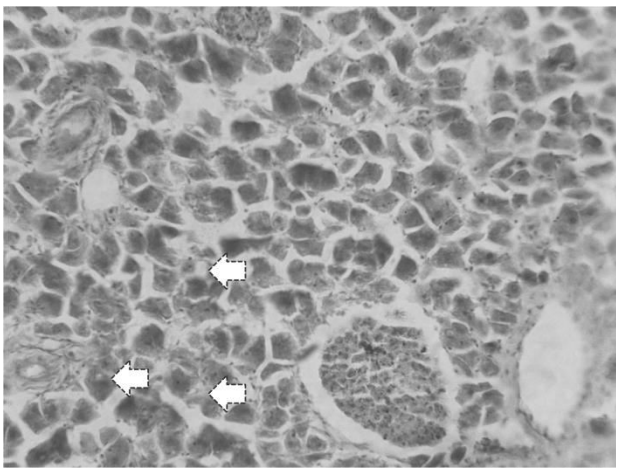


FT6



ST6

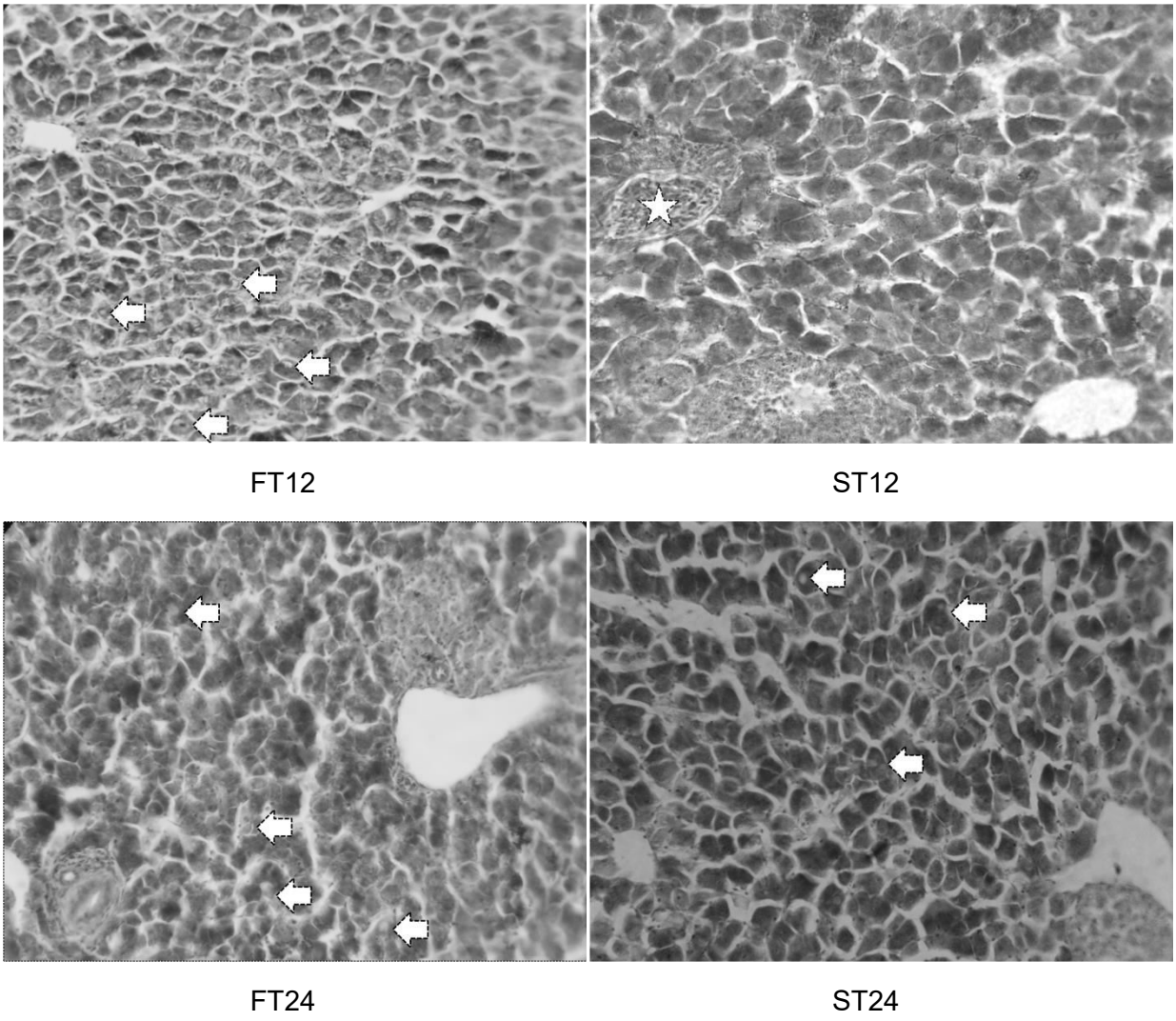


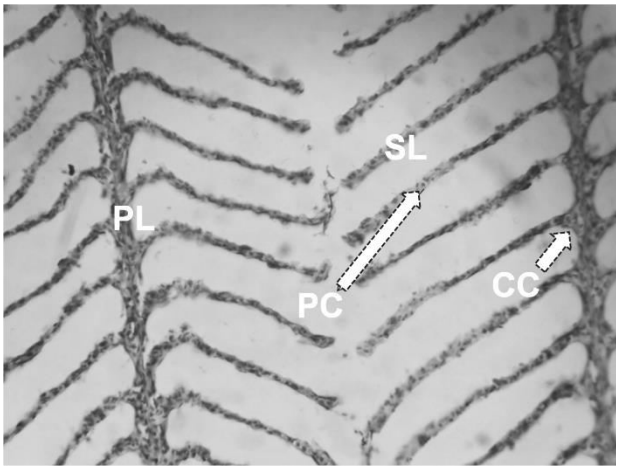
Figure 10: Representative photomicrograph of hepatopancreas tissue sections from control (C), toxic control (TC) and toxin/antioxidant pretreated fish groups. C: Control group (H) Hepatocyte; (V) Hepatic vein; (BD) Bile duct; (MMC) Melanomacrophage centres; (S) Sinusoid. TC: Toxin Control group treated with the LC_{50}: (1) Focal nicrosis and absence of nuclie; (2) Pyknotic nuclie; (3) Nuclier hypertrophy; (4) Cytoplasmic vacuolization; (5) Fatty Change; (6) Blood congestion. FT6, FT12, and FT24: Toxin/ F. hermonis pretreated group; (white arrow) Cytoplasmic vacuolization. ST6, ST12, and ST24: Toxin/ S. marianum pretreated groups; (star symbol) Congestion. Stain: H & E; Magnification 40X.

4.5.2. HISTOPATHOLOGICAL CHANGES IN GILLS

Representative sections of gills for all groups are shown in Figure 11. The control fish showed no signs of hyperplasia or other pathological symptoms; for instance, secondary lamellae layers, which are oriented perpendicular to the gill filament (primary lamellae). The surface epithelium primary lamellae is covered with stratified epithelium, which contains several cell types such as pillar cells, mucous cells, and chloride cells. The secondary lamellae are covered with thin epithelium and consist of a single or double layer of cells lying on a basement membrane supported by pillar cells. Similarly, none of the fish exposed to antioxidants alone (FC and SC) showed pathological changes.

Methidathion treated fish (TC) caused gill deformations and alterations. Epithelial lifting and clubbed tips were observed as well as thickening and shortening of secondary lamellae, proliferative tissue hyperplasia (PTH), partial fusion of the secondary lamellae (FS), blood congestion (BC) and curling of secondary lamellae (CSL), hypertrophy of chloride cells (HCC) and necrosis (N). Moreover, when fish were exposed to Methidathion, gill hyperplasia developed in all fish after 6 hours and was severe, with a fusion of secondary lamellae after 12 hours, which became progressively worse by 24 hours. (Fig. 11 TC).

On the other hand, fish received toxin, and antioxidant supplements showed less necrotic changes in the epithelial cells compared to both treated and untreated control groups. Likewise, *F. hermonis* and *S. marianum* photomicrographs revealed some blood congestion and proliferative tissue hyperplasia (PTH), some epithelium uplifting, curling of secondary lamellae and hypertrophy of chloride cells were observed.



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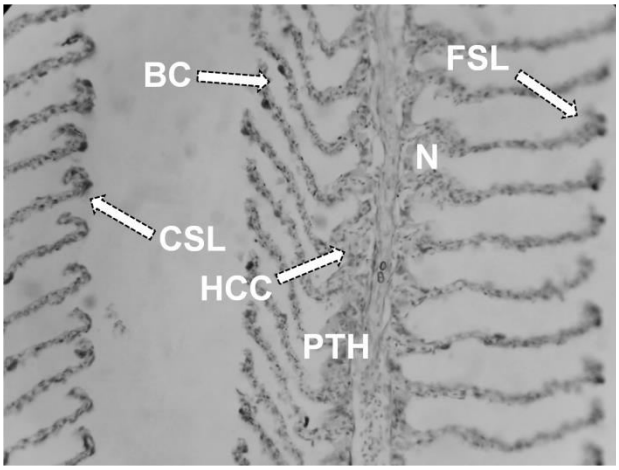

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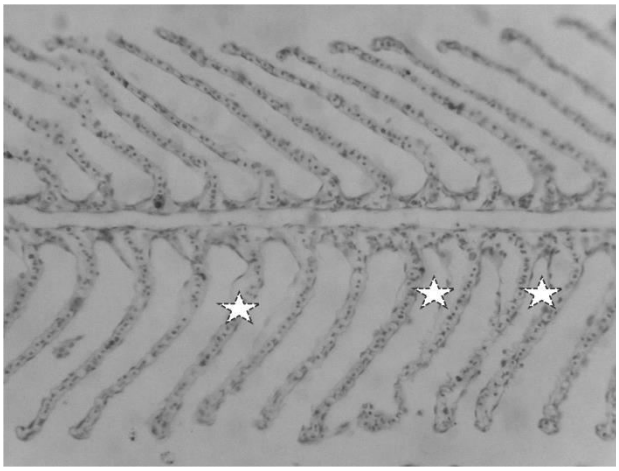

