“Unveiling the Therapeutic Potential of *Sarcopoterium spinosum* Fruits: A Comprehensive Exploration from Chemical Characterization to Cellular Models of Health Challenges.”

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Table of Contents

Acknowledgments 2

Table of Contents 3

List of abbreviations 5

List of figures 7

List of tables 8

Abstract 9

1. Chapter one: Introduction 12

1.1. History and Constituents of Medicinal Plants 12

1.2. Rosaceae Botanical Background 14

1.3. Sarcopoterium spinosum 15

1.4. Polyphenols 18

1.5. Metabolic disorders 20

1.5.1. Non-alcoholic fatty liver disease 21

1.5.2. Endothelial dysfunction 22

1.5.3. Insulin resistance 24

1.6. Cellular models 26

1.6.1. Cellular model of hepatic steatosis 26

1.6.2. Cellular model of endothelial dysfunction 27

1.6.3. Cellular model of insulin resistance 27

2. Chapter two: Aims of the study 29

3. Chapter Three: Polyphenol-enriched extracts of Sarcopoterium spinosum fruits for counteracting lipid accumulation and oxidative stress in an in vitro model of hepatic steatosis. 31

3.1. Introduction 34

3.2. Materials and methods 35

3.3. Results 41

3.4. Discussion 52

4. Chapter Four: The anti-inflammatory potential of an ethanolic extract from Sarcopoterium spinosum fruits for protection and/or counteraction against oxidative stress in dysfunctional endothelial cells 57

4.1. Introduction 60

4.2. Materials and methods 62

4.3. Results 67

4.4. Discussion 74

5. Chapter Five: Development of human models of insulin resistance in hepatoma HepG2 cells 80

5.1. Introduction 83

5.2. Material and methods 84

5.3. Results 87

5.4. Discussion 94

Conclusions 97

Bibliography 100

List of abbreviations

ABTS	2-2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonate)
APAP	N-acetyl-para-aminophenol
BSA	Bovine serum albumin
CAT	Catalase enzyme
Cg	Corilagin
DCF	2′-7′dichlorofluorescein
DCF-DA	2′-7′dichlorofluorescin diacetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
GAE	Gallic Acid Equivalents
LD	Lipid droplet
MDA	Malondialdehyde
NAFLD	Non-alcoholic fatty liver disease
OP	Steatotic cells
PCs	Phenolic compounds
QE	Quercetin equivalent
Qu	Quercetin
ROS	Reactive oxygen species
SBWE	Boiling water extract
SEE	Ethanol extract
SWE	Water extract
TA	Tormentic acid
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant activity
TG	Triglyceride
TPC	Total phenol content
DMEM	Dulbecco’s Modified Eagle’s Medium
FBS	Fetal Bovine Serum
GC	Glucose Consumption
HCL	Hydrochloric Acid
HepG2	Human Hepatoma Cell Line
H_2SO_4	Sulfuric Acid

IR	Insulin Resistance
KOH	Potassium Hydroxide
MetS	Metabolic Syndrome
NaOH	Sodium Hydroxide
ORO	Oil-RedO Staining
PB	Phosphate Buffer
OP	Oleate/Palmitate
T2D	Type 2 Diabetes
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H2O2	Hydrogen peroxide
HECV	Human endothelial cells
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
PPs	Polyphenols
SEE	Sarcopoterium spinosum fruits ethanol extract

List of figures

Figure-1 Thesis graphical abstract.....11

Figure 3- 1 Chapter three graphical abstract.....31
Figure 3- 2 Characteristics and distibution of Sarcopterium spinosum shrub 42
Figure 3- 3 HPLC-MS/MS analysis and cytotoxicity of the S. spinosum ethanolic extract 44
Figure 3- 4 Antisteatotic activity of the S. spinosum fruit ethanolic extract 47
Figure 3- 5 Antioxidant activity of the S. spinosum fruit ethanolic extract 49
Figure 3- 6 Antisteatotic and antioxidant activity of Corilagin and Quercetin.....52

Figure 4- 1 Chapter four graphical abstract.....57
Figure 4- 2 S. spinosum characteristics and distribution.....63
Figure 4- 3 Antioxidant activity of the S. spinosum fruit ethanolic extract 70
Figure 4- 4 Anti-inflammatory activity of the S. spinosum fruit ethanolic extract.....72
Figure 4- 5 Wound repair promoting effect of the S. spinosum fruit ethanolic extract.....73

Figure 5- 1 Chapter five graphical abstract.....80
Figure 5- 2 Steatotic activity of Oleate/Palmitate on HepG2 cells.....90
Figure 5- 3 Insulin effects with different concentrations and time exposure on HepG2 cells 91
Figure 5- 4 Comparative analyses between hyperinsulinemia and lipid accumulation to induced insulin resistance.....93

List of tables

Table 3- 1 Phytochemical content and antioxidant ability of S. spinosum fruit extracts.....43

Table 3- 2 Major components identified in S. spinosum fruit ethanol extract by using HPLC-MS/MS in the negative ionization mode.....45

Table 4- 1 Major components identified in the ethanolic extract from S. spinosum fruit by using HPLC-MS/MS in the negative ionization mode and their respective abundance 68

Abstract

The search for new bioactive compounds is of increasing interest, and it seems that despite new technical and scientific progress, most of the active ingredients have been known for centuries. Indeed, plants are an inexhaustible source of bioactive compounds that have been used since ancient times in both folk medicines and as preservatives of food. Medicinal plants have always been of interest for many kinds of industries because of their multiple applications for their antioxidant, antibacterial and cytoprotective properties. Plants, and plant-derived secondary metabolites may be applied in the management of a broad spectrum of metabolic dysfunction including obesity, fatty liver, cardiovascular disease. Metabolic diseases encompass a broad category of disorders characterized by disruptions of the body's metabolic homeostasis. These conditions impact on many metabolic pathways, such as glucose and lipid metabolism, with severe consequences for human health.

Many phytochemicals being found in plants may exhibit diverse properties that can modulate metabolic pathways, enhance insulin sensitivity, and regulate lipid metabolism. Among them, *Sarcopoterium spinosum* has emerged as a noteworthy medicinal plant with the potential to address metabolic diseases. Indigenous to the Mediterranean region and the Middle East, this botanical species boasts a rich history in traditional medicine. Recent research has spotlighted its anti-diabetic properties, particularly in the context of its root extracts exhibiting the capacity to regulate blood glucose levels and enhance insulin sensitivity. A flourishing interest has emerged regarding its role as a natural remedy for metabolic disorders, contributing to the expanding array of medicinal plants with the potential to promote metabolic equilibrium and overall well-being. Notably, ongoing investigations have predominantly focused on the roots rather than the fruits of *S. spinosum*.

In the context of this exploration, my PhD thesis aims to study different extracts derived from *S. spinosum* fruits, examining their bioactive compounds and their promising potential in ameliorating health and metabolic diseases. Three extracts were prepared from *S. spinosum* fruits, employing water, boiling water, and ethanol as extraction solvents. The ethanol extract, being distinguished by its superior radical scavenging potential, was selected for a detailed examination of the polyphenolic profile and the investigation of the potential beneficial effects. Two relevant cellular models were employed in this study: the rat hepatoma cell line (FaO) to mimic a cell model of hepatic steatosis, and the human endothelial cell line (HECV) to mimic a dysfunctional endothelium. The findings unveiled a rich profile of bioactive compounds for the ethanol extract, highlighting its abundance in ellagitannins, flavonoids, and terpenoids. Notably, this comprehensive chemical composition corresponded with the extract's demonstrated lipid-lowering, antioxidant, anti-inflammatory, and cytoprotective properties. In the final step of our investigation, our focus shifted to the design and develop a cellular model of insulin resistance utilizing the human hepatoma cell line HepG2. Employing hyperinsulinemia and fat accumulation as inducers, we successfully elicited the insulin resistance *in vitro* and this model will be tested using the *S. spinosum* extracts.

