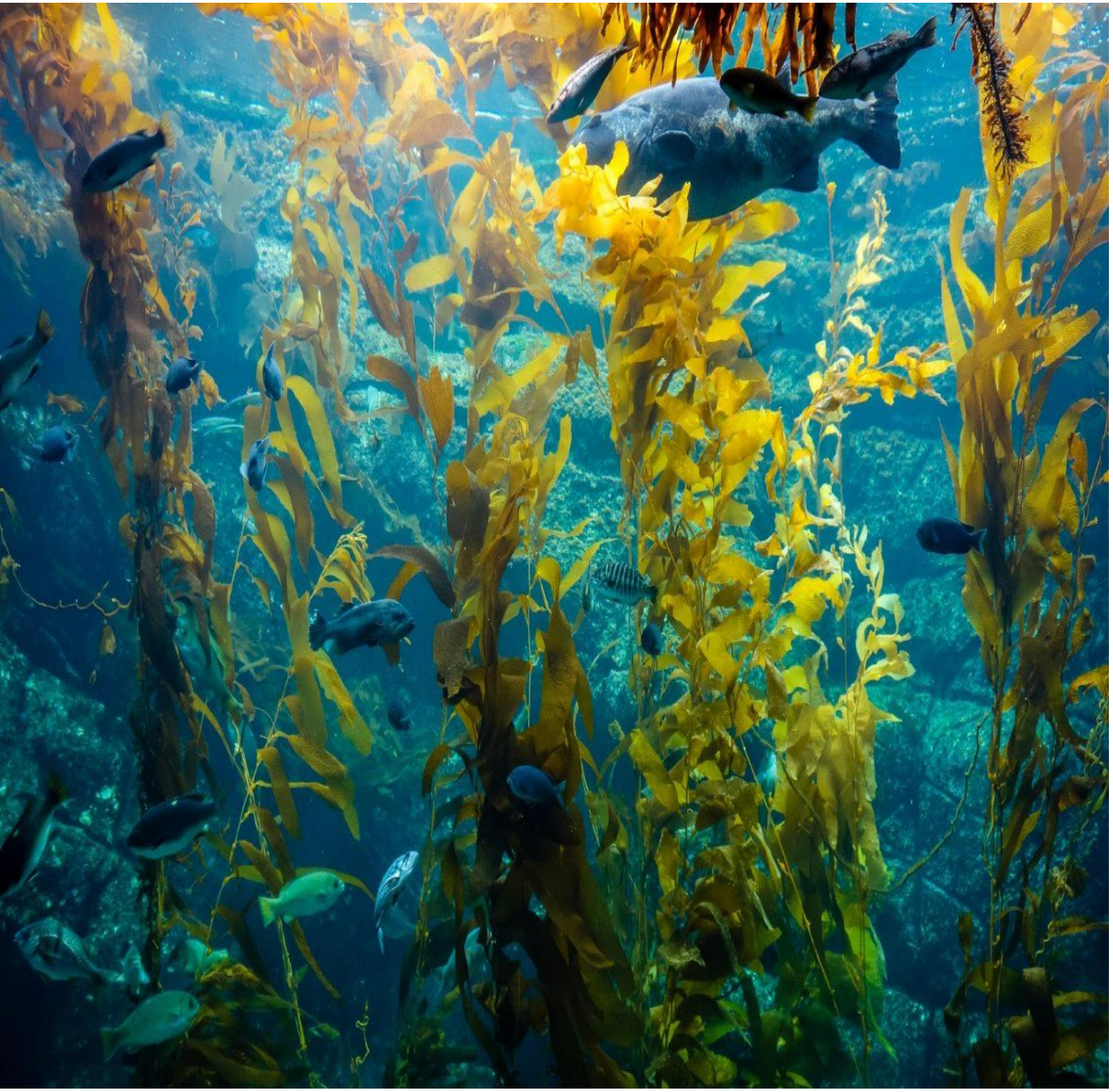
The Power of FUCOIDAN



Biological Activities of Some Algal Extracts from Lebanon; Antioxidant, Anti-inflammatory, and Anti-steatotic Effects

UNIVERSITÀ DEGLI STUDI
DI GENOVA

Scuola di Dottorato

Scienze e Tecnologie per l'Ambiente e il Territorio

By **Zeinab El Rashed**

A thesis submitted for the degree of

Doctor of Philosophy

March 2021

Certificate

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The Teaching Board of the doctorate studies of Technologies for the Environment and the Territory (STAT) “XXXIII cycle”

I would like to thank my committee for serving as my committee members even at this hard time. Thank you for your valuable comments and suggestions.

Dedication

To whom I missed the most

To my Father's Soul, Mohammed El Rashed

Anything good that has come to my life has been because of your love, support, guidance, safety, and patience.

All what I need is to make you proud of me and my sisters

We love you. we miss you….

May your soul rest in peace.

To my Dad

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I am most grateful to my family, your love and your prayers protected me and made me feel safe always. MAMA, you are great and strong, we are nothing without your love, and trust. My sisters: Diana, Samira, Elissar, Maram, Manessa; my brothers: Khoder and Youssef. you are the reason of my happiness. All what I need is to make you happy, I am sorry for not being beside you physically in the most difficult time. I promise I will always be with my heart. MY ALL
My BACKBONE, my partner “Ahmed basha Yehia” you understand me even when I don’t understand myself. With your wisdom, everything gets easier. Your guidance, patience and love made me grateful. Thank you for every single moment, I would like to thank your family also, they loved and treated me like their daughter. Ahmed, I promise to love you, take care of you, and give you the best of myself.
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With love

Team Expertise

University of Genoa

It was my pleasure to achieve my Ph.D. in the Laboratory of Molecular Physiology at the department of Earth and Life science of UNIGE. In this lab, I met

Prof ***Laura Canesi***: Prof Canesi is the head of the lab, she obtained her Ph.D. degree in Marine Environmental Sciences, and the scientific activity mainly concerned the study of different aspects of the physiology of marine organisms, in particular invertebrates, in relation to comparative and environmental aspects. This concerns the regulation of functions, from the cellular to the tissue and organism level, in response to both endogenous and environmental stimuli, using the Mytilus marine bivalve as the predominant model. Particular interest was addressed to comparative aspects in the main topics studied: pro-oxidant and antioxidant processes, aging processes, essential heavy metal homeostasis, calcium homeostasis, signal transduction mechanisms and effects of heterologous growth factors, immune response mechanisms innate, mechanisms of action of natural and environmental estrogens. Prof Canesi has published more than 183 articles.

Prof ***Adriana Voci:*** Prof Adriana Voci was an example of the researcher that makes you feel safe, and positive all the time even if some results are not going as you want. Prof Voci has more than 82 publications mainly related to nonalcoholic fatty liver disease.

Prof ***Ilaria Demori:*** Prof demori obtained her PhD degree in physiological sciences at the University of Milan and has been awarded scholarships for research activities at the Ludwig Institute for Cancer Research (London) and the University of Goettingen (Germany). Since 2001 she has been a full-time researcher in the Scientific Disciplinary Sector BIO / 09 Physiology. She teaches "Laboratory of physiology", "Endocrinology" and "Physiology of nutrition" at the iCCS of Biology and Dietetics. She is a professor of Psycho-Neuroendocrine Immunology (PNEI) in the 1st level Master "Complementary Medicine and Integrated Therapies", University of Siena. Prof Demori is a member of the Italian Society of Physiology (SIF) and is a founding member and deputy head of the Ligurian section of the Italian Society. di PNEI (SIPNEI). Demori et al has more than 44 publication. Research Lines and Fields of Interest: Hepatic steatosis and alterations of lipid metabolism: effects of treatment with iodithyronines and natural substances in animal and cellular models of hepatic steatosis. Nutrition and health. PNEI and integrated medicine.

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Lebanese University

Lebanese university has provided me the chance to graduate and complete my master's degree. More than six months of my thesis was carried in Rammal Rammal Laboratory (ATAC group), faculty of science-Lebanese University

Prof ***Hala Khalifeh***: Prof Hala obtained her PhD in Nutrition and Aging at the University of Camerino and has awarded a postdoctoral fellowship at the same University. Prof Hala has been a chief laboratory for four years before she started her educational career at Lebanese University. Prof Hala published 27 articles, participated in seven international conferences. Her research interests are related to the biological activities of medicinal plant extracts and bioactive compounds, such as antioxidant, anti-inflammatory, antiproliferative and cytotoxic activities.

University of Camerino

Prof ***Giulio Lupidi:*** Prof Lupidi is an associate professor at the School of Pharmaceutical Sciences and Health Products at the University of Camerino. He is interested in the pharmacological and chemical properties of natural bioactive compounds. He has more than 170 publication.

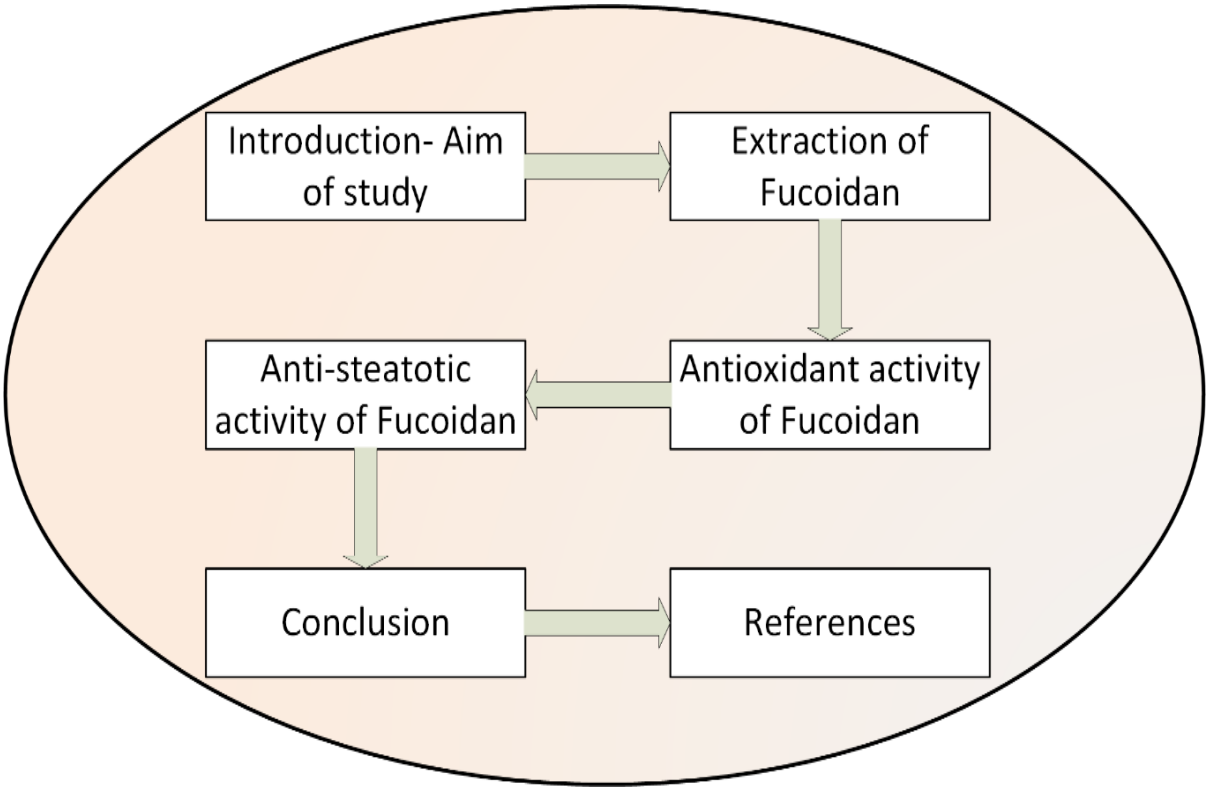
"Alone we can do so little; together we can do so much."

ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a metabolic manifestation of liver disease, characterized by lipid accumulation in hepatocytes. Despite starting as a benign disorder, NAFLD can progress to more severe pathologies such as nonalcoholic steatohepatitis, fibrosis, cirrhosis, and eventually hepatocellular carcinoma. The epidemiological prevalence of this disease is 25 % of the general population, and its worsening suggests that NAFLD is supposed to be the main cause for liver transplantation. NAFLD pharmacological treatment is still limited by unwanted side effects, whereas the best therapeutical approach is a lifestyle that depends on physical exercises and a healthy diet. The use of natural-derived compounds with therapeutic potential is advisable. Recently, medicinal plants applications have been focused on renewable sources as marine algae, especially seaweeds. Seaweeds are characterized with huge amounts of phytochemicals such as fucoidan (FUC) polysaccharide, which is known for its antioxidant, anti-inflammatory, anti-cancer, and hepatoprotective effects. In the present work, we extracted a water soluble fucoidan fraction from the brown algae Cystoseira compressa, and for the first time from the roots of the terrestrial shrub Ferula hermonis. These fucoidan fraction were termed CYS and FER, respectively. Then together with a previously purified fucoidan from the terrestrial plant Eucalyptus globulus "EUC" were studied and compared for their chemical features and biological activities. CYS, FER, and EUC contained fucose, glucose, sulfate, smaller amounts of monosaccharides such as galactose and mannose, and a minor quantity of proteins. FUCs structural features were investigated by FTIR, 1H NMR and 13C NMR spectroscopy. The antioxidant properties of FUCs were measured by DPPH, ABTS and FRAP assays, results revealed a high radical scavenging capacity that was confirmed in in vitro cellular models. In hepatic and endothelial cells, FUC reduced ROS production induced by intracellular lipid accumulation. Moreover, in our NAFLD model that consists of hepatic and endothelial cells treated with a fatty acid mixture prior to the FUCs treatment, FUCs purified from the three vegetal species exhibited a significant antisteatotic action; being able to reduce intracellular triglyceride content and to regulate the expression of key genes of hepatic lipid metabolism. Altogether, our results candidate CYS, FER, and EUC as possible bioactive compounds against fatty liver disease and related vascular damage. Results indicated that among the purified FUCs, that purified from the terrestrial plant *E. globulus* was the most biologically active extract.

KEYWORDS: Fucoidan, C. compressa, F. hermonis, E. globulus, antioxidant, and anti-steatotic.

Schematic Outline



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List of Abbreviations

13C NMR: Carbon NMR
1H NMR: Proton NMR
ABTS: 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid
ACC: acetyl-CoA carboxylase
ADP: Adenosine diphosphate
ADRP: Adipose differentiation-related protein.
ALG: Alginate
ApoB: Apoprotein B
ATGL: Adipose triglyceride lipase
ATP: Adenosine triphosphate
BAP: Brown algal polysaccharide
BSA: Bovine serum albumin
C: Control
CPT-1: Carnitine-palmitoyltransferase-1
CTP: Citrate transport protein
CYPs: Cytochrome P450
CYS: Fucoidan purified from C. compressa
DAG: Diacylglycerol
DAPI: 4′,6-diamidino-2-phenylindole
DCF: 2′-7′dichlorofluorescein
DMEM: Dulbecco's modified minimum essential medium
DNL: De novo lipogenesis
DPPH: 1,1-diphenyl-2-picrylhydrazyl
ED: Endothelial dysfunction
ER: Endoplasmic reticulum
ETC: Electron transport chain
EUC: Fucoidan purified from E. globulus
F12: Coon’s modified Ham’s medium
FA: Fatty acid
$FADH_2$: Flavin adenine dinucleotide
FaO: Rat hepatoma cells
FAS : Fatty acyl synthetase
Fe^{2+}: Ferrous ion
Fe^{3+}: Ferric ion
FER: Fucoidan purified from F. hermonis
FFA: Free fatty acid
FRAP: Ferric Reducing Antioxidant Power
FTIR: Fourier Transform Infrared spectroscopy
FUC: Fucoidan
GDP: guanosine diphosphate
GTP: guanosine triphosphate

H_2O_2: Hydrogen peroxide
HCl : Hydrochloric acid
HECV: Endothelial cells isolated from the umbilical vein
HFD: high fat diet
HSL: Hormone sensitive lipase
IFN-γ: Interferon gamma
IL-6: Interleukin 6
iNOS: Inducible nitric oxide synthase
LAM: Laminarin
LD: Lipid droplet
LMWF: Low molecular weight fucoidan
MAG: Monoacylglycerol
MGL: Monoglyceride lipase
MS: Metabolic syndrome
mtDNA: Mitochondrial DNA
MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NAFLD: Nonalcoholic fatty liver disease.
NASH: Non-alcoholic steatohepatitis
NMR: Nuclear Magnetic Resonance Spectroscopy
NO: Nitric oxide
OH: Hydroxyl group
OP: Oleate/Palmitate
OS :Oxidative stress
PBS: Phosphate-buffered saline
PKA: Protein kinase A
PPARs: Peroxisome Proliferated Activated Receptors
PUFA: Polyunsaturated fatty acid
qPCR: Quantitative polymerase chain reaction
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
ROS: Reactive oxygen species
RS: Reactive species
SCFA: Short chain fatty acid
SOD: Superoxide dismutase
SREBP-1: Sterol regulatory element binding protein-1
TAG: Triacylglycerol
TIP-47: Tail-interacting protein of 47 kDa
TNF-α: Tumor necrosis factor
VLDL: Very-low-density lipoprotein
β-oxidation: beta oxidation
ω-oxidation: omega oxidation

List of Supplementary Boxes

SUMMARY

Being first described in 1980's, NAFLD was defined as the accumulation of lipids in more than 5% of hepatocytes in the absence of excessive alcohol use or a direct cause for hepatic steatosis (Lindenmeyer et al., 2018). NAFLD is a widely spread disease, its prevalence is affected by several parameters such as body fat distribution, body composition, gender, age, and obesity (Souza et al., 2012). Studies have reported that NAFLD affects around 25-30% of the general population. However, this percentage increased to 57-74% in obese adults. Thus, patients with obesity, type 2 diabetes, dyslipidemia, and hypertension are at higher risk for NAFLD development compared to healthy people (Tarantino et al., 2007).

NAFLD pathogenesis initiates as hepatic triglyceride accumulation is considered to be a relatively benign condition, a second hit increases the susceptibility to liver injury mediated by inflammation, oxidative stress, and mitochondrial dysfunction leading to nonalcoholic steatohepatitis NASH, a severe form of steatosis. Moreover, a third hit is implicated in further pathogenesis due to inadequate hepatocytes infiltration which is strongly correlated with fibrosis, and hepatocellular carcinoma (DOWMAN et al., 2009).

Lipid homeostasis is regulated by different genes and pathways that are classified into four major sources leading to steatosis: increased *de novo* lipogenesis (DNL), increased free fatty acids (FFAs), reduced FFA oxidation, and decreased secretion of triacylglycerols (TAGs); any disruption can cause an imbalance between lipid acquisition and disposal resulting in fat accumulation (Vecchione *et al.*, 2016).

Several drugs have been used to treat NAFLD and ameliorate metabolic functions, such as thiazolidinediones, metformin, statins, and fibrates, but still limited due to their side effects. The most effective approach is a lifestyle depending on lowering calories intake by dietary modification and enhancing physical activities as well as searching for natural agents and phytochemicals that can be effective in treating NAFLD through their anti-steatotic, anti-oxidant and anti-inflammatory capacities as shown previously (Baselga-Escudero et al., 2017; Yao et al., 2016).

Due to the harsh environment that marine algae are forced to resist, they produce a wide range of biologically active compounds such as polysaccharides, vitamins, minerals, antioxidants, enzymes, and polyunsaturated fatty acids (Şükran *et al*., 2017). Fucoidan (FUC) is a fucose-rich sulfated polysaccharide with a backbone of α-(1–3)-linked fucose units or α-(1–3) - and α-(1–4) - alternating linked fucose residues. Some FUCs contain small amounts of monosaccharides such

as xylose, mannose, galactose, glucose and uronic acid. (Guangling *et al.*, 2011). FUC is abundant in the cell wall of marine brown algae, also found in marine invertebrates such as sea urchin and sea cucumber (Pomin, 2012). FUCs have gained a great interest in research due to their antioxidant, anti-inflammatory, anti-viral, and anti-steatotic effects (Fitton *et al.*, 2015).

FUC structure is highly variable depending on several factors such as extraction method, variation in season of collection, geographical location, population age and species type. Structural difference is characterized also by variation in monosaccharide composition, sulfate content, sugar binding residues and molecular weight, all leading to different biological potentials (Yu Wang et al., 2019).

Previously FUC has been purified from the brown algae Cystoseira compressa which is a brown algae species from Cystoseira genus, belongs to the family of Sargassaceae in the order of Fucales. However, Haddad *et al* identified FUC in the terrestrial tree *Eucalyptus globulus* (Haddad *et al*., 2017), also known as the blue gum, is an evergreen plant that belongs to the family of Myrtaceae. It rapidly grows especially in countries with Mediterranean-type climate. *E. globulus* is characterized by biological activities of potential medicinal value which make it of great interest (Qabaha *et al*., 2016).

AIM OF THE STUDY

1- The aim of this study is to extract FUC polysaccharide from the brown algae *C. compressa* (CYS) collected from the Lebanese coast and the terrestrial plant *F. hermonis* (FER) for the first time. Then to chemically characterize the purified FUC, and together with FUC purified from *E. globulus* (EUC) study their chemical composition.

2- Investigate and compare the antioxidant potentials of CYS, FER, and EUC; spectrophotometrically

3- After extraction and chemical features analysis we aimed to study and compare the anti-steatotic and antioxidant activity of FUC of marine algal source (CYS) with those extracted from terrestrial source (CYS and EUC).

METHODOLOGY

1- For the first aim, structural features were analyzed by Fourier Transform Infrared spectroscopy and Nuclear magnetic resonance; in addition to the chemical composition that was determined by sulfate, fucose, sugar, and protein content.

2- Antioxidant activities were determined by ; DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) assay, FRAP (Ferric Reducing Antioxidant Power) assay, and anti-hemolysis assay.

3- For the aim, two in vitro cellular models were used; rat hepatoma FaO cells exposed to an oleate/palmitate mixture representing a reliable *in vitro* model for hepatic steatosis as previously described by our group (Grasselli et al., 2017), and human endothelial HECV cells exposed to FAs were used also as an in vitro model of vascular endothelial damage.

RESULTS

Results indicated that CYS and FER extracts showed the structural features and functional groups that characterize fucoidan polysaccharide. CYS, FER, and EUC possess a great antioxidant activity. Furthermore, FUC purified from three different species ameliorated lipid accumulation in hepatic cells, regulate lipid metabolism, and reduce oxidative stress in hepatic and endothelial cells with fucoidan purified from *E. globulus* being the most effective.

CONCLUSION

Fucoidan purified from seaweeds or terrestrial plants displays a protective effect against oxidative damage in erythrocytes, hepatocytes, and endothelial cells. This candidate C. compressa, F. hermonis, and *E. globulus* species as sources for fucoidan extraction to be used in several applications as therapeutic agents.

CHAPTER I: EXTRACTION AND CHEMICAL CHARACTERIZATION OF FUCOIDAN POLYSACCHARIDE

Define the Problem

Side effects of several drugs in treating Oxidative stress and oxidative stress related diseases such as NAFLD

Research Questions

Replace synthetic drugs with natural derived compounds (Fucoidan polysaccharide).

Does the terrestrial plant *F. hermonis* contains FUC?

Determine the difference in structure of FUC derived from terrestrial and marine

ABSTRACT

Since ancient times, plants have been used as a medical source for treating human diseases. Nowadays, the search for natural compounds displaying therapeutic efficacy with less toxicity and side effects than synthetic drugs is still of great interest. A huge amount of phytochemicals are currently being studied for their potential benefits against the major threats to human health, i. e. non-communicable diseases including cardiovascular disorders, cancer, autoimmunity, neurodegenerative diseases, as well as metabolic impairments related to insulin resistance and liver disorders, such as Non-Alcoholic Fatty Liver Disease (NAFLD). The search for natural bioactive compounds has been focused on renewable resources such as marine algal seaweeds as they contain a wide range of phytochemicals such as pigments, vitamins, minerals, lipids, proteins, and polysaccharides . Seaweeds are classified based on their pigmentation into green, red, and brown algae. In this study, we will give a brief explanation on different brown algal polysaccharides such as "Alginate", "Laminarin", but focus mainly on **"FUCOIDAN**". Fucoidan, an example of marine phytochemicals, is a fucose-rich sulfated polysaccharide abundant in the cell wall of marine brown algae that gained a great interest in research due to its several biological activities including anti-viral, anti-cancer, antioxidant, anti-inflammatory, anti-diabetic effects.

In this study, fucoidan was extracted from the marine brown algae "Cystoseira compressa" and for the first time from the terrestrial plant, "Ferula hermonis" and chemically characterized by studying the structural features. Then together with a previously purified fucoidan from "Eucalyptus globulus" were investigated for more detailed chemical composition. Our results indicated that the yield of fucoidan purified *C. compressa* and *F. hermonis* was 3.6 % and 3.07%, respectively. Furthermore, the chemical structure analysis confirmed the presence of functional groups that mainly characterize fucoidan polysaccharide

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I. INTRODUCTION

TRADITIONAL MEDICINE

TRADITIONAL MEDICINE is defined as ***"the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses"*** (C.T. Che *et al.*, 2017).

Between 350,000 and almost half a million of plant species have been used as a source for the cure of human diseases, nobody knows exactly where and when the medicinal plants were used for the first time. The purpose was and still to improve overall health; in the beginning, plants were used to treat illnesses or even simply to feel better distinguishing useful plants with beneficial effects and then their use has been gradually refined over the generations to be known as traditional medicine.

Previously, the knowledge about medicinal plants was transmitted orally before the written texts. The first written text about the use of medicinal plants was 4000 years ago in a clay small board in the Sumerian culture, which would be equivalent to the current Iraq territory.

All civilizations have developed this form of medicine that varies among countries based on their own habitat, culture, history, and personal attitudes. There are even authors who claim that this transmitted knowledge is the origin of medicine and pharmacy.

Until the 18th century, all the knowledge related to traditional medicine was summarized in the species of the used plants, their therapeutic properties, effects on human health and their method of treatment, while the active compound was unknown. Medicinal plants have been used to treat several diseases; for example, chamomile flower is commonly used for anxiety and relaxation, ginger is used to ease nausea, tanacetum parthenium flower known as feverfew where it was traditionally used to treat fevers, and garlic that is used for lowering cholesterol and blood pressure. Until that time, the Canon of Medicine written by the Persian physician and scientist Avicenna (Ibn Sina) was used as a reference.

The origin of modern science, specifical improvement of chemical analysis and microscopic applications made a significant scientific transformation where it was possible to isolate the active principles of medical plants. Since then, these active compounds have been identified and used as a raw material in pharmaceutical medicine industry such as phenolic acids, oligosaccharides, polysaccharides, flavonoids, terpenoids and alkaloids (Thakur *et al.*, 2020). Today, due to the increased demand of herbal products, hundreds of higher plants are cultivated worldwide to obtain useful substances in medicine and pharmacy. Yet, due to the high cost of modern medicine with synthetic origin many countries of the underdeveloped world continue to use traditional medicine.

Traditional Medicine Drawbacks

It should be noticed that applications related to traditional medicine must be sufficiently researched due to two major drawbacks.

- The use of medicinal plants without sanitary control and without thinking about the possible harmful aspects for health. Some plants may have dangerous active principles. For example, the green seeds of Bitter melon (*Momordica charantia*) used to cure fever and in cases of malaria are very toxic as they can cause a sharp drop in blood sugar and induce a patient's coma (hypoglycemic coma).
- Products giving rise to false perspectives, as they are not well studied (Azaizeh *et al.*, 2006).

Global Traditional Medicine Evolution

Plants have been and still considered a significant source of pharmaceutical drugs. According to a recent study that analyzed a big number of publications, several categories have been developed based on the number of published articles, research area, countries, and keywords (Manzano *et al.,* 2020).

- ***Temporal Evolution of Medicinal Plants***

Since 1960, more than 100,000 articles related to medicinal plants have been published. This period was divided into three intervals based on the trend increase of publications. As shown in figure 1.

- From 1960 till 2001, the increase was continuous with just 1300 published studies.
- From 2001 till 2011, this trend increases faster with the maximum number of publications (6200).
- After 2011, the number of publications was stabilized over 5000 study per year.

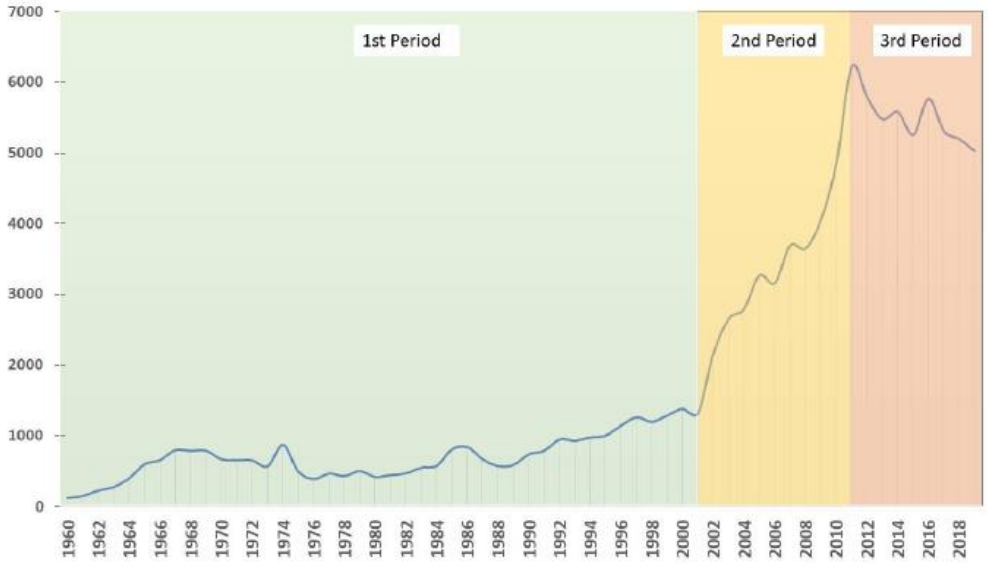


Figure 1: Worldwide temporal evolution of medical plants publications.

➢ *Traditional Medicine Scientific Categories*

Published studies related to medicinal plants can be divided based on the interested scientific major, for example most of the studies have been carried out in the Pharmacology, Toxicology and Pharmaceutics. Followed by other categories such as: Medicine, Biochemistry, Genetics and Molecular Biology, Agricultural and Biological Sciences, Chemistry, Immunology and Microbiology, Environmental Science, Chemical Engineering, and other categories (Nursing, Neuroscience, Earth and Planetary sciences, materials science, and Health Professions)

➢ *Medicinal Plants Publications by Countries*

Countries involved in traditional medicine research can be arranged in three groups based on the annual number of publications.

Group 1 (800 and 1100 publications per year)	Group 2 (200-400 publication per year)	Group 3 (100 publication per year)
• China • India	• Iran, Brazil, USA • South Korea, Pakistan	• Japan, Germany, UK • ***Italy,*** France

Figure 2: Diagram showing the classification of countries into three groups based on number of publications related to traditional medicine.

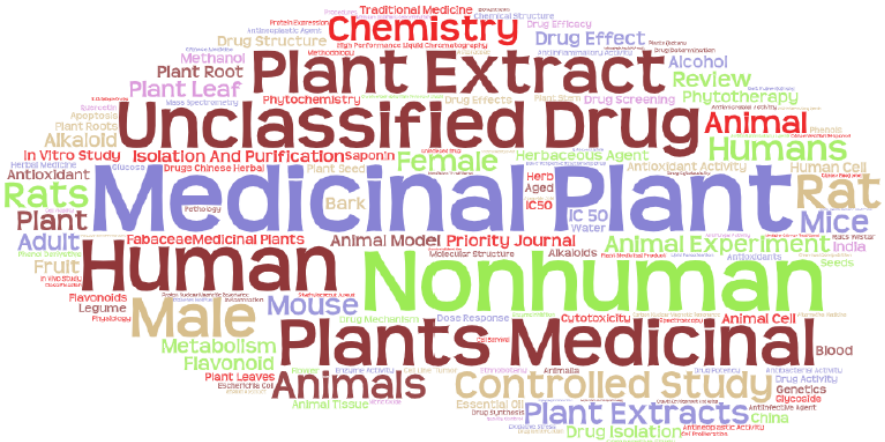


Figure 3: Cloud word of keywords related to medical plants publications. If keywords are extracted from the total number of publications, an overview can be made of the most used keywords in relation to traditional medicine. The most indexed terms are human, non-human, unclassified drugs, plant extract, plants, medicinal, mouse, animal, chemistry, and many other keywords.

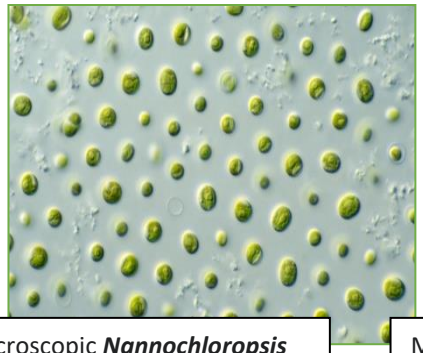
MARINE RESOURCES: ALGAE

Recently, interests have been focused on renewable natural sources, which shifted the search from terrestrial plants to marine resources such as sponge, bacteria, cyanobacteria, fungi, and algae. Algae are considered as a huge marine resource, defined as simple photosynthetic chlorophyll containing organisms, with great diversity in terms of morphological, structural, ecological, chemical, and physiological traits.

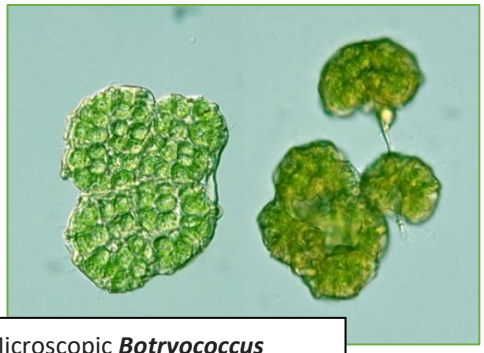
ALGAL CLASSIFICATION

Algae are divided into two main groups:

- **Microalgae:** are microscopic algae found in the freshwater and marine systems. They are unicellular organisms that exist individually or in groups ranging from a few micrometers (μm) to a few hundred micrometers. Microalgae do not have roots, stems, or leaves. They can perform photosynthesis producing approximately half of the atmospheric oxygen, used as bioactive compounds, food colorants pigments, cosmetics, and biofuel production (Priyadarshani *et al.*, 2012).
  Examples of the microscopic algae: ***Nannochloropsis*** and ***Botryococcus braunii.***



Microscopic ***Nannochloropsis*** microalgae



Microscopic ***Botryococcus braunii*** microalgae

- **Macroalgae:** known as **seaweeds**, are macroscopic multicellular organisms that range from few millimeters to 30 meters in size. For example, the brown algae "giant kelp" is the largest seaweed (30 meters). Seaweeds also can perform photosynthesis, used as human foods, cosmetics, fertilizers, and for the purification of chemicals with medicinal and industrial uses. In this research study, we therapeutic activities of macroalgae.

SEAWEEDS

Seaweeds Structure and Divisions

Seaweeds are still categorized as plants, but different in structure; they lack roots and have instead holdfasts for attachment rather than water and nutrient absorption, they have stripe as a stem used for support. Moreover, the leaves are called blades that function in providing a surface for sunlight absorption. Many Seaweeds have hollow, gas-filled structures called floats, such structures help in photosynthesis and energy absorption. The whole seaweed body refers as thallus (Mouritsen *et al.,* 2013).

Furthermore, they are divided into three groups (El Gamal *et al.*, 2009):

1. ***Chlorophyta,*** are green algae due to the presence of chlorophyll a and b; about 1200 species.
   Examples: chlorella, Ulva lactuca, and Aegagropila linnaei.
2. ***Phaeophyta***; contain around 1750 species, brown in color results due to the dominance of xanthophyll pigments and fucoxanthin over chlorophyll pigments, and the red algae.
   Example: Fucus vesiculosus, laminaria, cystoseira baccata, and cystoseira compressa.

3. ***Rhodophyta:*** about 6000 species; chlorophyll pigments are masked by other pigments such as phycoerythrin and phycothcyanin leading to red color dominance.
   Examples: Gigartinales, Nemaliales, and Gracilaria.