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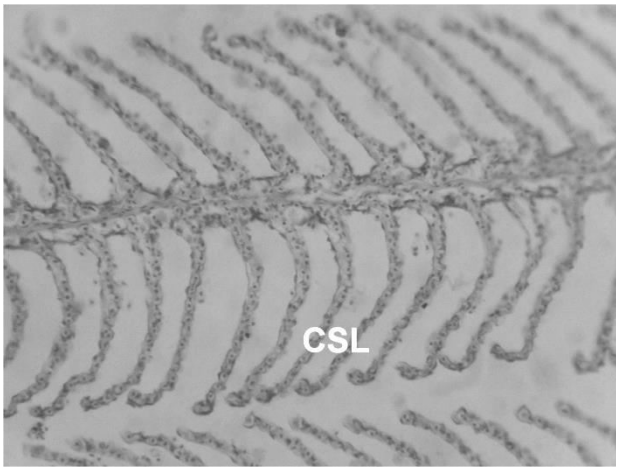

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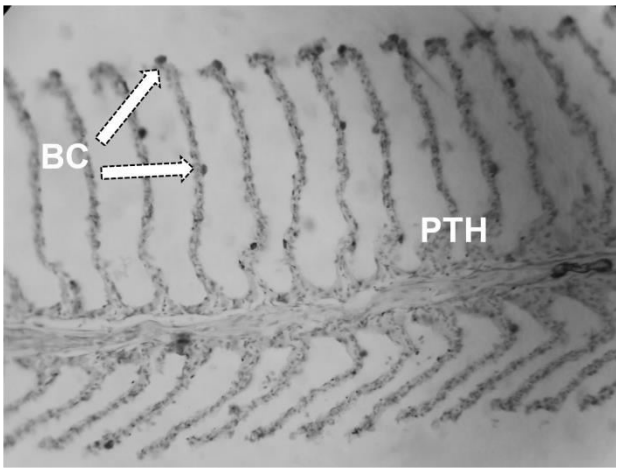

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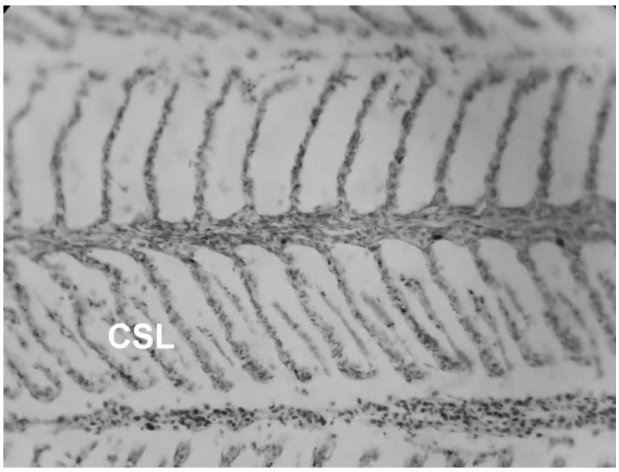

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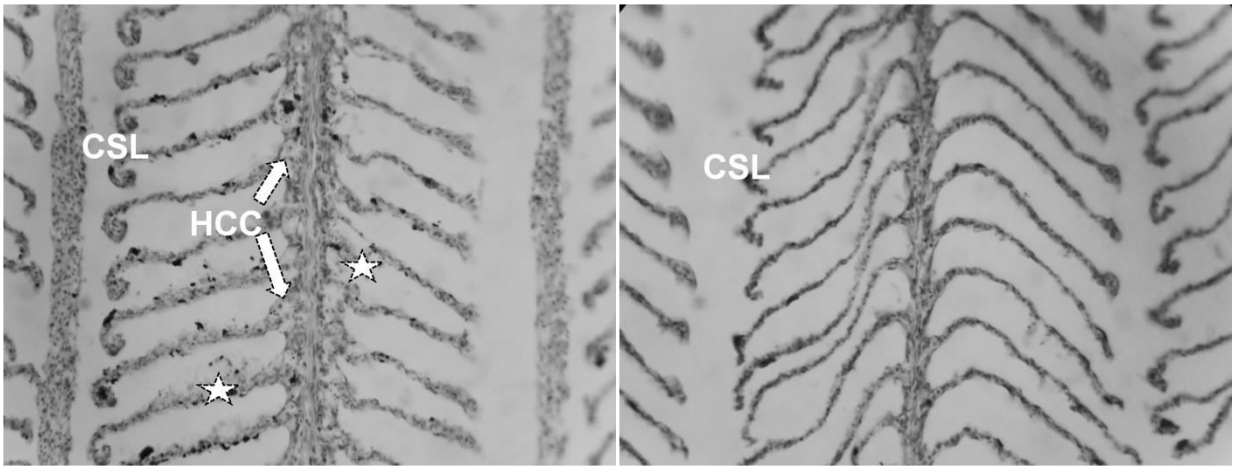
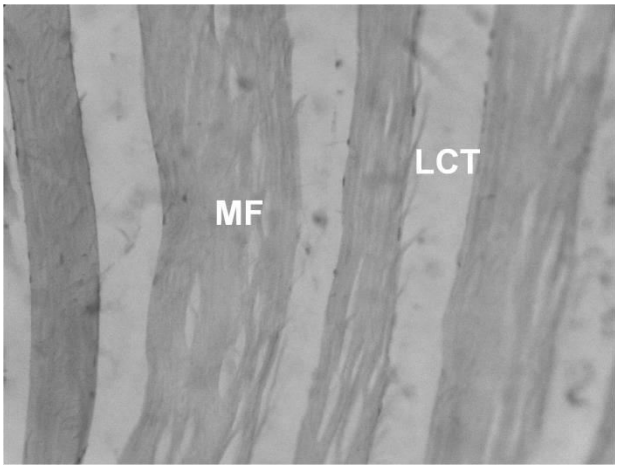


FT24 ST24

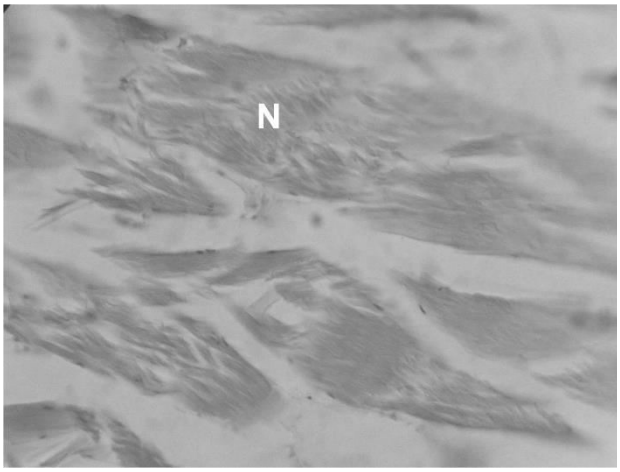
Figure 11: showing gill histoarchitecture from control fish (C), fishes exposed to toxin (TC), Toxin/antioxidant pre-treated fish (FT6, FT12, FT24, ST6, ST12, and ST24). C: Gills of control fish showed normal histology: (PL) Primary lamellae; (SL) Secondary lamellae; (PC) Pillar cell; (CC) Chloride cell. TC: Toxin treated fishes showed, proliferative tissue hyperplasia (PTH), partial fusion of the secondary lamellae (FS), blood congestion (BC) and curling of secondary lamellae (CSL), hypertrophy of chloride cells (HCC); Toxin/antioxidant pre-treated showed, FT6: epithelium uplifting (star); FT12: blood congestion (BC) and proliferative tissue hyperplasia (PTH); FT24: epithelium uplifting (star symbol), curling of secondary lamellae (CSL), hypertrophy of chloride cells (HCC); ST6, ST12, and ST24: curling of secondary lamellae (CSL). Stain: H & E; Magnification 40X.

4.5.3. HISTOPATHOLOGICAL CHANGES IN MUSCLES

The histoarchitecture of examined muscle tissues in all *S. rivulets* fish groups were displayed in Figure 12. The toxin treated fishes showed no histological alterations in the vital organs when compared to the control group. The typical architecture of muscle tissues was observed in the control fishes, whereas histopathological lesions were not found in the treated fishes. The main alterations observed in the toxin treated muscle tissues include histoarchitectural changes such as deformations in the muscle fiber along with intercellular oedema, necrosis, and atrophic myocytes. The intensity of the histological changes was further decreased (back to the control group) in the fishes exposed antioxidants pre-treatments.