In conclusion, the outcome of this PhD thesis shows that *S. spinosum* fruits could be a source of many bioactive compounds and the extracts represent a promising candidate to develop nutraceuticals or dietary supplements to treat/prevent obesity-related metabolic diseases.

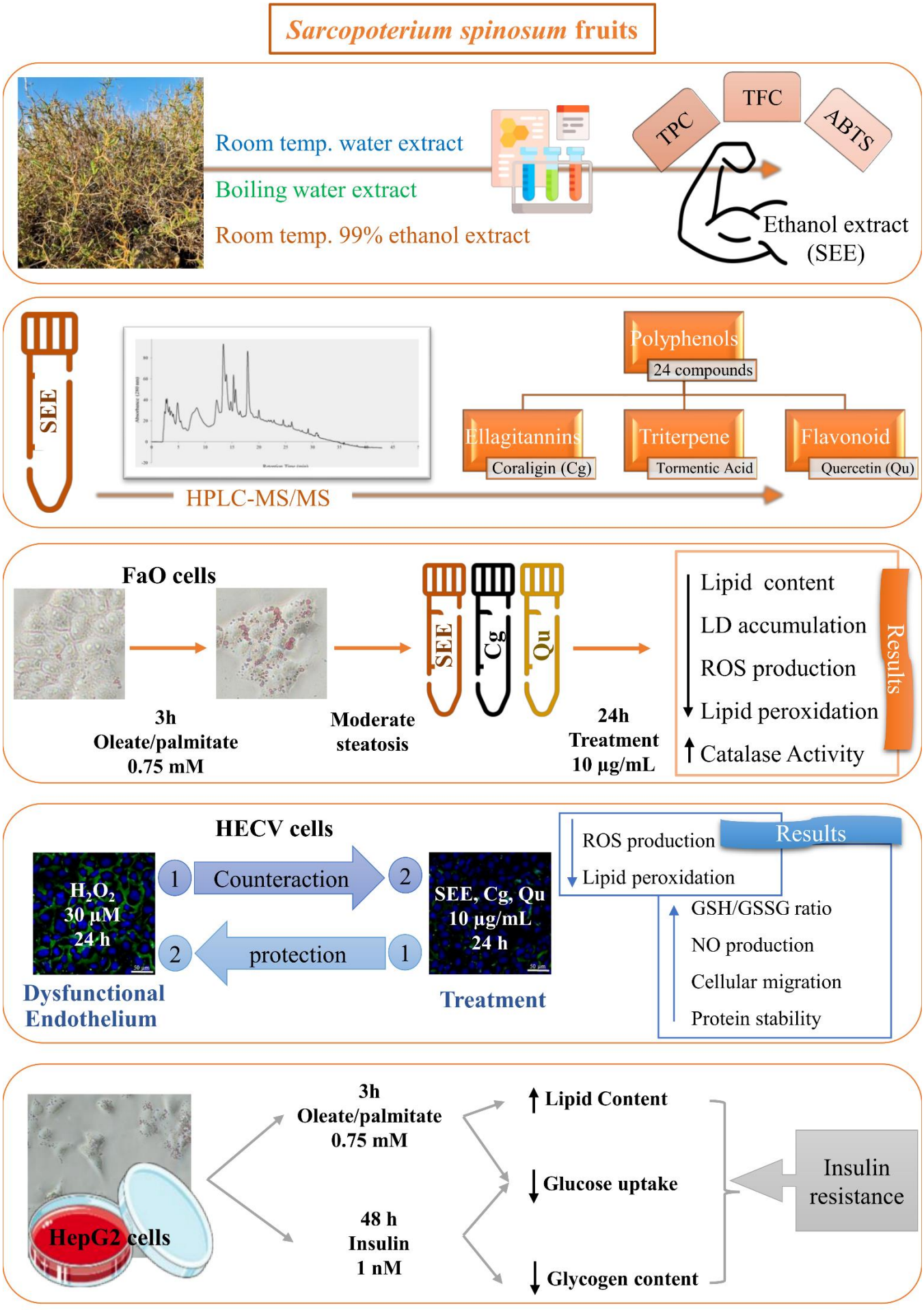

Figure-1 Thesis graphical abstract.

1. Chapter one: Introduction

Oxidative stress is a condition characterized by the disrupted balance between the generation of reactive oxygen species (ROS) or free radicals and the body's antioxidant defence mechanisms, tilting the scale in favour of oxidants. This phenomenon plays a pivotal role in numerous physio-pathological conditions including many chronic diseases, contributing to various pathological processes. Diabetes, cancer, arthritis, atherosclerosis, muscular dystrophy, pulmonary dysfunction, ischemia-reperfusion tissue damage, aging, degenerative diseases, and neurological disorders like Alzheimer and Parkinson diseases are associated with oxidative stress.

The detrimental impact of oxidative stress is evident in the oxidation of essential cellular components such as lipids, nucleic acids, and proteins. This oxidation interferes with the physiological cellular functions, ultimately leading to cell senescence, growth arrest, and cell death.

Throughout history, people have turned the interest to bioactive molecules derived from plants due to their natural, effective, and low side-effect properties. Notably, there has been extensive research on plants rich in polyphenols, which have been thoroughly studied and characterized for their diverse array of antioxidant properties. Therefore, polyphenols identified and extracted from various plants are being actively investigated as potential candidates for disease prevention and health maintenance. In essence, the exploration of polyphenol rich plants and their isolated bioactive compounds represents a promising avenue in the quest for natural solutions to combat oxidative stress and its associated chronic diseases.

1.1. History and Constituents of Medicinal Plants

Plants provide an important bio-resource of potential therapeutic compounds. Due to their wide therapeutic spectrum and minimal side effects, people count on plants to treat different diseases. The whole plant as well as the seeds, fruits, roots, leaves, skin and flowers are usually collected

at different seasonal times [1] and used as tincture, extract, infusion, inhalation, cream or active ingredients[2]. Active compounds isolated from medicinal plants and found to exert physiological effects on living organisms are used to synthetize drugs.

Since ancient time, plants have been treasured for their healing and pain relieving ability and 75% of the medicines rely on plants' curative property [3]. The selection and exact harvest time of plants have always been done randomly [4]. Pollens were collected from Neanderthal burial at Shanidar caves indicating that plants have been used as medicines almost 60000 years ago [5]. Initially, the knowledge about plants' application to treat certain ailment were only orally transmitted. Later, scripts were discovered dating back to almost 5000 years ago in China, Egypt, and India, and at least 2500 years ago in Central Asia and Greece. In fact, hundreds of medicinal plants, such as opium, were listed on clay tablets dating back to 5000 years ago from the Sumerian civilization in Nagpur [4]. The methods of preparation and use of medicinal plants including garlic, castor oil and myrrh were found listed on Egyptian Ebers Papyrus (approximately 1500 BCE) [6]. Although traditional Chinese medicine dates back at least to 2200 years, the earliest written record only dates to 300 BCE. In the western world, the science of medicinal plants related to the Roman and Greek cultures was documented in the "De Materia Medica" written by the Greek physician Dioscorides between 50 and 70 CE [3].

Until the 18th century, there was not a clear knowledge about the nature of the active compounds and their pharmacological activities. The clinical investigation of medicinal plants started when Anton von Storck studied the poisonous plants colchicum and aconite, and William Wuthering used the foxglove for the treatment of edema [7]. In the 19th century, German Friedrich Seturner isolated analgesic morphine from opium, which marked the beginning of plant derived drug discovery [7]. Since then, many plant constituents are being isolated from different medicinal plants and used in drug development.

Plant constituents referred to as phytochemicals, active compounds or secondary metabolites do not contribute to the growth and development of the plants but rather help them survive in

their environment by protecting them from microbial infections and herbivorous attacks, help them attract insects for pollination and give them colour, taste and odor [8]. Humans use plant constituents as agrochemicals, pharmaceuticals, Flavors, food additives, colouring agent and fragrances [9].