Chlorophyta

Phaeophyta

Rhodophyta

Seaweeds: A Brief History

Human and seaweed interaction seems to date back to many thousands of years and even before Christ. Some researchers suggest that seaweed has been used since 2700 BC in China. One of the oldest recorded writings in Iceland, refers to 961 BC related to regulations about coastal property rights with respect to sea vegetables. In 600 BC, Sze Teu wrote a book about seaweeds in China, followed by a book written by Chi Han in 300 BC related also to seaweeds. 100 BC, Greeks have used seaweeds to fed animals. In addition to the fact that some red algae were used since pre-Christian times as a source of medicine to treat parasitic worms.
2000 years ago, people from Japan have used seaweeds as a supportive food in their diet, to become later an everyday food where after 800 years it was reported at least six types of seaweeds used in everyday cooking. Then seaweeds gained much more interest as food and fertilizer; in Ireland, algae collection started 800 years ago (Buschmann *et al.*, 2017).

Global Seaweed Production

Despite their historical applications, only 70 years ago seaweed farming technology has been significantly developed mainly in Asia, then in America and Europe. According to the latest data adapted from Food and Agriculture Organization, in 2016 seaweed production reached 31.2 million tons (fresh weight). 96.5% of this weight was produced in aquaculture while just 3.5% was harvested from natural populations. The top seaweed-producing countries are Asian as we previously said: China, Indonesia, Philippines, Korea, and Japan. The demand of seaweed as food has spread, which lead to commercial farming projects for selected brown and red algae in many countries such as the USA, Canada, Brazil, France, and parts of Africa.

In 2000, world seaweed production reached around 10 million tons, as it increases greatly to reach 31.2 million tons after 16 years. This means that the global annual seaweed production does not stop growing (Poza *et al.*, 2020).

Industrial Seaweed Applications

Seaweeds have many applications in several fields. They have been considered as suitable fertilizers and soil conditioners thousands of years ago, especially the large brown seaweeds. Their effectiveness as fertilizer is attributed to the presence of trace elements while the large amounts of insoluble carbohydrates improve aeration and soil structure which explains their application as soil conditioners.

At the end of the 19th century and beginning of 20th-century seaweeds bath was widely spread in which they were used as a treatment for arthritis, rheumatism and other aches and pains. Moreover, seaweed powder has been involved in synthesizing body care products and other various cosmetic applications.

Seaweeds, specifically the red algae Gelidium genus is considered the main source of the polysaccharide agar, a natural vegetable gelatin counterpart used as a thickening agent and most important as a solid substrate for the growth of bacteria and fungi in a microbiological laboratory. Agar has been produced by most of the countries such as China, France, India, Indonesia, Japan, Madagascar, Mexico, Morocco, Namibia, Argentina, Canada, Russia, South Africa, Spain, Thailand, and the USA (Kılınç *et al.*, 2013).

Seaweeds Nutritional Value

Sze Teu wrote in his book "***Some algae are a delicacy fit for the most honored guests, even for the King himself***."

Sixty-six percent of seaweed applications are used directly in food (**Rhodophyta**: 79; **Phaeophyta**: 38; **Chlorophyta**: 28 species). The importance of seaweeds in the diet is attributed to minerals (sodium, calcium, magnesium, potassium, chlorine, sulfur, phosphorus), vitamin, protein, carbohydrates, and fiber content present at different concentrations in different species. Generally, protein contents is higher in green and red seaweeds (10–47 of dry weight—DW) than those in brown seaweeds (5–24% DW); lipids from 0.79% to 7.87% dry matter and dietary fiber proportion ranging from 36% to 60% of its dry matter. The most edible seaweeds are known as Nori, Kombu, Wakame, laverbread and Sea lettuce.

In the early 1980s, there has been a growing interest in functional food, simply defined as "processed foods having disease-preventing and/or health-promoting benefits in addition to their nutritive value." The nutrient-dense nature of seaweed candidates them as a proper

functional food usually produced as food supplements, rather than whole foods and are marketed as tablets and pills to provide health benefits. (Peñalver *et al.*, 2020)

Table 1: Source and nutritional value of the most known edible seaweeds

Edible seaweed	**Country**	**Class**	**Genus:species**	**Nutritional and health effects**
Nori, known also as "sea vegetable"	Japan	Red algae	Genus Pyropia: P. yezoensis and P. tenera	• Strong flavoring • Sufficient amounts of vitamins
Kombu	Japan Korea (known as Dasima) China (Known as Haidai)	Brown algae	Genus Laminaria: L. *japonica, L. Diabolica and L. longissima*	• Good source of glutamic acid • Rich in dietary fibers
Wakame	Japan China (known as *qúndài cài*) France (known as sea fern) Korea (kown as *miyeok)*	Brown algae	Genus Undaria: U. pinnatifida	• High content of calcium and nutrients • High energy • Dietary fibers, and omega-3 FA.
Laverbread	West coast of Great Britain and east coast of Ireland	Red algae	Genus Porphyra: P. umbilicalis	• High proportions of proteins, iron, iodine and Vitamin B_{12}.
Sea lettuce	Great Britain Ireland China Japan (known as aosa)	Green algae	Genus Ulva: U. anandii, U.bifrons and U. cornuta	• Rich in protein, soluble dietary fibers,vitamins and iron.

Marine Seaweeds Bioactive compounds

Besides their traditional use in Oriental cuisine and their unique nutritional properties, algae have recently gained great research interests based on their potential role as functional food or nutraceuticals. This is mainly due to the high presence of several bioactive compounds produced in the harsh environment that algae can resist, all leading to candidate algae as a source of

phytochemicals with beneficial effects against diseases. This wide range of biological compounds includes pigments, lipids, vitamins, minerals, proteins, and carbohydrates (most importantly polysaccharides).

- ***PIGMENTS*:** marine seaweed pigments are divided into three types: chlorophylls, carotenoids and phycoerythrins. Chlorophylls are greenish fat-soluble pigments found in terrestrial plants, algae, and cyanobacteria in which they play a key role in the photosynthesis phenomenon. Carotenoids are also called tetraterpenoids, are yellow, red, and orange pigments. Marine algal carotenoids include carotenes, fucoxanthin (the most abundant carotenoids), lycopene, astaxanthin, zeaxanthin and neoxanthin. Phycoerythrin is a red protein-pigment complex. These pigments have biological potentials, nutritional value and used as food colorants with antioxidant, anti-inflammatory, anticancer, antidiabetic, antiangiogenic and immunomodulatory properties.
- ***LIPIDS**:* usually lipid content of marine seaweeds does not exceed 5 % of their dry weight, varies among species, even within the same species according to several geographical conditions, climatic factors, and seasonal variations. Seaweeds fatty acid profile contains a wide range of polyunsaturated fatty acids (PUFAs). In addition to the sterols mainly represented by fucosterol, clionasterol and cholesterol with important nutritional and biological properties, such as anticancer, antioxidant, anti-obesity, antitumoral, antiviral, and are effective against cardiovascular diseases.
- ***PROTEINS:*** marine seaweeds are considered a good source of most amino acids such as proline, alanine, glycine, arginine, and especially aspartic and glutamic acids. This is due to the wide range of proteins that can vary from 5% to 47% of their dry weight (w/w) depending on several factors as we mentioned above. Generally, protein content in green and red is higher compared to brown algae, and proteins exhibit a large spectrum of bioactivities.
- ***VITAMINS and MINERALS*:** seaweeds are wealthy sources for vitamins and minerals. Both water soluble (such as vitamins B, and C) and lipophilic vitamins (vitamins A, D, E, and carotenoids). Furthermore, algal seaweeds contain significant amounts of macro-elements (Ca, K, P, Na, Mg, and Fe) and trace of elements (Pb, Cu, Zn, Sc, Sd, As, Sr and Cr).
- ***CARBOHYDRATES*:** marine macroalgae are a good source of carbohydrates that varies from 5 to 75% of their dry weight and consist mainly of polysaccharides with few amounts of disaccharides and monosaccharides. Algal Polysaccharides can be found in different forms sulfated and non-sulfated depending on different species. Green marine algae are rich in ulvans, red seaweeds contain carrageenans, agars, and sulfated galactans, while brown macroalgae are characterized by the presence of alginates (ALGs), fucoidans (FUCs) and laminarins (LAMs) (Hentati *et al.,* 2020).

Supplementary Box 1: Seaweed cultivation

Seaweed cultivation is becoming an increasingly competitive candidate for the increased demand on seaweeds due to their different uses such as food, industrial, cosmetical, and medicinal applications.….Seaweed production reached 30 million tons by 2016, which as mainly produced by Asian nations (China, Japan, Korea, and the Philippines). The most cultivated species are the brown algae *Laminaria japonica* and *Undaria pinnatifida*, the red algae *Porphyra, Eucheuma, Kappaphycus* and *Gracilaria* and the green algae *Monostroma* and *Enteromorpha* (Lüning et al., 2003) (FAO, 2018).

BROWN ALGAL POLYSACCHARIDES

Brown algae include about 250 genera for a total of over 1500 species that exhibit a great variety of shapes and sizes. They can be abundantly harvested, characterized by a high productivity rate and some species (such as *Hijikia fusiforme, Laminaria japonica*, etc.); cultivated on a large scale to be used as food or food additives. Brown algae prefer growing in cold water, able to grow underwater depth of 30-50 meters but mostly present in the intertidal and upper sublittoral zones of the oceans.

Brown Algal Polysaccharides

As for plant cells, algal cells are surrounded by a polysaccharide-rich cell wall. However, brown algal polysaccharide (BAPs) exhibit some unique structural features, such as the presence of sulfate, fucose, and uronic acid, that differentiate them from the polysaccharides of terrestrial plants. This seems to be related to marine algae adaptation to a high salt environment: as an example, sulfated polysaccharides facilitate water retention in extracellular matrices, thus preventing desiccation in low tide conditions. The most represented sulfated polysaccharide in brown algae is FUC, but major BAPs also include alginate (ALG) and laminarin (LAM). Structural BAPs diversity can be appreciated among different algal species or even within the same species, notably depending on the environment, seasonal variations, and reproductive status of the organism. Here we will give a brief explanation about ALG and LAM, then a detailed overview about FUC.

- **ALGINATE**: is a negatively charged polysaccharide isolated from the brown algal cell wall, as well as from some bacterial strains. ALG is a linear biopolymer that consists of 1,4-linked β-D-mannuronic acid (M) and 1,4 α-L-guluronic acid (G) residues arranged in homogenous (GG/MM) or heterogeneous (MG) block-like patterns. The well-known physical properties of ALG, such as viscosity and gel-forming, and its biological potentials are determined by molecular weight, M/G ratio and distribution, temperature, environmental pH, and extraction method. The non-toxic and biodegradable ALG has gained great importance due to its several applications in the food industry, as gelling and a thickening agent, in dermatological and cosmetic preparations, and in the textile industry. Furthermore, ALG is pharmaceutically used due to its antibacterial, antidiabetic, antitumor, and antioxidant potentials.

- **LAMINARIN:** LAM is a low molecular weight polysaccharide (approximately 5 kDa), composed of (1,3)-b-D-glucan with β (1,6) branches and showing a variety of bio-functional properties. LAM is a storage glucan used as a carbohydrate food reserve (El Rashed *et al.,* 2020).

- **FUCOIDAN:** Explained below in detail.

FUCOIDAN

In 1913, FUC was extracted for the first time from a species of brown algae, such as *Fucus vesiculosus* and *Ascophyllum nodosum* by Kylin *et al*. FUC is negatively charged, a highly hygroscopic and water-soluble polysaccharide. Recently the literature about the potential clinical applications of FUC is extremely rich, due to its wide spectrum of biological activities ranging from anticoagulant/antithrombotic, antiviral, immune potentiating, angiogenic, anticancer, antidiabetic, antioxidant, and anti-inflammatory.

Sources of Fucoidan

FUC polysaccharide is very abundant in the cell wall of Phaeophyta (mainly *L. digitata*, *A. nodosum*, *Macrocystis pyrifera* and *F. vesiculosus*), but also found in marine invertebrates, such as in the jelly coat from sea urchin eggs and in the sea cucumber body wall. More recently, FUC has been isolated also in terrestrial plants such as *Eucalyptus globulus* (Pomin *et al*., 2012) (Haddad *et al.,* 2017)

Structure of Fucoidan

FUC is a fucose-rich sulfated polysaccharide purified mainly from the brown algal extracellular matrix. FUC is characterized by the presence of fucose, sulfate groups and one or more monosaccharides (such as xylose, mannose, galactose, rhamnose, arabinose and glucose), glucuronic acid and acetyl groups in some species. FUC has two types of fucose backbone, either of α-(1−3)-linked fucose repeated units or α-(1−3)- and α-(1,4)-alternating linked fucose residues. The main commercially available form is extracted from *Fucus vesiculosus* and is composed of 44% fucose and 26% sulfate.

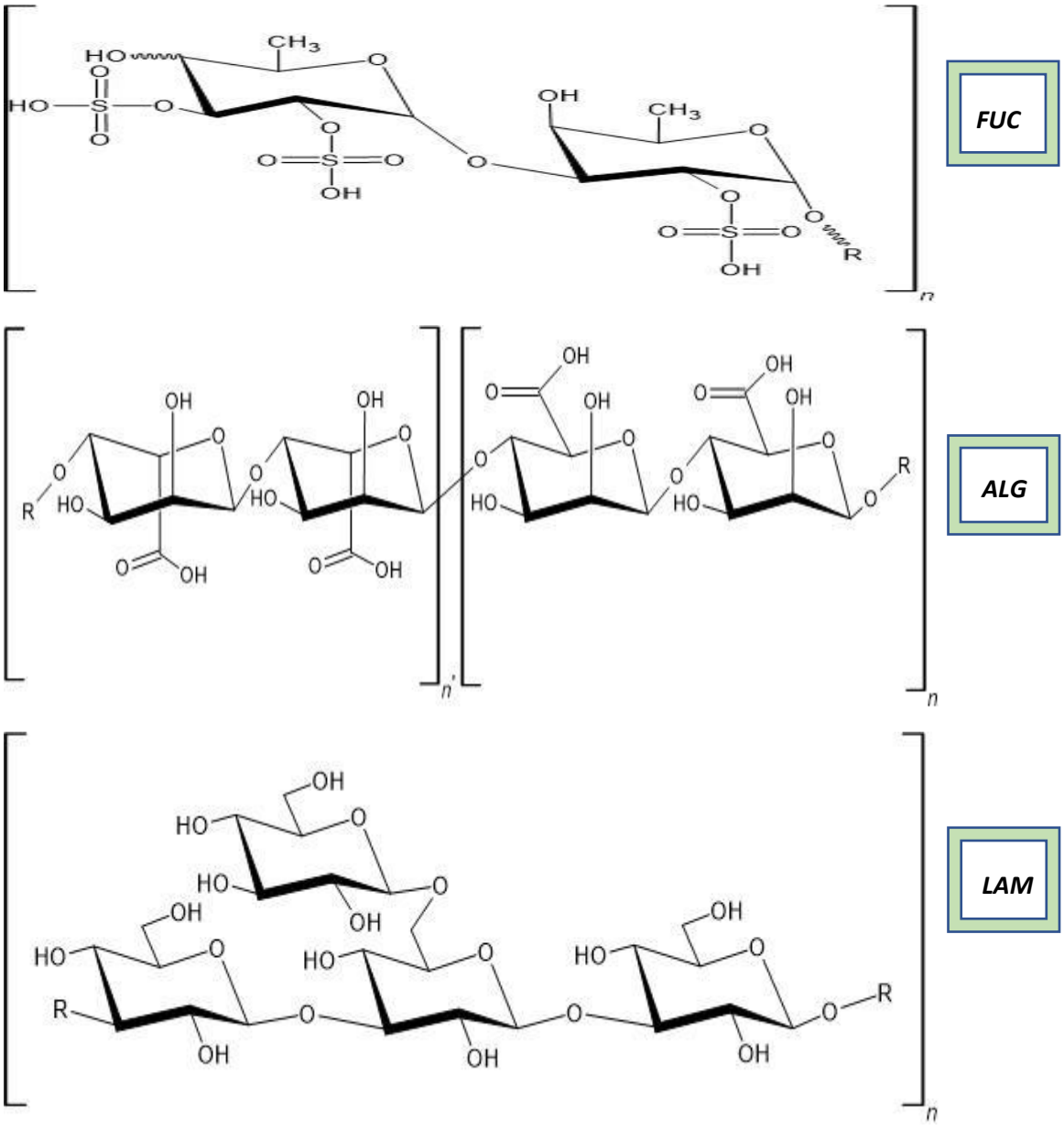


Figure 4: Chemical structure of brown algal polysaccharides: fucoidan, alginate, and laminarin (El Rashed et al., 2020).

Structural-Biological Diversity

FUC structure is highly variable depending on several factors such as extraction method, variation in a season of collection, geographical location, population age and species type. The structural difference is characterized also by variation in monosaccharide composition, sulfate content, sugar binding residues and molecular weight, all leading to different biological potentials.

The anti-tumor activity of two portions of FUC extracted from *Undaria pinnatifida* with different molecular weights (5100 and 490 kDa) with no significant difference in sulfate content, results showed that the anti-tumor activity of low molecular weight FUC was higher by two folds compared to the other FUC extract. Increased sulfate content usually yields an increased biological activity (Wang *et al*., 2019).

Fucoidan Bioavailability, Absorption and Pharmacokinetics.

In the last decade, several studies have investigated the biological activities of FUC; while only a few articles have reported FUC pharmacokinetic, intestinal absorption, tissue distribution, and excretion. Due to the high molecular weight and great structural diversity, several difficulties have been associated with understanding the uptake and fate of FUC.

In 2015, Nagamine et al. investigated intestinal absorption of FUC extracted from the brown seaweed, *Cladosiphon okamuranus* using human colorectal adenocarcinoma Caco-2 cell line as an *in vitro* model and *in vivo* Wistar rats. Results indicated that FUC transport across Caco-2 increased in a dose dependent manner to reach the maximum after a specific time (1 hour) and then decreased. This saturability of FUC transportation does not occur in simple diffusion, which suggests FUC absorption via transporter or pinocytosis. Furthermore, immunohistochemical and ELISA methods revealed the accumulation of FUC in jejunal epithelial cells, mononuclear cells in the jejunal lamina propria, Kupffer and sinusoidal non-parenchymal cells in the liver (Nagamine *et al.,* 2015).

Studying pharmacokinetics and tissue distribution of FUC is crucial for drug development as for understanding biological activities. FUC concentration purified from *F. vesiculosus* was analyzed in serum and tissues based on measuring anti-activated factor X (anti-Xa) activity. The highest concentration of FUC was found in kidneys followed by the spleen and liver, while the lowest was detected in plasma (Pozharitskaya *et al.,* 2018).

Recently, a new technique based on fluorescence detection was used to provide better knowledge about the mechanism of FUC absorption. FUC purchased from sigma aldrish (*F. vesiculosus*) were labeled with fluorescein isothiocyanate (FITC), then tested for their ability to

traverse Caco-2 monolayer cells and their tissue distribution after injection in mice. Results confirmed FITC-fucoidan transport across Caco-2 cells and their targeted accumulation in the kidney and liver (Bai *et al.,* 2020).

Furthermore, there are two clinical trials focusing on FUC; one on the biodistribution and tolerance of Fucoidan where healthy individuals or volunteers are involved in integrating FUC within their diet followed by tests that involve the biodistribution and safety of FUC while the other FUC is added to the chemotherapeutic treatment of patients with stage III-IV non-small cell lung cancer. This illustrates the importance of more studies to be performed related to FUC (Irhimeh *et al.,* 2005).

Supplementary box 2: Fucoidan Pharmacokinetics

Paracellular transport is defined as the transport of substances across an epithelium by passing through the intercellular space between the cells, while transcellular transport refers to the transfer of substances through the cell, passing through both the apical membrane and basolateral membrane.

Pinocytosis is a type of endocytosis where small particles suspended in extracellular fluid enter the cell through cellular membrane invagination.

Plasma anti-activated factor X (anti-Xa) activity assay : Factor Xa is one of the proteins involved in blood clot formation. Anti-Xa activity is used to measure plasma heparin levels and to monitor anticoagulant therapy. This assay is based on the inhibition of activated factor X (FXa) by Antithrombin (AT) in the presence of heparin as free Xa measured by a chromogenic Xa substrate. FUC shows certain structural similarities to heparin due to their polysaccharide nature and high negative charge density due to sulfation. In addition to the anti-coagulant effects of FUC. Thus, anti-Xa activity was used for the characterization of the pharmacokinetics and tissue distribution of fucoidan in plasma and tissues.

Biological Activities of Fucoidan

Anti-cancer:

Cancer is defined as a multifaceted process as several oncogene mutations in a group of cells end in an uncontrolled proliferation leading to tumor formation and spreading. It is a major cause of global mortality as the most used therapy “chemotherapy” still has side effects in addition to multidrug resistance. Thus, natural anti-tumor products with low toxicity are importantly required. FUC is a polysaccharide with anti-tumor potentials against a wide range of cancers. FUC purified from *C. okamuranus* was suggested to cause cell cycle arrest at G0/G1 phase in hepatocarcinoma cells (Sibusiso *et al.,* 2019). Another study on colon cancer showed that FUC purchased from *F. vesiculosus* inhibits tumor cell proliferation and induces apoptosis (Wang *et al.,* 2019) . Furthermore, Boo *et al* studied the anti-cancer effects of FUC obtained from *Undaria*

pinnatifida using human prostate cancer cells, where results showed that FUC induced apoptosis by activating both extrinsic and extrinsic pathways (Boo et al.,2013). Some clinical studies focused on combining FUC with other anti-cancer agents and results indicate a promising potential of FUC as a therapeutic agent *(*Sibusiso *et al.,* 2019).

Anti-viral:

FUC has been shown to alleviate some viral diseases symptoms and shorten the disease period, thus FUC can be used as an antiviral agent with low toxicity and resistance. Previous studies show that FUC inhibits the human immunodeficiency virus, herpes simplex virus, hepatitis B Virus, human cytomegalovirus, and influenza virus. The mechanism of antiviral activity of FUC is to inhibit virus adsorption onto host cells by interacting to the positively charged viral glycoprotein located on the envelope that is crucial for virus attachment *(*Wang *et al., 2019).*

Antioxidant:

Briefly, reactive oxygen species (ROS), a group of oxygen or nitrogen containing intermediates, unstable molecules that can easily react with cellular components and cause damage to DNA, RNA, and proteins. ROS types, production, effect, and elimination will be detailed in the next chapter. Oxidative stress OS resulted from high levels of ROS is common in a wide range of diseases such as neurodegenerative diseases , coronary heart disease, , diabetes, atherosclerosis, inflammatory diseases, and aging-related diseases. Thus, research focus on an intense identification of non-toxic antioxidant compounds. FUC is a well-known antioxidant compound acting as a ROS scavenger. FUC purified from *Laminaria japonica* and *Undaria pinnatifida* has been shown to exhibit Significant anti-oxidative properties (Wang *et al.,* 2019).

Anti-inflammatory*:*

The inflammatory response is explained as a reflection to numerous inflammatory factors, that when correctly regulated mediates the body's resistance to disease but can also cause tissue damage in case of overactivation. Inflammation is a common reaction in several diseases such as atherosclerosis, diabetes, neurodegenerative diseases, autoimmune diseases, liver diseases and those related to aging.

Previous studies demonstrated that FUC polysaccharide regulates inflammatory response in which FUC treatment inhibits nitric oxide (NO), intestinal inflammation, and leukocyte migration. On the other side, FUC synergistically enhanced the activity of anti-inflammatory drugs (Wang *et al.*, 2019).

Prebiotics:

Dietary prebiotics were first identified by Marcel Roberfroid in 1995 as compounds that induce the growth and activity have been used as functional food ingredients with health benefits due to the presence of indigestible dietary polysaccharides that pass undigested through the upper part of the gastrointestinal tract. The main source of polysaccharide in human's diet originate from terrestrial plant cell walls, and other less represented sources such as seaweeds. Due to the

fact that around 45% of the world's land surface is considered dry lands and lack of water is leading to 12 million hectares to be degraded yearly; recent studies suggest that in the next decades algae production will increase to replace or supplement plant food intake of terrestrial origin. Polysaccharides and oligosaccharides derived from marine origin have shown the potential to modulate intestinal metabolism, inhibit pathogen adhesion and invasion, stimulate the growth and activity of beneficial bacteria, and treat inflammatory bowel disease (Santamarina *et al.,* 2020).

Previous in vivo studies showed that FUC (purified from *A. nodosum*) administration resulted in microbiota diversity increasing the beneficial *Lactobacillus* strain, which produce lactic acid preventing harmful bacteria colonization and a decrease in serum levels of lipopolysaccharide-binding protein (Shang et al., 2016). Furthermore, FUC obtained from *Laminaria japonica* decrease the pathogenic bacterial *Enterobacter* species, increase *Lactobacillus,* increase lactic acid and short-chain fatty acids (SCFA) (Kong *et al.,* 2016).

Metabolic Syndrome Treatment: Metabolic syndrome (MS) refers to a cluster of interrelated factors and conditions leading to a pathophysiological state considered as a common feature of cardiovascular, cerebrovascular disease, insulin resistance, hypertension, and diabetes. MetS can be described also as a state in which proteins, fats, carbohydrates, and other substances in the body are metabolically disordered. Previous studies showed that FUC is attractive for improving and treating obesity in terms of lipid lowering effects that will be explained in detail within upcoming chapters (Wang et al., 2019).

Fucoidan Extraction Principle

Polysaccharides extraction and purification from algal cell wall is a complex and challenging process especially in the presence of a mixture of biopolymers. Fucoidan extraction involves five general steps:

1. The first step is considered as pre-treatment in which collected algae are dried and milled to have a higher surface-to-volume ratio; homogenous constant mass is obtained.
2. Lipids, terpenes, phenols, and pigments removal.
3. Crude FUC extraction by treating algal raw material different solvents (water, acidic or alkaline solution). This step may be repeated several times for a higher extraction yield.
4. FUC purification and its separation from other co-extracted contaminants.
5. The last step in extraction is known as dialysis, a process based on the utilization of different molecular weight cut-off membranes to separate FUC from smaller compounds depending on the high molecular weight of fucoidans, also to separate low molecular weight fucoidans (LMWF) from high molecular weight analogues.

Despite the difference in extraction method and the solved used, the main aim is to obtain an increased yield of FUC with high quality. Acidic solution extraction avoids alginic acid release with FUC and gives a better yield when compare to water, while water extraction maintains the stability and overall charge of FUC and alkaline solvent extraction usually co-extract alginic acid with FUC extract (Hahn et al., 2012).

Fucoidan Quality and Chemical Characterization

- ***Spectrophotometrical Analysis***

As above mentioned, structural and compositional differences in the purified sulfated polysaccharide result in a wide diversity of biological activities. This means that sugar, sulfate, and protein content determination are crucial for predicting FUC composition. Sugar, sulfate, and protein content were determined by colorimetric assays based on the utilization of standard curves. Sulfate and protein content were assessed spectrophotometrically by a simple and rapid assay based on their precipitation in the presence of agarose reagent and trichloroacetic acid (TCA).

Sugar content is analyzed by the most reliable and easiest colorimetric assay, based on the principle of sugar oxidation in the presence of sulfuric acid into a product that reacts with phenol. The final product light absorbance is determined by an ultraviolet spectrophotometer which is the oldest technique used for analyte quantification in a sample.

- ***Spectrometry***

FUC polysaccharides are characterized by their complex chemical structure, which means that several spectrometric methods have been used to elucidate FUC structural features such as the functional groups and the position of the glycosidic bond.

Fourier Transform Infrared spectroscopy FTIR: It is a technique for the identification of organic inorganic and polymeric material based on the absorption, emission of an infrared spectrum in the range of 400 and 4000 cm^{-1}. In this method, the mathematical process (Fourier transform) is utilized to translate the raw material into a spectrum that is used to identify specific functional molecular groups in the sample as compared to spectrum data in the automated software of spectroscopy. FTIR is useful also to identify the characteristics of unknown samples and detecting additives or contaminants in a material (Titus *et al.*, 2019). Previous studies showed that the preliminary identification of FUC functional groups is usually performed by studying the FTIR spectrum of the sample, in which typical IR bands correspond to FUC functional-structural main groups (such as O-H group of monomeric

monosaccharides, C-H, C-O-S of sulfate ester groups, S=O stretching, O-C-O and C-O-C of glycosidic linkage.

Nuclear Magnetic Resonance Spectroscopy NMR: It is the most significant analytical tool used in chemical research to investigate the dynamics and structural features of a compound in a solution or solid state. NMR is associated with energy absorption and emission. The theory behind this technique is based on the absorption of electromagnetic field by nuclei of the atoms. The NMR spectrum is a plot that measures relative resonance intensity as a function of chemical shifts in parts per million (ppm). Chemical shift is defined as the difference in ppm between the resonance frequency of the sample and a reference.

- Proton NMR **(^{1}H NMR)** is the application of NMR with respect to hydrogen atoms within the molecule to determine the structure.
- Carbon NMR **(^{13}C NMR)** is analogous to ^{1}H NMR, but instead applied with respect to carbon atoms, it gives information about the carbon skeleton and not just the proton attached to it. (Diehl et al., 2008)

Both forms of NMR spectroscopy have been widely used to study the structure of fucoidan in terms of sulfation pattern and glycosidic linkage.

II. AIM OF THE STUDY

In this study we aimed to extract FUC from the brown marine algae; Cystoseira compressa, and the terrestrial plant; Ferula hermonis. The purified polysaccharides were chemically characterized by FTIR and NMR techniques. Previously Kanaan et al. have extracted FUC for the first time from the terrestrial plant "Eucalyptus globulus". Sugar, sulfate, and protein content were determined in FUC extracted from the three species.

The difference in conditions between marine and terrestrial environments are important factors that may contribute to the formation of a wide scale of chemical compounds. Marine algae contain polysaccharides that have a different composition, substitutions, and linkage types than terrestrial polysaccharides, and some are unique for algal species such as sulfated polysaccharides. Brown seaweeds are considered as the main source of FUC polysaccharide, but recently FUC was identified in the terrestrial plant (Zheng *et al., 2020) (*Haddad *et al., 2017).* Briefly, we explained below about the three species.

CYSTOSEIRA COMPRESSA

C. compressa is a brown algae species from Cystoseira genus, belongs to the family of Sargassaceae in the order of Fucales. Cystoseira have highly differentiated basal and apical regions with floating air-vesicles like structures. This genus provides an essential habitat for several epiphytes, fish, and invertebrates; it is mostly found in the Mediterranean, Indian, and Pacific Oceans.Cystoseira genus consists of 293 species including C. compressa. The first publication related to *C. compressa* was in 1975 by Gerloff and Nizamuddin; *C. compressa* was first identified in the Adriatic Sea which is the arm of the Mediterranean Sea, lying between the Italian and Balkan peninsulas. (Gerloff et al., 1975).

Morphological variations of *C. compressa* is explained by thallus change in terms of size and appearance in relation to seasonal and environmental factors especially wave exposure variations. The vegetative development is observed in spring- presenting fresh algal material where thalli reach their maximum height and physiological activity in June; while in late summer, specifically August almost loses all upright branches (Falace et al., 2005).

In 2014, Kanaan et al. investigated the diversity of seaweeds growing on the Lebanese coasts. *C. compressa* was identified in three checked zones out of eight: Tyre, Barbara, and Naqoura (Kanaan et l., 2014).

Previous studies showed that *C. compressa* aqueous extracts have good anti-microbial, antioxidant, anti-inflammatory and anti-proliferative activities against HCT and MCF-7 cell lines (Mhadhebi *et al*., 2014). In 2017, Ammar *et al.* extracted alginates from *C. compressa* that showed gastroprotective effect and inhibited gastric lesions. Moreover, FUCs isolated from *C. compressa* exhibited a significant anti-inflammatory and antioxidant activity and a significant

decrease of gastric mucosal damage. Moreover, acetone extract of *C. compressa* was tested for its anti-microbial and cytotoxic properties (Kosanić M *et al*., 2015). In a recent study, *C. compressa* methanol extract showed high antioxidant potentials, and tested for its anti-diabetic activity as it exhibits a high α-glucosidase and pancreatic lipase inhibition rates (Çelenk et al., 2020).

Cystoseira compressa

Figure 5: Map showing the sites of collection of the seaweeds along the Lebanese Coast.

FERULA HERMONIS

Ferula hermonis, a small flowering plant that belongs to the Apiaceae family. It is commonly known as "Shirsh El Zallouh", endemic in Lebanon, Syria, Turkey, and Jordan. Roots are usually harvested from August to October, then extracted to be traditionally used in order to enhance sexual behavior, treat infertility and menopausal disturbances, in which it is commonly known as or "Lebanese Viagra" (Sattar *et al*., 2017). This plant is sold in many herbal shops in Lebanon, Syria, and other Arabic countries.

Moreover, it has been shown that *F. hermonis* exerts anti-bacterial, anti-inflammatory, anti-diabetic, hypolipidemic, and hepatoprotective effects. In 2008, Elouzi et al. investigated the cytotoxic effect of petroleum ether, ethyl acetate, and methanol *F. hermonis* extract against stomach cancer cell line. *F. hermonis* essential oil components were shown to exhibit strong anti-fungal activity particularly against the dermatophyte Tricophyton mentagrophytes (Al-Ja'fari et al., 2013). Another in vivo study demonstrated the antioxidant and antidiabetic effects of *F. hermonis* by reducing blood glucose concentration and body weight, as inducing catalase serum levels (Raafat et al., 2015).

These biological activities are related to the presence of a wide range of active compounds such as sulfur-containing compounds, sesquiterpenes, coumarins, ferutinin, α-pinene, camphene and carvacrol, as well as vitamins (A and E) and some minerals (Sattar *et al*., 2017). According to the chemical composition reported by Abdel-Kader *et al*. (2011), *F. hermonis* roots contain 38.38 % carbohydrates, 8.33 % lipids and 3.48 % proteins; however, to our knowledge, no investigation has been conducted on the water-soluble polysaccharide fractions.

Ferula hermonis

EUCALYPTUS GLOBULUS

E. globulus was first mentioned in 1800 by Jacques Labillardière in his book " Relation du Voyage à la Recherche de la Pérouse" where he described specimens collected in 1792 at Recherche Bay located on the extreme south-eastern corner of Tasmania, Australia (*La Billardière, 1800)*. It belongs to the family of Myrtaceae that include around 140 genera and 3800 species distributed in tropical and subtropical regions of the world. Among these genera, Eucalyptus is one of the world's important and most cultivated genera.

E. globulus is a tall aromatic tree, also known as the blue gum due to the green color of its foliage that remains through more than one growing seasons. This terrestrial tree has a height average of 45 meters (m), but under some conditions can grow as tall as 90-100 m. *E. globulus* green leaves are arranged in opposite pairs, with flower buds arranged singly or in groups of three or seven in leaf axis. Despite the native distribution of *E. globulus* in forests located in New South Wales, Victoria, and Tasmania; blue gum was planted in several countries with Mediterranean-type climate such as Algeria, Lebanon, and Italy where it rapidly grows (Hillis et al., 1984).

E. globulus flowers are a good source for honey production while leaves were used as herbal tea, in addition to essential oil extraction as this species is considered the primary source of global eucalyptus oil production; essential oil has been used as a flavoring, perfumery, anti-microbial, anti-oxidant agents with other therapeutical applications.

Eucalyptus Biological Activities

E. globulus is characterized by biological activities of potential medicinal value which make it of great interest (Qabaha *et al*., 2016). Previous studies showed that *E. globulus* has been used for the treatment of various diseases such as fungal infection, diabetes, influenza, and pulmonary tuberculosis. Crude extract of the fruits of *E. globulus* has shown antioxidant and anti-bacterial activity (Makhlouf *et al*., 2013). Another study shows that the extract of *E. globulus* leaves reduced lipogenesis, inflammatory cytokine expression, and OS, as it inhibited NASH induced by fructose ingestion in rats (Takahashi *et al*., 2015). Furthermore, aqueous extract and the essential oil have a good anti-bacterial and antioxidant activity due to polyphenols and flavonoid content (Pombal *et al*., 2014).

Eucalyptus globulus

III. MATERIALS AND METHODS

Algal and Terrestrial species

Collection

Cystoseira compressa: The brown seaweed *C. compressa* freshly collected from "Tyre Coast-South Lebanon" in the period of April-June 2018; were washed and cleaned with water, dried at room temperature, and grinded for further extraction.

Ferula hermonis: Roots of *F. hermonis* were harvested from "Mountains of Bekaa", Lebanon, in August 2017. The roots were washed and cleaned with water, then dried at room temperature and grinded for further extraction.

Eucalyptus globulus: purified FUC was provided by Professor Hussein Kanaan as it was extracted in 2017 at Laboratory of Chemical Synthesis and extraction of Polysaccharides from seaweed, Faculty of Pharmacy, Lebanese University.

CYS, FER and EUC correspond to FUC purified from C. compressa, *F. hermonis* and E. globulus, respectively.