C



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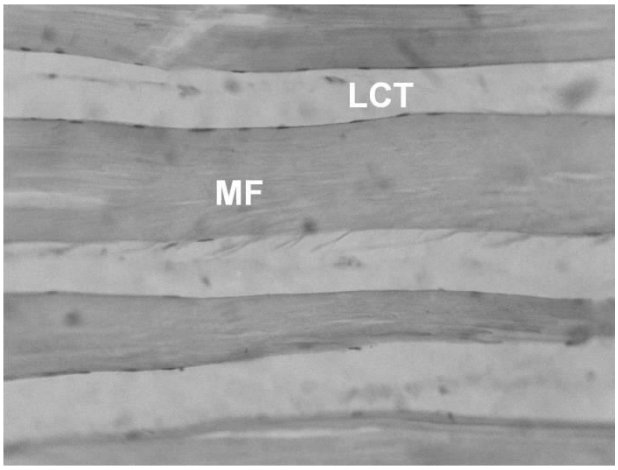
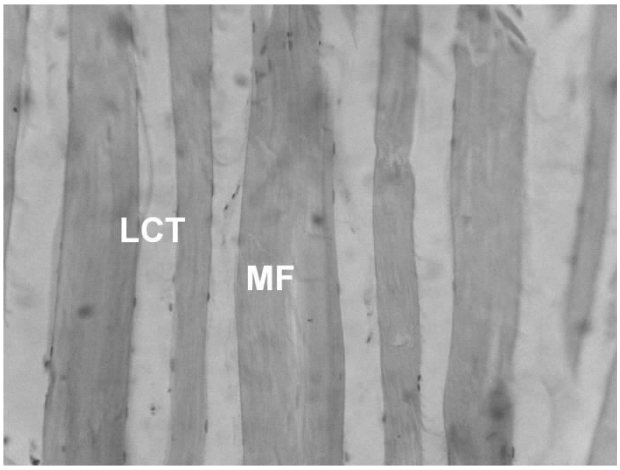


TF6



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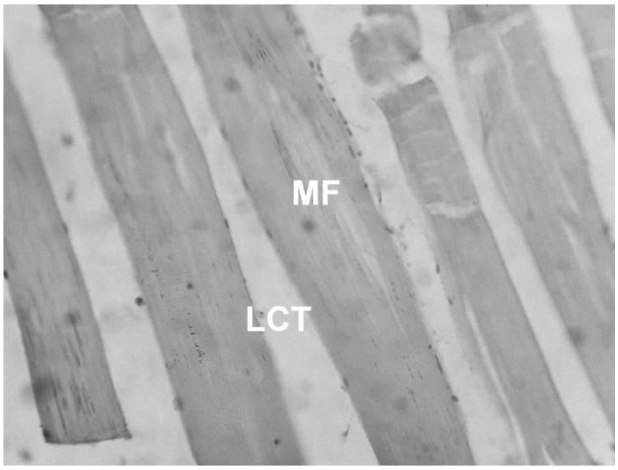

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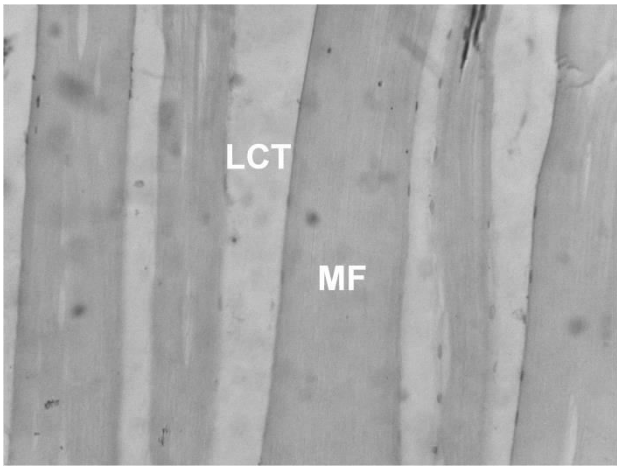

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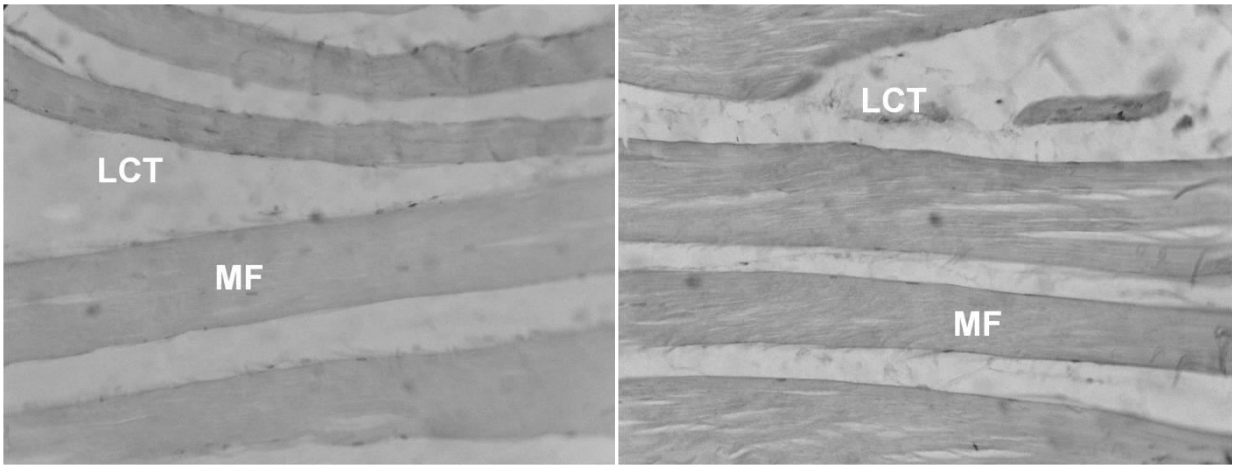

FT24 ST24

Figure 12: Representative photomicrograph of muscle tissues of control fish (, fishes exposed to toxin (TC), Toxin/antioxidant pre-treated fish (FT6, FT12, FT24, ST6, ST12, and ST24). Where C: Muscle of control fish showed normal architecture of muscle tissues. TC: Toxin treated muscle tissues showed necrosis. (MF) Muscle fibers, (LCT) Loose connective tissue, (N) necrosis. Stain: H & E; Magnification 40X.

CHAPTER

5

5. DISCUSSION

During the last decades, there are increasing shreds of evidence from different studies highlighting the involvement of organophosphate insecticides and in particular, Methidathion in inducing oxidative stress in fish (Özkan-Yılmaz et al., 2015). Despite a large number of scientists published on the harmful effects of organophosphates, their mischievous impact in Aquaculture has little attracted the interest of researchers worldwide. In the present study, symptoms of cytotoxicity were observed (using selected biomarkers and histopathology specimens) after Methidathion intoxication, suggesting the role of ROS in the pathogenesis of Methidathion, in agreement with previous findings by Kavitha and Rao (2008) and Kwonq (2002).

Methidathion ($C_6H_{11}N_2O_4PS_3$) is one of the most worldwide used and more toxic organophosphate insecticides. The LC_{50} concentration of Methidathion by bath administration was determined as 7.5 µg toxin/L by a modified Fawell's up and down method (Fawell et al., 1999), which is at variation with some earlier reports in aquatic organisms such as common carp (Balint et al., 1995), rainbow trout, bluegill, and

goldfish (USEPA, 2006). The wide range of LC_{50} values among various animal models may be depending on the species, age, and sex used (Güngördü, 2013; Özkan-Yılmaz et al., 2015).

During the experimental process, no mortality associated with Methidathion and Zallouh roots or Milk thistle seeds administration was observed. The behavior of the Methidathion treated fishes could be distinguished from those of the controls and antioxidants pre-treated fishes by dullness, loss of equilibrium and erratic swimming (Bálint et al., 1995; Uner et al., 2006; Banaee et al., 2011). In agreement with previous works by Altuntas et al. (2002) and Gokalp et al. (2003), our results indicate that severe hepatopancreas damage accompanied by a remarkable change in color and weight can occur after Methidathion exposure. Hepatocellular damage was first noticed by the increase in total hepatopancreas size, due to intrahepatic hemorrhage caused by the action of Methidathion toxicity (Dufour and Clavien, 2005). After the Methidathion exposure, histopathological examination showed some blood congestion in the hepatic vessel, due to the increase in the hydrostatic pressure leading a blood re-flows toward the hepatopancreas, causing its swelling and hepatomegaly (Carmichael, 1994). This observation was confirmed as outlined in the results (Chapter 4, section 5) for groups receiving only the toxin. The increase in hepatopancreas weight was less evident in all the antioxidant pre-treated groups that had received the toxin, due to the anti-hepatotoxicity effect of Zallouh (*Ferula hermonis*) roots and Milk thistle (*Silybum marianum*) seeds.

The primary acute mammalian toxicity associated with exposure to organophosphates results from inhibition of the acetylcholinesterase (Yavuz et al., 2005; Zimmerman and Soreq, 2006; Tripathi and Srivastava, 2008). However, recent findings indicated that oxidative stress could be an essential key factor to the toxicity

mechanism of organophosphates by enhancing the generation of reactive oxygen species (ROS) and disturb the equilibrium between ROS and antioxidant defense systems (Bagchi et al., 1995; Gultekin et al., 2000 and 2001). Consequently, organophosphate insecticides may increase the production of lipid peroxidation (LPO) by directly interacting with the cellular organic molecules, including polyunsaturated fatty acids (PUFA) in the cell membrane (Bachowski et al. 1997; Bagchi et al. 1995).

The cell has many mechanisms of mitigating the effects of oxidative stress damage, either by repairing the damage or by inhibiting or neutralize ROS using enzymatic and non-enzymatic antioxidant defense systems (Wahsha et al., 2010). Some of the most important antioxidant defense enzymes are superoxide dismutase, glutathione peroxidase, glutathione S-transferase, catalase and glutathione reductase (Sewald and Jakubke, 2002). On the other hand, polyphenols (such as flavonoids), act as non-enzymatic defense system (Alan and Miller, 1996; Jos et al., 2005) Plant flavonoids are emerging as potential therapeutic drugs effective against a wide range of ROS (Chaudhuri et al., 2007). Our findings suggest that Methidathion intoxication may induce the formation of extremely unstable free radicals that could attack lipids containing carbon-carbon double bonds, mainly PUFA. Some previous studies from our laboratory emphasized on the effects of milk thistle seed extracts on LPO and antioxidant enzymes (Wahsha and Al-Jassabi, 2009; Wahsha et al., 2012b). Gokalp et al. (2003) have shown that single dose treatment with alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C) after the administration of Methidathion can reduce liver damage in rats. Özkan-Yılmaz et al. (2015) showed that sub lethal dose concentrations of Methidathion could harm the antioxidant enzymatic defense system and increase lipid peroxide levels in the hepatopancreas of the Nile tilapia.

Yet, it is not known, up to our knowledge, that *Ferula hermonis* may lead to protect the damage induced by Methidathion.