They were found to carry antioxidant, antimicrobial, antiviral, anti-inflammatory, anti-fungal, anti-lipogenic, anti-hypertensive and anti-cancer activities [10]. Phytochemicals either interact with their target human proteins or alter the growth of commensal, parasitic or pathogenic organisms living inside the human body. Such compounds strengthen the human health by lowering the risk factors and preventing the onset of degenerative diseases including cardiovascular diseases, cancers and neural disorders [11].

Plant-derived phytochemicals or secondary metabolites are usually classified based on their chemical structure rather than their biological action [12]. They can be categorized into four major classes: alkaloids, terpenoids, phenolics and sulphur-containing compounds. Phytochemicals can be extracted using variable solvents with different polarities in order to be identified and investigated for potential biological effects [13].

1.2. Rosaceae Botanical Background

The Mediterranean region harbor a great biodiversity. It is highly rich in medicinal plants; about 25000 species are native to this region with many endemic and rare plants [14]. Medicinal plants are largely used for their therapeutic effects in the Middle East, specifically, Lebanon is one of the most important center of floral biodiversity [15] due to its geographic location, varied topography, definite soil types and climatic variations [16]. Lebanese flora counts 2607 species divided into 783 genera, 78 of which are endemic [2]. The list of Lebanese medicinal plants is continuously being updated as many studies focus on identifying and characterizing the biological and therapeutic values of these plants [16].

The Rosaceae family, also known as the rose family, stands out as one of the most diverse and

economically vital plant families globally, and can be classified into four subfamilies: Spiraeoideae, Maloideae, Rosoideae, and Amygdaloideae [17]. With over 3,000 recognized species and numerous horticulturally important cultivated varieties, this plant family holds a pivotal position in both natural ecosystems and human agriculture [18]. Rosaceae family members are distributed worldwide, with a particular prevalence in temperate regions of the Northern Hemisphere. This diverse family encompasses an array of plants, including trees, shrubs, and perennial herbaceous plants [19]. Among its ranks are some of the most iconic and economically significant fruits, including apples (*Malus domestica*), pears (*Pyrus*), cherries (*Prunus*), and strawberries (*Fragaria*) [20], they constitute a significant part of the human diet and are rich in various phytochemicals, including flavonoids, phenolic compounds, cyanogenic glucosides, phytoestrogens, and phenols. These compounds have the potential to offer numerous health benefits and may help combat various diseases [21]. Some notable antioxidants and cancer-inhibiting substances found in these fruits include L-ascorbic acid, quercetin, kaempferol, myricetin, p-coumaric acid, gallic acid, and ellagic acid.

1.3. *Sarcopoterium spinosum*

Sarcopoterium spinosum (L.) Spach, is a small, thorny shrub belonging to the Rosaceae family. In English, it is referred to as “Prickly Shrubby Burnet," while in Arabic, it goes by the names "Bilan" or "Natch". It typically reaches a height of 30 to 60 cm and features branches that terminate in thorns without leaves [22]. The flowers are small and typically greenish-pink and usually blooms during the spring and early summer [23]. In addition, its fruits are small and inconspicuous, usually measuring about 3-5 mm in diameter. They are typically round or slightly oval in shape, resembling small seeds or grains, when mature mainly from July to August, SS fruits can vary in color, often appearing shades of brown, reddish-brown, or even greyish [23]. These fruits are often referred to as "achenes" because they are dry, single-seeded fruits that remain intact when mature. Achenes are a common type of fruit found in plants of

the rose family [24], [25].

S. spinosum thrives in a wide range of climatic conditions, from the semi-arid areas between the Mediterranean and Irano-Turanian vegetation zones to the sub-humid Mediterranean regions, it can be found in various countries surrounding the Mediterranean Sea, including parts of Europe, North Africa, and the Middle East [26]. It had adapted to thrive in arid and semi-arid environments including coastal areas, hillsides, and disturbed soils, and is often found in dry, rocky, or sandy soils [26].

It is predominantly distributed in the Eastern Mediterranean and has been the subject of early studies in geobotany, eco-physiology, life cycle, and successional aspects. Long ago, within Arab villages, the entire shrub served a variety of practical purposes [22]. It was utilized as a source of fuel, for constructing fences and enclosures for sheep, for crafting brooms from its branches, and as filling material for mattresses. Furthermore, the branches were employed to provide protective cover for delicate young plants, shielding them from the threat of birds and animals [27].

Some ethnopharmacological studies reported the usage of *S. spinosum* extract for the treatment of several disorders. The majority of surveys indicate an aqueous extract made from the roots as the main treatment for diabetes [2], [28]. Other traditional uses of *S. spinosum* were also mentioned that include pain relief [29], particularly for toothache and disorders related to the digestive system, as well as applications for asthma, kidney stones, and poisoning [30].

Extensive literature underscores the promising potential of the aqueous extract from *S. spinosum* roots as a nutraceutical supplement. It demonstrates effectiveness in preventing and treating hepatic steatosis, enhancing insulin sensitivity, and impeding the early stages of non-alcoholic steatohepatitis (NASH), as well as alterations in gene expressions associated with inflammation, lipid, and carbohydrate metabolism have been observed in studies conducted on high-fat diet (HFD)-fed mice [31], [32]. In addition, it demonstrated its antidiabetic potential through inhibiting lipolysis and increasing insulin secretion thus stimulating glucose uptake and

glycogen synthesis and cell viability in AML-12 hepatocytes and L6 myotubes [33] and it improved insulin sensitivity, reduced fasting blood glucose, and prevented islet hypertrophy in diabetic mice [34]. The anti-inflammatory properties of *S. spinosum* root extract was also proved, it demonstrated the immunomodulatory effects in macrophages, inhibiting pro-inflammatory responses and promoting anti-inflammatory gene expression, and in obese mice, it reduced adipose tissue inflammation, suggesting its potential as a novel anti-inflammatory agent [35]. Lastly, the root extract demonstrated its potential as a novel agent for treating insulin resistance as it activated insulin signalling, evidenced by increased Glut4-facilitated glucose uptake and phosphorylation of key signalling proteins 3T3-L1 adipocytes [36]. In contrast, while the aerial parts of *S. spinosum*, particularly the fruits, have received limited investigation, a comparative analysis involving boiled extracts from leaves, fruits, and roots revealed that the antidiabetic activity of the leaves and fruits was observed, although with lower efficacy compared to the roots. This was evidenced by the inhibition of α-amylase and α-glucosidase activity, as well as the enhancement of basal and glucose-induced insulin secretion in 3T3-L1 adipocytes and L6 myotubes [37]. Moreover, *S. spinosum* fruit extracts, obtained through supercritical fluid and Soxhlet extractions, yielded an antioxidant-rich residual water with high polyphenol and flavonoid content, featuring potent tyrosinase inhibition and the presence of identified compounds such as quercetin glucuronide and luteolin 7-O-glucuronide [23]. Recently, an ethanol extract derived from *S. spinosum* leaves and thorns demonstrated its cytotoxic effects on cancer cells [38].

For this purpose, *Sarcopoterium spinosum* fruits were gathered from the wild in Haddatha, South Lebanon (Latitude: 33° 09' 60.00" N, Longitude: 35° 22' 59.99"), in August 2020. Subsequently, various extracts were prepared using distinct solvents: ethanol, water, and boiling water. This choice was guided by the expectation that different solvents might selectively enhance the extraction of hydrophilic compounds to varying degrees.

The three extracts underwent characterization in terms of phenol content (TPC), flavonoid

content (TFC), and radical scavenging ability. Although no significant differences were observed in TPC and TFC among the three extracts, the ethanolic extract exhibited superior potency in terms of radical scavenging capabilities.