Extraction and Purification of Fucoidan

Water-soluble polysaccharides were obtained as previously described using slightly modified methods (Imbs et al., 2009; Ermakova et al., 2013) . Briefly, 100 g of *C. compressa* and *F. hermonis* roots were extracted twice with 250 mL absolute ethanol for 3 h at 40 °C to remove low-molecular-weight compounds such as pigments, phenols, and proteins. The dried residues were extracted twice with aqueous hydrochloric acid (HCl) solution (pH = 2) at 60 °C for 3 h, then centrifuged at 1600× *g* for 20 min to obtain supernatant, containing the FLM complex (Fucoidan, Laminarin, Mannuronan). The supernatant was neutralized with 3% NaHCO3, evaporated in a Rotavac Vario Power Unit (Heidolph Instruments, Schwabach, Germany) to a final volume of 200 mL, which proceeded to 24 h dialysis (Spectra/Por Dialysis Tubing, MWCO 12000-14000) and subsequent lyophilization to obtain FLM powder. Fucoidan purification was obtained by adding 50 mL of aqueous HCl solution (pH = 2), followed by centrifugation at 1600× *g* for 20 min. The pellet was discarded whereas supernatant was lyophilized to obtain dry powdered fucoidan (CYS, FER), which was weighed to calculate yield.

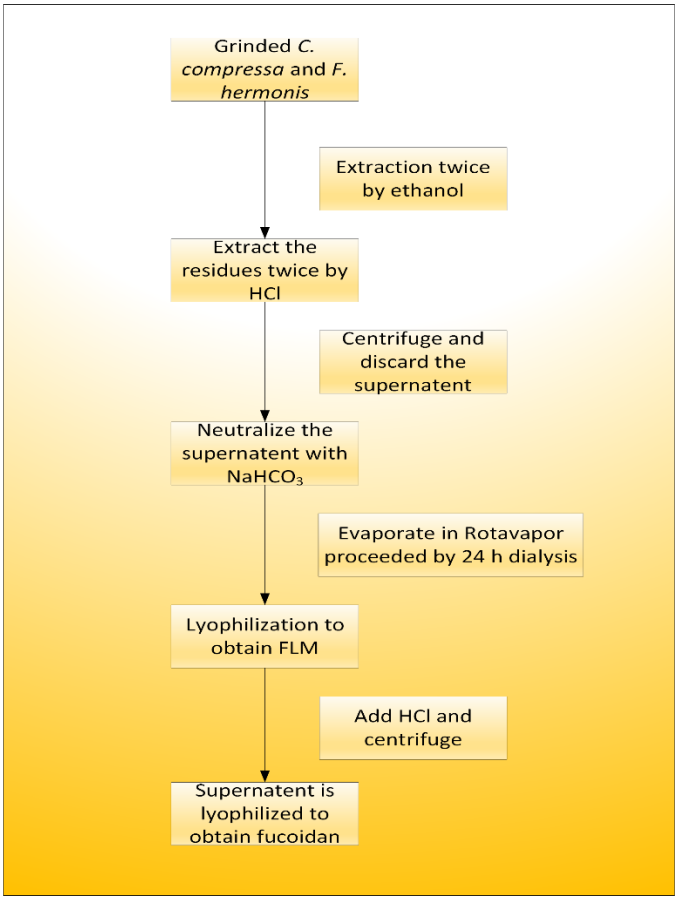


Figure 6: Extraction of fucoidan polysaccharide.

Chemical Characterization

A. **Sulfate and Protein Content:** Sulfate and protein content were determined by the turbidimetric assay described by Jackson and McCandless (1978). Reagents needed for these spectrophotometrical analysis are: 8% of trichloroacetic acid, and 0.01% agarose with or without Barium chloride for sulfate and protein content, respectively. FUC samples (CYS, FER, AND EUC) were incubated with TCA and the corresponding agarose for 40 minutes at room temperature RT, then turbidity was measured at 500 nm. Potassium Sulfate (K_2SO_4) and bovine serum albumin (BSA) were used as sulfate and protein standards, respectively.

B. **Sugar Content:** Sugar content was quantified using the phenol-sulfuric acid method developed by Dubois *et al*. (1956). 2 ml of the three purified FUC were incubated with 0.5 ml of phenol (3%) and 5 ml of H_2SO_4 for 15 minutes at RT followed by absorbance

recording at 520 nm. Fucose, glucose, galactose and mannose were used as sugar standards.

Functional Groups and Chemical Structure

A. **Fourier Transform Infrared spectroscopy (FTIR):** The FTIR spectra of CYS, FER and EUC were recorded on a Perkin-Elmer FTIR spectrometer Spectrum Two UAT. Data were collected in the range of 4000-400 cm^{-1}.

B. **Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectroscopy:** Proton and carbon positions in CYS, FER and EUC were determined by analyzing NMR spectra using a Bruker Ascend 500 AVANCE III HD spectrometer. The water-soluble polysaccharide was dissolved in 99% deuterium oxide (D_2O), and the spectra were recorded at room temperature (1H NMR: frequency 500 MHz, acquisition time 3.27 sec; 13C NMR: frequency 125 MHz, acquisition time 1.1 sec).

IV. RESULTS

Extraction Yield and Chemical Composition

The yield of the FUC extraction from *C. compressa* and *F. hermonis* roots were 3.6% and 3.07%, respectively. Both greater than that from *E. globulus*, which was 2.1% according to a previous study (Haddad *et al*., 2017). The chemical composition of purified FUCs is shown in Table 2 CYS,FER, and EUC contained significant amounts of fucose and sulfate groups, but also glucose, galactose, mannose, and a small amount of proteins were detected.

Table 2: Chemical composition (%) of fucoidan extracted from *C. compressa*, *F. hermonis*, and *E. globulus* Data are presented as mean ± SD of 3 experiments.

In (%)	Sulfate	Fucose	Glucose	Galactose	Mannose	Proteins
CYS	1.26 ± 6.1	22.3 ± 2.7	21.1± 5.3	1.99± 3.1	0.98±2.9	0.42 ± 6.3
FER	1.8 ± 1.52	31.5 ± 6.1	29.3 ± 6.32	2.6 ± 5.82	1.2 ± 4.95	0.46 ± 7.6
EUC	2.17 ± 6.2	33.5±3.4	32.2±4.7	2.89±2.6	1.39±3.7	0.58 ± 5.5

STRUCTURAL FEATURES

FTIR spectroscopic analysis:

- ***C. compressa***

The FT-IR spectrum of CYS (Figure 7A) showed a wide band at 3415.31 cm-1 corresponding to O-H stretching vibrations. Two weak signals at wavelengths 2927.41 and 2049.96 cm-1 are assigned to C-H stretching of the pyranose ring and of the methyl group of fucose (Saboural et al., 2014).Furthermore, the small peak at 1726.94 cm-1 is due to the C=O bond of carboxylate group (Liang et al., 2019). The band at 1619.91 cm-1 contributes to asymmetric vibrations of carboxylate anions O-C-O of uronic acid, glucoronate units (Huang et al., 2017). The band at 1079.94 is assigned to the sulfate ester group (Haddad et al., 2017). The band at 842.74 cm-1 corresponds to sulfate peak (Patankar et al., 1993). Moreover, the band at 618.074 cm-1 are almost contributed to the C-C-H stretching vibration (Myoung et al., 2011).

- ***F. hermonis***

As shown in Figure 7B, the FTIR spectrum of FER showed a wide band centered at 3301.02 cm-1, which was assigned to hydrogen bonded O-H stretching vibration. The weak signal at 2926.7 cm-1 indicated the presence of C-H (Sabourin et al., 2014). The band at 1595.49 cm-1 indicated the absorbance of uronic acid carbonyl group C=O, while that at 1410.07 cm-1 was assigned to carboxylate group vibrations (C–O). The weak band at 1273.21 cm-1 indicated the presence of S=O stretching vibration of the sulfate group, and that at 1028.88 cm-1 was due to a sulfate ester group (Myoung et al., 2011). Additional band at 530.98 cm-1 indicated the presence of C-O-S sulfate residues (Somasundaram et al., 2016).

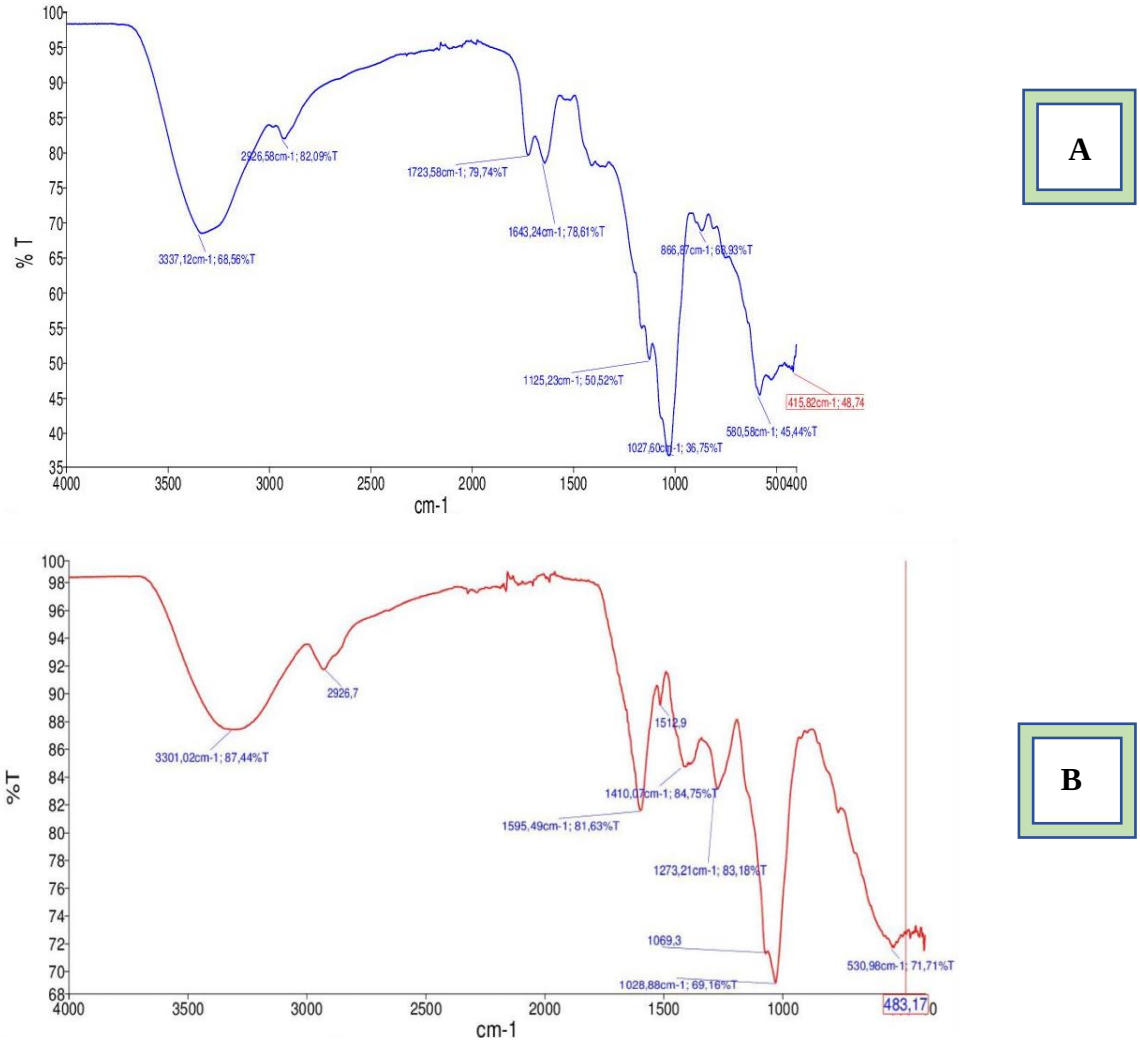


Figure 7: FTIR spectrum of fucoidan isolated from *F. hermonis* (A) and *C. compressa* (B). %T: % Transmittance. Fourier Transform

Nuclear Magnetic Resonance analysis:

- ***C. compressa***

As shown in figure 8A ^{1}H NMR analysis of CYS indicate the presence of a chemical shift between 0.61 and 1.15 ppm that corresponds to three hydrogen groups of a methyl group which is a main characterization of L-fucopyranose backbone. Another signal spread from 3.5 to 3.8 ppm are related to five ring protons indicating the presence of different fucose sulfate group types (Alwarsamy *et al*., 2016)

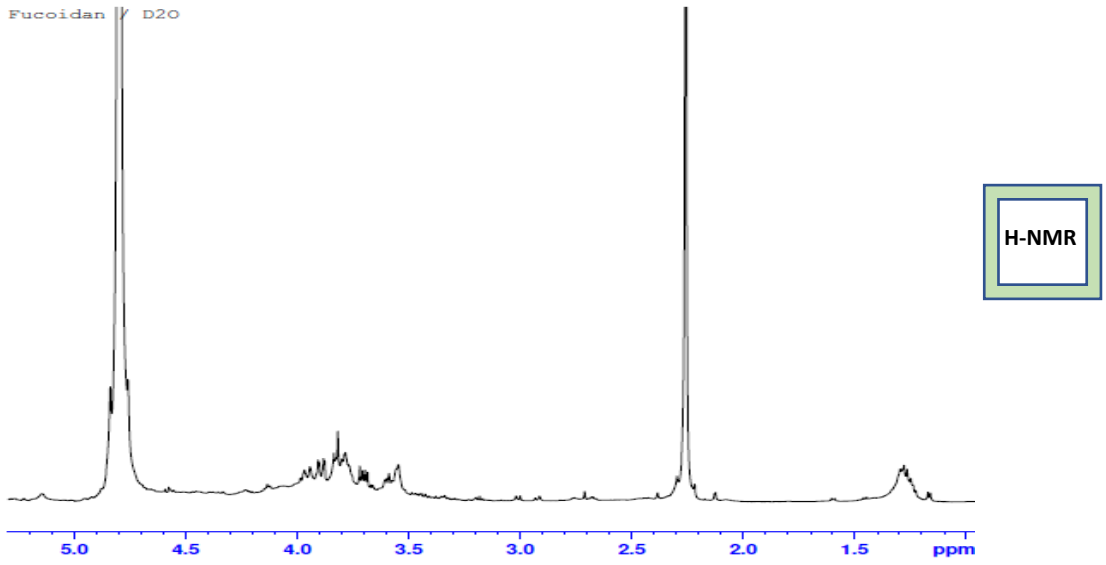


Figure 8A: 1H NMR of fucoidan purified from *C. compressa*.

- ***F. hermonis:***

The Proton (1H) NMR analysis of FER is shown in Figure 8B. The 1H NMR spectrum contained chemical shifts ranging from 5.0 to 5.7 ppm that are consistent with the presence of protons (H1α) of α-linked-l-fucose and β-linked sugars (H1β). Chemical shifts indicated between 0.75 and 1.81 ppm corresponded to the methyl group which is a main characterization of L-fucopyranose (L-FucP). The spectrum contained resonance characteristics of FUC with signals from ring protons (H-2 to H-5) at 3.46–4.20 ppm that support the presence of different types of fucosal sulfate groups with changes in glycosidic linkage positions and monosaccharide patterns (Alwarsamy et al., 2016) (Ajisaka et al., 2015). Furthermore, 1H signals due to linear unsubstituted L-fucose resonated at 5.07 to 5.22 ppm and the signals at 5.38–5.39 ppm could be supposed signals of substituted L-fucose and side chain of D-pyranose residues (Haddad et al., 2017) .

The 13C NMR spectrum of FER reported in Figure 8B showed major signals of sulfated L-fucan between 92.2 and 103.7 ppm (C1) and at 16.6 ppm (C6). The signals obtained in the region 60.2 and 82.0 ppm corresponded to pyranoid ring carbons (C2-C5) (Alwarsamy et al., 2016) (Haddad et al., 2017). In the 13C NMR spectrum of FER there are at least seven separate anomeric carbon signals. The C6 signal of presumably glucose is visible at 62.4 ppm.

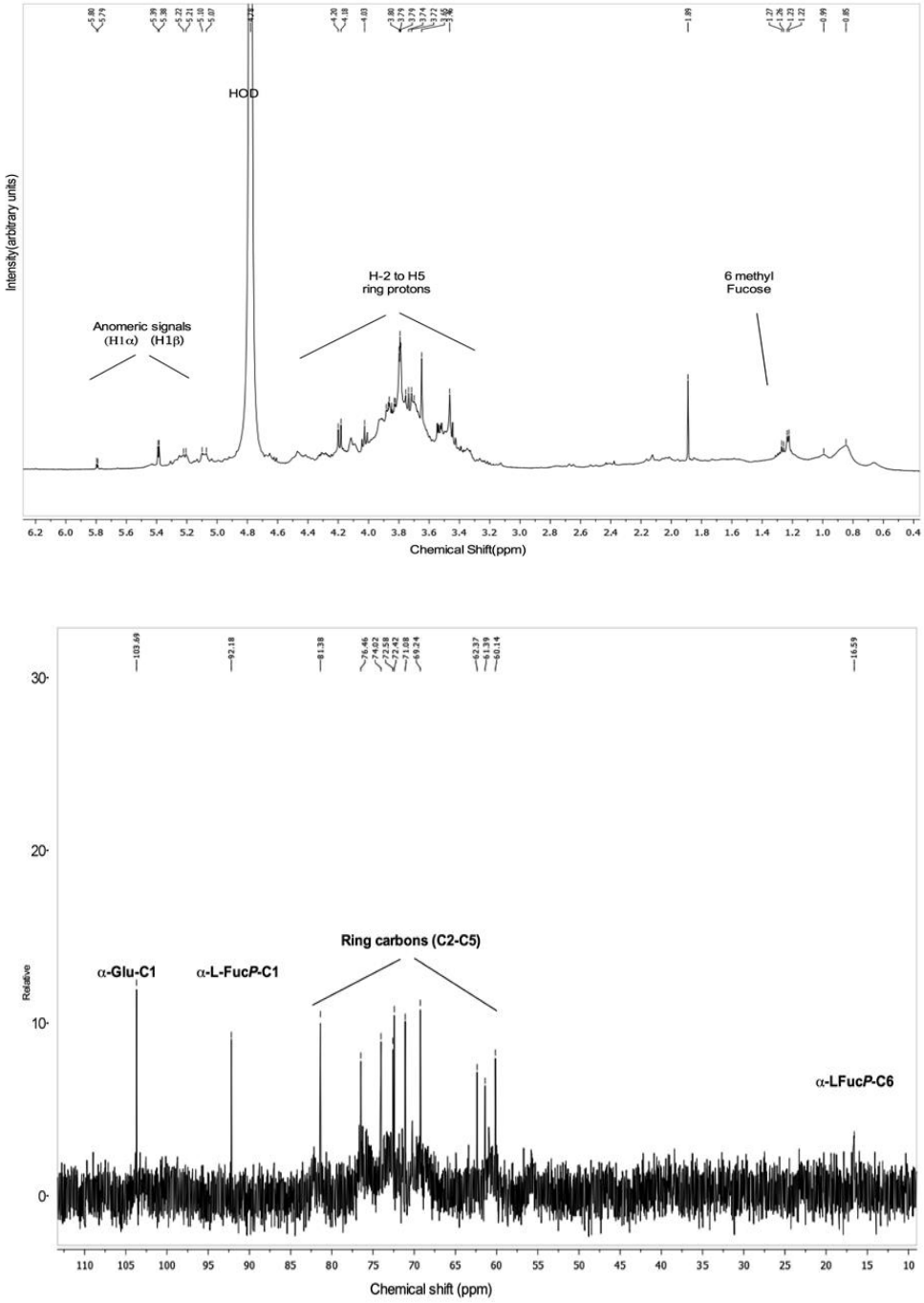


Figure 8B: [1]H NMR and [13] C NMR spectra of fucoidan isolated from *F. hermonis*.

V. DISCUSSION

Previously, several studies demonstrated that fucoidan polysaccharide possesses a wide spectrum of biological activities such as antitumor, antioxidant, anticoagulant, antithrombotic, immune-regulatory, anti-viral, anti-inflammatory and anti-steatotic; thus FUC can be considered a suitable candidate for several therapeutic and pharmaceutical applications (Wang *et al*., 2019 (Yokota *et al*., 2016). *B*rown algae are considered the main source of FUC (Kylin H. et al., 1913). Recently FUC has been isolated and characterized from the leaves of *E. globulus*, a terrestrial tree known to be used as a medicinal plant.

This inspired us to extract fucoidan from the brown algae Cystoseira compressa collected from the Lebanese coast, as well as from the terrestrial plant Ferula hermonis for the first time.

Our results indicated that the extraction yield of FUC from *C. compressa* (3.6%) was greater than that from *F. hermonis* which was 3.07% (El Rashed et al., 2021); while about the same yield obtained for FUC purified from *E. globulus* according to a previous study (Zein et al., 2018). Moreover, FUC yield from *C. compressa* was close to that obtained from the same species extracted in previous studies, where it was 5.2% (Hentati et al., 2018). The difference in FUC content varies between species and even in the same species it depends on the source of algae as well as the environmental changes.

Chemical composition analysis of CYS, FER and EUC extracts revealed the presence of sulfates, fucose, and glucose groups in high percentages, while smaller quantities of other monosaccharides and proteins were detected. EUC has shown to contain a higher amount of all components abovementioned as compared to FER and CYS. Thus, chemical composition as well as extraction yield can be affected by several factors including species difference, seasonal variations, environmental changes, and extraction methods. (Zhao *et al*., 2018).

The chemical structure of FUC extracted from *C. compressa* and *F. hermonis* investigated by FTIR and NMR confirmed the presence of functional groups that characterize fucoidans such as fucopyranosyl units and sulfate groups.
With the exception of peaks detected at 2049.96 cm-1, 1619.91 cm-1 and 618.074 cm-1, the FT-IR spectra of fucoidan purified from *C. compressa* showed similarities in their infrared absorption properties compared to that isolated from *E. globulus* as previously studied (Zein *et al*., 2018). Few differences are obtained also when comparing CYS and FER FTIR, where CYS show an additional peak at 618.074 cm^{-1} corresponding to C-C-H stretching vibrations while FER spectra indicated the presence of sulfate group at 1273.21 cm^{-1}. Moreover, IR spectra indicated a difference in sulfate substitutions in which CYS spectrum indicates the presence of absorption at 842.72 cm-1 corresponding to the sulfate groups at the axial C-4 position while sulfate substitution of EUC were located in the equatorial C-2 and/or C-3 positions (Patankar et al., 1993; Zein *et al*., 2018). The difference in species and growth environment diversity are

factors that can lead to chemical composition variation which in turn affect the bioactivity of the polysaccharide (Jiao *et al.*, 2012).

VI. CONCLUSION

In this study, we were able to purify fucoidan from the brown algae *C. compressa* collected from Lebanese cost, as well as from the terrestrial plant *F. hermonis* for the first time. The chemical features and functional groups of purified fucoidan were investigated by studying the spectra of FUC by Fourier Transform Infrared and nuclear magnetic resonance. Furthermore, the chemical composition of CYS, FER, and EUC was demonstrated by several assays that investigate fucose, sugar, sulfate, and protein content. Different yields, functional groups, and chemical compositions were obtained for the three vegetal species with different environments.

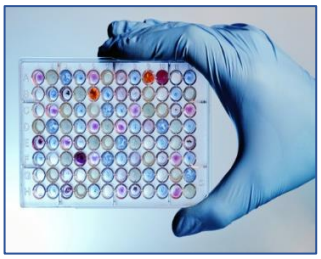
We confirmed that FUC structure is determined by the vegetal species and environmental factors, which encourages us to check if this will lead to the difference in biological activities associated with this water-soluble polysaccharide.

In the next study, the antioxidant activity of FUC will be investigated by several spectrophotometrical methods.

CHAPTER II: ANTIOXIDANT ACTIVITY OF FUCOIDAN EXTRACTED FROM C. compressa, F. hermonis, and E. globulus.

Define the problem:

- Oxidative stress is a common state among several
disease, especially metabolic diseases (MS).
- Search for new natural antioxidant compounds.



Chapter Research Question:

- Does FUC purified from three different species have antioxidant activities.
- Which FUC is the most active?

ABSTRACT

Oxidative stress (OS) is a common cause of most of the non-communicable diseases of the modern age and is caused by reactive oxygen species generated by biological systems through metabolism and environmental. Endogenously biological systems have their own natural defense antioxidant mechanisms, yet they cannot prevent the entire oxidative damage leading to the use of synthetic drugs that have several side effects. Therefore, there is an increase in the search for biologically active natural compounds that can repair OS by delaying or preventing the negative effect caused by free radicals. Fucoidan, the water-soluble polysaccharide is characterized by one of the main biological potentialities of phytochemicals, which is antioxidant activity. Antioxidant potentials for fucoidan purified from the three different species were investigated by several assays; DPPH assay, ABTS assay and FRAP assay. Furthermore, FUC protective role from oxidative damage to erythrocyte was assessed by anti-hemolysis assay. Our results indicated that CYS, FER, and EUC exhibit good antioxidant potentials, with EUC being the most active in all antioxidant activity testing assays.

Table of Content

I. INTRODUCTION

OXIDATIVE STRESS

"Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants , leading to a disruption of redox signaling and molecular damage"

Oxidants: Definition and Sources

Cancers, strokes, arthritis, aging, autoimmune disorders, diabetes, cardiovascular, neurodegenerative diseases, and liver diseases seem as different from one another as any diseases could be, which makes it hard to imagine them sharing a single feature. However, there is an integration of OS almost in all areas of human pathology. Oxidants collectively describes both free radical and other non-radical reactive derivatives.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly unstable molecules derived from oxygen and nitrogen molecules that are divided into free radicals and non-radical molecules.

- Free radicals are atoms, molecules or ions that are unstable and highly reactive with other molecules due to their unpaired electrons. These free radicals include hydroxyl (OH•), superoxide (O2•–), nitric oxide (NO•), nitrogen dioxide (NO_2•); as molecular oxygen being the main source of radicals in biological systems.

- Other molecules such as hydrogen peroxide (H_2O_2) and peroxynitrite are non-radical ROS but can give rise to free radicals upon further metabolisms in biological systems.

Reactive species (RS) are products of normal cellular metabolism with an important role in signaling pathways. Inside the cell, RS are produced as a result of extracellular or intracellular stimuli. Extracellular stimuli on plasma membrane receptors generate RS through tumor necrosis factor- (TNF-) α, hormones (insulin), and growth factors (platelet-derived growth factor (PDGF) and epithelial growth factor (EGF)). While intracellular stimuli generate (RS) by activating several are induced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), and cytochrome P450 (CYP).

Moreover, the exogenous source of oxidants results from the air and aqueous pollution, cigarette smoke, alcohol, heavy or transition metals (Hg, As), industrial solvents, cooking (smoked meat, used oil, fat), and radiation. After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals (Betteridge et al., 2000).

Oxidants: Biological Effects

Free radicals are initially produced by the body, but what makes such species play a dual role as both toxic and beneficial compounds is their level in the body. At low or moderate levels, ROS and RNS are safe for normal biological functions of mitochondria, cell, and tissues; in which they are implicated in vital roles of cell proliferation, differentiation, migration, and immune response. For example, phagocytes (neutrophils, macrophages, monocytes) release free radicals to destroy invading pathogenic microbes. Another example is the free radical nitric oxide (NO) which plays a role as an intercellular messenger for modulating blood flow, thrombosis, neural activity, and for killing intracellular pathogens and tumors (Zorov et al., 2014).

On the other side, excessive amounts of ROS can lead to deleterious effects when interacting with other molecules such as lipids, proteins, and DNA causing cell damage and DNA lesions.

As abovementioned, several organelles contribute to OS in specific ways that will be explained below.

Mitochondrial ROS Production

Within most mammalian cells, mitochondria are an important source for ROS production in which ROS contribute to mitochondrial damage and dysfunction in a range of pathologies. The first evidence on mitochondrial ROS (mtROS) production was recorded in 1961 by Jensen *et al* who observed that a small portion of oxygen consumed by the mitochondrial respiratory chain was converted to H_2O_2. Then, the mtROS research era was initiated in 1970's and since that time "artificial" systems such as isolated mitochondria, inside-out submitochondrial particles, reconstituted respiratory complexes, and pure enzymes have been used to study physiological processes. mtROS generation takes place at electron transport chain (ETC) located on the inner mitochondrial membrane where normally ATP is produced through cellular oxidative phosphorylation (detailed in chapter III). However, this process is not perfect in some cases where electrons leakage leads to a partial reduction of oxygen to form superoxide (O_2^-). Furthermore, O_2^- undergoes dismutation into H_2O_2 by superoxide dismutase enzyme; notice that there are two forms of SOD (SOD1 in mitochondrial intermembrane space and SOD2 in the mitochondrial matrix).

At the mitochondrial level, ROS can damage proteins, membranes, and lipids leading to defective protein synthesis and thus impairing the mitochondrial ability to allow a wide range of metabolic reactions such as Krebs cycle, fatty acid oxidation, amino acid metabolism, and the urea cycle. Moreover, OS activates apoptotic machinery by increasing the capacity of mitochondria to release intermembrane space proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) (Murphy et al., 2009).

Peroxisomal ROS Production

As mitochondria is the major source for ROS generation, peroxisomes are considered as central organelles within ROS generation and scavenging. Peroxisomes are involved in fatty acid α-oxidation, β-oxidation of very long chain fatty acids, catabolism of purines, and biosynthesis of glycerol-lipids and bile acids. They are rich in O_2 consuming oxidases , acyl-CoA oxidase, xanthine oxidase, D-amino acid oxidase, and nitric oxide synthase which result in the production of H_2O_2, O_2^- and nitric oxide $^{\cdot}NO$ as byproducts. (Bonekamp et al., 2009).

Endoplasmic Reticulum and ROS Production

Endoplasmic reticulum (ER) organelle is responsible for protein synthesis, folding, maturation, and assembly. Under several conditions unfolded or misfolded proteins are accumulated within ER lumen leading to ER stress which in turn induces ROS production and depletes reduced glutathione which is a scavenger of ROS (Ozgur et al., 2015).

ANTIOXIDANT SYSTEM

On the other side the body overcomes the oxidative stress; either by endogenous antioxidants produced by the body or by exogenous antioxidants supplied through food, they exert their effect by different mechanisms: suppress radical formation; act as scavengers and acting as their substrate (Huang et al., 2005).

The major endogenous antioxidants are superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase. In addition to exogenous antioxidants provided by diet and medicinal plants. Normally, prooxidant are in balance with antioxidant mechanisms, while in case of OS an imbalance occurs in favor to oxidants.

Supplementary Box 3: Antioxidant Enzymes

Superoxide Dismutase: It is an antioxidant defense enzyme that catalyzes the dismutation of O_2^- into O_2 and H_2O_2.

Catalase: This common antioxidant enzyme catalyzes the decomposition of H_2O_2 into H_2O and O_2.

Glutathione peroxidase: It is an enzyme with peroxidase activity whose main function is to prevent oxidative damage. It catalyzes the reduction of hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxides radicals to alcohols and oxygen.

Glutathione reductase: This enzyme catalyzes the reduction of glutathione disulfide to the reduced form glutathione.

Antioxidant Activities of Natural Compounds

Since the beginning of this century, the use of synthetic antioxidants that are inspired by natural scaffolds including vitamins C and E, and phenolic compounds have been increased. Used synthetic compounds are reported to be safe have been used in food products; although some studies indicate otherwise, such as the examples of butylated hydroxytoluene and butylated hydroxyanisole, which are the most widely used chemical antioxidants. However, several restrictions have limited the use of these synthetic compounds because of their long-term effects, and the focus of consumers on the reduction of synthetic additives or their replacement by natural products. Thus, studies have been directed to investigate the potential of natural products to serve as antioxidants for protection against free radicals. A simple search for antioxidant activities of natural compounds results in 43,800,000 articles. Such natural bioactive compounds can be purified from plants, fungi, and algae (Nunes et al., 2012).

Mechanisms of Action of Antioxidants

Plant extracts or their purified compounds show significant antioxidant activities. They act by several mechanisms listed below, all aimed to reduce OS by directly reacting with the reactive radicals and destroy them or producing new free radicals which are less active and less dangerous than those radicals they have neutralized. Moreover, such compounds can help to protect cellular damages from OS by inhibiting the activities or expressions of free radical generating enzymes such as NADPH oxidase and xanthine oxidase (XO) or inducing the expression and activity of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (Lü et al., 2010).

- Scavenging radicals, superoxide, and other ROS.
- Metal ion ((Fe2+, Fe3+, Cu2+ and Cu+) chelating.
- Inhibition of free radical generating enzymes.
- Activating intracellular antioxidant enzymes.
- DNA protection from damage.
- Prevention of lipid peroxidation.

METHODS for ANTIOXIDANT ACTIVITY QUANTIFICATION

Due to the contribution of OS in several pathologies and antioxidants significant role in scavenging ROS, several methods have been used to determine the antioxidant activity of phytochemicals. Based on the mechanism of action, and from a chemical point of view available methods for antioxidant capacity quantification are divided into three groups.

A. Electron-Transfer-Based Assays: antioxidants transfer an electron to reduce metal ions, carbonyls, and radicals.
Examples: DPPH free radical scavenging assay, Ferric reducing antioxidant power (FRAP), and Trolox equivalent antioxidant capacity (TEAC) assay.

B. **Hydrogen-Atom Transfer Reactions:** This type of assays depends on antioxidant's ability to "quench/intercept" free radicals by detaching a hydrogen atom.
Examples: Total radical trapping antioxidant parameter (TRAP), and Oxygen radical absorbance capacity (ORAC) method.

We will explain in a detailed way the principle and advantages of the three assays "DPPH, FRAP, AND TEAC"

DPPH ASSAY: DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable radical with a deep violet color that turns yellow when scavenged by a reducing agent (antioxidant), and this is monitored quantitively by spectrophotometrical reading of the absorbance decrease at 515 nm.

FRAP ASSAY: is a colorimetric redox-linked assay that measures the ferric reducing activity of the sample at low pH conditions, based on ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) reduction to a ferrous (Fe^{2+}-TPTZ) form by antioxidants present in tested material. Results are quantified as an increase in absorbance at 593 nm is proportional to the total Fe^{3+} reducing power of the tested sample which is quantitated at an absorption maximum of 593 nm.

ABTS ASSAY: this assay depends on $ABTS^{\bullet+}$ free radical which are generated when ABTS substrate is oxidized with potassium persulfate. $ABTS^{\bullet+}$ has a blue/green color, and decolorized when reduced in the presence of reducing agent. It is a spectrophotometrical method where $ABTS^{\bullet+}$ maximum absorption spectrum is monitored at 734 nm and called TEAC because he antioxidant capacity of the sample is measured in comparison to Trolox as a standard (Shalaby et al., 2013) (Ivanova et al., 2020) (Liang et al., 2014).

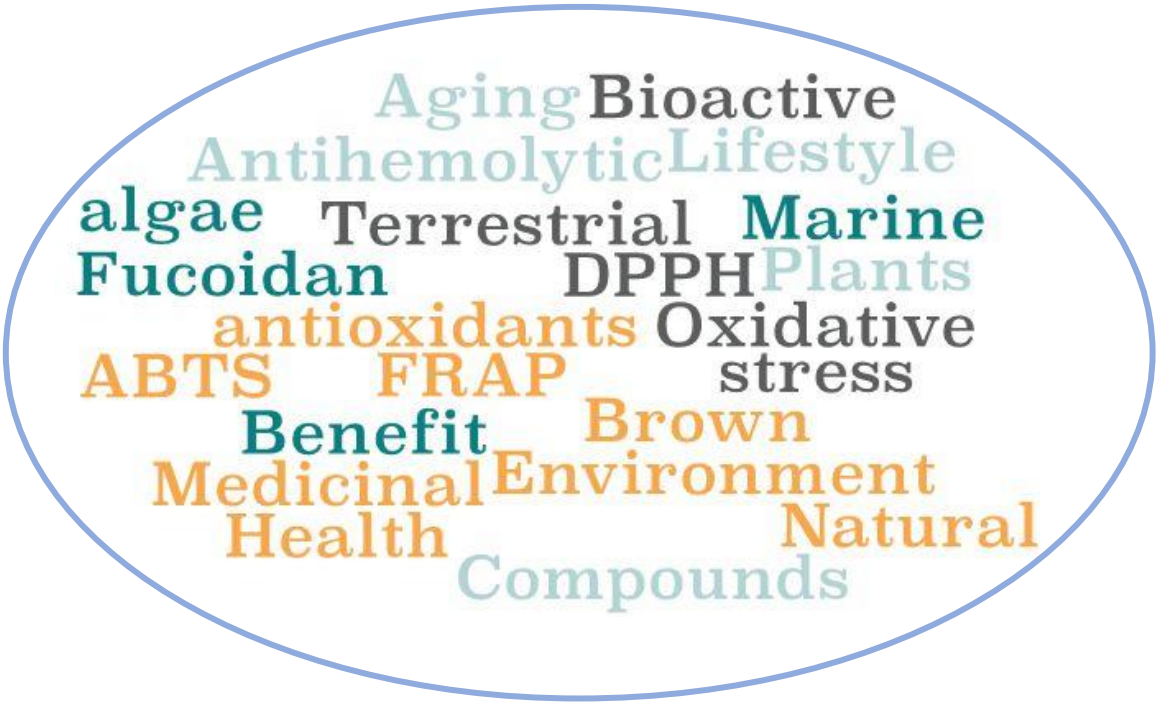
Table 3: Advantages and disadvantages of antioxidant quantification methods .