Evaluation of serum biochemical parameters including enzymes can be important to identify injuries of target organs, as well as the health status of the animal, and supports to provide early warning of potentially dangerous changes in stress conditions (Folmar, 1993; Oner et al., 2008). Thus, the specificity of essential hepatobiliary enzymes is of great importance to the diagnosis of hepatobiliary diseases (Asterisk et al., 2006). Damage to the hepatopancreas after Methidathion exposure is evident (as shown in chapter 4 section 5.1), and the apparent sign of hepatic injury is the leakage of hepatic enzymes into plasma (Xu et al., 2007). There is no doubt that the changes in the biochemical parameters in our study supported a diagnosis of liver damage. The increased levels of the serum enzymes ALT and LDH, and the decrease in total cholesterol were observed in Methidathion treated groups, indicating a notable increase in the permeability, injury or necrosis of hepatocytes. Elevated ALT is one of the most sensitive bio-indicator of hepatocellular damage (Vozarova *et al*., 2002). The determination of ALT had proved to be a reliable biomarker for liver injuries and disease. In the current study, ALT increased significantly ($P < 0.05$) after Methidathion exposure. Likewise, Gökalp et al. (2003) demonstrated increased activities of ALT in Wistar albino exposed to Methidathion. Nevertheless, Methidathion exposure slitly alter the levels of ALT in the pretreatment groups, and this in agreement with the findings of Altuntas et al. (2002). LDH is another biomarker was used in this study; it is a cytoplasmic enzyme that catalyzes the oxidation of lactate into pyruvate (Kato et al., 2006). LDH enters the blood circulation even in minor cell and tissue damage (Botha et al., 2004). LDH is predominantly located in the liver and less in cardiac and muscle tissues (Nidhi et

al., 2003). We could outline during this experiment that Zallouh roots and Milk thistle seeds could reduce the amount of LDH in serum compared to fish groups, which received only the toxin. Furthermore, lipids and cholesterol are the most susceptible to oxidative stress and several studies reported that Methidathion could initiate lipid peroxidation through the action of ROS (Agrahari et al., 2007).

The serum total cholesterol was significantly decreased ($P < 0.05$) in the experimental fishes that were exposed to Methidathion, in agreement with similar studies by (Choudhari and Chakraharti, 1984; Ryhanen et al., 1984). The observed decrease in the serum cholesterol is strongly interrelated to the detrimental effect of Methidathion on the hepatopancreas (as presented in chapter 4 section 2.3). However, as discussed previously, Methidathion can induce the generation of free radicals, which is believed to play an essential role in both physiological and pathological processes in many diseases such as cancer (Gallo and Lawryk, 1991). Unsaturated fatty acids and cholesterol are essential components of the plasma membranes in animal cells (Meer et al., 2008). ROS induces disturbance of the cell membrane structure, ultimately leading to loss of functions (Meo et al., 2016). Besides, organophosphate insecticide might inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, which are key enzyme in cholesterol production (Ryhanen et al. 1984). On the contrary, our analysis of serum cholesterol showed that antioxidants could bring a decrease in the formation of MDA through their ability to scavenge the hydroxyl radicals and therefore, reducing the amount of cholesterol damage. Some studies indicated that different organs might be affected by organophosphate insecticides intoxication (Yavuz et al., 2005 and references therein). In this study, we demonstrated in chapter 4, section 5 that bath administration of 7.5 µg/L Methidathion caused a significant alterations of the overall tissue structure. Likewise,

our findings are in accordance with a similar study by Yavuz et al. (2005), and Altinok and Capkin (2007). As suggested by Yavuz et al. (2005), the evidence for tissue deformation under the effect of Methidathion is due to the degeneration of collagenous and elastic fibers of vascular cell walls. Ejiri et al. (2003) indicated that one of the most striking histological characteristics of aneurysmal tissue is the medial lamellae fragmentation and decreased the concentration of elastic protein that allows tissues to resume their shape after stretching or contracting.

In this study, the protective antioxidant action against Mithidathion may be related to the secondary metabolite composition of the two plants used. The *Ferula hermonis* is rich in the terpenoids and the *Silybum marianum* presents an elevated concentration of terpenoids and flavonoids (see chapter 4, section 4.1). Moreover, flavonoids and terpenoids are a large group of plant derived compounds, that may enhance the overall physiological condition of fish (Chakraborty et al., 2013). Most of these phytoconstituents are redox active compounds and may inhibit the generation of ROS and scavenge free radicals (Engwa, 2018). The *Ferula hermonis* and *Silybum marianum* have biologically active metabolites such as ferutinin, teferidin, sinkiangenrin F, lehmannolol, silibinin, naringenin, eriodictyol, and luteolin. Some of which having a great variety of biological effects including antioxidant, antimicrobial, antimycobacterial, antifungal, anti-inflammatory (see appendix, annnex 1 and 2) (Narvaez-Mastache et al., 2008; Arghiani et al.,2014; Li et al., 2015; Sun et al., 2015; Csupor et al., 2016; Zavatti et al., 2016; Yin et al., 2018).

5.1. CONCLUSIONS

1. The present results show a strong correlation of Methidathion in inducing tissues lipid peroxidation and its role in the alterations of activity profile of certain enzymes in *vivo*.
2. Accordingly, our results showed a distinct increase in serum ALT, LDH and tissue homogenates MDA after the administration of the toxin in comparison to control groups.
3. To date, there is no effective natural treatment to the acute tissue injury induced by the Methidathion. In agreement with other similar studies, it is well confirmed that severe oxidative stress induced by Methidathion also contributes to the acute toxicity in several fish species. However, the protective effect of Zallouh roots and Milk thistle seeds used in this work could provide a new insight into the potential alternative therapeutic solution to aquaculture contamination by organophosphate insecticides in general.
4. The present work also highlights the importance of Zallouh roots and Milk thistle seeds as protective actions against Methidathion that induced oxidative stress, the protective action could be effective also for other toxic with ROS activity. This was supported by the biochemical and histological results in the pre-treated groups of fish by the two plants used.
5. Moreover, Zallouh roots and Milk thistle seeds treatments attenuated significantly the oxidative damage as indicated by reduced lipid peroxidation as well as they showed significant role in reversing the histopathological effect allowing tissue rehabilitation and recovery. Therefore, they have a potential of reducing the toxic effects of “insecticides”.

6. The protective antioxidant action can be related to the secondary metabolite composition of the two plant used for this study, the *Ferula hermonis* is rich in the terpenoids and the *Silybum marianum* presents elevated concentration of terpenoids and flavonoids.
7. A huge bulk of selected species (*Ferula hermonis* and *Silybum marianum*) remains untapped in terms of pharmacological constituents, and this is the research gap for future investigations. Further studies are required to thoroughly understand the molecular mechanisms of their biological action in vitro and in vivo and to assure the plant extracts are reliable for aquaculture and human use.

5.2. RECOMMENDATIONS

It is recommended to utilize the outcomes of this research and the adopted methodology by other researchers:

For knowledge dissemination to private enterprise promoting future intensive production of fish aquaculture.

To enhance regional cooperation by coordinating scientific efforts, collaboration in the Gulf of Aqaba and Mediterranean countries, and sharing information gained throughout this project.

Based on the above, further studies are still required to investigate more on the effectiveness of the used antioxidants on other fish species of marine origin.

5.3. ABSTRACT

The aim of this study was to investigate the protective effect of *Ferula hermonis* roots and *Silybum marianum* seeds on Methidathion induced oxidative stress in *Siganus rivulatus* fish. Different groups of fish were fed on daily bases with (2.5 g of *Ferula hermonis* or 2.5 g of *Silybum marianum* / Kg fish body weight), for 14 days. Then the fish were challenged with Methidathion LC_{50} for 24 hours. Fish health status was evaluated through stress biomarkers; Lipid peroxidation (LPO), Alanine Aminotransferase (ALT), and Lactate Dehydrogenase (LDH), in addition to total cholesterol. Normal and stressed fish tissue were subjected to histo-pathological investigation. Moreover, a holistic picture about all detectable metabolites including the known and unknown compounds in *F. hermonis* roots and *S. marianum* seeds was performed through HPLC-HRMS.

LC_{50} of Methidathion for *Siganus rivulatus* fish was determined via bath route for 24 hours, and it was about 7.5 ug/l. Our results showed a significant increase in the serum ALT, LDH activities, and lipid peroxidation products (MDA) in tissue homogenates (hepatopancreas, gills, and muscles) compared to control group. With exception to total cholesterol, which exhibit a significant decrease, in the toxin treated group.

The most frequent histo-pathological alterations in the gills of fish exposed to Methidathion were characterized by epithelial lifting, thickening and shortening of secondary lamellae, proliferative tissue hyperplasia, blood congestion, hypertrophy of chloride cells and necrosis. In the hepatopancreas, the most common observed histological changes were cellular infiltration, blood congestion and bile stagnation, nuclear hypertrophy, cytoplasmic vacuolization, and degeneration of hepatocytes. In the muscles tissues, the main alterations observed include histoarchitectural

changes such as deformations in the muscle fiber. The histological observation of this study support our research about the protective effects of *Ferula hermonis* roots and *Silybum marianum* seeds on Methidathion induce oxidative stress in *Siganus rivulatus* fish. Our study provides a new insight into the potential alternative therapeutic solution to Aquaculture contamination by organophosphate insecticides in general.

5.4. ABSTRACT ITALIANO

Lo scopo di questo studio era indagare gli effetti protettivi delle radici di *Ferula hermonis* e dei semi di *Silybum marianum* sullo stress ossidativo indotto dal pesticida Methidathion sul pesce *Siganus rivulatus*. Gruppi differenti di pesci erano alimentati giornalmente (2.5 g di Ferula hermonsis o 2.5 g di Silybum marianum/kg di peso corporeo del pesce) per 14 giorni; quindi i pesci erano trattati con Methidathion LC_{50} per 24 ore. Lo stato di salute del pesce è stato valutato mediante biomarker, perossidazione dei grassi (LPO), aminotrasferasi dell'alanina (ALT), and deidrogenasi del lattato (LDH), contenuto di colesterolo totale. Tessuti dei pesci stressati e non stressati sono stati sottoposti a indagini istopatologiche. Lo studio ha previsto una caratterizzazione metabolica per individuare componenti noti e sconosciuti delle parti di *Ferula hermonis* e *Silybum marianum* mediante analisi *untarget* HPLC-HRMS, con l'obiettivo di individuare le componenti con proprietà antiossidanti delle piante.