1.4. Polyphenols

Polyphenols (PPs) or phenolic compounds are natural molecules found abundantly in plants and showing various biological activities. They are the most essential non-nutrient bioactive compounds [73] because of their health-promoting properties including antioxidant, anti-cancer, anti-inflammatory, anti-microbial, anti-allergic, anti-atherogenic, anti-mutagenic, anti-lipogenic, anti-hypertensive and anti-thrombotic effects [19,71]. Polyphenols were described to slow down neurodegenerative diseases [74], cardiovascular diseases [75], diabetes [76] and metabolic disorders [77].

Polyphenols are structurally characterized by having at least one aromatic ring attached to one or more hydroxyl groups [71]. Natural PPs can be categorized according to their chemical structure into phenolic acids, flavonoids, and non-flavonoids [73]. These groups differ in their bioavailability, stability, and physiological function in humans [78]. Polyphenols can be associated with other polyphenols, organic acids and carbohydrates which increases their complexity, diversity, and ability to reach various molecular targets [19]. Flavonoids make the largest group among all polyphenols and are further divided into flavones, flavonols, flavanones, flavanols, isoflavones and anthocyanidins [19]. They have a chemical structure of 15 carbons (C6-C3-C6) consisting of two phenolic rings (A and B) linked by an oxygenated heterocyclic ring C [73]. Phenolic acids make 30% of the bound and free PPs [73] and are generally found in fruits and coffee beans [4]. They are derived from hydroxycinnamic (C6-C3) and hydroxybenzoic (C6-C1) acids, having one or more hydroxyl groups and a carboxylic acid function at the benzene ring [73]. Hydroxycinnamic acids (HCA) can be synthesized from tyrosine and phenylalanine, and fruits are considered their primary sources. Concerning non-

flavonoid polyphenols, they include resveratrol, ellagic acid and derivatives, lignans, curcumin, rosmarinic acid, gallic acid, caffeic acid and tannins [78]. The antioxidant property of polyphenols contributes significantly to disease prevention [79]. The structure of the functional groups dictates the efficiency of polyphenols' antioxidant activities. Indeed, the ability to act as radical scavengers and metal ion chelators is greatly influenced by the number and position of hydroxyl groups in a polyphenol [80]. For instance, flavonols such as quercetin that contain three hydroxyl groups showed higher antioxidant activity than those with less hydroxyl groups [80]. Moreover, it was found that the bigger the distance between the aromatic ring and carboxyl group in phenolic acids the more the antioxidant activity such as in cinnamic acids and derivatives versus benzoic acids [81].

In plants, polyphenols are the largest group of secondary metabolites. More than 8000 polyphenols (4000 of which are flavonoids) were identified with various chemical structures in edible plants [71,78]. PPs can be found in fruits (apples, grapes, cherries, pears, and berries), vegetables, beverages (tea, coffee, and red wine), chocolate, cereals, nuts, and legumes. Also, they are present in spices, flowers, herbs, leaves, barks, roots, seeds, and stems [71,82]. Several environmental factors affect the phenolic content of plants such as exposure to light, type of soil, culture method, rainfall, and fruit yield per tree. For instance, phenolic acids concentration varies according to the ripening process while flavonoids concentration depends on the exposure to light [82].

Polyphenols have many modes of action including radicals scavenging and ROS formation suppression [73,82]. They can donate an electron or hydrogen atom to a broad range of ROS, including superoxide and hydroxyl radicals thus neutralizing them [73]. They can disrupt lipid peroxidation chain reactions by donating an electron to the free radicals, making them more stable and less reactive radicals [78]. Moreover, polyphenols act as metal chelators, such as ferrous ions (Fe2+), thereby preventing hydroxyl radicals' production [73]. They can inhibit the oxidative damage caused by arachidonic acid metabolizing enzymes [82] and promote

antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) [78].

In the course of my thesis research, we started with the characterization of the phytochemical composition of the ethanolic extract from *Sarcopoterium spinosum* fruits, employing HPLC coupled with MS/MS in negative ionization mode for the first time. The ethanolic extract revealed a substantial richness in polyphenols. Notably, the ellagitannin family emerged as the most prevalent group of PPs in the extract, constituting approximately 50.8%. Additionally, notable quantities of triterpenes (around 11.9%) and flavonoids (approximately 7%) were discerned in the extract.

1.5. Metabolic disorders

A metabolic disease is a medical condition that disrupts the normal metabolic processes within the body, impacting the conversion of food into energy and the regulation of essential substances like carbohydrates, fats, and proteins [39]. These disorders often involve dysregulation in enzymes, hormones, or other metabolic pathways. Common examples include diabetes mellitus, obesity, and metabolic syndrome, high blood pressure, elevated blood sugar, and abnormal lipid levels that collectively heighten the risk of cardiovascular disease and type 2 diabetes [40]. Non-alcoholic fatty liver disease involves fat accumulation in the liver, and thyroid disorders can affect overall metabolic function. Management of these diseases often requires medical intervention, lifestyle adjustments, and ongoing treatment to control symptoms and prevent complications [41].

The prevalence of metabolic syndrome (MetS), characterized by a collection of interconnected metabolic disorders, has been consistently on the rise globally. The International Diabetes Federation reports that around 25% of the world's adult population is affected by MetS, with predictions anticipating a further increase in the coming decades [42]. This surge in MetS and its associated risk factors is attributed to a blend of genetic, epigenetic, and environmental factors [43], compounded by sedentary lifestyles marked by low physical activity and high-

energy diets [44]. Current management approaches primarily involve lifestyle modifications and pharmaceutical drugs targeting specific metabolic pathways. However, these drugs are costly, often result in poor patient compliance, and may yield undesirable side effects with prolonged use. Moreover, their focus tends to be limited, addressing only a subset of health outcomes related to metabolic dysfunction [45]. Consequently, there is a pressing need for research and development of alternative strategies to manage metabolic disorders. Medicinal plants emerge as a promising avenue for addressing MetS risk factors, defined as plants or plant preparations with health-promoting characteristics [46]. Healthcare practitioners increasingly recognize the potential of medicinal plants in managing and preventing metabolic disorders all over the world. Medicinal plants house pharmacodynamically active compounds with additive and synergistic effects that prove beneficial in treating metabolic disorders.

1.5.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a prevalent liver condition marked by the accumulation of fat in liver cells, unrelated to excessive alcohol consumption [47]. It encompasses a spectrum of disorders, ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH), which involves inflammation and liver cell damage [48]. Often associated with obesity, insulin resistance, and metabolic syndrome, NAFLD poses a significant health risk [49]. Risk factors include excess body weight, insulin resistance, and metabolic syndrome components. While early stages are usually asymptomatic, progression can lead to symptoms such as fatigue, upper abdominal discomfort, and, in severe cases, manifestations of liver failure like jaundice and confusion [50].

NAFLD is a complex and multifactorial disease, and its pathogenesis was initially explained by the "2-hit hypothesis." According to this hypothesis, the first hit involves hepatic triglyceride accumulation or steatosis, while the second hit is triggered by factors like inflammatory cytokines/adipokines, mitochondrial dysfunction, and oxidative stress, leading to

steatohepatitis and hepatic fibrosis [51]. It is now understood that free fatty acids (FFAs) directly contribute to liver injury. Various risk factors and mechanisms contribute to the accumulation of excessive triglycerides in hepatocytes, causing NAFLD. These factors include excess body fat, insulin resistance, inflammation, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, bacterial overgrowth, and genetic predisposition [52]–[55].

As of now, there is no ideal pharmacological treatment available for NAFLD. Consequently, lifestyle modification, encompassing a healthy diet, vigorous physical activity, and weight reduction, remains the primary approach for NAFLD treatment [56]. Despite its efficacy, long-term adherence to lifestyle modification can be challenging, and certain weight loss diets may have adverse effects on the liver. Recognizing the limitations and potential drawbacks of these approaches, there is a considerable interest in identifying therapeutic agents for preventing and treating NAFLD progression. Given the potential adverse effects associated with conventional medical therapies, attention has shifted towards exploring complementary and natural therapies, including herbal medicine and functional foods (e.g., fruits, vegetables) and their extracts [57].