Methods	Advantages	Disadvantages
DPPH	Easy, rapid, inexpensive, effective and the most widely used assay for plant screening.	Not an exact model of radical reactions in biological systems since DPPH is a long-lived radical that does not resemble the highly ROS
FRAP	Simple, highly reproducible, and inexpensive assay	The required low pH, acidic conditions is considered a strong deviation from body physiological conditions.
ABTS	Rapid, flexible to a wide pH range, and soluble in both aqueous and organic solvent which means that this assay is applicable to hydrophilic or lipophilic compounds.	ABTS reagent is quite expensive. More accuracy is required for an oxidizing agent used to generate ABTS•+; time and storage conditions of the solution; and temperature of analysis.

II. AIM of the STUDY

The aim of this study was to investigate and compare the antioxidant capacity of FUCs purified from C. compressa, F. hermonis, and *E. globulus* by different assays.

Furthermore, we aimed to test the protective effects of CYS, FER, and EUC on erythrocytes against H_2O_2 induced OS.



III. MATERIALS AND METHODS

RADICAL SCAVENGING ACTIVITY

DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) assay

The capacity of FUC to scavenge the free-radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was determined basically according to the method described by Haddad *et al* (2017). One mL aliquots of FUC purified from the three species, at different concentrations (50-500 µg/mL for CYS and FER; 2.5-500 µg/mL for EUC) were prepared and mixed with 1 mL of DPPH solution (0.05 g/L in methanol). After a 30 min incubation in darkness, the DPPH radical reduction was evaluated by reading the absorbance at 517 nm using a Gene Quant 1300 UV-VIS spectrophotometer.

Ascorbic acid was used as the reference standard. The results were calculated as follows: DPPH scavenging activity (%) = [(Absorbance of control – Absorbance of sample)/ (Absorbance of control)] X 100. The IC50 value, defined as the concentration of CYS,FER, AND EUC required to cause a 50% decrease in initial DPPH concentration, was estimated by a nonlinear regression algorithm.

ABTS assay

The ABTS assay was performed following the procedure described previously (Benelli et al. 2018), applied to a 96-well microplate. The ABTS·+ stock solution was prepared according to Miller and Rice-Evans (1997) to obtain a final solution with absorbance about 1 at 734 nm. FUC extracts at different concentrations (31.25-2000 µg/mL for CYS; 6.25-400 µg/mL for FER; 3.125-200 µg/mL for EUC) were incubated with ABTS·+ for 10 minutes then results were read at 734 nm using a microplate reader (FLUOstar Optima, BMG Lab-tech microplate reader).

Trolox was used as a reference and results were expressed as µmol Trolox Equivalents (TE)/g of extract (µmol TE/g). The IC50 value, defined as the concentration of CYS, FER, and EUC required to cause a 50% reduction in the assay was expressed in µg/mL and estimated by a nonlinear regression algorithm using GraphPad Software (San Diego, CA, USA).

FRAP assay

FRAP assay was performed as previously reported (Benelli et al., 2018) by using a 96-well microplate to monitor the reduction of Fe^{3+} tripyridyl triazine (TPTZ) to blue-colored Fe^{2+}-TPTZ. Fresh working solution was prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6), 1 volume of TPTZ (10 mM in 40 mM HCl), and 1 volume of FeCl3.6 H2O (20 mM). Different concentrations of FUC (31.25-2000 µg/mL for CYS; 6.25-400 µg/mL for FER; 3.125-200 µg/mL for EUC) were incubated with FRAP solution for 10 minutes and the absorbance of Fe^{2+}-TPTZ was read at 593 nm with Trolox as standard as described above for ABTS.

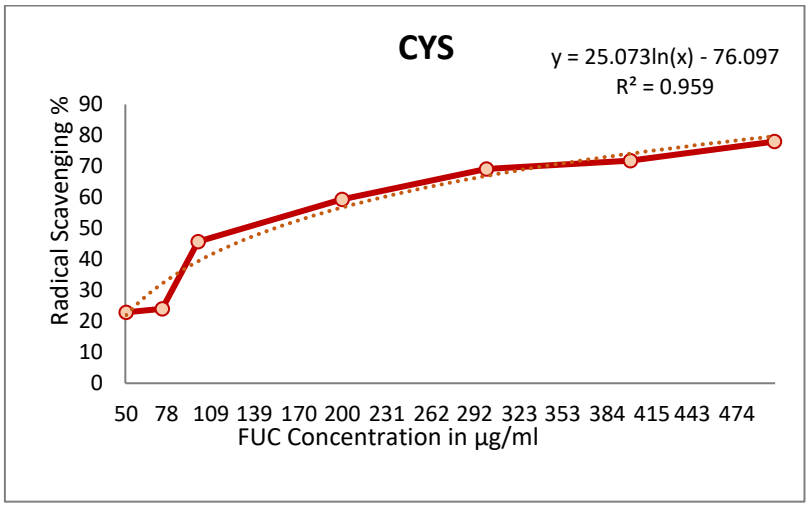
Anti-hemolytic activity

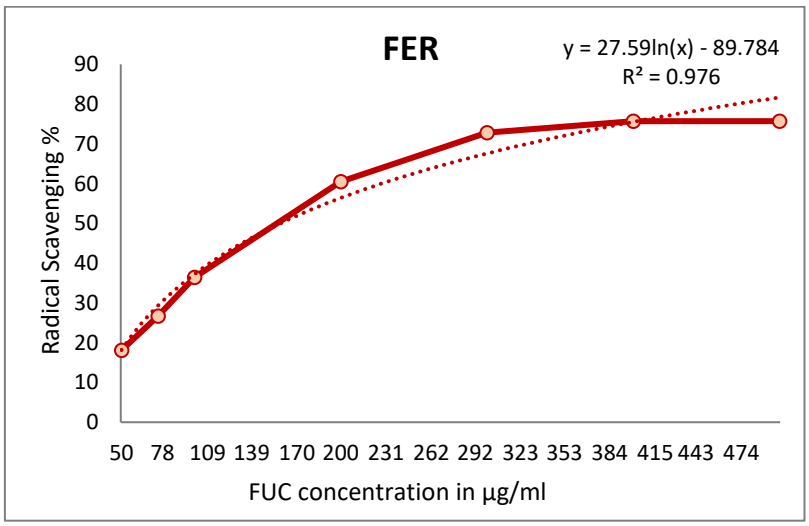
Human blood was obtained from healthy volunteers in the medical laboratory of Public Health Faculty- Lebanese University, washed three times in 9 volumes of Phosphate Buffered Saline (PBS) and centrifuged for 10 min (2500 rpm; 4 °C). Cell pellet was diluted 1:20 (v/v) in sterile PBS to form Red Blood Cell (RBC) suspension in which (1 mL) was mixed with purified CYS, FER, or EUC at 50 µg/mL. After 5 min, 30 µl of 30 % (w/w) H_2O_2 solution were added, incubated at 37°C for 1.5 h and then centrifuged for 10 min (2500 rpm; 4 °C). Results were recorded by reading the absorbance of the supernatant at 540 nm (James et al., 2014). Data were expressed as hemolysis percentage relative to H_2O_2 taken as 100%.

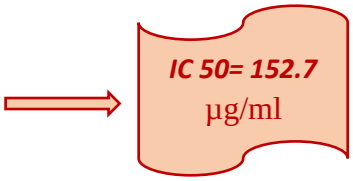
IV. RESULTS

DPPH:

DPPH assay results for the three FUC extracts indicated that radical scavenging activity increases as FUC concentration increase. As shown in figure 9, CYS and FER have approximately the same pattern of increase and same FUC concentration in which IC 50 was 152.62 µg/ml and 157.59 µg/ml, approximately. However, DPPH assay for FUC purified from *E. globulus* required to start with a lower EUC concentration due to its high antioxidant activity, where IC 50 obtained was 4.466 µg/ml.







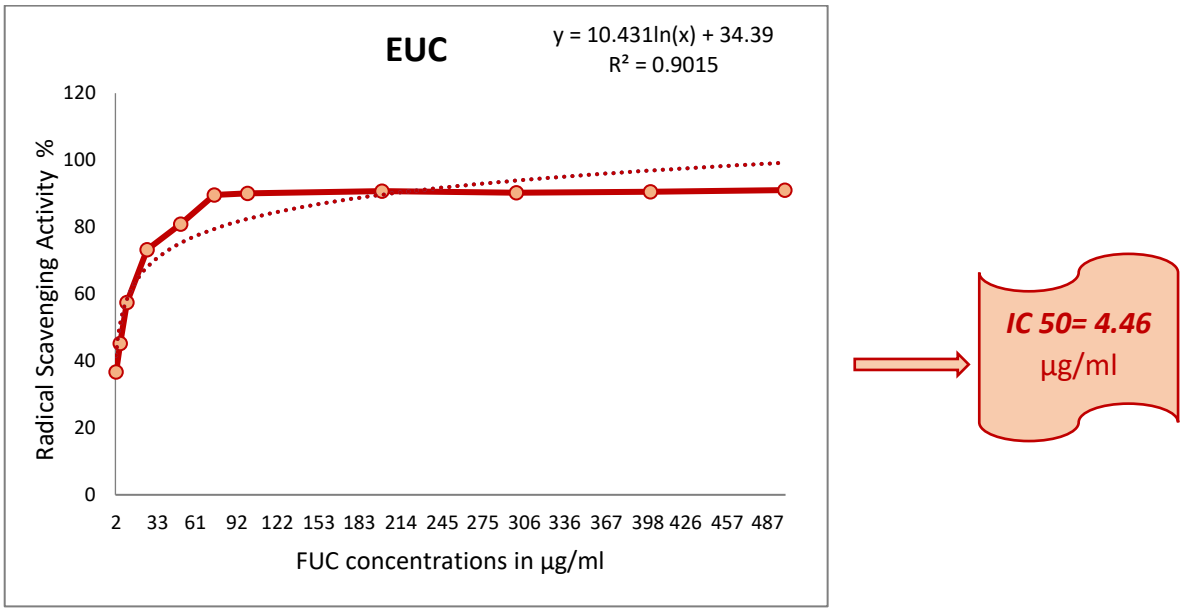


Figure 9: DPPH radical scavenging activity of CYS, FER, and EUC as function of increased FUC concentration in µg/ml.

ABTS and FRAP:

As shown in Table 4, FUC extracted from *C. compressa*, *F. hermonis*, and *E. globulus* showed high scavenging capacity when measured with both assays. For ABTS assay, the high TEAC obtained for FER and EUC, and the small IC50 suggested that FER and EUC are more active than CYS. IC50 of EUC and FER were higher than that of Trolox only by 3 and 10 times, respectively.

Same results obtained with FRAP, as TEAC values reflected the significant antioxidant activity of FUC.

Table 4: Antioxidant activities of CYS, FER and EUC measured by ABTS and FRAP assays.

	ABTS		FRAP
	TEAC (µmol TE/g)	IC_{50} (µg/g)	TEAC (µmol TE/g)
CYS	38.99 ± (3.7)	478.6 ± (39.24)	77.18 ± (9.1)
FER	410.0 ± (14.0)	44.26 ± (2.35)	81.30 ± (7.2)
EUC	1444.05 ± (11.5)	12.92 ± (0.81)	638.87 ± (17)
Trolox		4.53 ± (1.53)	

Anti-hemolytic Activity

Preliminary experiments in our study indicated that none of CYS, FER, or EUC show any hemolytic ability (data not shown). Then, anti-hemolysis activity of FUC at a concentration 50 µg/ml was tested in the presence of H_2O_2. Our results in Figure 10 show that CYS, FER, and EUC decrease the hemolytic percentage with respect to H_2O_2 treatment by 15.5%, 20.9% and 28%, respectively. This decrease was significant only for FER ($p \leq 0.05$) and EUC ($p \leq 0.01$).

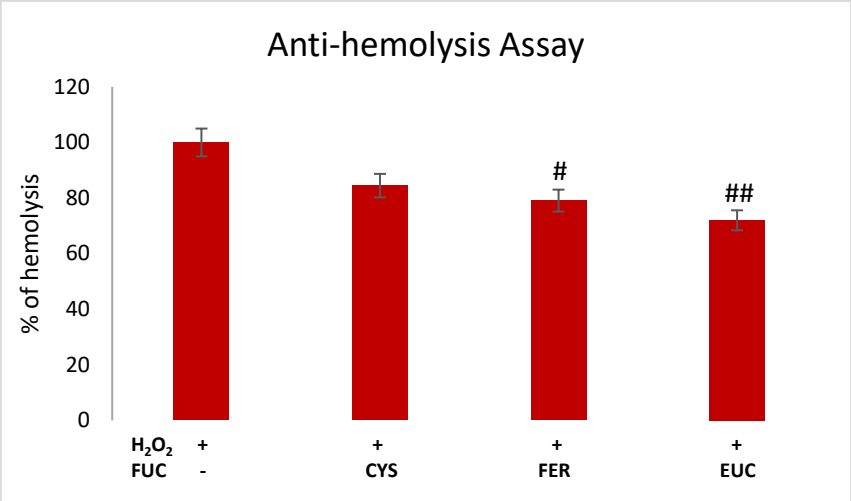


Figure 10: Anti-hemolytic activity of CYS, FER and EUC at a concentration 50 µg/ml. Data are expressed as percentage of hemolysis. Values are mean ± S.D. from three independent experiments. Significant differences are denoted by symbols: # $p \leq 0.05$, ## $p \leq 0.01$ vs H_2O_2.

V. DISCUSSION

DPPH, ABTS and FRAP assays were used as easy, rapid, and sensitive methods to determine the scavenging capacity of FUC against different radicals. In the three tested assays, EUC showed to be the most potent antioxidant purified fucoidan, followed by FER, and then CYS. The difference in antioxidant activity could be attributed to different mechanisms, such as complex with transition metal ion catalysts, break of chain initiation, radical scavenging prevention and increase of reductive capacity. All these properties have been described for other water-soluble polysaccharides and fucoidan fractions previously characterized from different sources (Zou et al., 2008; Laeliocattleya R.A. et al. 2020, Aji-saka K., et al. 2016, Wang Y. et al. 2019, Haddad et al., 2017). Previous studies indicated that FUC purified from *C. compressa* exhibited a significant anti-inflammatory and antioxidant activity and a significant decrease of

gastric mucosal damage (Hentati et al., 2018). Crude extract of the fruits of *E. globulus* have shown antioxidant and anti-bacterial activity (Makhlouf et al., 2013). Furthermore, sesquiterpene ferutinin, the main bioactive compound purified from Ferula genus is characterized by several biological activities, most importantly antioxidant effects (Macrì et al., 2020). In addition to other studies that demonstrated the antioxidant activity of *F. hermonis* extract.

The most plentiful cells in the human body are found to be the erythrocytes, which own copious biological and morphological characteristics, hence they have been widely exploited in drug transport. OS to erythrocytes can lead to their hemolysis. Our results indicated that FUC purified from the three species protected erythrocytes from hemolysis in the presence of any toxicant like H_2O_2.

VI. CONCLUSION

Being OS a common cause of most of the non-communicable diseases of the modern age, the antioxidant activity is perhaps one of the main biological potentialities of phytochemicals in general and particularly of FUC. In this study three different methods were used to characterize FUC antioxidant capacity, results showed that CYS, FER, and EUC exhibited a good scavenging activity against DPPH and ABTS radicals, as well as a ferric reducing power with that purified from *E. globulus* being the most potent polysaccharide. Moreover, the radical scavenging capacity of FUC was tested in a cellular model; erythrocytes, where protective and preventing the antioxidant ability of the polysaccharide decreased hemolysis percentage when incubated with H_2O_2.

These promising results encourage us more to continue studying the biological activities of FUC using two in vitro cellular models. In the next chapter will show the hepatoprotective and antioxidant effects of FUC in rat hepatoma cells, as well as in human endothelial cells.

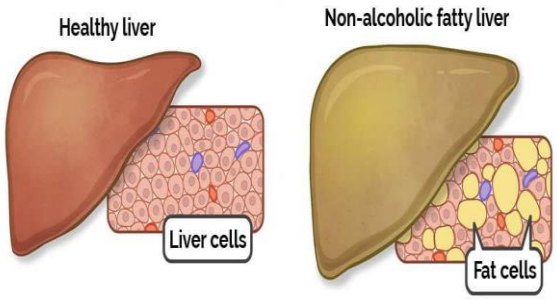
Chapter III: Anti-Steatotic and Antioxidant Activities comparison of marine derived Fucoidan (C. compressa) and terrestrial fucoidans (F. hermonis, and E. globulus)

Define the problem:

Nonalcoholic fatty liver disease is a metabolic manifestation of liver disease.

Its prevalence in increasing to reach 25% of general population. Several risks are associated with its pathogenesis.

Drugs used to treat NAFLD are still limited due to their side effects



Research questions:

FUC as a bioactive compound to treat NAFLD:

- Does FUC have any lipid lowering effects?
- Does FUC decreased ROS production in NAFLD models?
- Investigate the effect of FUC on endothelial damage.

ABSTRACT

Nonalcoholic fatty liver disease is a metabolic disorder characterized by lipid overloading in hepatocytes that can progress to more severe pathologies such as nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and eventually hepatocellular carcinoma. Such pathogenesis and worsening are complex and sustained by the synergetic action of a variety of nutritional, environmental, metabolic, and hormonal factors. NAFLD pharmacological treatment is still limited by unwanted side effects, whereas the use of food components with therapeutic potential is advisable. The culinary use of marine algae is traditional for some populations and reviving worldwide, with promising health outcomes due to the large number of bioactive compounds found in seaweeds. *In vitro* and *in vivo* studies demonstrate that fucoidan; a brown-algal derived polysaccharide exert beneficial actions on satiety feeling, caloric intake, fat absorption, and modulation of the gut microbiota, which could account for indirect effects on energy and lipid homeostasis, thus diminishing the fat overload in the liver. In this study, we aimed to investigate the anti-steatotic and antioxidant effects of CYS, FER, and EUC using *in vitro* cellular models of NAFLD and endothelial damage (rat hepatoma FaO cells and human endothelial HECV cell lines). Results showed that FUC purified from the brown algae and two terrestrial plants exert both antioxidant and anti-steatotic effects as they reduced the ROS production and triglyceride content and regulate the expression of genes implicated in lipid metabolism by inhibiting the expression of lipogenic genes. Fucoidan purified from *E. globulus* was the most effective extract almost in all experiments.

Table of Content

I. INTRODUCTION

THE LIVER

OVERVIEW

The liver is the heaviest internal organ and the largest gland in the body, being around 2-3% of body weight. It is located in the right upper quadrant of the abdominal cavity, beneath the right hemi-diaphragm, to the right of the stomach, protected by the rib cage and overlying gall bladder. It's close location to the spleen, pancreas, stomach, and intestine is appropriate for its importance as the body's chemical "factory". This factory is responsible for hundreds of separate functions, either in an exocrine or endocrine manner, such as bile production, protein and metabolite synthesis, metabolic reactions, hormone catabolism, drug detoxification, and many other important functions.

Liver structure

The liver is a reddish-brown organ, mainly divided into two lobes when viewed from above (right and left) or divided into 4 unequal lobes when viewed from below. The lobules are held together by a fine connective tissue known as Glisson's capsule. Furthermore, left and right lobes are divided by a thin fold of peritoneum called a falciform ligament. As mentioned above, the liver relates to the diaphragm by a triangular zone known as the bare area. Apart from this bare area, all the liver is covered by a thin, double layered mesothelial membrane derived also from the peritoneum to reduce friction with other organs.

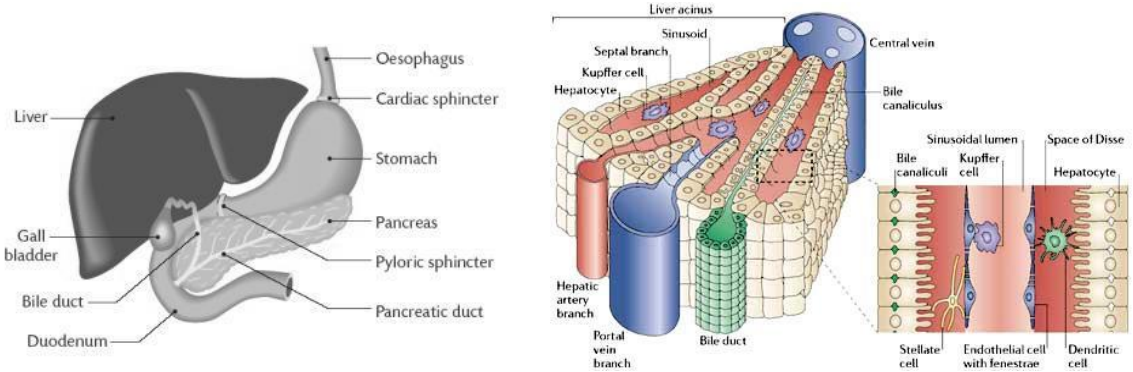


Figure 11: Anatomy of Liver (A); The structure of hepatic lobule (B).

The liver parenchyma is divided into polygonal basic units known as lobules, formed by numerous laminae of hepatocytes along with sinusoids, and a triad composed of the hepatic portal vein, hepatic artery, and bile duct. The hepatic artery and hepatic portal vein branches pass from the periphery toward the central lobule, forming sinusoids with thin pores called fenestrae. The center of the lobule is occupied by the central vein.

The hepatic sinusoids are thin-walled, broad-lumen blood capillaries characterized by the presence of pores and fenestrated endothelial cells. Between the endothelial cells of the sinusoids and the hepatocytes there is a space with a reticular connective texture called the Disse space. On the sides of the Disse space there are two laminae: that of the endothelial cells and that of the basal area of the hepatocytes, that have microvilli to increase the exchange surface. Plasma can pass through the fenestrations of the endothelial cells and partially travels in the space of Disse (Trefts et al., 2017).

BLOOD FLOW: Blood flow to the liver represents around 25% of total cardiac output and it reaches around 1500 ml/min. The hepatic artery, branching from the aorta, carries oxygenated blood, and provides around 25 % of liver blood input. However, the remaining 75 % is supplied by the hepatic portal vein, and it is rich in nutrients, hormones, and glucose.

At the level of sinusoids, blood delivers O2, nutrients, sugar, and simple amino acids to hepatocytes. As the blood circulation is centripetal, there is a gradient of O2 supply to hepatocytes from the portal triad to the central vein, which makes the cells close to the portal space the most metabolically active.

Concerning the hepatic output, after O2 and nutrient exchange at the sinusoidal level, blood drains into the central vein of each lobule, that all together form the hepatic vein, which drains de-oxygenated blood into the inferior vena cava (Yamanaka et al., 1976).

Supplementary Box 4: CELL TYPES OF THE LIVER

The liver is made up of different cell types which can be divided into two main categories: parenchymal and non-parenchymal cells.

- ***PARENCHYMAL CELLS*** Hepatocyte parenchymal cells make up about 60% of the liver cell population. They are highly specialized epithelial cells with the increased surface area for exchange with the vessels. The hepatocyte is one of the most metabolically active cells, very rich in organelles: smooth and rough ER, Golgi apparatus, lysosomes, peroxisomes, and most abundantly mitochondria.
- ***NON-PARENCHYMAL CELLS*** The remaining 40% of liver cells are non-parenchymal cells, which include the fenestrated epithelial cells lining the sinusoids, and Kupffer cells lining the sinusoidal walls (Kupffer cells form 3 % of liver cells and

are considered tissue macrophages that perform a protective role against bacteria, viruses, and immune complexes) (Lau et al., 2003).

LIVER FUNCTIONS

The liver is an essential organ with many functions. These important functions include endocrine and exocrine regulation, bile production, hematopoietic, and detoxifying actions. Moreover, the liver plays an important role in the central metabolic processes of nutrients (Trefts et al., 2017).

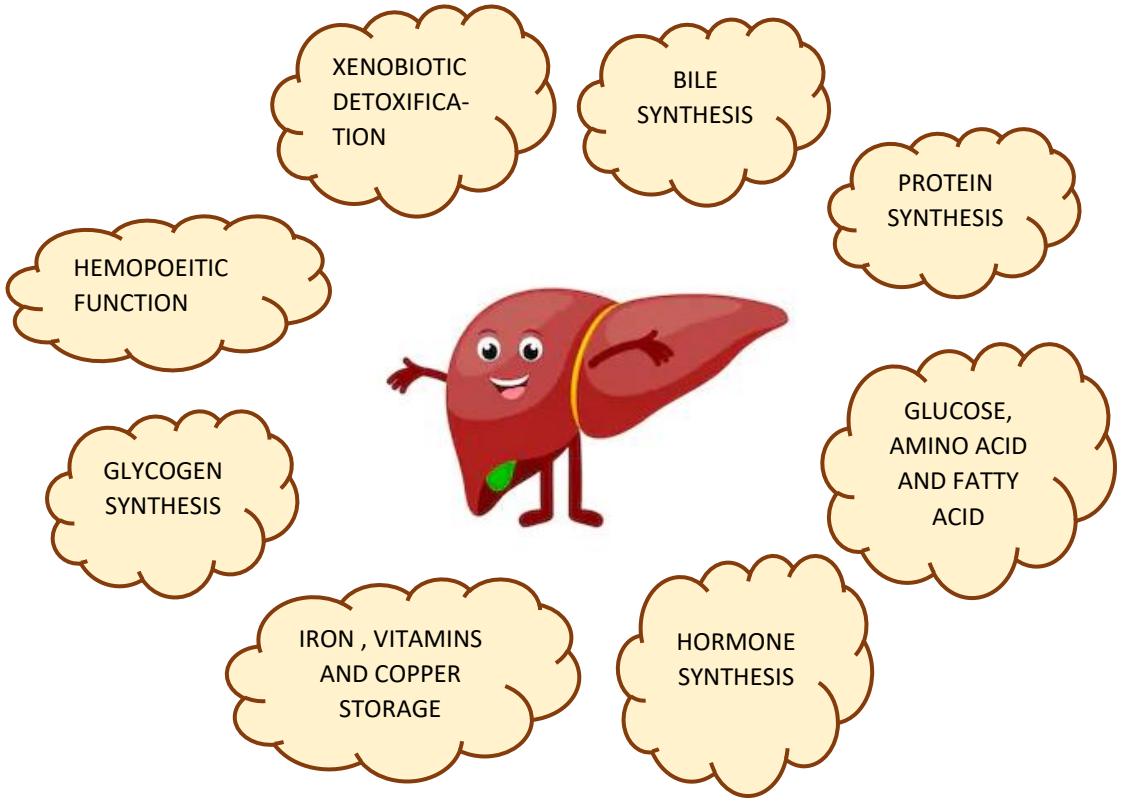


Figure 12: A diagram showing different liver functions.

XENOBIOTIC DETOXIFICATION

Xenobiotic is defined as any foreign substance, including natural and synthetic drugs, industrial chemicals, naturally occurring poison and environmental pollutants. They are almost hydrophobic, a characteristic that allows their absorption through the gastrointestinal tract, skin and lungs; once detected as a foreign substance, the body metabolically transforms such substances to more hydrophilic ones, in order to be secreted in urine, plasma, bile, sweat, or tears. Generally, during the detoxification process, the toxicity of the starting substance is also

reduced to produce completely or partially inactive metabolites. Around 75% of all biotransformation reactions occurring with the aim of detoxification take place at the level of the liver (McGinnity et l., 2017).

GLYCOGEN SYNTHESIS

Glucose can be phosphorylated into glucose 6-phosphate, and isomerized into glucose 1-phosphate

Then, glucose 1-phosphate is catalyzed into Uridine 5' diphosphate (UDP)-glucose by glucose 1-phosphate uridyltransferase in the presence of Uridine-5'-triphosphate (UTP) and Mg^{2+}. In the liver, UDP-glucose is involved in several metabolic pathways; UDP glucose is the glucose donor for glycogen synthesis by glycogen synthase enzyme, or it can be involved in UDP-glucuronidation that converts some endogenous and exogenous compounds into more polar metabolites, thus facilitating their metabolism and secretion. Furthermore, UDP-glucose can be utilized in the synthesis of UDP-galactose from galactose 1-phosphate; then UDP-galactose can be implicated in lactose, glycoproteins, glycolipids, and glycosaminoglycan synthesis by transferring/donating a galactose unit.

Around 50 % of ingested glucose is stored as glycogen showing the importance of this pathway that may lead to glucose metabolism disturbances in case of gene mutations or enzymatic dysfunctions.

BILE SYNTHESIS

Bile is a yellow-green fluid synthesized by the liver, made of bile acids, bilirubin, phospholipids, cholesterol, xenobiotics, water, electrolytes, glutathione, IgA antibodies and other components. Cholesterol is a main component of the bile, as it is also a precursor for bile acids. The liver produces around 900 ml of bile per day, which in part are stored in the gall bladder, and the remaining are distributed along with the biliary system. Bile stored in the gall bladder is concentrated, and it is critical as an emulsifying agent in the digestion and absorption of fats and fat-soluble vitamins in the small intestine. Bile secretion is considered also as a pathway by which cholesterol, pigments produced from hemoglobin breakdown, and other waste products are eliminated from the body. Cholesterol, bilirubin, and phospholipids synthesis is explained in supplementary box 5.

Supplementary Box 5: Cholesterol Synthesis

Cholesterol enters the smooth ER to undergo some hydroxylation reactions by cytochrome P450 (CYPs) enzymes, specifically CYP7E1 (7-α-hydroxylase) to give chenodeoxycholic acid or by CYP7E1 then CYP8B1 (12- α-hydroxylase) to give cholic acid. Both bile acids, also known as primary bile acids, can be either conjugated into more polar molecules or transported to biliary canaliculi by a bile salt export pump. In the intestine, they can be metabolized by bacterial enzymes producing the secondary bile acids deoxycholic acid and lithocholic acid through dihydroxylation reactions. Even if bile acids count for 70 % of bile components, their excess is hepatotoxic, which can explain the negative feedback that chenodeoxycholic acid and cholic acid can exert on the rate limiting enzyme CYP7E1.

Regarding bilirubin, it is the breakdown product of hemoglobin, knowing that hemoglobin breakage ends in globin and heme group production. The latter is degraded into biliverdin and Fe^{2+}. Biliverdin bounded to albumin protein is then transported to the biliary canaliculi as a component of the bile, but also it can pass to the intestinal tract, where it undergoes some metabolism by bacterial enzymes, resulting in two pigments, urobilin and stercobilin. Phospholipids are synthesized mainly from the fatty acids, and together with amino acids, water, electrolytes (sodium, chloride, and bicarbonate) and urea are transported to the biliary canaliculi by specific transporters (Boyer, 2013).

PROTEIN SYNTHESIS

The liver is known to be a protein FACTORY, in which hepatocytes are responsible to synthesize most of the plasma proteins. Below are some examples of such proteins with their function (De Feo, 2002).

Table 5: The function of several proteins synthesized by the liver.

Protein	Function
Albumin	Counts for 60 % of plasma proteins, regulates osmotic blood pressure and binds water, cations, hormones, biliverdin, as well as pharmaceuticals.

Coagulation factors	Factor XIII, XII, XI, X, IX, VII, V, II, and I also known as fibrin-stabilizing factor, Hageman factor, plasma thromboplastin antecedent, Stuart-Power factor, Christmas factor, stabilizing factor, proaccelerin, prothrombin, and fibrinogen. Control bleeding and their activation is vitamin K dependent.
Anticoagulants	• Plasmin: degrades many plasma proteins (factor V, VIII, platelet receptors), and mainly fibrin clot into fibrinogen. • Anti-thrombin: Deactivates factor II, IX, and X.
Alpha and beta globulins	Some globulins are made by the immune system, aid in the transport of lipid soluble substances like steroid hormones • Ceruloplasmin and transferrin for Cupper and Iron transport, respectively. • Haptoglobin which binds to excess free hemoglobin. • Retinol binding protein that binds retinol and delivers it to skin and eyes for further utilization. • Transcortin and thyroxin for corticosteroids and thyroid hormone transport, respectively.
Immune proteins	• Complement proteins (C1 to C9) that are involved in alternative and classical pathway, as well as opsonization in which bacteria and pathogens are marked for phagocytosis • C reactive peptide, elevated in active inflammation in which their production is stimulated by interleukin 6 (IL-6) and 1 and TNF-alpha.
Apolipoproteins	• Important for fat, cholesterol, and lipid soluble vitamins transport. • There are five main categories: ➢ ApoA: major component of HDL ➢ ApoB: component of LDL and VLDL ➢ ApoC: control lipid metabolism ➢ ApoD: a component of HDL ➢ ApoE: recognize lipoproteins

HORMONE SYNTHESIS

The liver is responsible for the production, transport and catabolism of some hormones, such as thrombopoietin (which plays an important role in platelet formation), Insulin-like growth factor I (IGF-I) that decreases blood glucose levels, acts on muscle as well as other tissues to stimulate amino acid uptake and protein synthesis and stimulates chondrocytes (cartilage cells) proliferation for bone growth. Furthermore, angiotensinogen is a pro-hormone released by the liver as a precursor for angiotensin II that is responsible for vasoconstriction of blood vessels and blood pressure increase (Langer et al., 1998).

Storage

The liver is considered to play a crucial role in storing glucose, vitamins, and minerals; providing all substances when needed.

- *Vitamins:*

Vitamin D: it is known that diet provides vitamin D, but the most significant amount is derived from our skin. Vitamin D is important for calcium homeostasis, neurodevelopment, bone and muscle growth, immunomodulation and regulation of cell growth and replication.

Vitamin K: Vitamin K exists in two forms: the natural form which is oxidized known as vitamin K epoxide, and the reduced vitamin K hydroquinone. Moreover, this vitamin is an important factor for bone growth and wound healing.

Vitamin A: As other lipid-soluble vitamins, vitamin A, also known as retinol ester, is absorbed into the liver in the form of chylomicrons. In the liver, retinol esters are transformed into retinol and then bound to retinol binding protein (synthesized in the liver). Furthermore, it can be stored in special specialized cells in the space of Disse, called stellate cells, to be used later.

Vitamin B12: Also known as cobalamin, it is transported to the liver by a specific protein produced by the liver itself, called transcobalumin II, then either stored or transported to peripheral tissues. In the bone marrow, vitamin B12 is a necessity for erythropoiesis. It is also important for DNA synthesis and for maintaining the proper functioning of the nervous system.

Vitamin E: It is related to antioxidant activities, and immune system support.

- ***Iron storage***

Iron is an important mineral that has an essential role in hemoglobin production, immune support, muscle metabolism, brain, and thyroid functioning. At the level of blood Fe2+ binds to the plasma protein transferrin where is distributed to be used mainly by muscle tissue and the erythroid compartment, or stored in the liver, spleen, and bone marrow . In the liver hepatocytes, an intracellular protein known as ferritin serves to store Fe^{2+} in a non-toxic form. Furthermore, a high concentration of Fe2+ in tissues reacts with hydrogen peroxide yielding to Fe3+ and hydroxyl radicals OH., a highly reactive oxygen species that can interact with lipids, proteins and DNA causing their damage. In this case, hepatocytes can produce a specific hormone known as hepcidin that inhibits ferroportin iron transporter at the level of the enterocyte.

- ***Copper storage***

The essential transition metal copper (Cu) is obtained from dietary sources including meats, shellfish, seeds, and beans. Due to its crucial role in health, it is important to maintain copper balance, knowing that the liver is the central regulatory organ of copper homeostasis. Copper is absorbed at the level of intestinal epithelium by a specific transporter. In the bloodstream, it is

most likely bound to albumin and delivered to the liver. In hepatic cells, copper binds to a specific protein known as ATP7B and it is used for mitochondrial respiration, radical detoxification, and other cellular processes. Copper deficiency has been linked to many diseases such as Alzheimer's disease, ischemic heart disease, obesity, and non-alcoholic fatty liver disease. It was shown that low copper diet induces lipogenic genes expression such as sterol regulatory element binding protein-1 (SREBP-1) that is involved in fatty acid synthesis (FAS), as well as the decrease of CYP7A1 enzyme, which is a key enzyme in cholesterol degradation. Furthermore, CuD downregulates enzymes related to mitochondrial and peroxisomal beta oxidation of fatty acids. Other consequences of copper accumulation in tissues are movement disorders and brown pigmentation around the eyes.