La dose letale 50 (LC_{50}) del Methidathion per il pesce *Siganus rivulatus* è stata determinata mediante immersione in bagno per 24 ore, il valore ottenuto è di 7.5μg/l. I risultati ottenuti mostravano un significativo incremento di ALT, attività LDH e dei prodotti della perossidazione dei lipidi (MDA) nel siero e in organi omogeneizzati (epatopancreas, branchie e muscoli) degli individui esposti a stress rispetto agli organismi di controllo. Il contenuto del colesterolo nel gruppo trattato con il pesticida era significativamente ridotto.

Le alterazioni istopatologiche più frequenti nelle branchie dei pesci esposti al methidathion erano caratterizzate da distacco epiteliale, ispessimento e accorciamento delle lamelle secondarie, proliferazione della iperplasia del tessuto, congestione sanguigna, ipertrofia delle cellule del cloruro e necrosi.

Nell’epatopancreas i cambiamenti più frequenti erano infiltrazioni cellulari, congestione sanguigna e stagnazione della bile, ipertrofia del nucleo, vacuolizzazione citoplasmatica e degenerazione degli epatociti. Nei tessuti muscolari le alterazioni più importanti includevano cambiamenti dell’architettura istologica come la deformazione delle fibre muscolari. Le indagini istologiche e sul contenuto di biomarker hanno evidenziato l’effetto protettivo derivante dall’uso di radici di *Ferula hermonis* e semi di *Silybum marianum,* come integratori alimentari, nello stress ossidativo indotto dall’esposizione a Methidathion. Lo studio fornisce evidenze della validità di un potenziale metodo terapeutico alternativo alla soluzione della contaminazione da insetticidi organo-fosfati e in generale da insetticidi in acquacultura.

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5.6. REFERENCES

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5.7. APPENDIX

Table S_1: Supplementary material S_1. Compounds that have been identified from roots extract of *Ferula hermonis.*

No	Rt	Mass(uD)	Formula	Putative compound	Class	References
1	32.47031	357.2064	$C_{22}H_{30}O_4$	Ferutinin	Sesquiterpene	Arnoldi et al., 2004.
2	33.61885	383.2221	$C_{24}H_{32}O_4$	Lehmannolol	Sesquiterpene coumarins	Yang et al., 2006.
3	25.66322	373.2019	$C_{22}H_{30}O_5$	6-(*p*-hydroxybenzoyl)epoxyjaeschkeanadiol	Diterpenes	Alkhatib et al., 2008.
4	27.71075	371.1853	$C_{22}H_{28}O_5$	Lancerodiol 6-(4-hydroxybenzoate)	Diterpene lactones	Razdan et al., 1989.
5	30.16764	399.2155	$C_{24}H_{32}O_5$	Sinkiangenrin F	Sesquiterpene coumarins	Li et al., 2015.
6	30.77069	415.2108	$C_{24}H_{32}O_6$	Lancerotriol 9-acetate-6-p-hydroxybenzoate	Carotane sesequiterpen	Ahmed et al., 2001.
7	22.1544	1117.537	$C_{54}H_{86}O_{24}$	Sandrosaponin IX	Triterpene saponins	Li et al., 2015.
8	36.02276	339.1964	$C_{22}H_{28}O_3$	14-(4'-Hydroxybenzoyloxy)dauc-4,8-diene	Daucane Sesquiterpenes	Galal et al., 2001.
9	25.20438	389.1976	$C_{22}H_{30}O_6$	Kuhistanicaol H	Sesquiterpene	Tamemoto et al., 2001.
10	36.59249	341.2108	$C_{22}H_{30}O_3$	Teferidin	Sesquiterpene	Lhuillier et al., 2005.
11	29.08895	403.2104	$C_{23}H_{32}O_6$	Tenuferin	Sesquiterpene	Saidkhodzhaev A.I., 1978.
12	31.73475	441.2268	$C_{26}H_{34}O_6$	8-O-acetyl sinkiangenorin	Sesquiterpene coumarins	Li et al., 2015.
13	34.97659	455.2404	$C_{27}H_{36}O_6$	10α-angeloyl ferutinin	Triterpenoids	Dall'Acqua et al., 2014.
14	28.36114	399.2155	$C_{24}H_{32}O_5$	Nevskin	Sesquiterpenes coumarine	Gonzalez et al., 1993.
15	23.13709	956.488	$C_{48}H_{76}O_{19}$	Sandrosaponin X	Triterpene saponins	Zedan et al., 2012.
16	26.45809	237.186	$C_{15}H_{26}O_2$	Ferutinol	Sesquiterpens	
17	27.42465	419.2051	$C_{23}H_{32}O_7$	Sinkiagenrin A	Steroidal esters	Li et al., 2014.
18	17.77212	433.2413	$C_{21}H_{38}O_9$		Sesquiterpene Glycosides	Fu et la., 2015.
19	18.10363	479.2481	$C_{21}H_{38}O_{9\ +\ F.A}$		Sesquiterpene Glycosides	Fu et la., 2015.
20	18.67176	509.2216	$C_{21}H_{36}O_{11\ +\ F.A}$		Terpene glycoside compounds	Wang et al., 2014.
21	17.3777	565.283	$C_{33}H_{42}O_8$		Terpinoid	Xie et al., 2008.
22	19.01831	361.1853	$C_{17}H_{30}O_8$		Terpen glycoside	
23	35.81727	525.319	$C_{32}H_{46}O_6$		Triterpenoids	
24	30.37365	663.4063	$C_{37}H_{60}O_{10}$		Triterpenoid	
25	18.22943	623.2896	$C_{27}H_{46}O_{13\ +\ F.A.}$		Terepene glycoside	
26	34.62826	293.1787	$C_{17}H_{26}O_4$		Sesquiterpenes	Ramos et al., 2006a.
27	37.97134	457.2946	$C_{28}H_{42}O_5$		Terpenoid	
28	41.79219	559.3757	$C_{37}H_{52}O_4$		Triterpenes	
29	18.86093	317.1957	$C_{15}H_{28}O_{4\ +\ F.A.}$		Sesquiterpenoids	Fu et la., 2015.
30	16.43062	569.2419	$C_{31}H_{38}O_{10}$		Diterpenoids	Jassbi A., 2006.
31	21.31439	575.2676	$C_{27}H_{44}O_{13}$		Triterpenoids glycoside	Zidorn C., 2008.
32	20.87091	553.2621	$C_{28}H_{42}O_{11}$		Sesquiterpenes	
33	15.59106	477.2324	$C_{22}H_{38}O_{11}$		Monoterpene glycoside	Uemura et al., 2013.
34	20.25291	607.2934	$C_{27}H_{46}O_{12\ +\ F.A.}$		Sesquiterpene	Huang et al., 2014.
35	14.0854	479.2471	$C_{22}H_{40}O_{11}$		Terpene glycosides	
36	30.64331	571.288	$C_{32}H_{44}O_9$		Triterpenoids	

37	19.08146	551.247	$C_{28}H_{40}O_{11}$		Diterpenoids	Shen et al., 2005.
38	40.68153	519.3436	$C_{34}H_{48}O_4$		Terpinoid	
39	20.36328	403.1954	$C_{19}H_{32}O_9$		Terpene glycosides	
40	16.19348	491.1754	$C_{21}H_{32}O_{13}$		Terpene glycosides	
41	16.00322	449.2371	$C_{21}H_{38}O_{10}$		Sesquiterpene Glycosides	Chang et al., 2001.
42	39.85613	813.4536	$C_{49}H_{66}O_{10}$		Terpenoid	
43	19.27111	317.1954	$C_{16}H_{30}O_6$		Terpene glycosides	
44	21.26725	599.2697	$C_{29}H_{44}O_{13}$		Terpene glycosides	
45	23.26407	407.2054	$C_{22}H_{32}O_7$		Diterpenoids	
46	33.79263	311.1671	$C_{20}H_{24}O_3$		Diterpene	
47	40.20529	961.6062	$C_{57}H_{86}O_{12}$		Triterpenoids	
48	43.50741	677.4952	$C_{40}H_{70}O_8$		Terpene glycosides	
49	30.92874	353.1745	$C_{22}H_{26}O_4$		Terpenid	
50	29.81779	595.2871	$C_{41}H_{40}O_4$		Terpenid	
51	14.0057	569.2253	$C_{27}H_{38}O_{13}$		Sesuiterpenes glycoside	Zhan, et al. 2005.
52	41.72915	651.4233	$C_{40}H_{60}O_7$		Triterpenes	Kaweetripob et al., 2013.
53	22.80369	651.3203	$C_{37}H_{48}O_{10}$		Diterpenoids	Zhan et al., 2005.
54	39.82444	565.3528	$C_{34}H_{48}O_{4 + F.A.}$		Terpenoid	
55	38.07187	619.4189	$C_{36}H_{60}O_8$		Triterpenoids	Zhou et al., 2017.
56	38.2155	621.4366	$C_{36}H_{62}O_8$		Triterpenoids	
57	20.07849	535.2524	$C_{28}H_{40}O_{10}$		Terpenoid glycoside	
58	26.20418	541.2632	$C_{27}H_{42}O_{11}$		Terpenoid glycoside	
59	18.18203	825.4241	$C_{42}H_{66}O_{16}$		Triterpenoids	
60	26.66412	793.4321	$C_{42}H_{66}O_{14}$	Tibesaikosaponin II	Triterpene saponins	Fang et al., 2017.
61	20.34788	461.2372	$C_{22}H_{38}O_{10}$		Monoterpenoid glycoside	Tian et al., 1998.
62	18.45049	391.178	$C_{21}H_{28}O_7$		Terpenoids	
63	38.89534	507.3092	$C_{32}H_{44}O_5$		Terpenoid	
64	13.38727	379.1597	$C_{16}H_{28}O_{10}$		Monoterpenoid	
65	44.03194	937.5184	$C_{49}H_{78}O_{17}$		Pregnane glycoside (steroidal)	Warashina et al., 2011.
66	30.4526	397.2003	$C_{24}H_{30}O_5$	13-hydroxyfeselol	Coumarins and Derivatives	Ahmed et al., 2007.
67	25.50516	391.2107	$C_{22}H_{32}O_6$	Kuhistanol C	Prenylated Benzoic Acid Derivatives	Chen et al., 2000.
68	33.80891	381.2068	$C_{24}H_{30}O_4$	(E)-omega-Hydroxyferulenol	Coumarins and derivatives	Arnoldi et al., 2004.
69	15.54391	353.0856	$C_{16}H_{18}O_9$	5-Caffeoylquinic acid	Phynolic compound	Alkhatib et al., 2008.
70	19.61974	515.1181	$C_{25}H_{24}O_{12}$	1,5-dicaffeoylquinic acid	Phenol glycoside	Alkhatib et al., 2008.
71	29.64362	369.1701	$C_{22}H_{26}O_5$		Isoflavonoids	Kırmızıbekmez et al., 2015.
72	16.66757	553.229	$C_{27}H_{38}O_{12}$		Lignan glycosides	Marchal et al., 2015.
73	14.54527	387.0915	$C_{16}H_{20}O_{11}$		Coumarins glycoside	
74	24.28248	577.1335	$C_{30}H_{26}O_{12}$	4-O-8',5'-5"-Dehydrotriferulic acid	Biphenyls and derivatives	Antognoni et al., 2011.
75	35.68998	545.2883	$C_{34}H_{42}O_6$		Xanthones	
76	21.77475	551.2106	$C_{27}H_{36}O_{12}$		Lignan glycosides	Long et al., 2016.
77	10.26417	393.1758	$C_{17}H_{30}O_{10}$		Phenolic compounds	Gadetskaya et al., 2015.
78	36.02276	679.3978	$C_{44}H_{56}O_6$		Flavonoid	
79	20.45846	487.1798	$C_{22}H_{32}O_{12}$		Phenolic glycosides	Ma et al., 2011.