Chapter one of this thesis investigates the potential lipid-lowering effect of an ethanolic extract from *Sarcopoterium spinosum* fruits using an *in vitro* model of moderate hepatic steatosis. FaO hepatoma cells were subjected to a fatty acid mixture to simulate the physiological conditions of a high-fat diet. Subsequently, the steatotic cells underwent incubation with the *S. spinosum* extract at varying concentrations. The results proved the extract's efficacy in attenuating lipid accumulation and its antioxidant capabilities in mitigating oxidative stress induced by the accumulation of fatty acids.

1.5.2. Endothelial dysfunction

The vascular endothelium consists of a single layer of specialized cells known as endothelial cells, creating a boundary between the underlying smooth muscle cells in the vascular lumen. The functionality of endothelial cells can vary significantly depending on their environment

[58]. For instance, the endothelium forming the blood–brain barrier differs markedly in functional characteristics from that lining the aorta. Despite these variations, all endothelial cells share common functions, such as regulating haemostasis, maintaining vascular permeability, mediating acute and chronic immune responses to injuries, and controlling vascular tone [59]. Endothelial cells synthesize various significant vasoactive compounds, such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF), carbon monoxide, endothelin, vasoactive prostanoids, and superoxide [60]. Alongside other substances generated by the endothelium, these factors play a role in regulating local thrombotic and inflammatory pathways, thereby impacting the initiation and advancement of atherosclerosis and its associated complications [61].

Impairment of the endothelium represents a multifaceted pathophysiological occurrence encompassing heightened endothelial cell activation and the initiation of endothelial dysfunction [62]. This activation involves an inflammatory and coagulation-promoting state within the endothelial cells, marked by the emergence of surface adhesion molecules necessary for attracting and bonding with inflammatory cells [63], [64]. This state of activation is triggered by cytokines released by tissues and organs during inflammatory situations.

Numerous studies have demonstrated the significant role of oxidative stress in arranging the production and release of cytokines, establishing a connection between reactive oxygen species (ROS), inflammation, and the activation and dysfunction of endothelial cells [65], [66]. Within endothelial cells, nitric oxide (NO) governs the maintenance of vascular homeostasis. The decline in NO availability, stemming from diminished production and/or heightened degradation by the superoxide anion, signifies the initiation of ED [67]. Specifically, the interaction of superoxide anion with NO results in the formation of peroxynitrite ($ONOO^-$). Subsequently, peroxynitrite fosters protein nitration, contributing to the dysfunction and demise of endothelial cells [68]. Superoxide anion is produced by various enzymes, including NADPH oxidase, xanthine oxidase, and uncoupled eNOS. Moreover, alterations in mitochondrial

respiratory chain complexes significantly contribute to the generation of chemical species associated with oxidative stress [69].

Diverse pathological conditions, such as hyperglycemia, hyperlipidemia, hypertension, mental stress, aging, and exposure to certain drugs, have the potential to disrupt the molecular mechanisms regulating NO bioavailability, thereby influencing endothelial function [70]–[72]. Consequently, conventional therapeutic approaches targeting improvements in insulin sensitivity, glycemic control, lipid profiles, and blood pressure often prove effective in ameliorating endothelial dysfunction.

In the second chapter of this thesis, we explored the prospective anti-inflammatory and cyto-protective aspects of the ethanol extract derived from *Sarcopoterium spinosum* fruits, utilizing an *in vitro* model of endothelial dysfunction. Human endothelial cells (HECV) were exposed to hydrogen peroxide to mimic the oxidative conditions that occur physiologically. Following this, the stressed cells were subjected to incubation with the ethanol extract. The outcomes conclusively demonstrate the extract's effectiveness in averting and ameliorating endothelial dysfunction, attributing these beneficial effects to its anti-inflammatory and antioxidant properties.

1.5.3. Insulin resistance

Insulin is a polypeptide hormone produced by the β-cells in the pancreatic islets of Langerhans; its secretion is triggered by the presence of high concentrations of glucose and amino acids in the bloodstream, often in response to a meal. Insulin plays a central role in regulating glucose levels in the body, contributing to the maintenance of glucose homeostasis. Of note insulin isa central hormone in the body energy homeostasis being key regulator of the anabolic processes, including tissue growth and development [73].

In a healthy individual, the blood glucose level (glycemia) is tightly controlled within a narrow range of values through the regulation of glucose synthesis/release primarily by the liver, and

to a lesser extent by the kidney, and the uptake of glucose by peripheral tissues, notably skeletal muscle, liver, and adipose tissue [74]. Insulin also controls the lipid metabolism by promoting lipid synthesis in the liver and adipose tissue, and by inhibiting lipolysis (the breakdown of triglycerides into fatty acids) [75].

Insulin resistance (IR), also known as impaired insulin sensitivity, happens when cells in your muscles, fat and liver don't respond as they should to insulin. Therefore, IR can be described as the inability of insulin to effectively stimulate the disposal of glucose. For several reasons, muscle, fat, and liver cells respond inappropriately to insulin, which means they cannot efficiently take up glucose from your blood or store it. As a result, the pancreas makes more insulin to try to overcome your increasing blood glucose levels leading to e condition called hyperinsulinemia, which, over time, leads to prediabetes and type 2 diabetes (T2D) [76]. Because of this heightened demand for insulin production, the β-cells in the pancreas undergoes hypertrophy [77].

Insulin acts on the tissues by binding to its receptor (called insulin receptor) thus initiating a complex signalling cascade. This involves autophosphorylation of the insulin receptor and subsequent activation of the insulin receptor substrate (IRS 1/2). Activated IRS sets off three pivotal pathways: PI3K/AKT, facilitating glucose transport; TSC1/2-mTOR, critical for protein synthesis and responsive to insulin and nutrient factors; and the RAS-MAPK pathway, supporting cell survival and division by regulating gene expression, mitogenesis, and differentiation. Overall, insulin signalling plays a central role in glucose metabolism, protein synthesis, and cell survival [78]–[80]. Kinases and phosphatases delicately balance this cascade, with tyrosine phosphorylation activating and serine/threonine phosphorylation inactivating insulin receptor and IRS proteins [81]. Also, factors like free fatty acids, oxidative stress, inflammatory cytokines and altered adipokine function contribute to these influences, hindering IRS signalling [82]–[84].

IR consistently associates with obesity and related complications, including type 2 diabetes

mellitus, cardiovascular disease, certain cancer types, infertility, non-alcoholic fatty liver disease, and cognitive impairment [49], [85], [86]. While surgical and pharmacological strategies have shown utility, energy-reduced diets, as part of a healthy lifestyle, almost invariably facilitate weight loss and reduce IR in these patients.

In the final chapter of this thesis, I described the studies aimed to design and develop an *in vitro* model of human insulin resistance applicable for further studies of the beneficial effects of S. spinosum and other bioactive compounds. To achieve this, we utilized HepG2 cells sourced from the American Type Culture Collection (ATCC). These cells were subjected to varying concentrations of insulin over different time frames or exposed to a fatty acids' mixture over different time frames. Our findings demonstrate that insulin resistance in HepG2 can be induced by both elevated insulin levels (hyperinsulinemia) and the accumulation of lipids.

1.6. Cellular models

Three distinct cell lines were utilized in my studies presented throughout this project.

1.6.1. Cellular model of hepatic steatosis

FaO cells, a rat hepatoma cell line, chosen to replicate and mimic the onset and progression of NAFLD. These FaO rat hepatoma cells, obtained from the European Collection of Cell Cultures (ECCC-Sigma-Aldrich Corp.), express a diverse range of liver-specific mRNAs. They possess the capability to assemble and secrete very-low-density lipoproteins (VLDL) and respond to stimuli activating transcription factors like PPARs [87]. Consequently, this cell line serves as a well-established and widely employed cellular system, maintaining key features of rat hepatocytes.