HEMOPOIETIC FUNCTION

During fetal life, the liver participates directly in blood cell production. Kupffer cells are responsible for the synthesis of erythropoietin hormone, that promotes the production, proliferation, differentiation, and maturation of red blood cells. During the postnatal life erythropoietin is mainly produced by the kidneys, and the liver contribution to this hormone production is between 5-10%. Moreover, normal liver function is essential for hematopoiesis due to iron, folic acid, and vitamin B12 supply.

LIVER METABOLISM

Blood delivered to the liver through the hepatic portal vein is rich in nutrients; glucose, amino acids, and fatty acids ready to be metabolized.

HEPATIC GLUGOSE METABOLISM

Glucose entry to hepatocytes is controlled by the transporter "glucose transporter-2". Within hepatocytes, glucose is phosphorylated into glucose 6-phosphate by hexokinase enzyme with adenosine triphosphate (ATP) dephosphorylation into adenosine diphosphate (ADP). Other studies showed that this glucokinase accounts for 95 % of the glucose phosphorylation activity in cultured hepatocytes. Glucose 6-phosphate may follow three different routes that are explained below (Andany *et al.*, 2016).

1. Glucose 6 phosphate can be isomerized into glucose 1-phosphate by the phosphoglucomutase enzyme. Glucose 1-phosphate then is implicated within the glycogen synthesis pathway.

2. Pentose phosphate pathway: It is an alternative pathway of glycolysis that takes place in the cytosol and is considered as an anabolic pathway and not catabolic. Glucose 6-phosphate is oxidized by glucose 6-phosphate dehydrogenase into gluconolactone and

carbon dioxide with the reduction of $NADP^+$ into NADPH. Gluconolactone is converted then oxidized and isomerized into ribulose 5-phosphateribose 5-phosphate or xylulose 5-phosphate. Ribose 5-phosphate is essential for the synthesis of nucleotides and nucleic acid and released NADPH is essential for the synthesis of fatty acids, cholesterol, steroid hormones and of two non-essential amino acids (proline and tyrosine) and for the reduction of antioxidant enzymes such as glutathione.

3. Glucose 6-phosphate can be reversibly isomerized into fructose 6-phosphate by glucose 6-phosphate isomerase. Fructose 6-phosphate is involved in two different pathways explained below.
   - **Hexosamine pathway:**
     In this pathway fructose 6-phosphate leads to the production of UDP-N-acetylglucosamine is essential for glycosylation of proteins, lipids, immune receptors, immunoglobulins, cytokines, and other molecules that contribute to immune system functioning.

   - **Glycolysis:**
     Glycolysis is a process that takes place in the cytosol, in which fructose 6-phosphate undergoes further phosphorylation producing fructose 1,6-biphosphate that splits into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, two reversible trioses. GA3P is then converted into a pyruvate molecule in some reactions producing two pyruvate molecules, two ATP and two nicotinamide adenine dinucleotide (NADH) molecules. Pyruvate is transported into the mitochondria by pyruvate translocase and decarboxylated to produce acetyl-CoA that enters the Krebs cycle (Andany et al., 2016; Iynedjian P. et al., 1995).

Supplementary Box 6: KREBS CYCLE

Also known as citric acid cycle CAC or tricarboxylic acid cycle, a series of reactions that take place in the mitochondrial matrix to produce energy in the form of ATP. Acetyl-CoA derived from carbohydrates, fats, and proteins metabolism enters Krebs cycle where it is condensed with oxaloacetate to produce a 6-carbon molecule known as citrate that is isomerized into isocitrate. The latter is oxidized and decarboxylated into 5-carbon α-Ketoglutarate and CO_2 by Isocitrate dehydrogenase where NAD^+ is reduced into NADH. Then α-Ketoglutarate undergoes another oxidation-decarboxylation reaction by α-Ketoglutarate Dehydrogenase producing 4-carbon Succinyl-CoA, NADH molecule, and CO_2. Succinyl-CoA is converted into succinate by Succinyl-CoA synthetase with phosphorylation of guanosine diphosphate (GDP) into guanosine triphosphate (GTP) which is equivalent to an ATP molecule. Then succinate is oxidized into fumarate along with FAD reduction into $FADH_2$. The product fumarate is hydrolyzed by fumarase enzyme to produce L-Malate which is then oxidized by malate dehydrogenase into oxaloacetate generating another NADH molecule. This means that one acetyl-CoA

molecule following the Krebs cycle can produce 1 ATP, 3 NADH, and 2 $FADH_2$ molecules (Haddad et al., 2020).

OXIDATIVE PHOSPHORYLATION

NADH and $FADH_2$ molecules produced from the Krebs cycle in the mitochondrial matrix are utilized to produce ATP in a process known as oxidative phosphorylation that involves electron flow through the ETC, a series of proteins and electron carriers within the mitochondrial membrane. ATP is essential for the function and all the reactions taking place within the cell.

- $FADH_2$ and NADH Delivery: It should be clear that NADH is considered to be more electron donating than $FADH_2$ since NADH can transfer its electron into complex I where the energy released is used to pump protons from the matrix into the intermembrane, while $FADH_2$ is delivered to complex II which cannot pump proton across the membrane. This means that $FADH_2$ pumps less protons yielding less ATP production.
- Then electrons from both molecules follow the same route, electrons from complex I and II are transferred to an electron carrier called ubiquinone (Q). The latter delivers the electron to complex III where more protons are pumped into the intermembrane, then electrons are transferred to another mobile carrier known as cytochrome C.
- Cytochrome C carries the electrons to complex IX pumping more protons across the membrane. At the level of complex IX, in the mitochondrial matrix electrons are quenched by oxygen, the final acceptor of ETC, forming H_2O. At this step the ETC role ends, resulting in an electrochemical gradient across the inner mitochondrial by proton accumulation within the intermembrane. H^+ move down the gradient across the membrane by the help of a membrane spanning protein called complex V, also known as ATP synthase. As ATP synthase turns, it catalyzes the addition of phosphate group to ADP resulting in ATP production. This process by which ATP is produced from a proton gradient is known as chemiosmosis (Cardol, 2009).

AMINO ACID METABOLISM

The liver absorbs up to 60 - 70% of **amino acids** present in the portal vein through sodium dependent transporters. Amino acid metabolism only accounts for 10 to 15% of our total energy production. In the liver amino acids are utilized for the synthesis of liver protein and serum proteins, hormones, porphyries, and nucleotides. In case of amino acid excess, some amino acids, such as alanine, cysteine, serine, glycine, threonine, and tryptophan can be metabolized into pyruvate that follows several routes as explained above. Pyruvate can either undergo gluconeogenesis for glucose synthesis or transformed into acetyl-CoA than in turn enters Krebs cycle or used for fatty acid synthesis in case of ATP excess (Hou et al., 2020).

FATTY ACID METABOLISM

Biological lipids are defined as a diversity of chemical groups sharing a common feature, which is their insolubility in water. Fatty acid is a carboxylic acid with a hydrocarbon chain of 4 to 36 carbons, it can be fully saturated with no double bonds, unsaturated with one double bond, and with two or more double bonds, known as polyunsaturated fatty acids. Length of hydrocarbon chain and the degree of unsaturation determined by a number of double bonds define the physical properties of fatty acids, for example as the hydrocarbon chain gets shorter the ability of hydrocarbon chains to interact is reduced therefore; the molecules are not tightly packaged and the solubility in water increases. In case of double bonds number increase more kinks in the hydrocarbon chains are formed.

➢ *FATTY ACID FUNCTIONS*

FAs biological functions are diverse as shown in table 6, fats and oils are considered the principal storage form of energy in many organisms, in addition to their role in hormones, pigments, cofactors and detergents synthesis.

Table 6: Examples on different functions of fatty acids.

Function	Example
Energy storage	Fatty acids, triglycerides
Hormones	Sex hormones: estrogen, testosterone Adrenal hormones: cortisol, aldosterone
Electron carriers	Ubiquinone (known as coenzyme Q)
Pigments	Retinal
Messengers	Extracellular: eicosanoids Intracellular: phosphatidylinositol
Cofactors	Vitamin K
Detergents	Bile salts

FATTY ACIDS SOURCE, DIGESTION AND UPTAKE

Cells obtain FAs mainly from three different sources: dietary fats, lipolysis of FAs stored in LDs and de novo lipogenesis where FAs are synthesized from a non-lipid source like carbohydrates.

- ***DIETARY FATTY ACIDS:***

Concerning dietary FAs, after digestion FAs are emulsified and converted into micelles in the small intestine by the action of bile salts stored in the gall bladder. Then intestinal lipases degrade these micelles into free molecules of fatty acids and monoglycerides that can diffuse through epithelial cells lining intestinal mucosa where they are packaged together with cholesterol and apoproteins into chylomicrons. Chylomicrons move through the lymphatic system and bloodstream to reach specific tissue; inside the capillary ApoC-II, which is an apoprotein on the surface of chylomicrons, activates lipoprotein lipase. The latter hydrolyses triacylglycerols allowing the uptake of FFA and glycerol by cells of target tissues.

- ***LIPOGENESIS:***

It is a process by which fatty acids are synthesized from acetyl-CoA derived from lipids or a non-lipid source such as carbohydrates, which is known as de novo lipogenesis. Fed state, high blood glucose level yielding to ATP excess stimulate fatty acid synthesis. Actually, high levels of ATP exert a negative feedback on the isocitrate dehydrogenase enzyme thus increasing isocitrate concentration which is reversibly converted to citrate. High levels of citrate negatively regulate acetyl-CoA from completing the Krebs cycle, and citrate is transported to cytoplasm via citrate transport protein (CTP), then broken down into oxaloacetate and acetyl-CoA. Produced acetyl-CoA is carboxylated by an important enzyme known as acetyl-CoA carboxylase (ACC) into malonyl-CoA.

Acetyl-CoA and malonyl-CoA are the substrates of fatty acid synthesis, but this requires NADPH as a reducing agent provided as discussed above from malic enzyme activity and pentose phosphate pathway. Fatty acyl synthetase (FAS) is the enzyme responsible for FA synthesis through several repeated reactions. This process takes place in the cytoplasm of adipose tissues and the liver mainly. Synthesized fatty acids are cellular key components that have essential functions.

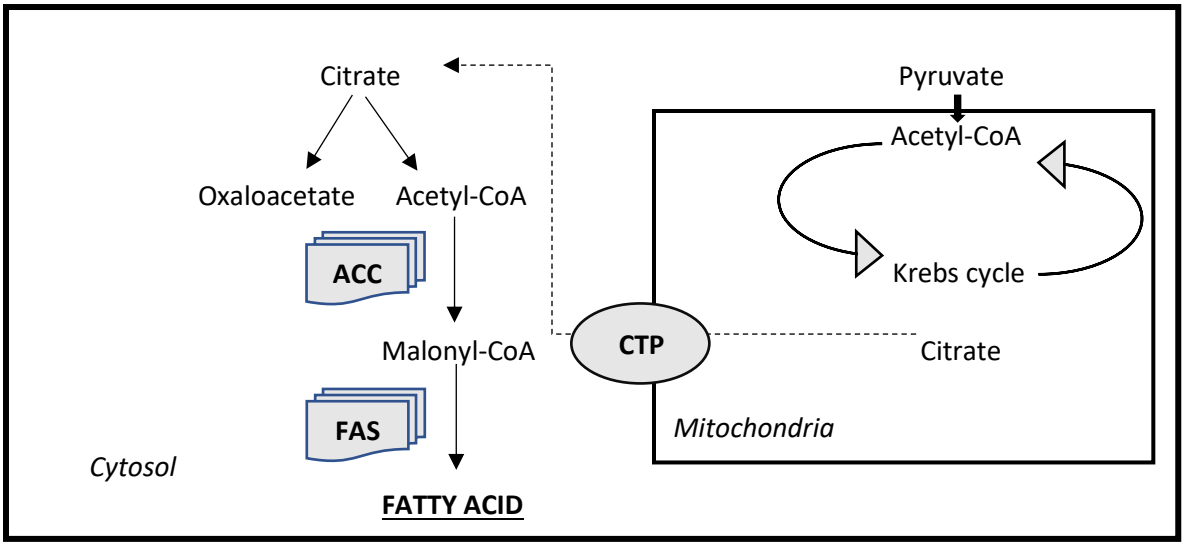


Figure 13: Hepatic lipogenesis. Malonyl-CoA is condensed with several acetyl-CoA moieties by FAS to produce fatty acid in the presence of NADPH as reducing agent.

- ***LIPOLYSIS***

Fatty acids can be derived from stored fats by lipolysis which is a catabolic process that involves the breakdown of fat stored in the form of TAGs within adipose tissue, where three fatty acid chains and a glycerol backbone are formed as products. This process requires three different steps, each step liberates one fatty acid chain.

- 1st step: Adipose triglyceride lipase (ATGL) hydrolyses the first bond resulting in one FA and a diacylglycerol (DAG).
- 2nd step: second ester bond is cleaved by hormone sensitive lipase (HSL), liberating FA and a monoacylglycerol (MAG).
- 3rd step: MAG undergoes hydrolysis by monoglyceride lipase (MGL) releasing its last FA and glycerol.

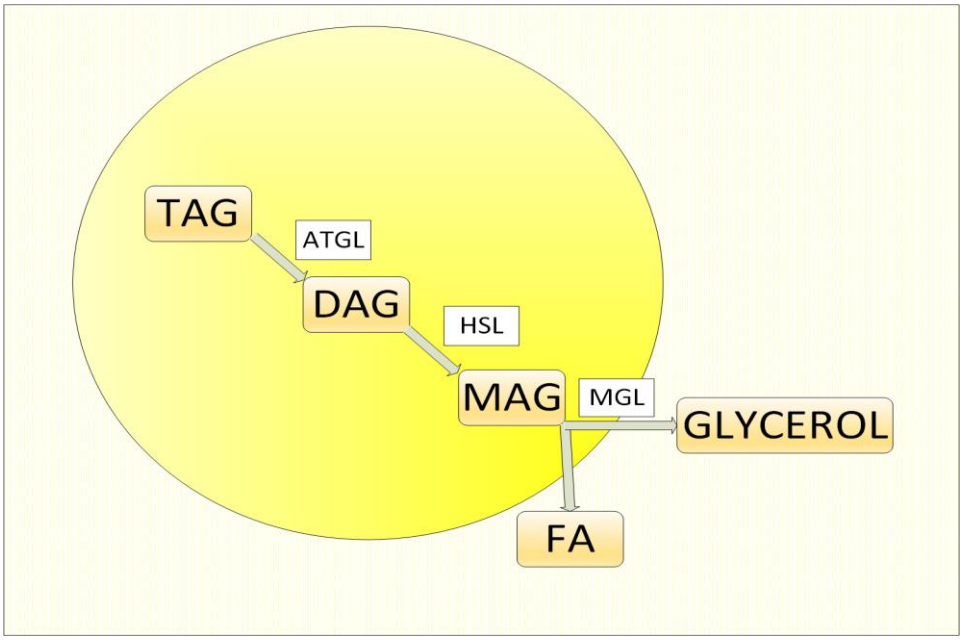


Figure 14: Diagram showing lipolysis steps and enzymes.

TRIGLYCERIDE SYNTHESIS

Triglyceride synthesis takes place in the endoplasmic reticulum of cells in an esterification process of fatty acids to glycerol. Three fatty acid chains are bonded to each glycerol molecule in which each of the OH group of the glycerol reacts with the carboxyl end of a fatty acid chain

(-COOH). The three fatty acid chains can be of the same kind or of two or more different fatty acid types to form simple or mixed triacylglycerol, respectively. As in fatty acid synthesis, triglyceride synthesis can occur in adipose tissue and liver; those synthesized in the liver are secreted into the blood in the form of very-low-density lipoproteins (VLDL) to be delivered to peripheral tissues.

Supplementary Box 7: LIPOGENESIS AND LIPOLYSIS REGULATION

ACC enzyme is heavily regulated by two ways: allosteric regulation, where it is stimulated by citrate and inhibited by long chain fatty acids, hormonal regulation, as insulin can stimulate malonyl-CoA formation through phospho-protein phosphatase that dephosphorylates ACC lowering its enzymatic activity, while adrenalin, cortisol and glucagon inhibit FA synthesis and trigger lipolysis mediated by protein kinase A (PKA) activation, which in turn phosphorylates ACC and inactivates its enzymatic activity.

Moreover, insulin stimulates pyruvate dehydrogenase which converts pyruvate into acetyl-CoA, thus stimulating the lipogenic pathway. Leptin, a hormone released by adipocytes, negatively regulates lipogenesis through glucose intake inhibition and down regulates the expression of genes involved in fatty acid synthesis. Growth hormone prevents the stimulation of lipogenesis through insulin signaling modulation, thereby decreasing insulin sensitivity, and down regulating fatty acid synthase expression.

Lipolysis is triggered by several hormones: adrenalin, cortisol, growth hormone and glucagon, which activate adenylyl cyclase to release cyclic adenosine monophosphate cAMP; PKA activated by cAMP phosphorylates perilipin (also known as LD-associated protein), which in turn stimulates HSL.

FATTY ACID OXIDATION

MITOCHONDRIAL BETA-OXIDATION

Fatty acid oxidation is the catabolic phase of FA metabolism to generate energy. In a eukaryotic cell, FA oxidation takes place mainly within the mitochondria in three steps: removal of successive two- carbon units in the form of acetyl-CoA, then acetyl-CoA are oxidized into CO_2 via Krebs cycle and finally products obtained from the first two steps donate electrons to the mitochondrial respiratory chain for ATP production. It is called beta oxidation since oxidation occurs at β carbon.

- *EVEN NUMBER OF CARBON FATTY ACID CHAIN*

SATURATED FATTY ACID:

The most naturally occurring fatty acids found in various animal and plant fats are with even number of carbon atoms such as stearic acid, oleic acid, and palmitic acid. Fatty acid is broken into acetyl-CoA in 4 steps, after each cycle acetyl-CoA, $FADH_2$, and NADH molecule are produced. After that, $FADH_2$ and NADH electron carriers move to ETC to generate 2 and 3 ATP molecules each, respectively. Furthermore, acetyl-CoA enters Krebs cycle to be oxidized into CO_2 and H_2O producing 12 molecules of ATP.

UNSATURATED FATTY ACID CHAIN

Oxidation of unsaturated fatty acid requires an additional isomerization step while it does not generate as many ATPs molecules produced from saturated fatty acid with the same number of carbons. This is explained by the fact that the presence of double bond eliminates the first step usually done in saturated fatty acids, which is dehydrogenation for double bond formation, along with this step an $FADH_2$ molecule is produces. Thus, for every double bond 2 ATP are less formed.

- *ODD NUMBER OF CARBON FATTY ACID CHAIN*

Fatty acids with odd number of carbon atoms are usually found in the lipids of plants and some marine organisms. They are oxidized in the same pathway as the even-carbon acids in the beginning, but differ in the substrate for the last step, where a three carbon propionyl-CoA is produced. Then propionyl-CoA is carboxylated, converted and undergoes intramolecular rearrangement to form succinyl-CoA that can enter the citric acid cycle. The difference in terms, succinyl-CoA when oxidized into CO_2 and H_2O via citric acid cycle generate 6 ATP molecule, less than those produced from the last acetyl-CoA produced from an even-carbon chain.

PEROXISOMAL β-OXIDATION

The major site of fatty acid beta oxidation is mitochondria, but it still can occur in other compartments within certain cells. Very long chain fatty acids (longer than 22 Carbons), branched fatty acids, and some prostaglandins undergo oxidation inside peroxisomes. Peroxisomes are membrane bound organelles that contain enzymes involved in fatty acid oxidation, in a slightly different process from mitochondrial β oxidation. The difference is in the first step where FA is oxidized, and one FAD molecule is reduced to $FADH_2$. In mitochondrial oxidation electrons are carried by $FADH_2$ into ETC for ATP production while in peroxisomes they are transferred directly to O_2 molecule forming H_2O_2 (detoxified by catalase

enzyme into H_2O and O_2). Subsequent steps are the same as their mitochondrial counterparts. Sometimes peroxisomal oxidation is considered as a shortening process where the yielded shortened fatty acyl continues its oxidation in the mitochondria.

MICROSOMAL OMEGA OXIDATION

Omega (ω) oxidation is important in some animals, and an alternative pathway in humans when defects in β-oxidation occur. ω-oxidation takes place in the smooth ER of liver and kidney instead of the mitochondria and peroxisome , it is involved in the oxidation of medium-chain (10-12 carbon atoms). It is called so because ω-carbon (the most distant carbon from the carboxyl group) undergoes hydroxylation by P450 where a hydroxyl group (OH) is oxidized to produce fatty aldehyde. The latter is oxidized into a carboxylic acid. Thus, a fatty dicarboxylic acid with a carboxylic acid at each end is obtained that undergoes several rounds of β-oxidation to give acetyl-CoA molecules. It can end also in four carbon chain "succinyl-CoA" which as an intermediate in citric acid cycle. Succinyl enzyme also provide substrate for gluconeogenesis under starvation or diabetic conditions.

ALPHA-OXIDATION OF FATTY ACID

Another form of fatty acid oxidation is known as alpha-oxidation not common in humans, but in other mammals that feed on grasses. α- oxidation is involved in the oxidation of branched fatty acids at the beta carbon where the attached group on the beta carbon prevents this fatty acid to undergo β-oxidation. α- Carbon is hydroxylated and oxidized, followed by the removal of one carbon unit adjacent to the α carbon from the carboxylic end in the form of CO_2 (Lehninger et al., 1993).

LIVER DISEASES

Thus, the liver is an important organ with vital and multi-functions, exposed to multiple metabolic disorders, toxic substances and microbes leading to disease development. Liver disease is defined as any potential disturbance that causes loss of liver function resulting in illness. Several diseases can be associated to the liver, some are asymptomatic while others show symptoms after weeks, months or even years. For example, cholestasis, in which bile flows are obstructed, cell inflammation in case of hepatitis, inadequate blood flow, tissue damage by chemicals as well as minerals or due to abnormal cell infiltration such as cancer cells, viral infections such as Hepatitis (A, B and E) and steatosis.

STEATOSIS DEFINITION

It is a pathological process characterized by triglycerides accumulation within the parenchymal cells, especially at the level of the liver to end in hepatic steatosis, but it can occur also at the level of the heart, kidney, and muscle (Figure 15). The steatotic liver is characterized by an increased size, and at the microscopic level by the presence of intracellular lipid droplets.

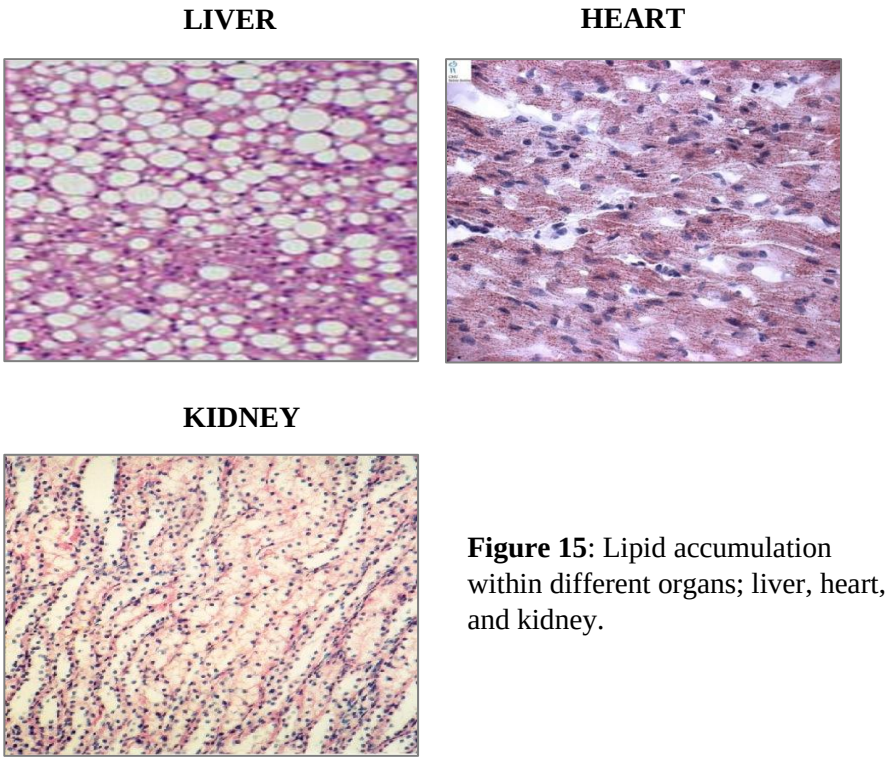


Figure 15: Lipid accumulation within different organs; liver, heart, and kidney.

Several factors can lead to **steatosis**:

- Toxic substances such as poisons, dyes and ethanol can cause liver toxicity.
- Metabolic imbalance leading to mitochondrial function impairment.
- Diabetes or hyper-lipidic diet

CLASSIFICATIONS OF STEATOSIS

From a **morphological point of view**, steatosis is classified into 3 different classes: micro-vesicular, macro-vesicular and mixed.

Micro-vesicular steatosis: It is the case where triglycerides accumulate in numerous tiny cytoplasmic LDs forming a foamy cytoplasm, and the nucleus remains central. Micro-vesicular steatosis is less common but more severe. This pattern is usually associated with disorders of mitochondrial β-oxidation leading to decreased synthesis of apoproteins and lower production of VLDL .

Macro-vesicular steatosis: It is usually the predominant form in which cytoplasmic LDs accumulate within a single large vacuole displacing the nucleus to the periphery which leads to hepatocyte enlargement. Macro-vesicular steatosis is typically linked to alcohol consumption, diabetes, obesity, drug impairment, toxins, and protein malnutrition.

The third is the *mixed droplet pattern* (micro-vesicular and macro-vesicular) which has a higher risk of progression to cirrhosis than macro-vesicular steatosis. (Stefan G. Hübscher et al., 2018; Apica et al., 2014).

Another classification is based on **the involvement of alcohol in disease development**.

- ***ALCOHOLIC FATTY LIVER*** is a pathological condition mainly caused by excessive alcohol intake, where alcohol degradation and oxidation yield to acetaldehyde formation in a reaction that produces NADH. Further oxidation of the former produces NADH also at the mitochondrial level. Increased NADH production stimulates lipid synthesis, decreases mitochondrial lipid oxidation, and prevents VLDL release. Furthermore, acetaldehyde is a very reactive molecule that can react with the amino groups of proteins causing damage and lead to ROS production.

- ***NON-ALCOHOLIC FATTY LIVER DISEASE:*** Explained in detail below.

NON-ALCOHOLIC FATTY LIVER DISEASE

DEFINITION

Another form of steatosis is known as ***NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)***. NAFLD, was first described in 1980's as the accumulation of lipids in more than 5% of hepatocytes, in the absence of excessive alcohol use or a direct cause for hepatic steatosis. It is a metabolic dysfunction associated with a wide spectrum of liver diseases, ranging from simple asymptomatic steatosis, also known as NAFLD, that can progress over a 10 years period to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, up to hepatocellular carcinoma (Lindenmeyer et al., 2018).

Lipids accumulate in the form of cytosolic Lipid Droplets (LDs) that contain mainly triacylglycerol (TAGs). Fat accumulation may be due to:

i. Increased de novo lipogenesis (DNL) from the non-lipid source like carbohydrates.
ii. Increased FFAs due to excessive lipolysis or increased dietary fat intake.
iii. Reduced FFA oxidation.
iv. Decreased secretion of TGs as very low-density lipoprotein (VLDL) (Masarone et al., 2018).

EPIDEMIOLOGY

Studies have reported that NAFLD affects around 25% of the general population and around 3% of the pediatric population, knowing that the highest prevalence is in the Middle East and South America while the lowest is in Africa. This prevalence is affected by several parameters such as body fat distribution and body composition. Moreover, patients with obesity, type 2 diabetes, dyslipidemia, and hypertension are at higher risk of NAFLD development compared to control groups (Souza et al., 2012). Previous study showed that NAFLD prevalence increases to 57-74% and 22 to 58 % in obese adults and children, respectively (Tarantino et al., 2007). It is shown that NAFLD prevalence in adolescents is higher than in children, which can be explained by several factors including sex hormones and insulin resistance in puberty, in addition to the increased unhealthy food choices and changed lifestyle in adolescence. Moreover, NAFLD is more common in males than in females which suggests the intervention of estrogens in liver protection (Barshop et al., 2008; Schwimmer et al., 2005).

DIAGNOSIS

A major problem associated with steatosis is being liver biopsy, the gold standard procedure used for diagnosis. This method is invasive and risky due to complications during surgery (such as bleeding, pain, and infection), it results in a lot of bias where the biopsy is only a little specimen, which limits data attribution to the entire liver status. It is difficult to monitor progression to fibrosis since it requires multiple repetitions of the biopsy procedures.

Several tests and screening methods candidate to provide an alternative approach to replace liver biopsy.

BLOOD AND SERUM TESTS

Since NAFLD is a complex multi-factorial disease, it is difficult to identify a single marker to assess hepatic steatosis. Thus, several markers and scores that can provide a good diagnostic measurement will be detailed in the table 7 below. These multiple-parametric panels are cost effective and not available in all clinical applications.

Table 7: Parameters tested in NAFLD diagnosis

Panel	Parameters tested
NAFLD ridge score	ALT, HDL, cholesterol, triglycerides, HbA1c, leukocyte count, hypertension
NAFLD Liver Fat Score (NLFS)	Liver fat content, metabolic syndrome, type 2 diabetes, AST, AST:ALT, fasting insulin level
Fatty liver index (FLI)	BMI, waist circumference, triglycerides, -GT
Lipid accumulation product (LAP) Index	sex, triglycerides, weight circumference

IMAGING TECHNIQUES

Thus, several Imaging techniques offer direct evaluation of liver status. We will briefly list available Image-based methods for NAFLD diagnosis.

- **Ultrasound**: It is an **imaging** method that uses high-frequency sound waves to produce images of structures within your body, it was first reported as a diagnostic tool for hepatic steatosis in 1981 in patients with the alcohol-related disease. This method analyses parenchymal alterations, fatty infiltration, and hepatic veins dilatation. However, the sensitivity of this method is still limited when hepatic steatosis content is below the threshold.

- **Controlled Attenuation Parameter CAP:** It is a non-invasive method for hepatic steatosis diagnosis, a novel physical measure based on ultrasonic signals acquired by the FibroScan device. Hepatic steatosis is detected in patients with 10% of hepatocyte degeneration. CAP application is limited in obese patients since abdominal fat may increase the skin to liver distance resulting in overestimation of the CAP data.
- **Magnetic Resonance Imaging MRI:** It is a medical imaging technique that uses a strong magnetic field and does not require ionizing radiation to obtain anatomical pictures and illustrate physiological processes within the body. A non-invasive advanced MRI technique known as MRI-proton density fat fraction (MRI-PDFF) was developed to accurately quantify fat over the entire liver. MRI-PDFF is based on the measurement of chemical shifts cause by mobile protons from triglyceride and water, where PDFF is a ratio of the density of mobile protons from triglycerides and the total density of protons from mobile triglycerides and mobile water that ranges from 0-100%. PDFF score is related to the percentage of cells containing intracellular fat droplets. Even though, MRI-based techniques are still not suitable due to high cost and limited availability (Pu et al., 2019) (Caussy et al., 2018) (Drescher et al., 2019).

PATHOGENESIS.

Multiple factors involved in NAFLD pathogenesis create a network of interactions that participate in pathogenesis mechanism. NAFLD initiates as hepatic triglyceride accumulation, considered to be relatively benign condition; then, a second hit increases the susceptibility to liver injury mediated by pro-inflammatory cytokines, OS, mitochondrial dysfunction and gut endotoxins which can develop into NASH, a severe form of steatosis. This mechanism was known over a decade ago as “Two-hit theory” (Giorgio et al., 2013). Recently, a third hit has been suggested to be implicated in further pathogenesis, due to inadequate hepatocyte infiltration Normally, cell death stimulates the replication of mature hepatocytes to replace dead cells. OS associated with NAFLD inhibits mature hepatocytes replication resulting in expansion of the hepatic progenitor cell population. The latter differentiates into hepatocyte-like cells that are strongly correlated with fibrosis and hepatocellular carcinoma (DOWMAN et al., 2009).

MECHANISMS INVOLVED IN NAFLD PATHOGENESIS.

As we mentioned above, NAFLD is considered as the hepatic manifestation of Metabolic Syndrome (MS) and is highly correlated with obesity, diabetes, insulin resistance and cardiovascular risk. Despite being a benign and reversible condition at the first stages, it can progress to more severe pathologies such as nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and eventually hepatocellular carcinoma (Vecchione et al., 2016) (Mello et al., 2015).

Several factors have shown a contribution to NAFLD pathology progression, mainly by OS and inflammation development such as mitochondrial dysfunction, endoplasmic reticulum (ER), gut-liver axis, insulin resistance, and peroxisomes.

- **MITOCHONDRIAL DYSFUNCTION**: earlier studies pointed that primarily NAFLD was characterized by the presence of mitochondrial dysfunction. Mitochondrial activities including fatty acid β-oxidation and ETC regulate fat homeostasis and energy production within hepatocytes. Mitochondrial abnormalities may be due to multiple mechanisms ranging from mitochondrial DNA (mtDNA) damage to enzymes imbalance, all detailed in the diagram below. (Deng et al, 2007).

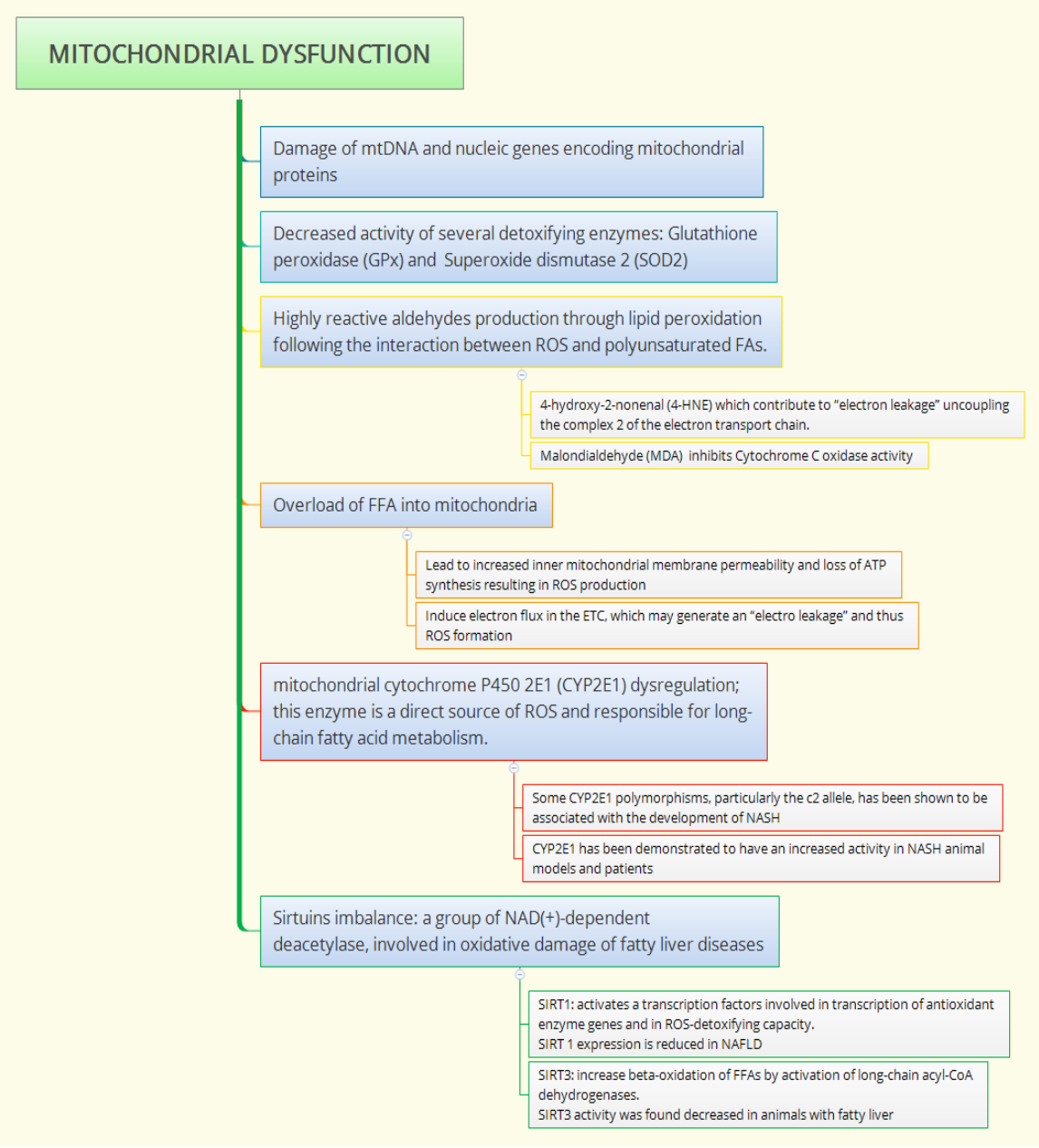


Figure 16: Mitochondrial contribution to NAFLD pathogenesis.