80	21.72716	329.1051	$C_{18}H_{18}O_6$		Isoflavonoid	
81	39.91957	713.4019	$C_{44}H_{58}O_8$		Xanthones	
82	42.36415	575.3717	$C_{37}H_{52}O_5$		Coumarine	Chen et al., 2006.
83	15.16298	417.1006	$C_{17}H_{22}O_{12}$		Phenolic derivative of benzoic acid	Starka et al., 2017.
84	18.57668	493.227	$C_{22}H_{38}O_{12}$		Glycoside	
85	19.68283	475.179	$C_{20}H_{30}O_{10 + F.A.}$		O-glycosyl	
86	37.13213	776.548	$C_{40}H_{77}NO_{10 + F.A}$		Glucocerebrosides	
87	42.93585	786.4554	$C_{31}H_{65}N_9O_{14}$		Glucopyranoside	
88	17.04587	367.1023	$C_{17}H_{20}O_9$	5-Caffeoylquinic acid methyl ester	Quinic acids and derivatives	
89	19.16088	543.1512	$C_{27}H_{28}O_{12}$		Quinic acids and derivatives	Wenzl et al., 2000.
90	15.73387	509.2222	$C_{22}H_{38}O_{13}$		Glucopyranose	
91	19.30282	361.1857	$C_{17}H_{30}O_8$		Glycoside	
92	15.14753	417.1024	$C_{17}H_{22}O_{12}$		Glucosides	
93	16.46238	685.2708	$C_{32}H_{46}O_{16}$		Glycosides	
94	18.32424	581.2558	$C_{29}H_{42}O_{12}$		Glycoside	
95	16.87229	803.366	$C_{38}H_{60}O_{18}$		Glycoside	
96	14.90971	427.1809	$C_{16}H_{30}O_{10 + F.A.}$		Glucosides	Uemura et al., 2013.
97	13.84721	425.1649	$C_{17}H_{30}O_{12}$		Glycoside	
98	14.46554	417.1025	$C_{17}H_{22}O_{12}$		Glycoside	
99	14.76657	447.1484	$C_{18}H_{26}O_{10 + F.A.}$		O-glycosyl compounds	
100	21.91697	543.1501	$C_{27}H_{28}O_{12}$		Quinic acids and derivatives	Wenzl et al., 2000.
101	20.71278	517.1317	$C_{25}H_{26}O_{12}$		O-glycosyl compounds	
102	37.14761	730.5397	$C_{40}H_{77}NO_{10}$		Glycosphingolipids	
103	42.50666	791.5252	$C_{45}H_{76}O_{11}$		Glycolipid	
104	21.42542	529.1332	$C_{26}H_{26}O_{12}$		Quinic acids and derivatives	Rodrigues and Bragagnolo, 2013.
105	12.97298	341.0872	$C_{15}H_{18}O_9$		O-glycosyl compounds	
106	9.355997	399.1496	$C_{15}H_{28}O_{12}$		Glucoside	
107	15.81255	461.1646	$C_{20}H_{30}O_{12}$		O-glycosyl compounds	
108	22.37504	645.2775	$C_{30}H_{46}O_{15}$		Trisaccharide	
109	41.30054	963.6191	$C_{49}H_{90}O_{15 + F.A.}$		O-glycoside	
110	31.92122	295.2278	$C_{18}H_{32}O_3$		Lineolic acids	
111	36.67198	279.2323	$C_{18}H_{32}O_2$		Long-chain fatty acids	
112	16.95156	401.1798	$C_{19}H_{30}O_9$		Fatty acyl glycosides	
113	24.17082	329.2322	$C_{18}H_{34}O_5$		Long-chain fatty acids	Trana et al., 2017.
114	35.59492	277.2167	$C_{18}H_{30}O_2$		Linolenic acid	Moura et al., 2005.
115	38.05759	593.3839	$C_{37}H_{54}O_6$		Lineolic acids and derivatives	
116	37.48097	255.2325	$C_{16}H_{32}O_2$	Palmitic acid	Long-chain fatty acids	Moura et al., 2005.
117	9.483479	429.1591	$C_{16}H_{30}O_{13}$		Fat acyl glycoside	
118	12.14434	411.1851	$C_{17}H_{32}O_{11}$		Fatty acyl glycosides	
119	34.09238	767.4505	$C_{48}H_{64}O_8$		Unkown	
120	17.66137	865.3464	$C_{49}H_{54}O_{14}$		Unkown	
121	39.0854	579.4212	$C_{34}H_{60}O_7$		Unkown	

122	38.7048	518.2898	$C_{28}H_{37}O_3N_7$		Unkown	
123	24.39316	373.2062	$C_{12}H_{26}N_{10}O_4$		Unkown	
124	19.65132	492.169	$C_{28}H_{23}N_5O_4$		Unkown	
125	17.99262	459.2218	$C_{22}H_{36}O_{10}$		Unkown	
126	20.52181	588.2567	$C_{29}H_{39}O_{10}N_3$		Unkown	
127	25.37904	1018.486	$C_{61}H_{69}N_3O_{11}$		Unkown	
128	41.47513	750.4179	$C_{50}H_{57}NO_5$		Unkown	
129	26.72708	747.4077	$C_{44}H_{60}O_{10}$		Unkown	
130	35.13493	453.2261	$C_{27}H_{34}O_6$		Benzodilactones	Huang et al., 2007.
131	40.47467	493.3292	$C_{32}H_{46}O_4$		Prenylated tricyclic	Winkelmann et al., 2001.
132	19.9204	595.2953	$C_{26}H_{46}O_{12 + F.A}$		Unkown	

Table S_2: Supplementary material S_2. Compounds that have been identified from seeds extract of *Silybum marianum*