In our experimental setup, FaO cells at 70-80% confluence underwent 3 h incubation in a high glucose starvation medium, F12 Coon's modified medium supplemented with 0.25% BSA and with mixture of oleate and palmitate (0.75 mM with molar ratio 2:1). It is noteworthy that the

basal lipid content of FaO cells is considerably lower than that of primary hepatocytes. As a result, the fatty acids treatments led to a more rapid and pronounced accumulation of triglycerides compared to primary cultured hepatocytes. The exposure to oleate and palmitate mixture resulted in a significant increase in both the number and size of cytosolic lipid droplets. These observations establish lipid-loaded FaO cells as a reliable and convenient *in vitro* model for studying NAFLD [88].

1.6.2. Cellular model of endothelial dysfunction

HECV cells, a human endothelial vascular cell line, were utilized to emulate human endothelium dysfunction. The human endothelial cell line (HECV) was sourced from the Cell Bank and Culture (GMP-IST- Genoa, Italy) and derived from the human umbilical vein, preserving a substantial degree of endothelial function.

Widely employed for in vitro studies, these cells were cultured in Dulbecco's modified Eagle's medium high glucose (D-MEM) supplemented with L-glutamine and 10% FBS. To simulate and mimic endothelial dysfunctional, at 70-80% confluency HECV cells were incubated with 30 µM H_2O_2 for 24 h. As a result, hydrogen peroxide exposure led to a more rapid and pronounced ROS production, lipid peroxidation and decreased NO availability. These observations establish H_2O_2-exposed HECV cells as a reliable and convenient *in vitro* model for studying endothelial dysfunction.

1.6.3. Cellular model of insulin resistance

HepG2 cells are a human hepatoma cell line obtained from the American Type Culture Collection (ATCC). HepG2 cells are maintained in EMEM supplemented with L-glutamine and 10% FBS.

In our experimental model of insulin resistance, HepG2 cells at 70-80% confluence underwent 6 h incubation in a low glucose DMEM supplemented with 0.4% FBS. After that cells were

incubated with high glucose DMEM supplemented with 0.4% FBS and 10 nM insulin for 48 h.

2. Chapter two: Aims of the study

The experimental study that I carried on during the three years of my PhD project was mainly focused on investigating the effects of *Sarcopoterium spinosum*, a significant component in Lebanese traditional medicine, particularly for managing diabetes and alleviating pain. While the roots of this shrub have been extensively studied, the fruits have been poorly investigated. Initially, our research aimed to conduct a comprehensive chemical characterization of three distinct extracts from *S. spinosum* fruits. This involved evaluating their total polyphenol and flavonoid content, alongside assessing their *in vitro* radical scavenging ability. This initial phase aimed to identify the most potent extract among the three, setting the stage for further investigation into their biological effects.

Following the chemical characterization, our study delved into the characterization of the phytochemical profile of the selected extract, aiming to identify and quantify its bioactive compounds. Understanding the specific compounds present in this extract is crucial to unravelling the mechanisms behind its potential therapeutic properties.

Subsequently, our investigation extended to exploring the biological effects of the selected extract using cellular models relevant to metabolic disorders such as hepatic steatosis, dysfunctional endothelium, and insulin resistance. By employing these cellular models, we aimed to elucidate how the extract from *S. spinosum* fruits might impact these dysfunctions at a cellular level, providing insights into its potential therapeutic applications.

This sequential approach, from chemical characterization to exploring biological effects on specific cellular models, was designed to comprehensively evaluate the therapeutic potential of *S. spinosum* fruit extracts. This research is particularly relevant and timely, considering the escalating global prevalence of the metabolic disorders, which have become a growing concern. Hepatic steatosis, dysfunctional endothelium, and insulin resistance are on the rise globally,

contributing significantly to the burden of chronic diseases. Therefore, understanding the potential of *S. spinosum* fruit extracts in mitigating these conditions could have far-reaching implications for public health, offering a novel and natural approach to addressing health challenges that have become increasingly prevalent on a global scale.

Furthermore, the identification and characterization of bioactive compounds in *S. spinosum* fruit extracts pave the way for the potential development of these extracts and their main components as valuable candidates for nutraceutical formulations. The findings from this investigation may contribute to the growing body of evidence supporting the utilization of *S. spinosum* derived compounds in nutraceutical products, providing a natural and plant-based alternative for managing prevalent health conditions and promoting overall well-being.

3. Chapter Three: Polyphenol-enriched extracts of *Sarcopoterium spinosum* fruits for counteracting lipid accumulation and oxidative stress in an in vitro model of hepatic steatosis.

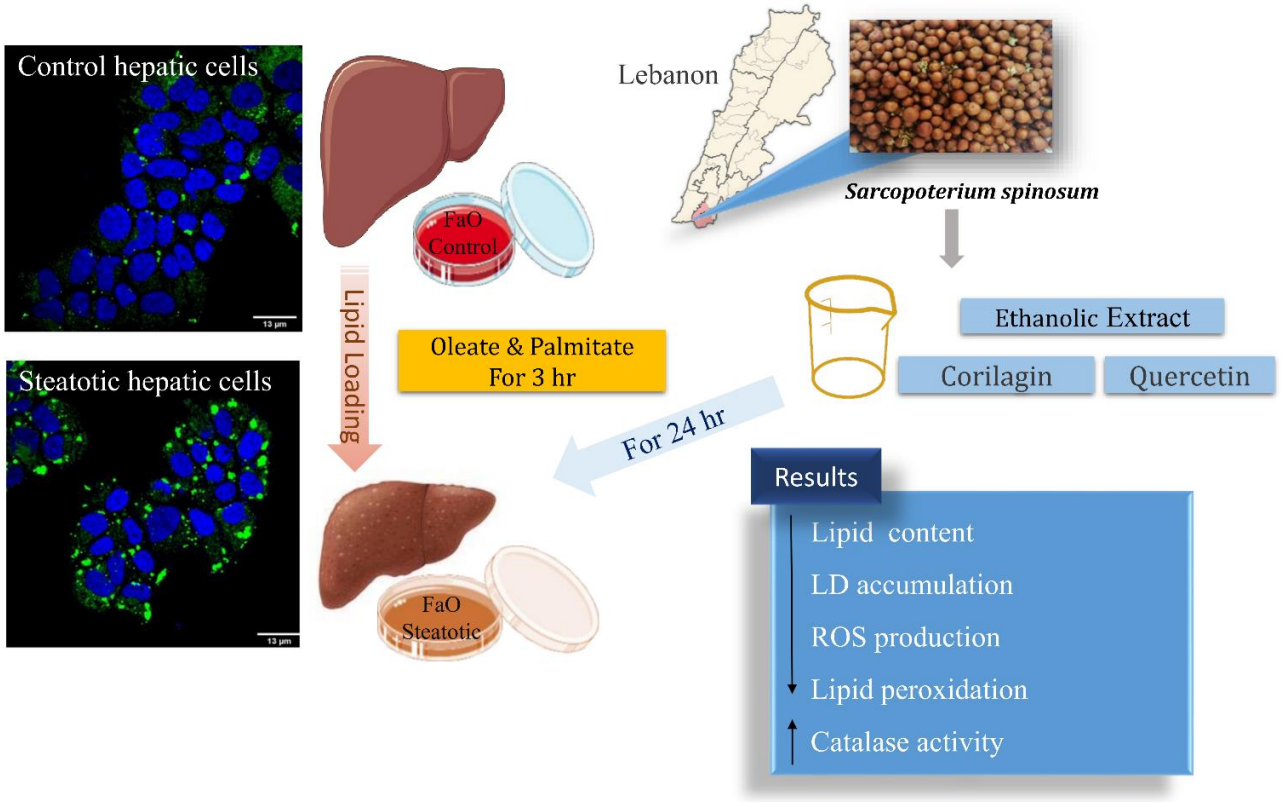

Figure 3- 1 Chapter three graphical abstract

Polyphenol-enriched extracts of *Sarcopoterium spinosum* fruits for counteracting lipid accumulation and oxidative stress in an *in vitro* model of hepatic steatosis