- **ENDOPLASMIC RETICULUM:** In the same way, FFA overload induce ER stress illustrated by unfolded and misfolded proteins accumulation in ER lumen, activating a specific signaling response "unfolded protein response", which in turn activates the expression of pro-inflammatory genes and suppresses those coding for antioxidant enzymes. In addition, ER is a potent source of ROS counting for about the 25% of all cellular ROS generation.
  ER lumen is the main site of calcium storage that plays a critical role in ER stress. Saturated fatty acids may induce ER calcium store disruption leading to ETC mitochondrial membranes blocking through the formation of permeability transition pores for cytochrome C, which finally results in an increased ROS production and apoptosis induction.

- **PEROXISOMES:** Moreover, peroxisomes are implicated in long chain FFA oxidation, producing H_2O_2 as an end-product, which means an additional source of radicals. This oxidative process may result in lipid peroxidation that in turn activates Kupffer cells to release inflammatory cytokines such as TNF-α and IL-6. All together aid in NAFLD pathogenesis and its progression to NASH. (Ucar et al., 2013) (Masarone et al., 2018).

- **ENDOTHELIAL DYSFUNCTION (ED):** Inducible isoform of nitric oxide synthase (iNOS) is upregulated under stress conditions through proinflammatory cytokines (TNF-α, IL1, and interferon gamma IFN-γ) that are upregulated also in obesity and insulin resistance. iNOS overexpression that contributes also for ED leads to high amounts of NO that are involved in several inflammatory and autoimmune diseases. Impaired generation of NO has been shown to promote the production on superoxide and hydroxyl radical intermediates increasing OS state. Altogether, it explains the role of ED in pathophysiological progression.

- **INSULIN RESISTANCE (IR):** IR is a major factor implicated in NAFLD and MS pathogenesis. In the case of IR within the liver, suppression of hepatic glucose production by insulin is reduced and synthesis of apolipoprotein B-100 is decreased, while hepatic synthesis of FFAs is increased, all leading to triglyceride accumulation in the liver. Thus, IR is correlated to increased FFA flux as a result of lipolysis suppression; since FFAs can directly cause toxicity by triggering OS and inflammatory pathways, they are accumulated in the form of triglyceride as a protective mechanism to avoid toxicity. Furthermore, FFAs contribute to NAFLD pathogenesis toward NASH and more severe status (Yamaguchi et al., 2007).

- **GUT-LIVER AXIS**: Bacterial population hosted in adult human intestine is enormous, almost around 100 trillion, variable and dynamic during human life, being influenced by several factors including diet, environmental hygiene, and antibiotic use. The gut

microbiota has several functions, including digestion of otherwise inaccessible nutrients, vitamin synthesis, and resistance to colonization by pathogens. However, any modifications (either quantitative or qualitative) of "normal" gut microbiota, also known as dysbiosis, may be involved in the development and pathogenesis of NAFLD and other metabolic diseases. Thus, the new concept of gut-liver axis involvement in NAFLD progression refers to the interaction between the liver, the intestinal barrier, and the gut microbiota. An unbalanced diet, particularly rich in fats and fructose, may lead to dysbiosis and intestinal barrier impairment, which favors metabolic endotoxemia and low-grade inflammation. It's known that intestinal dysbiosis may affect energy intake and whole-body metabolism. Microbiota-derived metabolites, namely Short Chain Fatty Acids (SCFAs), are known to be both a source of energy, particularly for colonocytes, and signaling molecules able to interfere with the host metabolism at different levels, comprising the regulation of de novo lipogenesis. Moreover, different strains of gut bacteria can produce other metabolites such as neurotransmitters, neuropeptides which can interfere with the mechanism regulating energy homeostasis and food intake. Immunological and inflammatory signaling deriving from the disruption of the integrity of the intestinal barrier (the so-called "leaky gut") may be part of the hits that trigger the worsening of simple steatosis to steatohepatitis and further liver damages. Interestingly, germ-free animals seem to be resistant to diet-induced obesity, liver steatosis and insulin resistance. When these animals are transplanted with a microbiota from a healthy donor, the hepatic TAG content is doubled. When the donor for microbiota transplantation suffer from NAFLD, this phenotype is replicated in wild-type recipients. Previous studies stated the association of some microbial strains with NAFLD phenotype in mice, for example Lachnospiraceae bacterium 609 and a relative of Barnesiella intestinihominis (Bäckhed et al., 2004) (Le Roy et al., 2013) (Kirpich et al., 2015)

GENES REGULATING LIPID HOMEOSTASIS

Biological processes are regulated by several genes, for example lipid homeostasis is controlled by a panel of genes regulating either lipogenesis or lipolysis.

1. **Peroxisome Proliferated Activated Receptors (PPARs):** PPARs were originally named for the ability of ligands to induce hepatic peroxisome proliferation, which is a phenomenon specific to rodents (Issemann et al., 1990). PPARs are the most known genes implicated in lipid homeostasis. PPARs are ligand-activated transcription factors activated by different FAs, naturally occurring FA-derived molecules and synthetic compounds. They share common structural features that include an amino-terminal domain, DNA binding domain and a carboxyl-terminal ligand binding domain. Once activated by an endogenous or exogenous ligand, PPARs form a heterodimer with retinoid X receptor and bind peroxisome proliferator response elements that regulate the expression of a panel of genes encoding enzyme and proteins implicated in beta oxidation, fatty acid uptake, adipogenesis, adipocyte differentiation, carbohydrates and protein metabolism, cellular differentiation,

tumorigenesis and inflammation (Varga et al., 2011). There are three different isoforms for PPARs, differentially expressed in various tissues.

- PPARα in humans is expressed equally among most tissues with higher expression in liver, kidney, heart, muscle, and adipose tissue. This isoform has a critical role in metabolism regulation as it induces the expression of proteins involved in FA uptake, trafficking, and oxidation (**β** and **ω-oxidation)** as well as in glucose uptake. Furthermore, it exerts anti-inflammatory effect through NF-kB pathway regulation.
- PPARϒ is the second isoform of PPARs, highly expressed in adipose tissue and induced mainly when energy storage is required. It has a crucial role in regulating adipocyte differentiation, adipogenesis, and lipid metabolism in which it is implicated in the anabolic esterification of FAs to TAGs and activation of adipogenic gene expression program, leading to accumulation of lipid storage within the liver. PPARϒ overexpression is a main feature of steatotic liver in which its expression was observed to be upregulated in all experimental models.
- PPARβ, also known as PPARδ, is expressed in gastrointestinal tract, kidneys, skin, adipose tissue; PPARβ is well expressed in hepatocytes, in addition to its presence in Kupffer cells and hepatic stellate cells, which explains the role in inflammation and fibrosis. Even if the role of this PPAR is still unclear in NAFLD progression, it has been shown that PPARβ expression ameliorate hepatic steatosis and inflammation by promoting hepatic FA oxidation (Vergani et al., 2017).

  As shown in Table 8 due to the critical role that PPARs have in regulating lipid and glucose metabolism, several studies have targeted this family of nuclear receptors for drug development aimed to treat metabolic diseases including NAFLD. ((Grasselli *et al.*, 2017) (Liss et al., 2017)

LIPID DROPLET ASSOCIATED PROTEINS

In mammals, excess lipids are stored in the form of triglycerides within LDs that are present in adipose tissue, hepatocytes, heart, and skeletal monocytes. Several models for LD biosynthesis have been suggested by Walther and Farese, and the most accepted model is that triglyceride accumulated at the ER membranes bud and form a vesicle that increases by size via LD fusion (Farese *et al.*, 2009). Thus, LD structure is composed of a neutral lipid core, which consists mainly of triacylglycerols (TAGs) and cholesterol esters, surrounded by a phospholipid monolayer decorated by several structural and functional proteins. The most studied group of LD-associated proteins are known as the PLINs family that comprises several members detailed below. Despite the sequence difference among PLINs, they all share a common feature that is coating LD surface with a main function to regulate LD metabolism by recruiting lipases and other proteins to LD surface which enhances lipolysis thus increasing FA and glycerol concentrations.

- **PLIN1:** known as perilipin, expressed in hepatocytes isolated from humans, while it was absent in normal and steatotic livers of mice. The most studied role of PLIN1 is the control of lipolysis by activating ATGL. Previous study showed that PLIN1 expression is

synchronized with a key regulator of de novo lipogenesis known as sterol regulatory element-binding protein (SREBP)-1c.

- **PLIN2:** was originally named as adipose differentiation-related protein (ADRP) due to its high expression during adipocyte differentiation. PLIN2 in expressed in human and murine liver. PLIN2 promotes TAG accumulation, inhibits FA oxidation, increases insulin resistance, and impairs glucose tolerance. PLIN2 polymorphism in NAFLD patients was linked to decreased VLDL secretion. Previously, Imai *et al* showed that antisense oligonucleotide against ADRP reduced steatosis and VLDL secretion, and enhanced hepatic insulin sensitivity in *ob/ob* and diet-induced obese mice (Imai *et al.,* 2007) Furthermore, PLIN2 knock-out mice were protected from hepatic steatosis when fed with a high fat diet.
- **PLIN3:** also named as tail-interacting protein of 47 kDa (TIP-47) that is ubiquitously expressed among tissues. TIP-47 and ADRP are the major LD proteins in hepatocytes. It has been identified in human and mouse liver, as well as in rat hepatocytes. PLIN3 has shown to be upregulated in the liver of HFD-fed mice. Another study showed that the use of PLIN3 anti-sense oligonucleotide in mice reduces hepatic TAGs and improves insulin sensitivity (Carr *et al.,* 2011).
- **PLIN4:** originally called S3–12. This protein is present at cytosol and LDs. PLIN4 knock-out mice showed a significant reduction of TAG content in heart tissue.
- **PLIN5:** known as oxidative tissue-enriched PAT protein (OXPAT), has been widely documented in human and mouse protein, as in rat hepatocytes. PLIN5 expression is increased in fatty livers of dystrophic mice, plays a crucial role in regulating cellular FA oxidation and stabilizes LDs. Moreover, PLIN5 knock-out mice were from hepatic steatosis by increased lipolysis and FA oxidation.

Genes coding for LD- associated PLINs are under the control of different transcription factors such as PPARS. Especially PPARϒ that appears to regulate the expression of PLIN1, PLIN2, PLIN4 and PLIN5. (Grasselli *et al.,* 2017) (Minehira *et al.*, 2017).

Table 8: Several studies targeting PPARs to ameliorate NAFLD in different models.

Targeted PPAR Isoform	Treatment	NAFLD Model	Results	Reference
PPARα	PPARα agonist (Wy-14643)	*In vivo* (mice) (Methionine/Choline Deficient Diet)	• reversed induced steatohepatitis via increased fatty acid oxidation • decreased number of activated hepatic stellate cells	IP *et al.,* 2003
PPARα	PPARα agonist (Fenofibrate)	Humanized APOE2 knock-in (APOE2KI) mice fed western diet (high levels of	• decreased hepatic steatosis, hepatic macrophage accumulation, and	Shiri-Sverdlov *et al.*, 2006

		sucrose and cholesterol)	inflammatory gene expression • Upregulation of genes involved in beta oxidation	
PPARβ	PPARβ agonist (GW501516)	HFD mice	• Improved insulin sensitivity • Prevention of hepatic lipid accumulation	Tanaka *et al.*, 2003.
PPARβ	PPARβ agonist (GW501516)	*In vivo* (mice) (Methionine/Choline Deficient Diet)	• decreased hepatic triglyceride content • increased expression of genes involved in FA beta oxidation such as acyl-CoA oxidase, and carnitine palmitoyltransferase-1	Nagasawa *et al.*, 2006.
PPARβ	PPARβ agonist GW0742	OLETF (Otsuka Long Evans Tokushima Fatty) rat model	• improved insulin signaling • Reduced hepatic steatosis • Decreased expression of inflammatory genes.	Lee *et al.*, 2012.

Supplementary box 8: Definitions

- Methionine/Choline Deficient Diet (MCD) mice: A well-accepted NAFLD model, based on the deficiency of two essential amino acids: methionine and choline; essential elements for hepatic mitochondrial β-oxidation (VLDL) synthesis (Anstee *et al.*, 2006). (Choline for VLDL secretion and methionine for regulatory enzyme activity)
- Humanized APOE2 knock-in (APOE2KI) mouse fed with a high fat diet, a model that has a markedly reduced affinity for the LDL receptor, leading to hyperlipidemia.
- Fenofibrates are a part of a drug class known as fibrates; a medication used to treat abnormal lipid levels by activating PPARα.
- OLETF (Otsuka Long Evans Tokushima Fatty) rat is a model of non-insulin dependent diabetes mellitus, are also used as a model for NAFLD.

2. **GENES INVOLVED IN FA OXIDATION:** As explained above, PPARα plays a critical role in regulation of lipid mitochondrial β-oxidation that in turn activates the expression of PPARα-target genes involved in lipid catabolism such as:

- Carnitine-palmitoyltransferase-1 (CPT-1), which is an enzyme in the outer mitochondrial membrane considered as rate limiting step in long chain fatty acid oxidation. Long chain fatty acids, unlike short chain fatty acids that diffuse freely through mitochondrial inner membrane, require transport shuttle system to be transported into the mitochondrial matrix. CPT-1 as we said is the first component of carnitine palmitoyltransferase system, catalyzing the transfer of the acyl group from coenzyme A to carnitine to form acylcarnitine. A translocase then shuttles the acyl carnitine across the inner mitochondrial membrane ensuring its entrance into the mitochondrial matrix (Grasselli *et al.*, 2016).

- Another alternative pathway for FA metabolism is ω-oxidation controlled by several microsomal cytochromes P450, mainly CYP2E1 and CYP4A1 detailed above . In addition, exocytosis is a way for TAGs secretion; in which they are packaged with apoprotein B (ApoB); the primary apolipoprotein component of VLDL (Grasselli *et al.*, 2014).

II. AIM OF THE STUDY

In this study, we aimed to investigate the antioxidant and anti-steatotic effects of FUCs purified from *C. compressa*, *F. hermonis*, and *E. globulus*. To this aim, we used two in vitro model consisting of rat hepatoma FaO and HECV cells exposed to an oleate/palmitate mixture or H_2O_2 for steatosis and OS induction, respectively.

- ***Cytotoxic effect of FUC***
- ***Quantifying ROS production***
- ***TAG content quantification***
- ***Regulation of lipid metabolism genes expression***
- ***Effect on NO production***

III. MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

FaO Cells

FaO cell line (European Collection of Authenticated Cell Cultures, Sigma-Aldrich) are cells of rat hepatoma that grow adherent to the growth surface. FaO cells are widely used as a cellular model to mimic liver function as they maintain hepatocyte-specific markers and liver functions including VLDL secretion. Cells were grown in Coon's modified Ham's F12 medium supplemented with L-Glutamine and 10% fetal bovine serum (FBS) with a low glucose concentration and incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Prior to the experiment by 24 hours, cells were incubated in with F-12 with 10% FBS and 1% glucose 1X (HGC) to supply more glucose for cell growth and TAG synthesis.

To induce intracellular lipid accumulation and mimic hepatic steatosis and endothelial damage, FaO cells were treated for 3 h with a mixture of fatty acids oleate/palmitate (OP) to form steatotic cells at a final concentration of 0.75 mM (2:1 molar ratio; which means 0.5 mM for oleate and 0.25 for palmitate) (Grasselli *et al*. 2017, Vergani *et al*., 2017). All treatments (both OP and FUC treatment) were performed in F-12 medium deprived from FBS but supplied with 1% glucose and 0.25% of BSA.

HECV Cells

HECV cells are endothelial cells isolated from the umbilical vein that grow also in an adherent way. Endothelial cells are used as a model for endothelial damage which is a main metabolic abnormal characteristic observed in NAFLD. The culture medium used for the HECV growth is DMEM (Dulbecco's modified minimum essential medium) with 10% FBS and 1% L-glutamine, incubated at 37°C, 5% CO2.

To mimic in vitro effect of a high fat diet and induce steatosis, HECV cells were treated as FaO cells with OP mixture using the same concentration.

Fucoidan Treatment

Fucoidan were purified as previously explained. The aqueous stock solution of 1 mg/mL was prepared diluted in the proper treatment culture medium to the desired working concentration. FaO and HECV cells were treated for 24 h.

CELL VIABILITY

Cell viability was estimated using a colorimetric test is known as the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of the yellow tetrazolium MTT into purple formazan crystals by metabolically active and alive cells. Cells were seeded in 96-well plates and cultured to sub-confluence. At the end of the different treatments, cells were incubated for 3 h with 0.5 mg/mL MTT. After discarding the supernatant, formazan crystals in each well were dissolved by acidified isopropanol (0.04N HCl in 2-propanol). Results were obtained by reading the absorbance at 570 nm in a Varian Cary 50 UV-VIS spectrophotometer.

According to Lambert-Beer's law, absorbance value is directly proportional to the concentration which is cell viability. Control (untreated) cells were considered to represent 100% viability, and results were expressed as compared to control (Van Meerloo *et al.*, 2011).

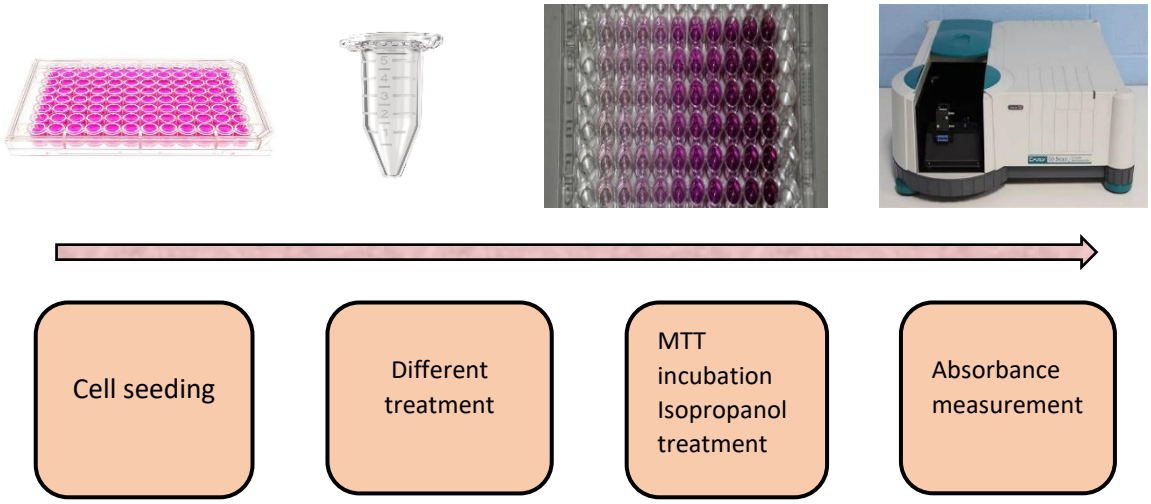


Figure 17: MTT cell viability assay steps.

❖ ***Varian Cary 50 UV-VIS spectrophotometer***

The spectrophotometer is a crucial instrument used in most of the research fields; biological, chemical, clinical, and environmental used for quantitative substance measurement. Its principle is based on four principle components.

LIGHT SOURCE: that should be stable during the measurement and provide intensity and adequate amount of energy

↓

MONOCHROMATOR: functions to separate the light into component wavelengths

↓

CUVETTES: are containers where the solution is placed to determine the absorbance; can be made from quartz or silica

↓

DETECTOR: transform output of the spectrometer into an electrical signal that can be analyzed using a computer.

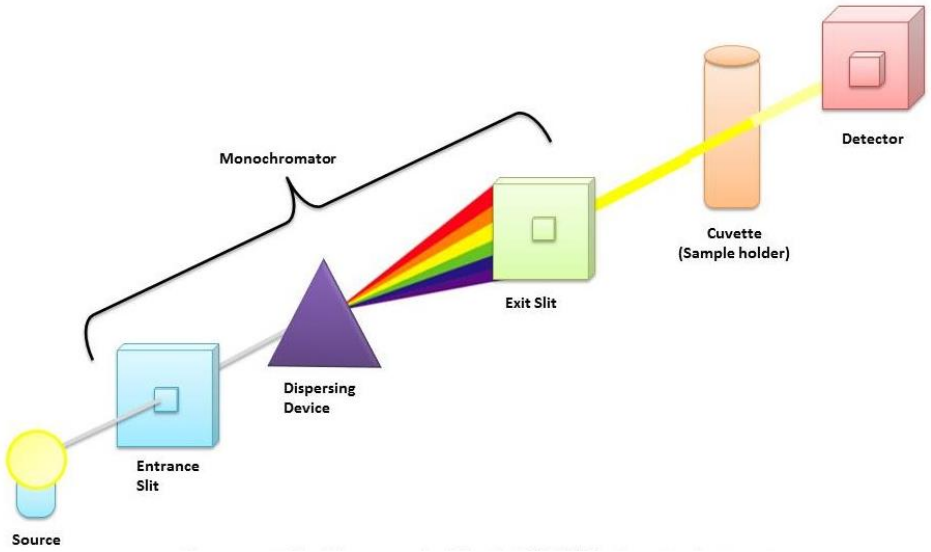
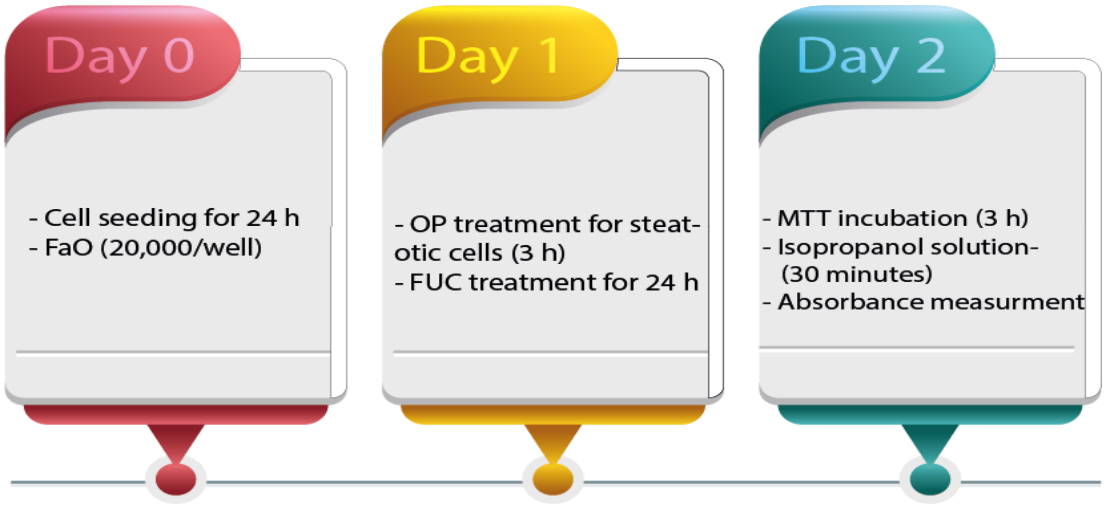


Figure 18: Representative Diagram of a simple UV-Visible Spectrophotometer.

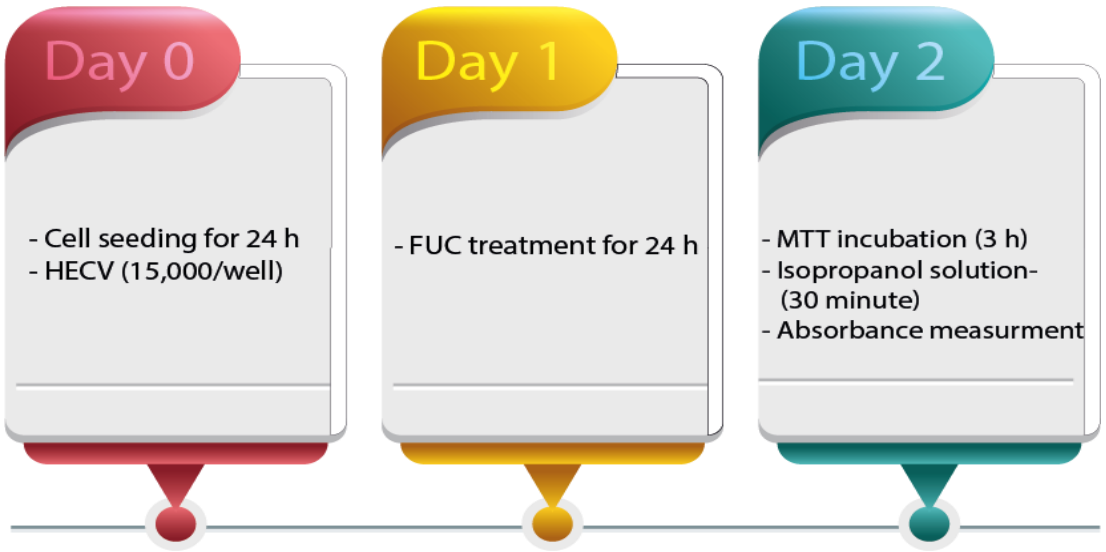
- **Effect of FUC on FaO cell viability**

FUCs were tested for their cytotoxic effect on FaO cells, both steatotic and normal. 20,000 FaO cells per well were seeded in 96 well plate, 200µl/well, and incubated for 24 h to grow. For steatotic cells, cells were treated with OP for 3h prior to FUC treatment.



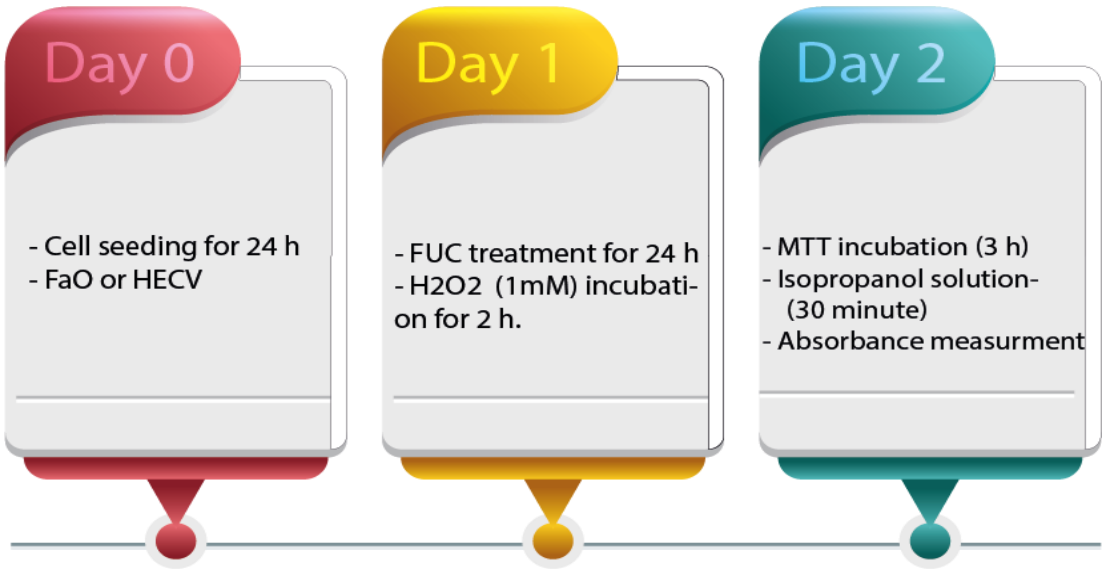
- **Effect of FUC on FaO cell viability**

Cytotoxic effects of FUC on HECV cell viability was measured by MTT assay. 15,000 HECV cell per well were seeded in 96 well plate for 24 h, then treated with FUC for another 24 h before absorbance recording.



- **Protective effect of FUC against H_2O_2 treated cells.**

  Another MTT assay was performed to check the protective effect of FUCs against hydrogen peroxide (H_2O_2) induced OS in both FaO and HECV cells. Sub-confluent cells were treated with 50 µg/mL of FUCs for 24 h. Then, the medium was replaced by new medium containing H_2O_2 (1 mM) and left for 2 h. MTT was added and results were recorded as previously explained (Xie *et al*., 2015).



QUANTIFICATION OF TRIGLYCERIDES (TAGs)

FUC anti-steatotic activity was tested by TAG quantification using the "Triglycerides liquid" kit after their extraction in chloroform/methanol (2:1) (Sentinel, Milan, Italy). FaO cells were grown as previously explained, incubated for 24 hours to reach sub-confluence. Then steatosis was induced by OP treatment followed by FUC treatment.

This assay is based on the principle of triglyceride hydrolysis by lipoproteinlipase (LPL) to glycerol and fatty acids; glycerol is phosphorilized to glycerol-3-phosphate in the presence of glycerolkinase (GK) and adenosine 5-triphosphate (ATP) and then converted by glycerol-3-phosphate oxidase (GPO) into dihydroxy-acetone-phosphate and hydrogen peroxide. In the presence of hydrogen peroxide, peroxidase (POD) oxidizes the chromagen 4-amino antipyrine and TOOS to form a red compound which color intensity is proportional to the concentration of an analyte in the sample. The used kit contains all the enzymes required for this hydrolysis, in addition to the substrate (chromagen 4-amino antipyrine and TOOS).

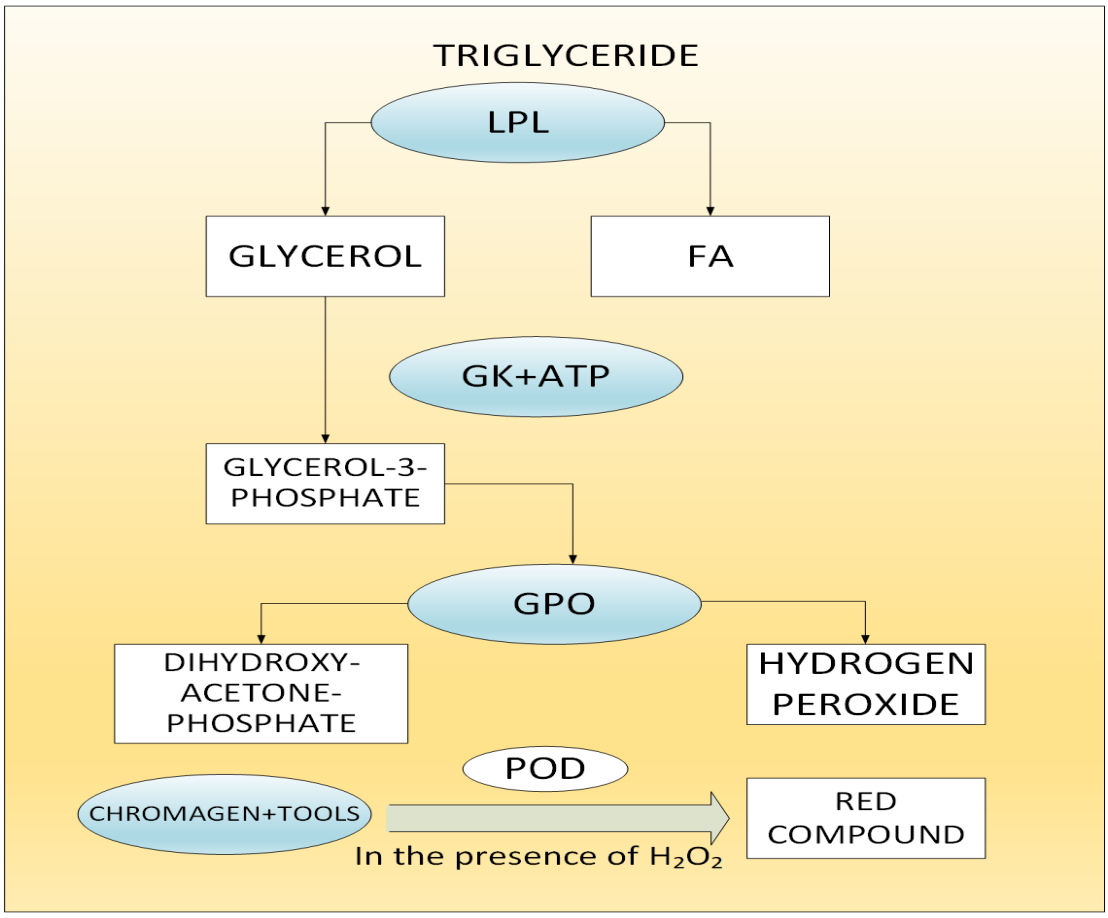


Figure 19: Principle of "Triglyceride kit". Compound represented in pink color are all reagents within the kit.

EXPERIMENTAL SETUP.

1. After treatment of the steatotic (OP) cells with FUC for 24 h, the medium was removed and stored for extracellular TAG measurement.
2. Cells were washed twice with 3 ml of non-sterile phosphate-buffered saline PBS 1X solution
3. Add 3 ml of PBS 1X, scrape the cells and transfer to 15 ml falcon tube.
4. Centrifuge at 720×g , 4 °C for 10'.
5. Discard the supernatant and resuspend the cells in 1 ml of PBS.
6. Transfer to an Eppendorf and centrifuge at 845×g , 4°C for 10'.
7. Discard the supernatant, add 300 µl of PBS to the pellet and lyse cells using a gauge needle.
8. The measurements of TAG for every treatment are done in triplicates; in a TAG specific Eppendorf put 250 µl of methanol and then add 70 µl of cells.
9. Add 500 µl of chloroform, vortex for 1 h and then add 250 µl of H2O.
10. Centrifuge at 300×g , 4 °C for 25'.

11. Remove the upper phase (methanol that contains salts, carbohydrates, and some proteins), quantify the volume of the lower phase (which contains extracted lipids), and keep overnight under the hood for chloroform evaporation.
12. The next day, add 600 µl of "triglyceride kit", incubate for 15' at 37 °C.
13. Results were obtained by reading the absorbance at 546 nm.

Recorded absorbance should be compared to a standard, for this we did a series of glycerol concentrations as TAG standard. Glycerol stock solution was diluted to obtain a final concentration of 1.24839 mg/ml. which was used diluted to obtain the standards listed in the Table 9.

Table 9: Concentrations of glycerol standard for TAG quantification. Volumes in µl.

Concentration	Volume of glycerol (1.24839 mg/ml)	Volume of H_2O	Total volume
0.13	20	180	200
0.063	20	380	400
0.031	20	780	800
0.025	20	980	1000
0.017	20	1480	1500
0.013	20	1980	2000
0.007	20	3980	4000
0.0035	20	7980	8000

14. From each glycerol concentration, take 10 µl and put in a TAG Eppendorf, add 590 µl of "triglyceride kit", incubate for 15' at 37 °C.
15. Results were obtained by reading the absorbance at 546 nm. (Grasselli *et al*., 2014) (Grasselli *et al*., 2011).

Values were normalized to protein content as measured by Bradford assay (detailed below) and data are expressed as percent TAG content relative to controls.

Extracellular TAG content

For ***extracellular TAG*** content, the culture media were processed according to the same method.

BRADFORD ASSAY

TAG measurements were normalized to protein concentration to overcome the differences in cell number among treatments. For this, proteins were quantified by Bradford assay, which is based on the binding of protein molecules to Coomassie dye under acidic conditions, resulting in a shift in maximum absorbance from 465 to 595 nm.

Bradford reagent preparation:

1. Weigh 0.003 grams of comassie G-250.
2. Add 2.4 ml of absolute ethanol and vortex.
4. Add 5 ml of phosphoric acid and vortex.
6. Complete the volume to 50 ml by milli-Q H2O.

BSA (0.1 mg/ml) was used to prepare a standard for protein quantification as indicated in the table.

Table 10: Preparation of BSA concentrations used for protein quantification.