No	Rt	Mass(uD)	Formula	Putative compound	class	Ref
1	24.02019	481.1124	$C_{25}H_{22}O_{10}$	Silibinin(silymarin)	Flavonolignans	Lee et al., 2006.
2	8.465792	333.0397	$C_{19}H_{10}O_6$	6,10-Dihydroxy-1-methylbenzo[h]chromeno[5,4,3-cde]chromene-5,12-dione	Flavonoid	
3	24.36862	271.0602	$C_{15}H_{12}O_5$	Naringenin	Flavanones	Harborne and Baxter, 1999
4	20.97473	287.0555	$C_{15}H_{12}O_6$	Eriodictyol	Flavanones	Abenavoli, and Milic, 2017.
5	19.17665	303.0501	$C_{15}H_{10}O_{6\ +\ H2O}$	Luteolin	Flavonoids	Pereira et al., 2015.
6	20.52971	329.1017	$C_{18}H_{18}O_6$	4'-Hydroxy-5,6,7-trimethoxyflavanone	Flavonoids	Harborne and Baxter, 1999
7	23.67357	337.1069	$C_{20}H_{18}O_5$	5,7-Dihydroxy-8-C-(gamma-methyl-gamma-formylallyl)flavanone	Isoflavonoids	Harborne and Baxter, 1999
8	25.59069	367.1169	$C_{21}H_{20}O_6$	4'-Hydroxy-5-methoxy-7-(3-methyl-2,3-epoxybutoxy)flavone	Flavonoids	Norbedo Norbedo et al., 1984
9	11.39784	413.1643	$C_{23}H_{26}O_7$		Flavonoid	
10	19.55767	433.1113	$C_{21}H_{22}O_{10}$		Flavonoid	
11	22.30436	453.1167	$C_{24}H_{20}O_8$		Flavonoid	
12	27.0403	479.0956	$C_{25}H_{20}O_{10}$	2,3- Dehydrosilybin	Flavonolignans	
13	21.65457	481.1129	$C_{25}H_{22}O_{10}$	Silymarin	Flavonolignans	Lee et al., 2006.
14	27.15258	523.1218	$C_{27}H_{24}O_{11}$		Flavonoid	
15	18.89108	565.1557	$C_{26}H_{30}O_{14}$		Flavonoid glycuside	
16	17.78307	579.1723	$C_{27}H_{48}O_{14}$	Naringin	Flavonoid-7-o-glycosides	Abenavoli, and Milic, 2017.
17	26.51485	603.0761	$C_{30}H_{20}O_{14}$		Flavonoid	
18	17.27545	617.1169	$C_{28}H_{26}O_{16}$	Taxillusin	Flavonoid glycoside	
19	23.48311	659.173	$C_{35}H_{32}O_{13}$		Flavonoid	
20	18.3995	661.1761	$C_{31}H_{34}O_{16}$		Flavonoid diglycoside	
21	18.54204	691.1859	$C_{32}H_{36}O_{17}$		Flavonoid glycoside	
22	15.11005	759.2319	$C_{32}H_{42}O_{18\ +\ F.A.}$		Chalcone glycosides	
23	13.53254	329.0858	$C_{14}H_{18}O_9$	Vanillic acid 4-O-β-d-glucopyranoside	Phenolic glycosides	Yu et al., 2017.
24	16.03411	206.0817	$C_{11}H_{13}NO_3$	2-Acetamido-4-methylphenyl acetate	Phenol esters	
25	13.53254	329.0858	$C_{14}H_{18}O_9$	Vanillic acid 4-*O*-*β*-d-glucopyranoside	Phenolic glucoside	Yu et al., 2017.
26	15.2056	353.0868	$C_{16}H_{18}O_9$	Chlorogenic acid	Polyphenolic	Lucini et al., 2016.
27	14.61606	371.0958	$C_{16}H_{20}O_{10}$	Dihydroferulic acid 4-O-glucuronide	Phenolic glycosides	
28	12.65619	389.1431	$C_{17}H_{26}O_{10}$	Loganin	Iridoid glycoside	Jiang et al., 2012.
29	14.10654	417.1376	$C_{18}H_{26}O_{11}$	Oleoside dimethyl ester	Terpene glycosides	
30	14.31373	475.1799	$C_{21}H_{32}O_{12}$	Darendoside B	Iridoid Glycoside	Murata et al., 2008.
31	33.04464	309.1733	$C_{17}H_{26}O_5$	Hymenolide	Sesquiterpene	
32	33.14027	357.2052	$C_{22}H_{30}O_4$	18-Oxoaustrochaparol acetate	Diterpene	Bohlmann et al., 1980.
33	32.15192	363.2159	$C_{21}H_{32}O_5$	6alpha-Formyloxygrindelic acid	Diterpenes	
34	39.65066	383.1896	$C_{23}H_{28}O_5$	2alpha-Anisoyloxy-9-oxoisoanhydrooplopanone	Sesquiterpenoid	Jakupovic et al., 1988
35	34.08034	383.2205	$C_{24}H_{32}O_4$		Terpinoid	
36	14.10654	417.1376	$C_{18}H_{26}O_{11}$		Terpene glycosides	

37	33.26728	433.2343	$C_{28}H_{34}O_4$		Terpenoid	
38	36.16726	447.2505	$C_{29}H_{36}O_4$		Terpenoid	
39	38.5831	449.2667	$C_{29}H_{38}O_4$	Celastrol	Triterpene	
40	16.41553	475.1787	$C_{21}H_{32}O_{12}$		Terpenoid glycoside	
41	27.51874	477.2472	$C_{26}H_{38}O_8$		Terpenoid	
42	32.12008	507.2718	$C_{31}H_{40}O_6$		Terpenoid	
43	16.92582	557.2573	$C_{27}H_{42}O_{12}$	Ixerisoside N	Sesquiterpene	Zidorn, ., 2008.
44	30.64143	571.2864	$C_{32}H_{44}O_9$		Triterpenoids	
45	40.63898	605.4024	$C_{35}H_{58}O_8$		Terpenoid glycoside	
46	36.59776	619.418	$C_{36}H_{60}O_8$		Triterpenoid	
47	38.07786	621.4372	$C_{36}H_{62}O_8$		Terpenoid glycoside	
48	22.67082	639.3002	$C_{32}H_{48}O_{13}$		Terpenoid glycoside	
49	31.45347	653.4227	$C_{36}H_{62}O_{10}$		Triterpenoids	
50	43.50678	677.4951	$C_{40}H_{70}O_8$		Triterpenoid glycoside	
51	44.38162	679.5107	$C_{47}H_{68}O_3$		Terpenoid	
52	23.4192	685.3044	$C_{33}H_{50}O_{15}$		Terpenoid glycoside	
53	23.73703	765.3111	$C_{41}H_{50}O_{14}$		Terpenoid	
54	20.03609	813.3138	$C_{44}H_{48}O_{12 + F.A.}$		Terpenoid	
55	40.20836	961.6074	$C_{57}H_{86}O_{12}$		Terpenoid	
56	37.50504	255.2324	$C_{16}H_{32}O_2$	Palmitic acid	Long-chain fatty acids	Pelter & Hänsel, 1975.
57	35.57763	277.2163	$C_{18}H_{30}O_2$	Alpha-Linolenic acid	Lineolic acids and derivatives	Pelter & Hänsel, 1975.
58	36.67667	279.2325	$C_{18}H_{32}O_2$	Linoleic acid	Linoleic acid	Pelter & Hänsel, 1975.
59	30.91162	293.2114	$C_{18}H_{30}O_3$	Helenynolic acid	Fatty acid	Powell, G.R., 2009.
60	31.93158	295.2278	$C_{18}H_{30}O_{2 + H2O}$	Alpha-Linolenic acid	Lineolic acids and derivatives	
61	32.56621	297.2436	$C_{18}H_{34}O_3$	Ricinoleic acid	Unsaturated fatty acid	Perdomo et al., 2013.
62	40.00159	309.2797	$C_{20}H_{38}O_2$		Fatty acid acyle	
63	29.04839	313.2369	$C_{18}H_{34}O_4$		Fatty acids	
64	25.8134	327.2161	$C_{18}H_{32}O_5$		Lineolic acids	
65	26.64193	343.2475	$C_{19}H_{36}O_5$		Fatty acid	
66	35.81666	591.4099	$C_{30}H_{58}O_8$		Acyl glycoside	
67	10.55288	443.1745	$C_{16}H_{30}O_{11 +F.A.}$		O-glycosyl	
68	11.39784	413.1643	$C_{15}H_{28}O_{10 + F.A.}$		O-glycosyl	
69	9.708273	267.073	$C_9H_{16}O_9$		Sugar acids and derivatives	
70	18.3995	337.0919	$C_{16}H_{18}O_8$	3-O-p-Coumaroylquinic acid	Quinic acids and derivatives	
71	38.01436	381.1732	$C_{16}H_{30}O_{10}$		O-glycosyl compounds	
72	17.06882	459.1852	$C_{21}H_{32}O_{11}$		Glycosides	
73	15.81004	489.1594	$C_{20}H_{28}O_{11}$		Glycoside	
74	18.14703	499.1433	$C_{22}H_{28}O_{13}$		Glycoside	
75	16.60671	505.1892	$C_{22}H_{34}O_{13}$		O-glycosyl compound	
76	16.78202	509.2219	$C_{21}H_{36}O_{11}$		O-glycosyl compound	
78	38.9977	577.3734	$C_{33}H_{54}O_8$		Steroid glycoside	
79	35.81666	591.4099	$C_{30}H_{58}O_8$		Acyl glycoside	
80	20.32236	675.3206	$C_{31}H_{50}O_{13}$		Glycoside	

81	20.62528	681.2744	$C_{33}H_{46}O_{15}$		Glycoside	
82	15.11005	759.2319	$C_{32}H_{42}O_{18}$ + F.A.		Chalcone glycosides	
83	44.82819	859.5308	$C_{52}H_{76}O_{10}$		Glycoside	
84	14.39297	191.0556	$C_7H_{12}O_6$	Quinic acid		
85	13.22984	194.0816	$C_{10}H_{13}NO_3$	N-Acetyldopamine	Catechols	
86	8.226768	203.082	$C_{11}H_{12}N_2O_2$	D-Tryptophan	Indolyl carboxylic acids and derivatives	
87	17.52968	245.0927	$C_{13}H_{14}O_3N_2$	Nigellicine	Pyridazinoindazoles	
88	14.42489	274.0382	$C_{16}H_7O_3N_2$		Quinoxaline	
89	35.24303	325.1828	$C_{21}H_{26}O_3$		Retinoids	
90	18.06746	330.0841	$C_{14}H_{13}O_5N_5$			
91	21.00651	353.149	$C_{20}H_{22}N_2O_4$			
92	34.30343	364.2841	$C_{22}H_{39}NO_3$			
93	34.62161	399.2732	$C_{22}H_{40}O_6$			
94	29.31877	452.2767	$C_{23}H_{39}N_3O_6$			
95	28.85673	476.2766	$C_{25}H_{39}N_3O_6$			
96	29.94069	478.2923	$C_{25}H_{41}O_6N_3$			
97	34.06393	535.3463	$C_{34}H_{48}O_5$			
98	30.35407	540.329	$C_{28}H_{43}N_7O_4$			
99	42.37577	543.4153	$C_{27}H_{54}O_4N_4$			
100	29.70186	564.3271	$C_{30}H_{43}N_7O_4$			
101	31.00769	566.3443	$C_{30}H_{45}N_7O_4$			
102	30.19476	595.2873	$C_{41}H_{40}O_4$			
103	29.65337	632.3155	$C_{28}H_{43}N_9O_8$			
104	31.02324	634.3309	$C_{40}H_{41}N_7O$			
105	20.7687	677.1854	$C_{35}H_{34}O_{14}$		Benzoate	
106	17.68802	699.2949	$C_{44}H_{44}O_8$			
107	45.24207	714.5051	$C_{41}H_{69}N_3O_7$			
108	16.51109	727.2419	$C_{40}H_{40}O_{13}$			
109	42.64642	738.5047	$C_{43}H_{69}O_7N_3$			
110	46.03884	740.5203	$C_{39}H_{67}N_9O_5$			
111	21.87495	765.3094	$C_{49}H_{42}N_4O_5$			Qin et al., 32017.
112	19.74887	769.3255	$C_{44}H_{50}O_{12}$			
113	37.17003	776.5489	$C_{47}H_{73}NO_5$			
114	26.33925	781.1345	NA			
115	24.89003	781.1365	NA			
116	27.34347	781.1375	NA			
117	24.25681	783.1492	NA			
118	22.86198	783.1545	NA			
119	22.86198	783.1545	NA			
120	22.03357	783.3192	NA			
121	22.60748	817.3455	NA			
122	43.85657	833.5157	$C_{50}H_{74}O_{10}$			
123	23.05366	839.2527	$C_{40}H_{44}N_2O_{18}$		Butylamide	

124	43.07654	841.5099	$C_{48}H_{74}O_{12}$			
125	41.54722	855.4982	$C_{43}H_{68}N_8O_{10}$			
126	42.615	857.5137	$C_{43}H_{70}N_8O_{10}$			

Annex 1: Compounds of particular interest have been identified from root extract of *Ferula hermonis* (the compound number is referred to the complete list of identified compounds in supplementary material: Table S_1).