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Abstract

Sarcopoterium spinosum (L.) Spach is a Rosaceae shrub employed in the folk medicine in the Eastern Mediterranean basin. The previous few studies have focused on the *S. spinosum* roots, while the fruits have been poorly investigated. The present study aims to assess the biological properties of ***S. spinosum*** fruits collected in Lebanon and subjected to ethanolic, water or boiling water extraction. The extracts were compared for the phenol and flavonoid contents, and for the *in vitro* radical scavenging ability. The ethanolic extract (SEE) was selected and characterized by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) showing a phenolome rich in tannins (ellagitannins), flavonoids (quercetin derivatives), and triterpenes. The biological activity of SEE was tested on a cellular model of moderate steatosis consisting of lipid-loaded hepatic cells treated with increasing concentrations of SEE (1-25 µg/mL), or with corilagin or quercetin as comparison. In steatotic hepatocytes the SEE was able (i) to ameliorate the hepatosteatosis; *(ii)* to counteract the excess ROS and lipid peroxidation; *(iii)* to restore the impaired catalase activity. The results indicate that the ethanolic extract from *S. spinosum* fruits is endowed with relevant antisteatotic and antioxidant activities and might find application as nutraceutical product.

Key Words:

Sarcopoterium spinosum fruit extracts; corilagin; phenolome profile; anti-steatotic activity; antioxidant acitvity.

3.1. Introduction

Medicinal plants are a source of bioactive compounds and are largely investigated for functional food applications. Moreover, their study may help to direct and optimize the identification of new drugs or nutraceuticals [89]. The phenolic compounds (PCs) are phytochemicals characterized by the presence of phenolic rings and they are found in most edible plants. Although they are not classified as nutrients, their dietary intake provides health-protective effects. PCs are classified into phenolic acids, flavonoids, anthocyanins, and tannins [90] that are largely studied for their efficacy in preventing many diseases, including diabetes, obesity and cancer [91]. The interest for plant PCs depend on their wide availability, variety, and negligible side effects [92].

Lebanon notably boasts one of the highest densities of floral diversity in the Mediterranean basin as it includes various climates allowing the growth of many endemic valuable medicinal plants [15]. Rosaceae is one of the most important plant families worldwide, with more than 3,000 members [20] which includes many plant fruits, such as apples, strawberries and almonds. *Sarcopoterium spinosum* (L.) Spach is an Eastern Mediterranean shrub being mentioned as a medicinal plant in a large number of ethnobotanical surveys [23]. Most studies on *S. spinosum* have been performed on the roots which are described to play anti-inflammatory [35], antidiabetic [33], and insulin-sensitizing [34] activities. However, in Lebanon, the folk medicine preferentially uses *S. spinosum* fruits for treating diabetes and for weight loss [15], also because fruits are extremely rich in phenolic compounds. This is the reason of our study on the extracts from *S. spinosum* fruits.

Non-alcoholic fatty liver disease (NAFLD) has emerged as a leading cause of chronic liver disease worldwide, with an estimated global prevalence of 25% in the adult population [93]. NAFLD describes hepatic fat deposition as part of the metabolic syndrome. NAFLD spectrum ranges from simple hepatic steatosis which may progress to non-alcoholic steatohepatitis with inflammation and hepatocellular ballooning, up to liver fibrosis, cirrhosis, and hepatocellular

carcinoma [93]. Obesity, type 2 diabetes and dyslipidemia are leading risk factors for NAFLD [94]. To date, no effective pharmacological treatment is available for NAFLD, and the most effective approach is the weight loss through a balanced diet and appropriate physical activity. Therefore, the use of natural compounds for treating NAFLD and other metabolic disorders is currently gaining interest [95].

In the present study, we explored the alleged beneficial properties of the *S. spinosum* fruits from South Lebanon plants in terms of antisteatotic and antioxidant effects. After a preliminary characterization of three different extracts, we focused on the ethanolic extract to assess the phenolome, the lipid-lowering, antioxidant and cytoprotective activities using a cellular model of hepatic steatosis. The effects of the *S. spinosum* ethanolic extract were compared to those of corilagin (Cg) and quercetin (Qu) representing the most abundant and more promising PCs in the extract. Our findings seem to indicate that the ethanol extract showing significant *in vitro* antisteatotic and antioxidant effects could find nutraceutical applications for treatment of moderate hepatic steatosis.

3.2. Materials and methods

3.2.1. Chemicals

Reagents were purchased from Sigma-Aldrich (Italy) unless otherwise specified.

3.2.2. Plant materials and extract preparation

Sarcopoterium spinosum (L.) Spach fruits (Figure 3-2) were collected from the wild in the Haddatha in South Lebanon, (Latitude: 33° 09' 60.00" N, Longitude: 35° 22' 59.99"), during the August 2020. They were identified by Prof. George Tohme, a taxonomist president of Lebanese CNRS, and classified according to his book (Illustrated Flora of Lebanon, 2014) based on morphological and binocular analyses. A voucher specimen (R5.36) was deposited in the Lebanon National Herbarium at the Lebanese University, Faculty of Sciences. The fruits

were dried at room temperature in the shade for three weeks. Then, 50 g of ground material were extracted with 1 L of ethanol (99%) for 3 hours with agitation at room temperature. After ethanol evaporation at 42°C in a rotary evaporator (Heidolph Instruments, Schwabach, Germany) we collected the dried pellet representing the SEE. Two different aqueous extracts were prepared by incubating 50 g of ground materials with either 1 L of distilled water at room temperature for 3 h with agitation (SWE) or with 1 L of distilled boiling water (100°C) for 3 h with agitation (SBWE). Then, SWE and SBWE separetly were freeze-dried in an Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany) to obtain the water-soluble extracts.

3.2.3. *Phytochemical profiling of the extracts*

Total phenol content (TPC) was quantified according to Folin–Ciocalteu method [96]. Briefly, 100 µl of sample (1 mg/mL) were mixed with 0.5 mL of 10% Folin–Ciocalteu reagent and incubated in the dark for 5 min. After addition of 1.4 mL of 10% Na2CO3, the samples were incubated for 30 min at room temperature. Absorbance (λ_{760}) was read with a UV-VIS spectrophotometer (Model U-2900, Hitachi High Technologies, Japan) against a blank. Gallic acid (0–100 µg/mL) was used as reference and results were expressed in mg Gallic Acid Equivalents (GAE) per gram dry extract.

Total flovonoid content (TFC) was quantified according to a standard method [97]. Briefly, 0.3 mL of sample (1 mg/mL) were mixed with 1.2 mL distilled water and 90 µL 5% $NaNO_2$. After incubation for 5 min in the dark at room temperature, 90 µL of 10% $AlCl_3$ were added. Then, 0.6 mL NaOH (1M) and 0.72 mL distilled water were added and after 25 min the absorbance (λ_{510}) was read with a UV-VIS spectrophotometer against a blank. Quercetin dissolved in ethanol (5–100 µg/mL) was used as reference and results were expressed as mg Quercetin equivalent (QE) per g dry extract.

3.2.4. *Protein Quantification*

The protein content of each sample was quantified by Bradford assay using bovine serum albumin (BSA) as a standard [98].

3.2.5. *Antioxidant activity determination*

The ABTS assay was performed according to R. Re [99]. Briefly, 200 µL of ABTS solution were added to 50 µL of sample (1–5000 µg/mL) and incubated at room temperature for 20 min in the dark. Absorbance (λ_{734}) was read against equal amount of ABTS solution using a UV-VIS spectrophotometer microplate reader (Model FLUOstar Optima, BMG Labtech). Trolox was used as a reference and reults were estimated as Trolox equivalent antioxidant activity (TEAC) (µg TE/mg dry extract).

The DPPH assay was performed according to W. Brand-Williams [100]. Briefly, 50 µL aliquot of sample (0–2 mg/mL) was mixed to 200 µL of DPPH solution (0.1 mM in methanol). After incubation in darkness for 30 min at room temperature, the absorbance (λ_{490}) was measured using a UV–VIS microplate reader against DPPH solution as a blank. Trolox was used as a reference and results were expressed as TEAC (µg TE/mg dry extract).