BSA standard	Volume H_2O µl	Volume BSA in µl (0.1 mg/ml)	Volume of Bradford reagent (ml)
B0	100	0	1
B10	90	10	1
B20	80	20	1
B40	60	40	1
B80	20	80	1

For cellular protein quantification, cell lysate was diluted 1:5. Two measurements were done for Bradford assay, named sample 10 and sample 20 which means 10 µl or 20 µl of diluted sample mixed to 90 µl and 80 µl of H_2O, respectively. Followed by the addition of

Samples and standard were incubated for 15' in dark with 1 ml of Bradford reagent. Absorbance was read at 595 nm. (Bradford, 1976).

BODIPY STAINING

BODIPY 493/503 is a lipophilic fluorescent probe with its nonpolar structure used for neutral lipid staining. For this experiment, FaO cells were grown on collagenated coverslips for 24 h,

then steatosis was induced by OP incubation followed by FUC treatment to monitor their effect on intracellular LDs (Grandl and Schmitz, 2010).

Qualitative lipid droplet analysis

After treatment cells were rinsed three times with non-sterile PBS 1X, fixed with 4% paraformaldehyde for 20 min at room temperature, and then washed another three times with PBS.

Neutral lipids were stained with **BODIPY** as follow:

1. Incubation with 1 μg/mL BODIPY 493/503 (Molecular Probes, Life technologies, Monza, Italy) prepared in PBS for 30 min.
2. Wash with PBS (3 times).
3. After neutral lipids staining, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/mL, (ProLong Gold medium with DAPI; Invitrogen) which is a fluorescent stain that binds strongly to adenine – thymine -rich regions in DNA.

Mounted slides were examined at 10X magnification by Olympus IX53 light microscope (Olympus, Milano, Italy), equipped with the standard epifluorescence filter set up. Representative images were captured with a CCD UC30 camera and a digital image acquisition software (CellSens Entry).

Quantitative lipid droplet analysis

For quantitative lipid measurement using BODIPY staining, the same experiment was done in 96 well plate, where cells were seeded, treated, and stained with BODIPY and DAPI. Results were obtained fluorometrically in a LS50B fluorimeter (Perkin Elmer, USA) at 25 °C using a microplate reader. Results were normalized for DAPI measurements.

ROS PRODUCTION

ROS (mainly H_2O_2) production was quantified based on the oxidation of the cell-permeant 2′-7′ dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2′-7′dichlorofluorescein (DCF). Stock solution of DCF-DA (10 mM in DMSO) was prepared and stored at −20°C in the dark.

- **QUANTITATIVE ANALYSIS**

FAO CELLS: At the end of treatments FaO cells were scraped and centrifuged at $300\times g$ rpm for 10’ at 4°C. The supernatant was discarded and cells were resuspended in 1 ml PBS-1X and incubated for 30’ in dark with 1 µl of DCF-DA stock solution (prepared in DMSO at a concentration 10 mM and stored at −20 °C) Then, cells were centrifuged, resuspended in PBS

and the fluorescence was measured fluorometrically (λex = 495 nm; λem = 525 nm) (Halliwell *et al.*, 2004). All measurements were performed in a LS50B fluorimeter (Perkin Elmer, USA) at 25 °C using a water-thermostated cuvette holder. Results were normalized for total proteins (Bradford, 1976).

HECV CELLS: ROS production was measured as described by Rottmann et al. with slight modifications. Cells were seeded in 96 well plate for 24 hours, then treated with FUC for another 24 hours. After FUC treatment, OS was induced by H_2O_2 (0.5 mM for 30'). Cells were stained with DCF for 10' in the dark and washed three times in PBS-1X. Results were recorded fluorometrically with excitation wavelength at 485 nm and emission at 530 nm (Rottmann et al., 2002).

- **QUALITATIVE ANALYSIS**

HECV cells: The same principle of DCF was used to qualitatively evaluate ROS production using a fluorescent microscope. In a 24 well plate, 40,000 cell/well were seeded for 24 hours, then treated with FUC purified from different species for 24 hours. At the end of the treatment, cells were treated cells were incubated with 1 mM H_2O_2 solution prepared in DMEM medium for 10 min. After that cells were stained with DCF for 10 min, washed 4 times with PBS1X, and images were recorded at the same spot both in the visible and in the fluorescence. The time as the fluorescence exposure was the same for all at 150 ms. Cells were examined by Olympus IX53 light microscope (Olympus, Milano, Italy), equipped with the standard epifluorescence filter set up. Representative images were captured with a CCD UC30 camera and a digital image acquisition software (CellSens Entry).

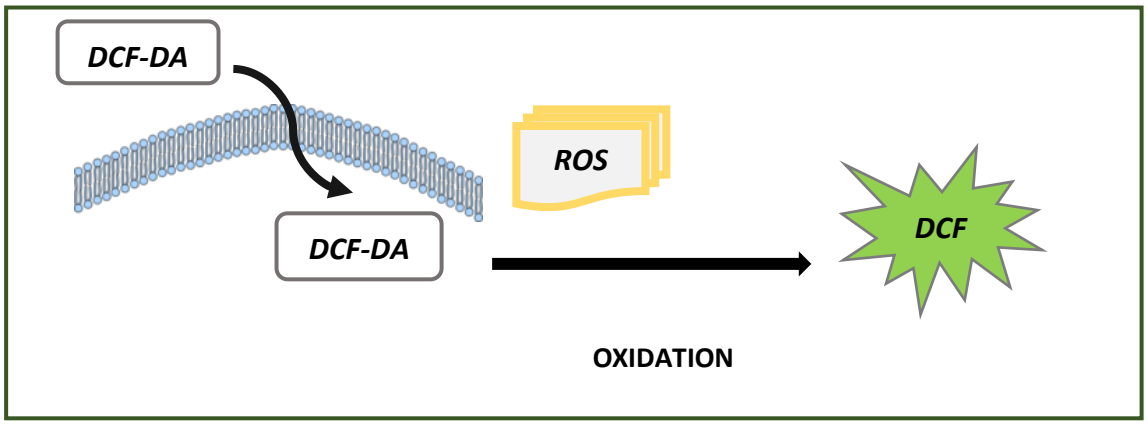


Figure 20: DCF assay based on the oxidation of DCF-DA by ROS (mainly H_2O_2) into a fluorescent DCF molecule.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

The effect of FUC treatment at the molecular was tested by monitoring the expression of genes implicated in lipid metabolism regulation. For this, quantitative polymerase chain reaction

(qPCR) analysis was used, also known as real-time PCR or quantitative real-time PCR. It is a PCR-based technique that correlates amplification of a target DNA sequence with quantification of the concentration of that DNA species in the reaction.

Gene expression analysis requires several steps:

- RNA EXTRACTION
- REVERSE TRANSCRIPTION
- REAL-TIME PCR

RNA EXTRACTION: Total RNA was extracted using Trizol Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

- Cells were scrapped in PBS-1X as previously explained and the cell pellet was resuspended in 1 ml of Trizol reagent for 5' at room temperature
- Add 200 µl of chloroform, vortex and wait for 15'.
- Centrifuge at 12400×g , 4 °C for 15'. Three phases are obtained: the upper aqueous phase containing RNA, the intermediate white phase which contains DNA, and the lower pink phase containing proteins.
- Add to the RNA containing phase 500 µl of isopropanol, vortex and wait for 10'.
- Centrifuge at 12400×g, 4 °C for 10' to get RNA pellet and wash with 1 ml of 75% ethanol.
- Centrifuge at 4800×g , 4 °C for 5'; discard the supernatant and wait for ethanol evaporation.
- Store in 20 µl of Diethyl pyrocarbonate (DEPC) water.

Nanodrop was used to quantify extracted RNA in (ng/µl) and test the purity by measuring the ratio (260/280) of absorbance at 260/280 nm.

REVERSE TRANSCRIPTION RT: it is an enzyme-mediated synthesis of a DNA molecule using RNA as template. The resulting DNA is known as complementary DNA (cDNA). RT is done also in 2 main separate steps.

The first step is required to denature RNA from its secondary structure and anneal polyA of mRNA to the PolyT primer. For this, 10 µl of a mixture of PolyT (1µl), RNA sample (volume corresponding to 1µg) and Rnase free H_2O were denatured for 5' at 70 °C and then held at 4 °C.

In the next step, each mRNA sample is mixed with RT mix that contains Buffer 4 µl, H_2O 3.5µl, DNTPs 1 µl, RNase inhibitor 0.5 µl, and 1 µl of RevertAid H-Minus M-MuLV Reverse Transcriptase (Fermentas, Hannover, MD, USA) . PCR elongation step was triggered by running the machine on 42 °C for 1 h and then on 72 °C for 10' so that the polymerase enzyme is inactivated (Grasselli et al., 2016).

For each sample add 80 µl of RNase free H_2O so to have 100 µl of non-diluted cDNA sample.

REAL-TIME PCR

Real-time quantitative (qPCR) reactions were performed in triplicate in a final volume of 15 µl using 2X SybrGreen SuperMix and Chromo4TM System apparatus (Biorad, Monza, Italy) as previously described (Grasselli et al., 2012). Amplification conditions were as follows: 3 min at 95 °C, followed by 5 s at 95 °C and 1 min at 60 °C or 64°C for 40 cycles. A melting curve of qPCR products (65–94◦C) was also performed to ensure the absence of artifacts. The relative quantity of target mRNA was calculated by the comparative CT method using glyceraldehyde 3-phosphate dehydrogenase as housekeeping gene and expressed as fold induction with respect to controls (Pfaffl, 2001).

Table 11: Primer pair sequences and annealing temperature.

Primer name	Annealing temperature (°C)
PPARα	60
PPARγ	60
PLIN2	64
PLIN5	64
CYP4A1	60
CPT-1	60
ApoB	64

MEASUREMENT OF NITRITE/NITRATE (NOx) LEVELS

As Nitric Oxide (NO) has a short half-life, its production was measured indirectly by its end products: nitrites and nitrates (collectively referred as NOx), using the Griess reaction (Green *et al*., 1982). NOx accumulation in cell culture media was calculated against a standard curve of sodium nitrite ($NaNO_2$) and normalized by protein content (µmol $NaNO_2$/mg sample protein). All spectrophotometric analyses were carried out at 25 °C recording absorbance at 540 nm with a Varian Cary 50 UV-VIS spectrophotometer.

The principle of this assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. In acidic medium, nitrite reacts with sulfanilic acid (a component of Griess reagent) to produce the diazonium ion. The ion is then coupled to the other main component of the reagent; *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm.

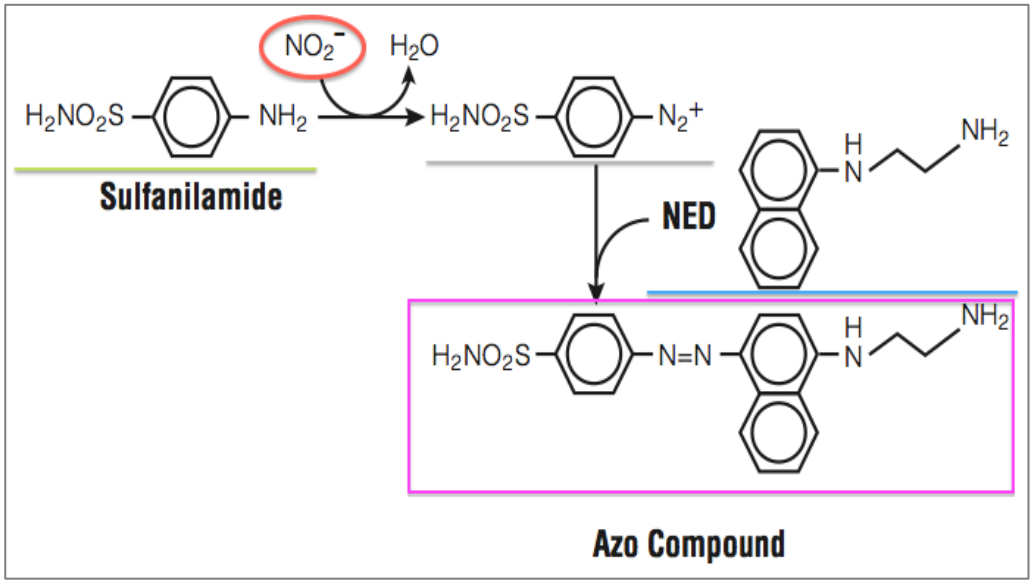


Figure 21: The mechanism for the nitrite, sulfanilamide, and NED reaction to form Azo compound in the presence of NO_2.

NO production was stimulated in HECV cells in two different ways:

1. HECV cells were incubated with OP for 3 h.
2. HECV cells were incubated with 25 µM H_2O_2 for 24h

In both cases, the cells were then treated for 24 h in absence or presence of 50 µg/mL FUC.

Table 12: Griess reagent preparation.

Sulfanilamide (in grams)	0.5
N-(1-naphthyl) Ethylenediamine (in grams)	0.05
Phosphoric Acid (volume in ml)	2.94
H_2O (total volume in ml)	50

EXPERIMENTAL PROTOCOL

1. After FUC treatment, culture medium was collected and centrifuged at 16900×*g* for 10' at 4 °C, whilst the cells were scrapped and tested for protein quantification using Bradford assay.
2. 400 µl of the supernatant were incubated with 400 µl of Griess reagent for 10' in dark at room temperature.
3. Sodium nitrate ($NaNO_2$) stock solution (30 mM) was used to prepare a standard for values comparison (500 µM, 50 µM, 10 µM, 5 µM and 1 µM)
4. Results were obtained by reading the absorbance of chromophoric azo-derivative at 543 nm.

IV. RESULTS

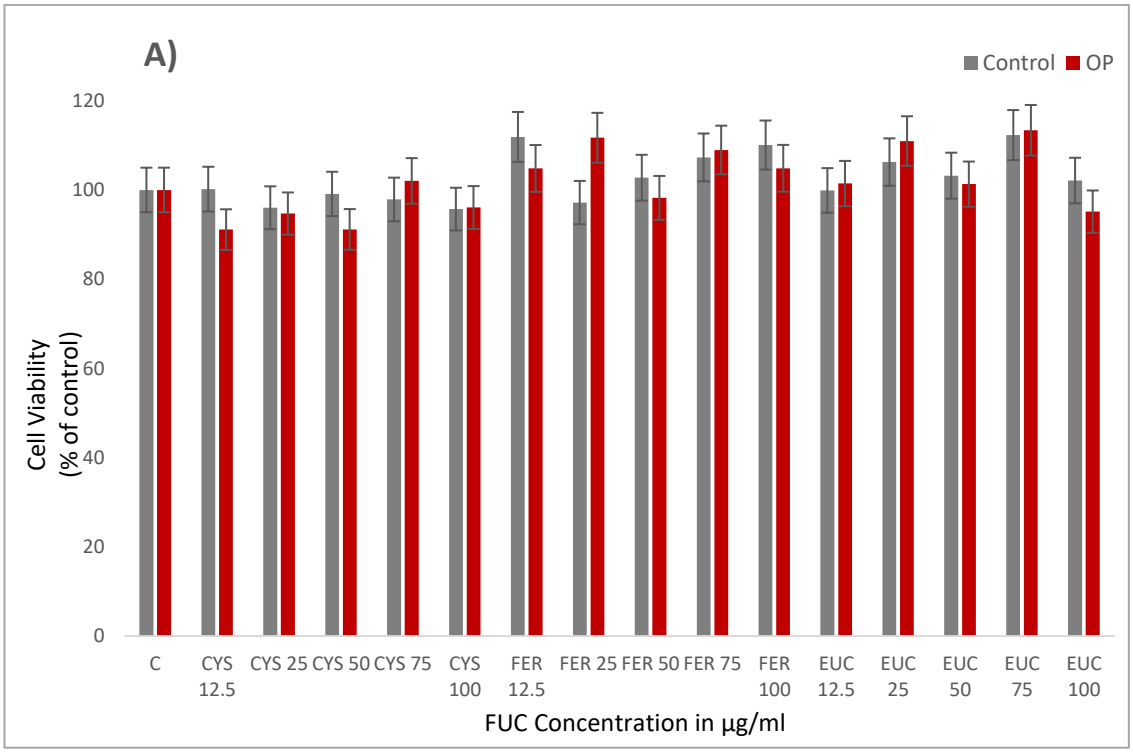
FaO CELLS (rat hepatoma cell line)

ANTIOXIDANT EFFECTS in FaO CELLS

Cell Viability

Results of MTT assay demonstrated that purified FUC tested at different concentrations (12.5-100 µg/mL) did not affect cell viability both in the absence and in the presence of OP. The intermediate concentration 50 µg/mL of CYS, FER, and EUC was selected for further experiments.

MTT assay was used also to test cell viability in FaO cells pretreated with CYS, FER or EUC for 24 h and subsequently with 1 mM H_2O_2 for 2 h to induce oxidative cell damage. As shown in Figure 22B, 1mM H_2O_2 alone/ in the absence of FUC significantly reduced cell viability by 38% ($p \leq 0.001$ with respect to control), whereas FUC pretreatment prevented this decrease in a significant way only for FER, and EUC ($p \leq 0.01$ with respect to H_2O_2) in which cell viability was restored to 90.38% and 93.46%; respectively, compared to the control (100%).



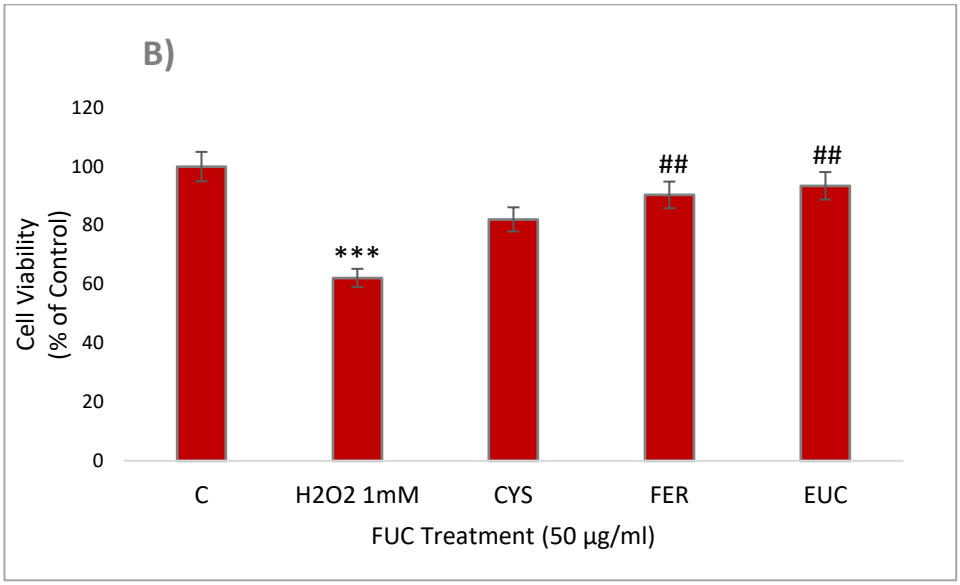


Figure 22: MTT assay results on FaO cells; **A**: *control and steatotic FaO Cells*; **B:** *control and H_2O_2 treated FaO cells*). ***$p \leq 0.001$ with respect to control; ## $p \leq 0.01$ with respect to H_2O_2.

ROS PRODUCTION

The principle of the cell-permeant 2′-7′ dichlorofluorescin diacetate oxidation to 2′-7′dichlorofluorescein (DCF) is used for quantifying in situ the production of H_2O_2 and other ROS. Antioxidant potential of FUC was confirmed by DCF assay for ROS detection in steatotic FaO cells (Figure 23). OP treatment resulted in a significant increase in ROS production (+ 30%, $p \leq 0.01$ with respect to control), while ROS production was decreased in steatotic FaO cells after FUC treatment:

- Slight decrease with CYS treatment (8.6 % compared to OP).
- Significant decrease with FER (by 55.9 %) and EUC (by 64.2 %) with respect to OP treatment, respectively ($p \leq 0.001$ vs OP).

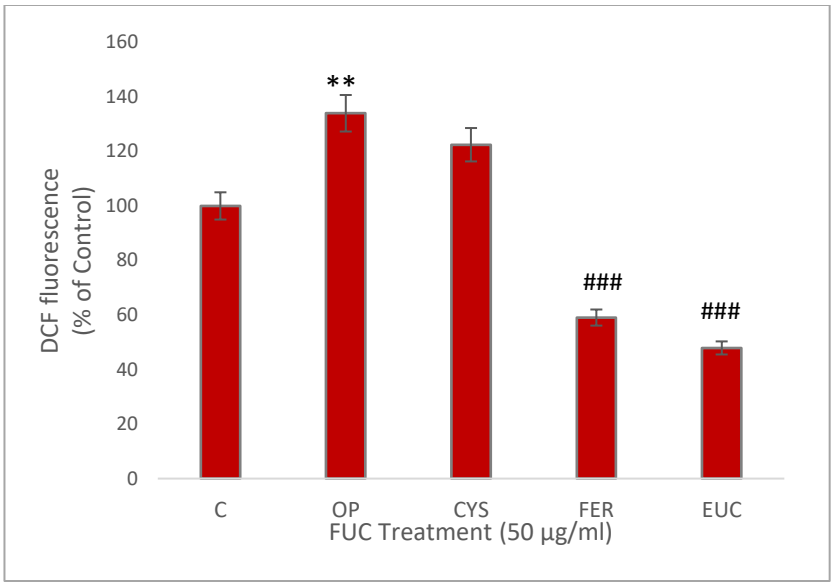


Figure 23: Results of ROS production in steatotic FaO cells measured by DCF assay. (**p ≤ 0.01 with respect to control; ### p ≤ 0.001 vs OP).

ANTI-STEATOTIC EFFECTS ON FaO CELLS

Spectrophotometrical analysis

Intracellular TAG content was quantified in both control (C) and steatotic cells in absence (OP) or presence of FUC (CYS, FER or EUC). Results reported in figure 24A indicated that OP treatment significantly increased TAG content with respect to control (+175%; p ≤ 0.001) as previously described (Grasselli *et al*., 2011). However, incubation with 50 µg/ml of CYS, FER or EUC after OP treatment decreased TAG content by 35%, 36% and 56%, respectively as compared to OP (p ≤ 0.01 for CYS and FER; p ≤ 0.001 for EUC) (Fig 24A).

Extracellular TAG content in cell culture medium was measured as an indication of TAG secretion by FaO cells. Figure 24B shows that TAG secretion was significantly increased in OP cells with respect to control (+40%, p ≤ 0.01), and subsequent CYS or FER treatment did not cause any significant change in TAG content while EUC treatment enhanced its secretion in a significant way with respect to OP (p≤0.05).

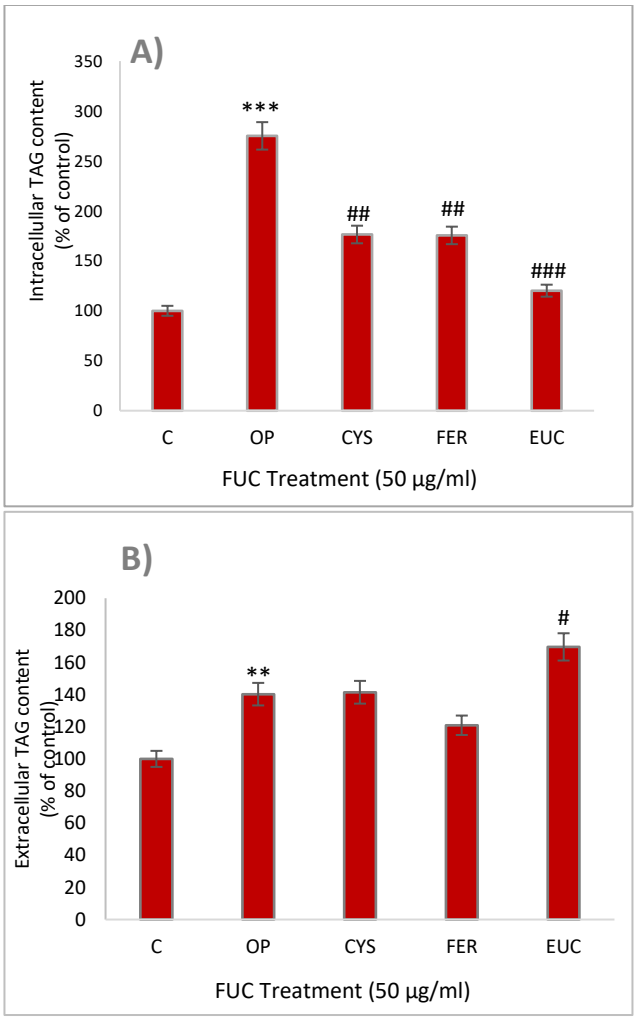
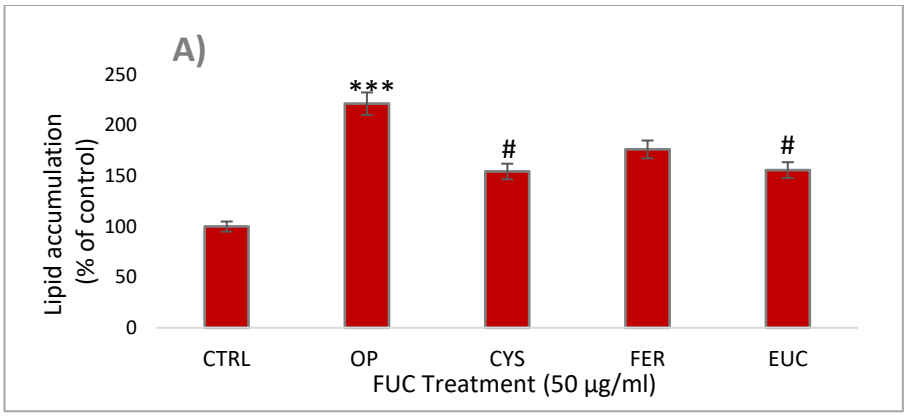


Figure 24: TAG content quantification in FaO cells. (**A:** *Intracellular TAG*; **B:** *Extracellular TAG*). (***p ≤ 0.001, **p ≤ 0.01 with respect to control; ### p ≤ 0.001, ## p ≤ 0.01, and # p ≤ 0.05 vs OP).

BODIPY STAINING

Lipid lowering property of FUC was confirmed by BODIPY/DAPI staining in which OP treatment resulted in a significant increase of neutral lipid content with respect to control (+121%; p ≤ 0.001). Both CYS and EUC treatment significantly decreased lipid content in steatotic FaO cells by 30.2% and 29.6% with respect to OP (p≤0.05), while this decrease was not significant for FER treatment (Figure 25A). Qualitatively, same results were obtained with fluorescence microscopy visualization with BODIPY 493/503 staining (Figure 25B).



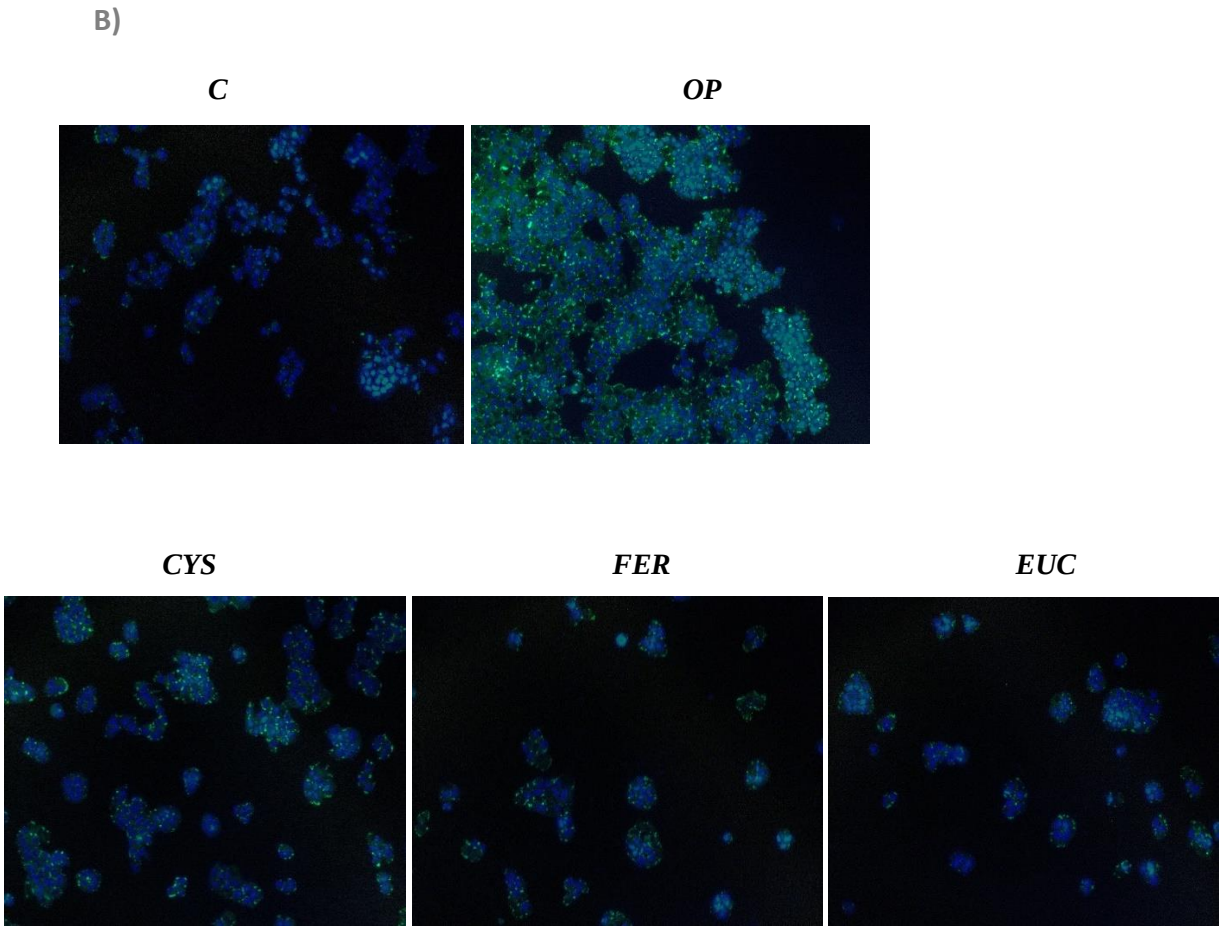


Figure 25: A-Lipid droplet analysis by BODIPY staining in FaO cells (***p ≤ 0.001 with respect to control; # p ≤ 0.05 vs OP). **B-** Representative images of BODIPY (green)/DAPI (blue) staining of FaO cells showing cytosolic LDs.

EFFECTS OF FUC ON GENES OF HEPATIC LIPID METABOLISM

FaO cells express a wide range of liver specific mRNA which makes this cell line suitable for investigating the effect of FUC on key genes regulating hepatic lipid metabolism. Using qPCR, the expression of several genes was investigated; both lipogenic genes and genes implicated in FFA oxidation.

Figure 26A shows the expression profile of lipogenic genes such as those coding for PPARs (Peroxisome Proliferator-Activated Receptors) transcription factors and for a family of LD-associated proteins (perilipins, PLINs). The most abundant PPAR isoforms expressed in FaO cells are PPARα and PPARγ; results indicated that OP treatment causes a slight increase in PPARα expression in steatotic FaO cells compared to control cells, and a return to control values after FUC treatment was apparent, but the results did not reach statistical significance. However, PPARγ expression was significantly induced by OP treatment (1.7-fold induction, $p \leq 0.01$) and a significant down-regulation to control levels was measured after incubation with CYS, FER, and EUC by 42%, 50%, and 60%, respectively ($p \leq 0.001$ with respect to OP). PLIN2 and PLIN5 hepatic expressions are regulated by PPARγ. Accordingly, the mRNA levels of both PLINs are significantly increased in steatotic FaO cells with respect to control by (1.5- and 2.2- fold induction, $p \leq 0.01$ and $p \leq 0.001$, respectively). FUC treatment significantly downregulated the expression for PLIN2 and PLIN5 to control values: CYS ($p \leq 0.01$, $p \leq 0.05$); FER ($p \leq 0.01$, $p \leq 0.001$); EUC ($p \leq 0.001$. $p \leq 0.001$), respectively.

On the contrary, as shown in figure 26C genes encoding for proteins implicated in FFA oxidation such as CPT-1 and CYP4A1 were slightly overexpressed in OP cells by comparing to control (1.45 and 1.42 folds). CPT1 expression in steatotic cells did not show any difference when treated with FUC extracted from C. compressa, F. hermonis, and E. globulus, compared to untreated steatotic cells, while for CYP4A1, a further increase was observed only with EUC ($p \leq 0.05$ with respect to control).

Moreover, expression of ApoB gene showed a slight increase upon OP treatment, and a subsequent treatment with EUC significantly increased its expression ($p \leq 0.001$, $p \leq 0.05$ with respect to control and OP, respectively), while CYS or FER treatment for steatotic cells did not show any significant changes.

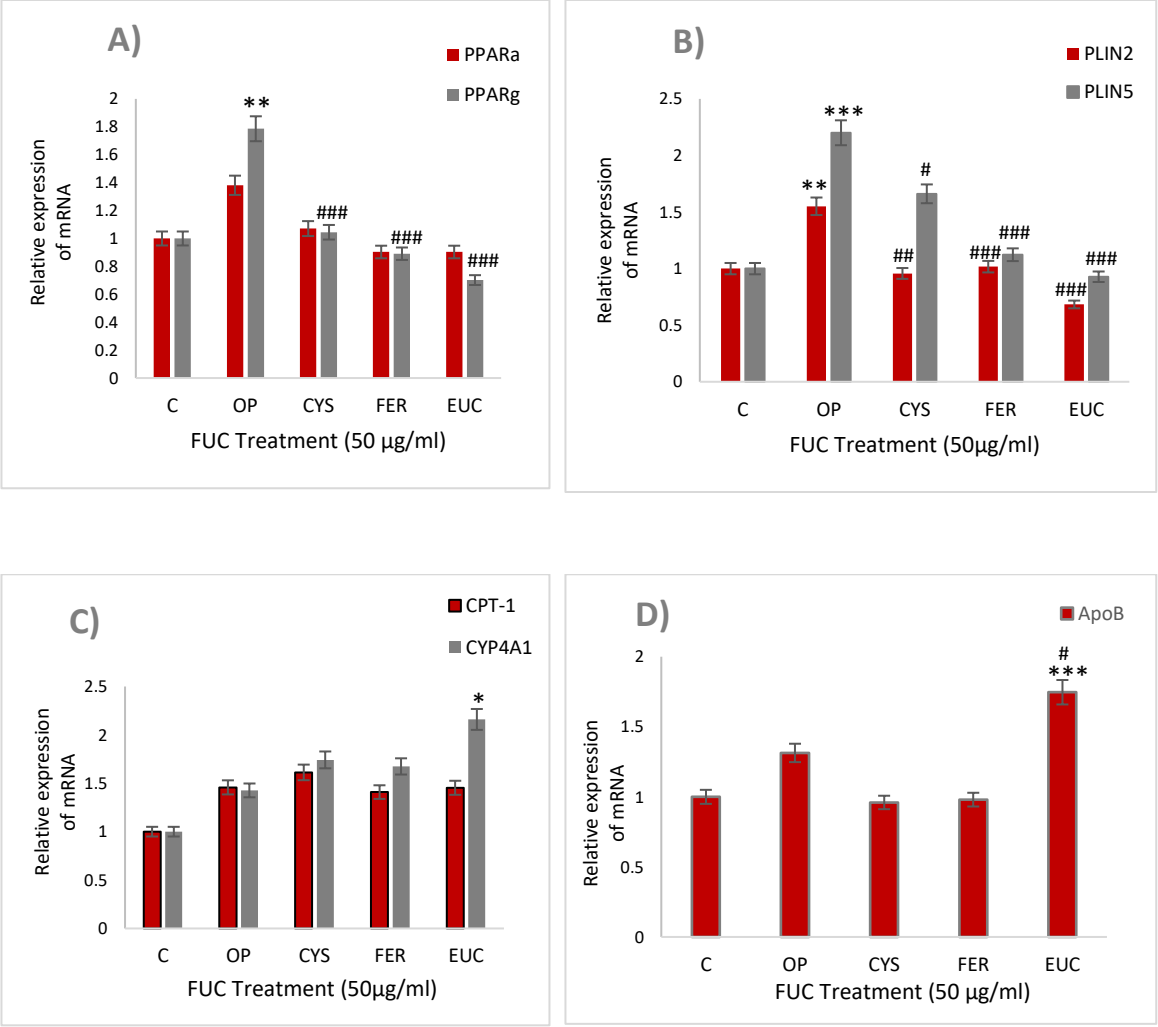


Figure 26: Effects of CYS, FER and EUC on hepatic gene expression. The relative mRNA expression of **A)** *PPARα and PPARγ*, **B)** *PLIN2 and PLIN5*, **C)** *CPT-1 and CYP41*, and **D)** *ApoB* was quantified by qPCR in control (C) and steatotic FaO cells incubated in the absence (OP) or in the presence FUC of 50 µg/mL for 24 h. Data are expressed as fold induction with respect to controls (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs C; # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ vs OP).