No	formula	Putative compound	Biological activity	References
1	$C_{22}H_{30}O_4$	Ferutinin	Antiproliferative activity, Anti-inflammatory, Antifungal activity, Anticancer, Bone formation	Zavatti et al., 2016; Arghiani et al., 2014.
2	$C_{24}H_{32}O_4$	Lehmannolol	Cytotoxic activity human cancer cell lines	Li et al., 2015.
3	$C_{22}H_{30}O_5$	6-(*p*-hydroxybenzoyl) epoxyjaeschkeanadiol	Cytotoxic activities against cancer cell	Alkhatib et al., 2008.
5	$C_{24}H_{32}O_5$	Sinkiangenrin F	Cytotoxic activity human cancer cell lines	Li et al., 2015; Guangzhi, et al., 2015.
7	$C_{54}H_{86}O_{24}$	Sandrosaponin IX	Antioxidant activities	Dini et al., 2009.
8	$C_{22}H_{28}O_3$	14-(4'-Hydroxybenzoyloxy)dauc-4,8-diene	Antimicrobial activity	Galal et al., 2001.
9	$C_{22}H_{30}O_6$	Kuhistanicaol H	Antibacterial activity	Tamemoto et al., 2001.
10	$C_{22}H_{30}O_3$	Teferidin	Antimycobacterial, Antifungal, Anti-inflammatory and Cytotoxic activities	Arghiani et al., 2014; Geroushi et al., 2010.
12	$C_{26}H_{34}O_6$	8-O-acetyl sinkiangenorin	Cytotoxic activity against human cancer cell lines	Li et al., 2015.
16	$C_{15}H_{26}O_2$	Ferutinol	Antimicrobial activity	Galal et al., 2001.
60	$C_{42}H_{66}O_{14}$	Tibesaikosaponin II	Antiviral activity	Fang et al., 2017.
66	$C_{24}H_{30}O_5$	13-hydroxyfeselol	Cytotoxicity activity against human colon cancer cell lines	Jabrane et al., 2010.
68	$C_{24}H_{30}O_4$	(E)-omega-Hydroxyferulenol	Antimicrobial, Anticoagulant, Antifeedant, and Antiproliferative activities	Akaberi, et al., 2015.
69	$C_{16}H_{18}O_9$	5-Caffeoylquinic acid	Anti-inflammatory	Liu et al., 2015.
70	$C_{25}H_{24}O_{12}$	1,5-dicaffeoylquinic acid	Antioxidant activities	Slanina et al., 1999.
116	$C_{16}H_{32}O_2$	Palmitic acid	Antimicrobial activity	Huang et al., 2011

Annex 2: Compounds of particular interest have been identified from seed extract of *Silybum marianum* (the compound number is referred to the complete list of identified compounds in supplementary material: Table S_2).

No	Formula	Compound	Biological/Pharmacological Activities	Reference(s)
1	$C_{25}H_{22}O_{10}$	Silibinin(silymarin)	Antioxidant, Anti-inflammatory, Antiviral, Antitumor and Hepatoprotective.	Csupor et al., 2016; Polyak et al., 2010; Scambia et al., 1996.
3	$C_{15}H_{12}O_5$	Naringenin	Hepatoprotective, Anti-inflammatory, Anticancer, Antimutagenic, and Antimicribial agent	Yin et al., 2018; Karim et al., 2018.
4	$C_{15}H_{12}O_6$	Eriodictyol	Antioxidant and Anti-inflammatory effects	Narvaez-Mastache et al., 2008.
5	$C_{15}H_{10}O_6$	Luteolin	Antioxidant, Antitumor, Anti-inflammatory, and Antiapoptotic efficacy	Sun et al., 2015; Zhang et al., 2016.
6	$C_{18}H_{18}O_6$	4'-Hydroxy-5,6,7-trimethoxyflavanone	Antimycobacterial activity	Suksamrarn et al., 2004.
12	$C_{25}H_{20}O_{10}$	2,3- Dehydrosilybin	Antioxidants	Reina and Martínez, 2015.
16	$C_{27}H_{48}O_{14}$	Naringin	Antioxidant, Lipid-lowering, Antimicrobial, Anti-inflammatory and Anticancer	Jeon et al., 2004.
18	$C_{28}H_{26}O_{16}$	Taxillusin	Antimicrobial activities	Fukunaga et al., 1989.
23	$C_{14}H_{18}O_9$	Vanillic acid 4-O-β-d-glucopyranoside	Antioxidants	Chemam et al., 2017.
26	$C_{16}H_{18}O_9$	Chlorogenic acid	Antioxidant and Anti-inflammatory activities	Farah et al., 2008.
28	$C_{17}H_{26}O_{10}$	Loganin	Cognitive enhancing and Free radical scavenging capacity	Lee et al. 2009.
29	$C_{18}H_{26}O_{11}$	Oleoside dimethyl ester	Antioxidant activity	Wang et al., 2010.
30	$C_{21}H_{32}O_{12}$	Darendoside B	Antioxidant activity	Pan et al., 2003.
31	$C_{17}H_{26}O_5$	Hymenolide	Phagostimulant activity	Juárez et al., 2014.
56	$C_{16}H_{32}O_2$	Palmitic acid	Antimicrobial activity	Huang et al., 2011.
57	$C_{18}H_{30}O_2$	Alpha-Linolenic acid	Neuroprotective and Anti-inflammatory	Nicolas et al., 2015.
58	$C_{18}H_{32}O_2$	Linoleic acid	Antibacterial activity	Zheng et al., 2005.
61	$C_{18}H_{34}O_3$	Ricinoleic acid	Antibacterial activity, Anti-inflammatory	Kuppala et al., 2016; Abdul et al., 2018.

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DICHIARO

1) che la tesi, in quanto caratterizzata da vincoli di segretezza, non dovrà essere consultabile on line da terzi per un periodo di 12 (dodici) mesi a partire dalla data di conseguimento del titolo di dottore di ricerca;
2) di essere a conoscenza del fatto che la versione elettronica della tesi dovrà altresì essere depositata a cura dell'Ateneo presso le Biblioteche Nazionali Centrali di Roma e Firenze dove sarà comunque consultabile su PC privi di periferiche; la tesi sarà inoltre consultabile in formato cartaceo presso l'Archivio Tesi di Ateneo;
3) di essere a conoscenza che allo scadere del dodicesimo mese a partire dalla data di conseguimento del titolo di dottore di ricerca la tesi sarà immessa in rete e comunicata al pubblico tramite servizio on line entro l'Archivio Istituzionale ad Accesso Aperto.

Specificare la motivazione:
☒ motivi di segretezza e/o di proprietà dei risultati e/o informazioni sensibili dell'Università Ca' Foscari di Venezia.
□ motivi di segretezza e/o di proprietà dei risultati e informazioni di enti esterni o aziende private che hanno partecipato alla realizzazione del lavoro di ricerca relativo alla tesi di dottorato.
□ dichiaro che la tesi di dottorato presenta elementi di innovazione per i quali è già stata attivata / si intende attivare la seguente procedura di tutela:

.....;

□ Altro (specificare):

.....

.....

.....

A tal fine:
- dichiaro di aver consegnato la copia integrale della tesi in formato elettronico tramite auto-archiviazione (upload) nel sito dell'Università; la tesi in formato elettronico sarà caricata automaticamente nell'Archivio Istituzionale ad Accesso Aperto dell'Università Ca' Foscari, dove rimarrà non accessibile fino allo scadere dell'embargo, e verrà consegnata mediante procedura telematica per il deposito legale presso la Biblioteca Nazionale Centrale di Firenze;
- consegno la copia integrale della tesi in formato cartaceo presso la segreteria didattica del dipartimento di riferimento del corso di dottorato ai fini del deposito presso l'Archivio di Ateneo.

Data 4/12/2019 **Firma** .....

La presente dichiarazione è sottoscritta dall'interessato in presenza del dipendente addetto, ovvero sottoscritta e inviata, unitamente a copia fotostatica non autenticata di un documento di identità del dichiarante, all'ufficio competente via fax, ovvero tramite un incaricato, oppure a mezzo posta.

Firma del dipendente addetto .....

Ai sensi dell'art. 13 del D.Lgs. n. 196/03 si informa che il titolare del trattamento dei dati forniti è l'Università Ca' Foscari - Venezia.
I dati sono acquisiti e trattati esclusivamente per l'espletamento delle finalità istituzionali d'Ateneo; l'eventuale rifiuto di fornire i propri dati personali potrebbe comportare il mancato espletamento degli adempimenti necessari e delle procedure amministrative di gestione delle carriere studenti. Sono comunque riconosciuti i diritti di cui all'art. 7 D. Lgs. n. 196/03.