HECV (Human Endothelial Cord Vein Cells)

ANTIOXIDANT EFFECTS in HECV CELLS

CELL VIABILITY

Results obtained by MTT assay showed that none of the purified FUCs tested at different (12.5-100 µg/ml) exerts any cytotoxic effect on HECV cells; both in the absence and in the presence of OP. The intermediate concentration 50 µg/mL of CYS, FER, and EUC was selected for further experiments.

H_2O_2 treatment reduced cell viability by 50.6 % with respect to control untreated cells ($p \leq 0.001$ with respect to control). Furthermore, FUCs pretreatment slightly restored the percentage of viable cells upon H_2O_2 treatment.

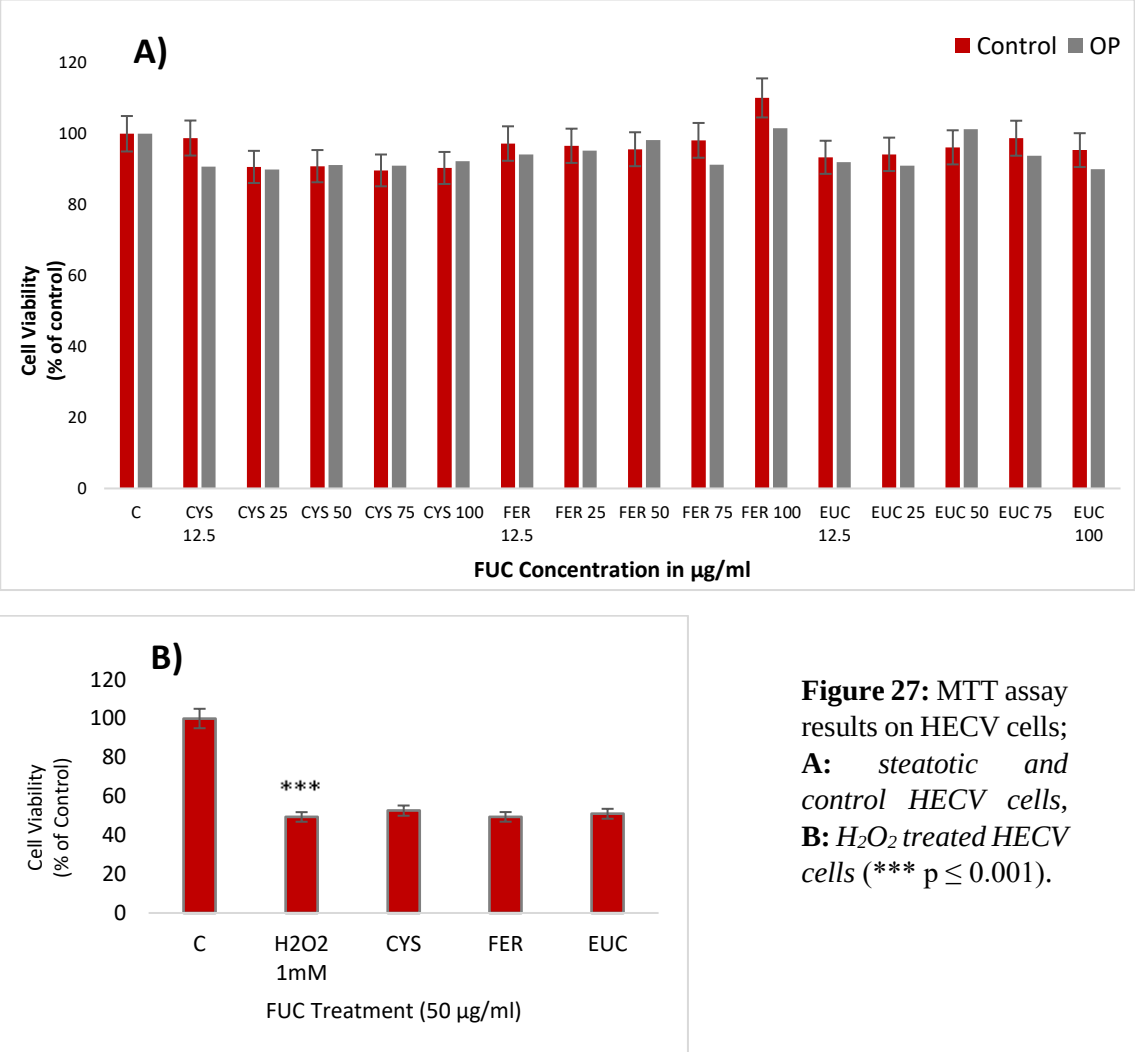


Figure 27: MTT assay results on HECV cells; **A:** *steatotic and control HECV cells*, **B:** *H_2O_2 treated HECV cells* (*** $p \leq 0.001$).

ROS PRODUCTION

QUANTITAIVE ANALYSIS

- ***Steatotic HECV:*** Antioxidant capacities of FUC were confirmed by monitoring ROS production in steatotic HECV cells where ROS production was significantly increased by 46 % compared to control cells ($p \leq 0.05$ with respect to control). Steatotic FaO cells pre-treated with FUC purified from CYS and FER significantly decreased ROS production to become about the same as control cells ($p \leq 0.05$ with respect to OP). EUC treatment lead to a further decrease in ROS concentration where this reduction was by 42 % compared to OP ($p \leq 0.01$ with respect to OP).

- ***H_2O_2 treated HECV cells:*** H_2O_2 treatment induces ROS production in HECV cells more than the steatotic model. H_2O_2 significantly increased ROS production by 91 % compared to control cells ($p \leq 0.001$ with respect to C), while FUCs purified from C. compressa, F. hermonis, and *E. globulus* reduced H_2O_2 induced ROS production by 31.6 %, 35.2 %, and 42.6 % ; respectively, as compared to only H_2O_2 treated cells. ($p \leq 0.01$ with respect to H_2O_2).

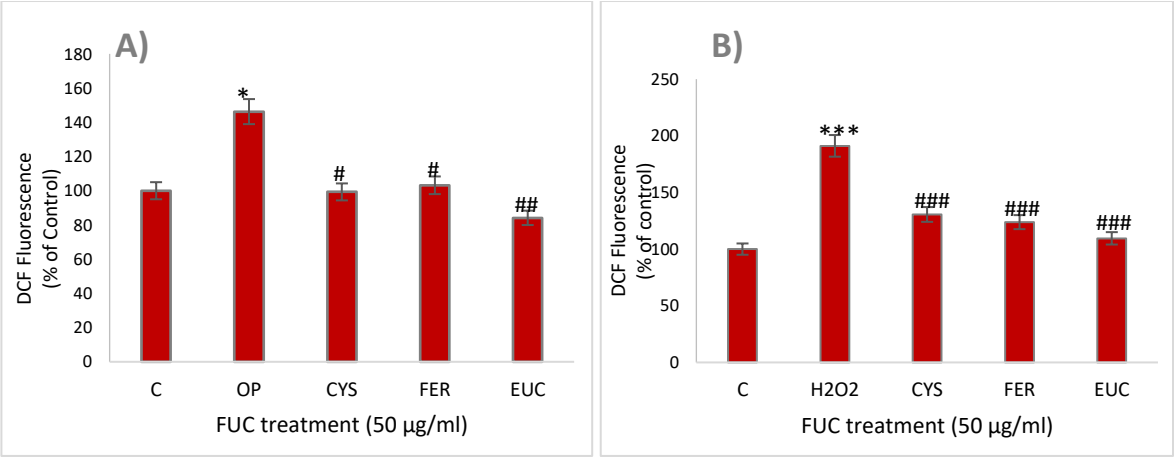


Figure 28: ROS production measured by DCF assay in HECV cells. **A:** *Steatotic HECV cells*; **B:** *H_2O_2 treated HECV cells.* (* $p \leq 0.05$, *** $p \leq 0.001$ vs C; # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ vs OP or H_2O_2).

QUALITATIVE MEASUREMENTS

H_2O_2 treated HECV cells showed an increased ROS production by 90 % compared to control cells ($p \leq 0.001$ with respect to control). CYS, FER and EUC significantly suppressed ROS production by 31.8 %, 35.3 %, and 42.8 %, respectively ($p \leq 0.001$ with respect to OP). These results were confirmed by microscopic qualitative analysis for the fluorescence emitted by DCF. As shown in figure 29 recorded images demonstrated that H_2O_2 treated cells have fluorescence

intensity greater than that observed in control cells. Moreover, H_2O_2 did not induce ROS production in FUC treated cells as it was in case of H_2O_2 treatment alone since CYS, FER, and EUC treated cells showed a reduced intensity as compared to H_2O_2 treated cells.

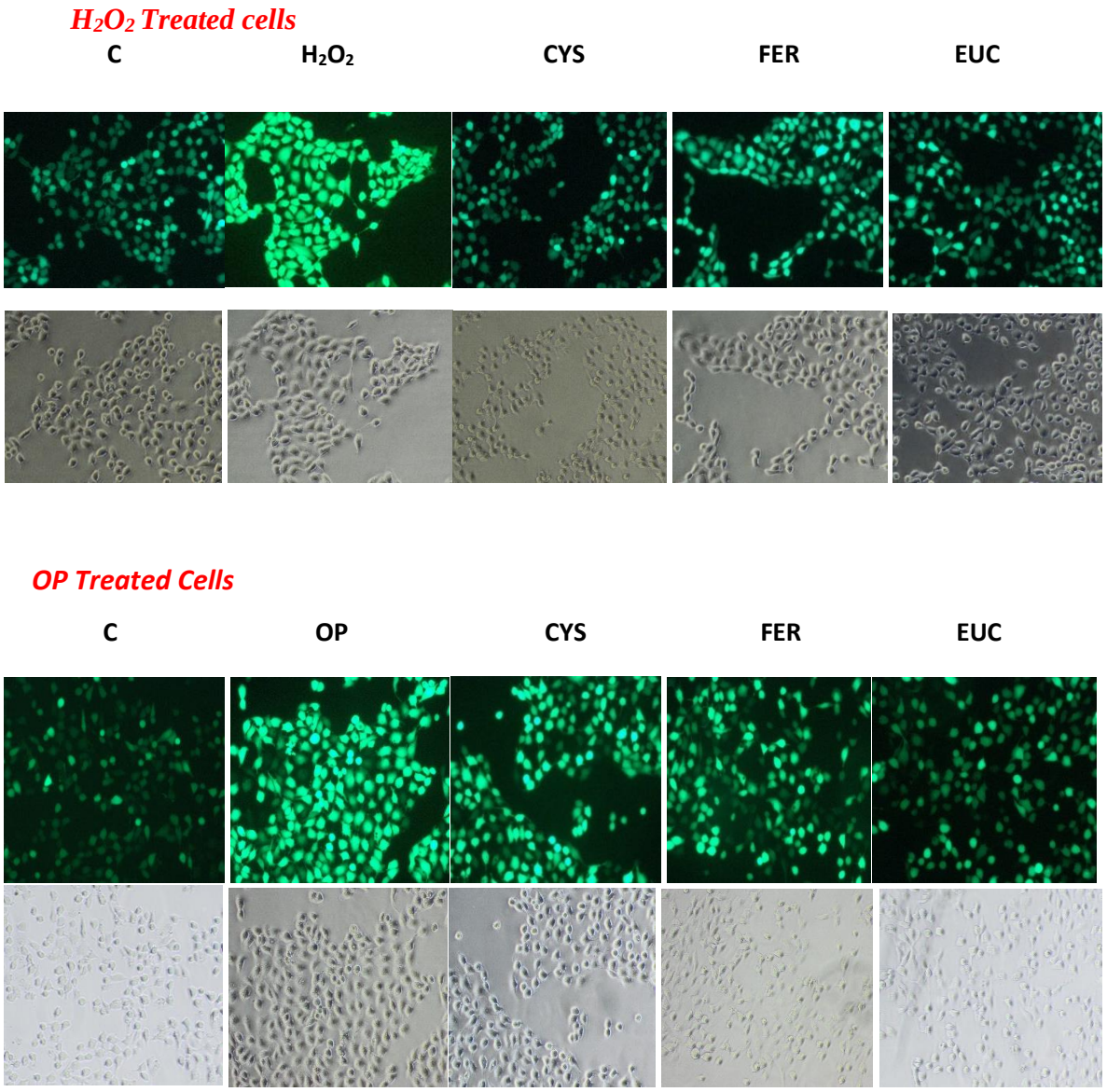


Figure 29: Representative images of DCF staining (green) to monitor ROS production in steatotic HECV and H_2O_2 treated HECV cells. Images were captured in the fluorescence and visible.

ANTI-STEATOTIC EFFECTS ON HECV CELLS.

Anti-steatotic effects of FUC on endothelial cells were investigated by TAG content quantification in the same way as FaO cells.

Spectrophotometrical analysis

- ***Intracellular TAG*** content was quantified in both control (C) and steatotic HECV cells in the absence (OP) or presence of FUC (CYS, FER or EUC). As shown in Figure… OP treatment for 3 h significantly increased TAG content with respect to control (+200%; $p \leq 0.01$). However, steatotic cells treated with FUC showed a slight decrease in TAG content for CYS and FER as compared to OP by 5.7% and 14.3 %; respectively, while FUC purified from *E. globulus* results in a significant TAG content decrease by 30.2% ($p \leq 0.05$ with respect to OP).

- ***Extracellular TAG*** content was significantly increased in steatotic cells by (+72%; $p \leq 0.01$) with respect to control cells. However, subsequent FUC treatment from the three different species did not significantly affect TAG secretion in HECV steatotic cells.

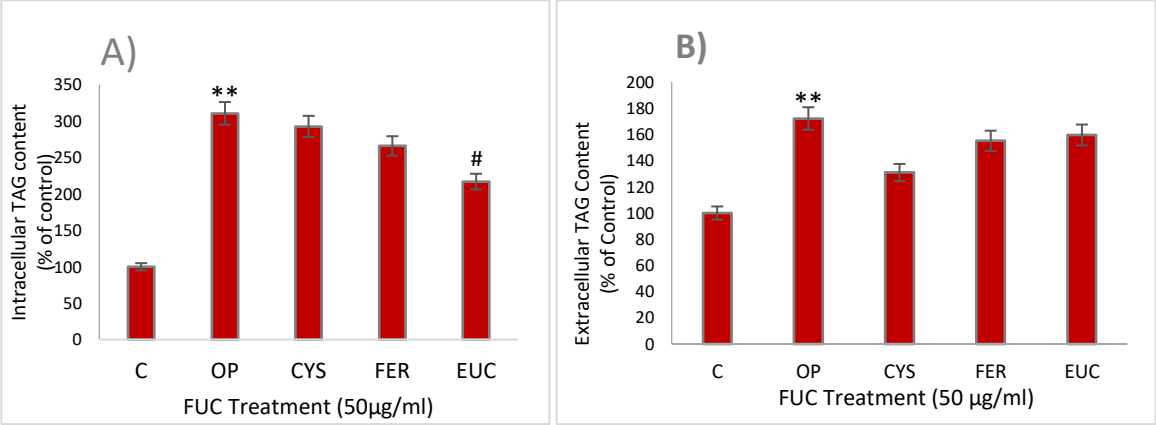


Figure 30: TAG content quantification in HECV cells. (**A:** *Intracellular TAG*; **B:** *Extracellular TAG*). (**$p \leq 0.01$ with respect to control; # $p \leq 0.05$ vs OP).

BODIPY Staining

BODIPY/DAPI staining method for TAG quantification was validated also in steatotic HECV cells. As shown in figure 31 results indicated that OP treatment significantly increased TAG content by 35 % as compared to control cells ($p \leq 0.05$). Steatotic endothelial cells pre-treated with FUC from CYS, FER, and EUC showed a slight non-significant decrease with respect to OP.

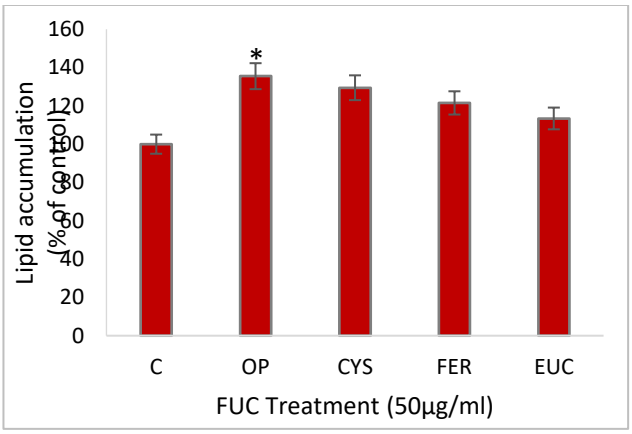


Figure 31: Lipid droplet analysis by BODIPY staining in HECV cells (*p ≤ 0.001 with respect to control).

NITRIC OXIDE PRODUCTION

NO production in HECV cells was measured indirectly by spectrophotometric quantification of the end products (nitrites and nitrates, collectively referred as NOx) using the Griess reaction as explained above. NO production in HECV cells was induced by OP or H_2O_2 treatment.

- **Steatotic HECV cells:** As shown in Figure 32A, OP treatment also significantly enhanced NO production in HECV cells compared to control (+ 118%, $p \leq 0.001$) and this increase was effectively reduced by FER, and EUC treatment by 32.5 and 40.7, respectively ($p \leq 0.001$ with respect to OP), while that caused by CYS treatment was not significant (16.5 % with respect to OP).
- **H_2O_2 Treated HECV Cells:** NO production was induced also by H_2O_2 treatment, in which NO release was increased by 60% as compared to control ($p \leq 0.001$), and significantly reduced in HECV treated by CYS, FER, and EUC by 51%, 30% and 54%, respectively by comparing to only H_2O_2 treatment ($p \leq 0.001$ for both).

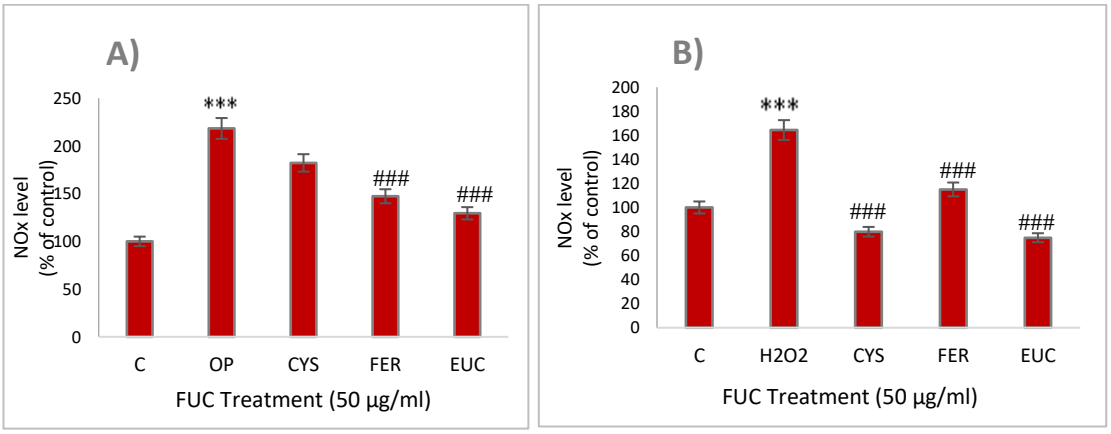


Figure 32: NOx production measured by Griess assay in HECV cells. **A:** *Steatotic HECV cells*; **B:** *H_2O_2 treated HECV cells.* (*** $p \leq 0.001$ vs C; ### $p \leq 0.001$ vs OP or H_2O_2).

V. DISCUSSION

NAFLD is a physio-pathological condition affecting 25% of the population worldwide; it does not simply represent a fatty liver, because its worsening is a burden to the liver and extra-hepatic tissues as well, being closely associated with cardiovascular diseases (Adams et al., 2017). Previous studies showed the impact of a lipid- rich diet during pregnancy as it predisposes offspring for the later development of NAFLD (Hughes et al., 2014). Apart from dietary and lifestyle interventions, there is still a lack of therapies for NAFLD, and research is addressed to discover new natural compounds able to counteract NAFLD onset and progression, possibly without the side effects of synthetic drugs. The literature about algae as nutritional and functional food sources, is extremely rich (Wells et al., 2018) and FUC from different seaweeds are the most extensively studied algal polysaccharides in relation to NAFLD, showing efficacy both *in vivo* and *in vitro* experimental models, as we recently reviewed (El Rashed et al., 2020).
Rat hepatoma FaO cells treated with a mixture of fatty acids (OP) represents an in vitro model of NAFLD, that we previously validate to study the molecular mechanisms underlying NAFLD onset and progression as well as putative therapeutic agents (Grasselli et al., 2017). Our results indicated that FUC purified from three different species at low concentration (50 µg/mL) exhibited an anti-steatotic and anti-oxidant potentials illustrated by lowering intracellular TAG content in lipid-overloaded cells and down-regulating ROS production induced by excessive fat, respectively.

Increased ROS production in steatotic hepatocytes due to ER stress, mitochondrial and peroxisomal dysfunction contribute to OS and inflammation development. The interaction of produced ROS with polyunsaturated fatty acids (PUFA); known as lipid peroxidation ends in formation of highly reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) that have longer half-lives than ROS. Furthermore, lipid peroxidation activates kupffer cells to release inflammatory cytokines such as TNF-α and IL-6. Thus, ROS might contribute to immunological dysfunction and inflammation, triggering NAFLD progression steatohepatitis, fibrosis cirrhosis, leading hepatocellular carcinoma in some cases (Ucar et al., 2013) (Masarone et al., 2018)
FaO cells are suitable to monitor lipid metabolism regulation as they express a wide range of liver specific genes which are involved in lipogenesis and FAs oxidation. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors playing a key role in the regulation of hepatic lipid balance, and their expression is targeted in NAFLD therapies (Mello 2015; liss et al., 2017).
In previous studies, lipid over-loaded FaO steatotic cells showed an increased PPARγ expression (Grasselli et al., 2017). PPARγ is a marker of fatty liver and it is highly expressed in NAFLD to induce energy storage, as it mediates the activation of lipogenic genes (Rahimian et al., 2001). So, the mechanism of action for several drugs in down-regulating PPARγ expression was detected also by CYS, FER, and EUC treatment in which the results explain the decrease in intracellular TAG content.

PPARγ regulate the expression of other genes such as PLIN2 and PLIN5 important for TAGs storage within LDs (Schadinger et al., 2005; Wolins et al., 2006). PLINs (perilipins) are a family

of LD-associated proteins that stabilize LDs formation and play important roles in their metabolism, thus trafficking lipid inside and outside the cell (Bickel et al., 2009; Kimmel et al., 2010). In accordance with PPARγ expression profile, PLIN2 and PLIN5 expression was significantly enhanced in steatotic cells and decreased after FUC treatment from the three different species. Furthermore, accumulated neutral lipid percentage obtained from BODIPY staining measured by the fluorimeter, as well as their representative images indicate less LDs in FUC-treated steatotic cells.

Concerning genes implicated in oxidative and/or secretion pathways, neither PPARα nor CPT-1 expression was modified upon FUC treatment which means that FAs transport into mitochondria and their oxidation respectively were not affected. CYP4A1 plays a central role in microsomal ω- oxidation of fatty acid, and its expression does not significantly change in OP treated cells as obtained before by Vecchione et al. (Vecchione et al., 2016), while EUC treatment only cause a slight increase in CYP4A1 expression as compared to control, which means induction of FAs oxidation. Moreover, the secretion of TAGs as very low-density lipoproteins was altered only by EUC treatment in steatotic FaO cells, as it significantly induced ApoB gene expression, which codes for the main component of VLDL.

Taken together, these results confirm the anti-steatotic action of FUC purified from C. compressa, *F. hermonis* and *E. globulus* realized by mechanisms that involve energy storage down-regulation and changes in LD trafficking, while only EUC stimulate microsomal oxidation of FAs and promote their secretion.

Endothelial cells serve as a permeable barrier between blood vessels and tissues to regulate blood flow and allow energy supply, thus their proper functioning is pivotal while endothelial dysfunction plays a central role in different metabolic disorders such as obesity, impaired glucose metabolism, hypertension and dyslipidemia (Tziomalos et al., 2010).Moreover, endothelial cells are the most abundant non parenchymal cells in the liver where they are crucial for nutrients, lipids, and lipoproteins transfer. Miyao *et al* indicated that endothelial injury might have a "gatekeeper" role in NAFLD pathogenesis toward NASH (Miyao et al., 2015). Atherosclerosis defined as fat and cholesterol build up in the artery wall, initiates by a number of events being the most important is endothelial damage, and significantly associated with NAFLD (Thakur et al., 2012) .Therefore, anti-steatotic and anti-oxidant effects of CYS, FER, and EUC were investigated also in human endothelial cells (HECV). As shown previously, fatty acid treated HECV cells accumulate cytosolic lipids and could be considered as atherosclerosis model (Vergani et al. 2018). Our results confirmed lipid accumulation within HECV in which a significant increase in intracellular and extracellular TAG content in OP- treated cells. Exposure of fat enriched HECV cells to FUC purified from *E. globulus* significantly decreased intracellular TAG content and did not affect extracellular TAG content, while CYS and FER treatment did not modify lipid accumulation, neither intracellular nor extracellular one.

OS in HECV cells was induced by OP and H_2O_2 treatment where ROS production was significantly increased in both cases. CYS, FER, and EUC treatment after OP or H_2O_2 incubation significantly reduced ROS production in HECV cells.

NO is a key cellular signaling molecule involved in a variety of biological functions throughout the whole body, at low concentrations it regulates leucocyte adhesion, platelet aggregation, vascular tone and blood flow by activating soluble guanylate cyclase in the vascular smooth muscle. Under pathological conditions like hypertension, atherosclerosis, and angiogenesis-associated disorders, inducible nitric oxidase synthase expression is upregulated resulting in vascular NO overproduction and transport abnormalities (Luiking et al., 2010). Furthermore, previous studies indicated increased iNOS levels in NAFLD patients with significant hepatic steatosis as compared to those with lower score of steatosis (Elsheikh et al., 2017). In this study, FER and EUC significantly decreased NO production, after it was significantly induced by OP or H_2O_2 treatment. However, for CYS this reduction was significant only in H_2O_2 treated HECV cells.

The difference in biological activities is related to structural variation which can be attributed to the environmental changes between plants and brown algae, species diversity, molecular weight, monosaccharide composition, extraction method, harvesting season, sulfate content, and sulfate content (Wang et al., 2019). At species level, *E. globulus* have gained more interest and more studies were done than *C. compressa* and *F. hermonis* to investigate the medicinal applications of these species. Previous studies showed that *E. globulus* has been used for treatment of various diseases such as fungal infection, diabetes, influenza, and pulmonary tuberculosis. Another study shows that the extract of *E. globulus* leaves reduced lipogenesis, inflammatory cytokine expression and OS, and inhibited NASH induced by fructose ingestion in rats (Takahashi et al., 2015). While for cystoseira compressa, previous studies have showed that *C. compressa* aqueous extracts have good anti-microbial, antioxidant, anti-inflammatory and anti-proliferative activities against HCT and MCF-7 cell lines (Mhadhebi et al., 2014). FUC purified from *C. compressa* that showed gastroprotective effect and inhibited gastric lesions (Hadj Ammar et al., 2015). Moreover, FUCs isolated from *C. compressa* exhibited a significant anti-inflammatory and antioxidant activity and a significant decrease of gastric mucosal damage. However, regarding *F. hermonis*, it has been shown that it exerts antibacterial, anti-inflammatory, antidiabetic, hypolipidemic, and hepatoprotective effects. These biological activities are related to the presence of a wide range of active compounds such a sulfur-containing compounds, sesquiterpenes, coumarins, ferutinin, α-pinene, camphene and carvacrol, as well as vitamins (A and E) and some minerals (Sattar et al., 2017). According to the chemical composition reported by Abdel-Kader et al. (Abdel-Kader et al., 2011), *F. hermonis* roots contain 38.38% carbohydrates, 8.33% lipids, and 3.48% proteins. FUC obtained from *E. globulus* was characterized with the highest sulfate content as compared to the other two species, as FUC with higher sulfate content exerts more anti-oxidant effect (li et al., 2008), confirming our results which states that EUC is the most potent antioxidant as compared to FUC and FER. Furthermore, the difference in sulfate position can affect FUC biological properties. Therefore, all these factors that mediate the therapeutic effects of the extracted polysaccharide results in making FUC purified from *E. globulus* with more antioxidant and anti-steatotic potentials than that purified from *C. compressa* and *F. hermonis*.

VI. CONCLUSION

In this study we were able to study the antioxidant and lipid lowering effect of FUC purified from Cystoseira compressa, Ferula hermonis, and Eucalyptus globulus. These effects point to candidate FUC as a possible bioactive compound against NAFLD progression towards more serious conditions and counteract the progression of OS disorders related to NAFLD such as atherosclerosis. . Fucoidan purified from *E. globulus* exhibited the most antioxidant and anti-steatotic potentials using two cellular models. The biological properties of FUC are possibly linked to the pharmaceutical properties of the three vegetal sources of FUC that we analyzed (CYS, FER and EUC), which are widely used in popular medicine for a variety of diseases, including OS-associated pathologies such as inflammatory disorders, neurodegenerative conditions, diabetes, atherosclerosis, stroke, asthma and cancer.

GENERAL CONCLUSIONS

In this thesis, we purified FUC, the water soluble fucose sulfated polysaccharide, from the brown algae (C. compressa) collected from the Lebanese coast and the terrestrial plant (F. hermonis) harvested from the "Mountains of Bekaa" for the first time. Some structural and chemical compositional differences were determined between CYS, FER, and fucoidan purified previously from the terrestrial plant (E. globulus). *E. globulus* showed the highest content of fucose, sulfate and sugar. The structural difference may refer to the vegetal species and environmental conditions variation.

FUC purified from the three species possess a potent antioxidant activity measured by different in vitro spectrophotometrical assays.

Our cellular in vitro models were composed of hepatoma FaO cells and endothelial HECV cells incubated with Oleate/Palmitate mixture to induce steatosis and endothelial damage.

The protective effect of FUC against OS was confirmed by preventing cell viability decrease caused by H_2O_2 incubation, and through lowering ROS production in FUC- treated steatotic FaO cells. Furthermore, ROS increased production in steatotic FaO, steatotic HECV, and H_2O_2 treated HECV cell was reduced upon CYS,FER, and EUC treatment.

FUC reduces the lipid content in steatotic liver cells and in fat-enriched endothelial cells. The antisteatotic action exhibited in hepatocytes points to candidate FUCs as possible bioactive compounds against NAFLD onset and progression towards more serious conditions. The effect of counteracting lipid accumulation in endothelial cells may be associated with effective actions against atherosclerosis.

In liver cells, the anti-steatotic effect of FUC is associated with a decrease in the expression of PPARγ, a known marker of hepatic steatosis and is implicated in the stimulation of lipogenesis. In accordance, the hepatic expression of ADRP and OXPAT, which are under the control of PPARγ and stabilize LDs, is decreased. These results suggest that the anti-steatotic effect of FUC is realized through an inhibition of DNL and an increase in hepatic LD turnover. Excess TAGs contained in LDs could be directed to secretion and/or oxidative pathways. Our results on gene expression pointed to a stimulation of oxidative pathway and secretion mechanisms for FUC purified from *E. globulus* where CYP4A1 and ApoB expression were induced.

Furthermore, results indicated an increased level of nitric oxide production in fat enriched and H_2O_2 treated HECV endothelial cells. CYS, FER, and EUC significantly decreased NO production, after it was significantly induced by OP or H_2O_2 treatment. This means that FUC ameliorates endothelial integrity and function.

The difference in algal species, environmental variations, monosaccharide composition, molecular weight, and extraction method all together affect the structure and biological activities

of purified FUCs with EUC being the most potent polysaccharide in all experiments tested in this thesis.

FUC displays a protective effect against oxidative damage in erythrocytes, hepatocytes, and endothelial cells. This is possibly linked to the pharmaceutical properties of the three vegetal sources of FUC that we analyzed (CYS, FER and EUC), which are widely used in popular medicine for a variety of diseases, including oxidative stress-associated pathologies such as inflammatory disorders, neurodegenerative conditions, diabetes, atherosclerosis, stroke, asthma and cancer.

Thus, it can be concluded that an increased intake of FUC in the diet, either as dietary supplements or through an increased consumption of FUC-rich edible algae and plants, may help to counteract the onset or progression of oxidative stress-related disorders such as NAFLD and atherosclerosis.

FUTURE PERSPECTIVES

In the next steps, we would like to:

- Investigate the anti-inflammatory effects of the three purified fucoidan.
- Study the absorption of FUC, using human intestinal Caco-2 cells for example.
- Anti-cancer activity of FUC (mainly EUC).
- Study the biological activities using an *in vivo* model.

PUBLICATIONS

Masters

- "Peptides for Skin Protection and Healing in Amphibians". Molecules Journal, 2019, 24, 347. **SECOND AUTHOR**
- "Use of an in vitro model of hepatic steatosis for studying the anti-oxidant and anti-steatotic effects of fucoidan polysaccharides**".** Biomedical Science and Engineering, 2019. Vol. 3(s3): 109. **FIRST AUTHOR**
- "The Antioxidant, Antibacterial, Antihemolytic, and Epithelial Ovarian Cancer Antiproliferative Activities of the Lebanese Plant Salvia Libanotica". Asian Journal of Science and Technology, 2018. Vol. 09, Issue, 03. **IN COLLABORATION**
- "'EPIDEMIOLOGY OF HELICOBACTER PYLORI INFECTION AMONG THE HEALTHY POPULATION IN LEBANON". World Journal of Pharmacy and Pharmaceutical Sciences; 2017. Volume 6, Issue 6. **IN COLLABORATION**

Ph.D.

- "Peptides for Skin Protection and Healing in Amphibians" Review paper, Molecules, 2019; 24: 347. **IN COLLABORATION.**
- " Brown-Algae Polysaccharide as Active Constituents against Nonalcoholic Fatty Liver Disease". Review paper, PLAMED Journal, 2020. **FIRST AUTHOR.**
- **"** Synthesis, Photoisomerization, Anti-oxidant Activity, and Lipid-Lowering Effect of Ferulic Acid and Feruloyl Amides" Molecules, 2021; 26: 89. **IN COLLABORATION.**
- " Anti-oxidant and anti-Steatotic activities of a New fucoidan Extracted from Ferula hermonis roots harvested on Lebanese mountains" Molecules, 2021. **FIRST AUTHOR.**
- " Anti-oxidant and anti-Steatotic activities of fucoidan polysaccharide extracted from Eucalyptus globulus and Cystoseira compressa". In preparation **FIRST AUTHOR.**

POSTERS

- Poster in 69th SIF National Congress Italian Physiological Society**. "Anti-steatotic effects of an antimicrobial peptide isolated from amphibian skin''** Ilaria Demori, L.Queirolo, L.Rovegno, K. Cortese, Z. El Rashed, D.Granglia, E. Millo, A. Salis, S.Salvidio, L.Canesi, E.Crasselli. Italy-Florence- 21, 22-September 2018.
- Poster in 2nd Centro 3R (Reduce, Replace, and Refine) Annual Meeting**. "Use of an in vitro model of hepatic steatosis for studying the anti-oxidant and anti- steatotic effects of fucoidan polysaccharides"**. El Rashed Z, Khalife H, Voci A, Grasselli E, Canesi L, Demori I. Italy-Genoa, 20-21, 2019.
- Poster in joint meeting of the Federation of European Physiological Societies and the Italian Physiological Society. **"Anti-steatotic and anti-oxidant effects of fucoidan polysaccharide".** El Rashed Z, Khalife H, Voci A, Grasselli E, Canesi L, Demori I. Italy – Bologna, September 10th to 13th, 2019.
- Poster in joint meeting of the Federation of European Physiological Societies and the Italian Physiological Society. **"Amphibian skin peptide macrotympanain A1 powerfully reduces lipid accumulation in a cellular model of hepatic steatosis".** Demori I, Lobasso S, Lopalco P, Corcelli A, Bellese G, Queirolo L., El Rashed Z, Millo E, Salvidio S, Canesi L, Cortese K, Grasselli E. Italy- Bologna, September 10th to 13th, 2019.
- Poster in symposium of the Zoological Society of London **"Mitigating single pathogen and co- infections that threaten amphibian biodiversity". "Wound healing as a possible mechanism contributing to resistance to chytridiomicoses".** Grasselli E. El Rashed Z, Rovegno L, Queirolo L, Canesi L, Catenazzi L, Salvidio S, Demori I. London, 24th-25th April 2019.

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