The FRAP assay was performed according to I.F.F. Benzie [101]. Briefly, 25 µL of the sample (0–2 mg/mL) were mixed with 175 µL of FRAP working solution (300 mM acetate buffer (pH 3.6), 20 mM ferric chloride) and 10 mM TPTZ (2,4,6- tri (2-pyridyl)—S-triazine) made up in 40 mM HCl. The three solutions were mixed (10:1:1 ratio, v:v:v). The mixture was incubated at 37°C in the dark for 30 min and then the absorbance (λ_{593}) was read using a UV–VIS microplate reader against FRAP solution as a blank. Trolox was used as reference and results were expressed as TEAC (µg TE/mg dry extract).

3.2.6. *HPLC-MS/MS analysis*

The ethanolic exctract was analysed by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS and MS/MS) using an Agilent 1100 HPLC-MSD Ion Trap XCT

system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). For HPLC-MS/MS analyses the ethanol extract was analysed both as crude extract and after fractionation to improve the compound identification according to P. F. P. dos Santos [102]. Briefly, 1.7g of the SEE was re-suspended in 200 mL water:methanol mixture (H_2O-MeOH, 8:2, v/v) and partitioned with 100 mL of hexane to obtain the free hexane-soluble extract (fSEE). The remained H_2O-MeOH solution was dried in rotary evaporator. The component separation of the SEE and fSEE was performed on a Jupiter C18 column 1 mm × 150 mm with 3.5 μm particle size (Phenomenex, USA). For eluents: water (eluent A) and MeOH (eluent B) were used, both added with 0.1% formic acid. The gradient applied was: 15% eluent B for 5 min, linear to 100% eluent B in 35 min, and finally, hold at 100% eluent B for another 5 min. The flow rate was set to 50 μL/min with a column temperature of 30°C. The injection volume was 8 μL. Ions were detected in the positive and negative ion mode, in the 100–1000m/z range, and ion charged control with a target ion value of 100,000 and an accumulation time of 300 ms. A capillary voltage of 3800 V, nebulizer pressure of 20 psi, drying gas of 8 L/min, dry temperature of 360°C, and 3 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound. Once the total ion current was obtained, the m/z signals with a significant signal-to-noise ratio (5:1) were extracted. Both full-scan and tandem spectra were acquired.

3.2.7. *Cell culture and treatments*

Rat hepatoma FaO cells (European Collection of Authenticated Cell Cultures-ECACC, Salisbury, UK) were cultured in Coon's modified Ham's F12 medium with low glucose (1.8 g/L), supplemented with 2 mM Glutamine and 10% Fetal Bovine Serum (Euroclone, Italy) at 37°C in a humidified atmosphere with 5% CO_2. Stock solutions of SEE, corilagin (Cg) (MedChemExpress, USA) and quercetin (Qu) were prepared in DMSO with final concentration of 5 mg/mL. For treatments, cells were plated in cell culture dishes and grown for 24 h in high-

glucose (4.5 g/L) medium with 0.25% BSA until reaching 80% confluence. Then, cells were exposed for 3 h to a mixture of oleate/ palmitate at a final concentration of 0.75 mM (2:1 molar ratio) in order to mimic *in vitro* the effect of high-fat diet [88]. After exposure to oleate/palmitate mix, medium was changed and the steatotic cells were incubated for 24 h with the extract at different concentrations (1, 10, and 25 µg/mL). For comparison, steatotic cells were treated with either 10 µg/mL corilagin (16 µM) or 10 µg/mL quercetin (33 µM). Each experiment was performed at least in quadruplicate. The results represent the average of at least four independent experiments in triplicate. The potential cytotoxicity of the extracts, was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test [103].

3.2.8. *Quantification of the intracellular triglyceride content*

At the end of each treatment, cells were scraped and lysed by passing cell suspension through a 25 gauge needle. Lipids were extracted using the chloroform/methanol method [104]. The TG content was quantified by adding 500 µL of Triglycerides liquid kit reagent (Sentinel Diagnostics, Italy) for 15 min at 37˚C. Absorbance (λ_{546}) was read with a UV-VIS spectrophotometer against a blank. Glycerol (0.007–0.2 µg/mL) was used as reference. The TG content was normalized to protein content and expressed as percent TG content relative to control.

3.2.9. *Optical Microscopy and morphometric analyses*

Cells grown on coverslips were fixed with 4% paraformaldehyde and visualized both with absorption and fluorescence microscopy [105]. For absorption microscopy, Oil-RedO (ORO) staining was employed, and cells were stained with 0.3% ORO solution. Images are acquired by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany). For fluorescence microscopy, LDs were stained with 1 µg/mL BODIPY 493/503 (Molecular Probes, Life Technologies, Italy) , and DNA with 2 µg/mL Hoechst 33342 (Thermofiher Scientific, Italy). Images were acquired at 100X magnification by A1R Nikon

inverted CONFOCAL microscope. All images were processed by using ImageJ software for quantification of lipid droplet size and number [106].

3.2.10. *Quantification of ROS production*

The production of ROS was quantified *in situ* using the oxidation of the cell-permeant 2′-7′dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2′-7′dichlorofluorescein (DCF) [107]. Cells were grown in plates, scarped and collected by centrifugation. Then, cells were loaded with 1 µM DCF-DA in PBS prepared from stock 10 mM stock prepared in DMSO for 30 min at 37°C in the dark. Then, cells were centrifuged and resuspended in 2 mL PBS. The fluorescence was read with a LS50B fluorimeter (Perkin Elmer, USA) at 25°C using a water-thermostated cuvette holder (□ex=495 nm; □em=525 nm). Results were normalized to protein content and expressed as percent flourescence intensity relative to control. The results represent the average of at least three independent experiments in triplicate.

3.2.11. *Determination of lipid peroxidation*

Thiobarbituric acid reactive substances (TBARS) assay allowed spectrophotometrically to determine lipid peroxidation which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA)[108]. Briefly, 250 µL of cell suspension was incubated for 45 min at 95 °C with 500 µL of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 750 µL of N-butanol was added , centrifuged and the absorbance of the organic phase (upper phase)was measured at 532 nm in a UV-VIS spectrophotometer. Results were normalized to protein content and expressed as percent MDA levels relative to control

3.2.12. *Determination of catalase activity*

Catalase (CAT, EC 1.11.16) activity was evaluated in the 12000 × g supernatant of cell lysates following the consumption of hydrogen peroxide (H_2O_2) at 25°C [109]. Briefly, 10 µL of the supernatant was added to 487 µL of phosphate buffer (50mM K_2HPO_4, 50mM KH_2PO_4, PH 7).

Instantly after 3 µL of H_2O_2 (30%) was added to the sample the acquistion started for 3 min (acqusition was recorded every 15 seconds). The absorbance was read at 240nm with a UV-VIS spectrophotometer at 25 °C using a Peltier-thermostated cuvette holder. Enzyme activity was calculated as µmoles of decomposed H_2O_2 per min/mg protein, presented as percentage values to controls and normalized for total proteins.

3.2.13. *Statistical analysis*

Data are means ± S.D. of at least three independent experiments. The differences among the groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post-test (version 8.0, GraphPad Software, Inc., USA).

3.3. Results

3.3.1 Chemical characterization of three different *S. spinosum* extracts

S. spinosum fruits collected in South Lebanon (Figure 3-2) to prepare the ethanol (SEE), water (SWE) and boiling water (SBWE) extracts. The three extracts were characterized for their phenol and flavonoid contents, and the radical scavenging activity. The extracts showed similar total phenol content (TPC) and total flavonoid content (TFC). In details, TPC ranged from 147.85 to 162.22 mgGAE/g dry extract, and TFC from 38.81 to 45.39 mgQE/g dry extract (Table 3-1).

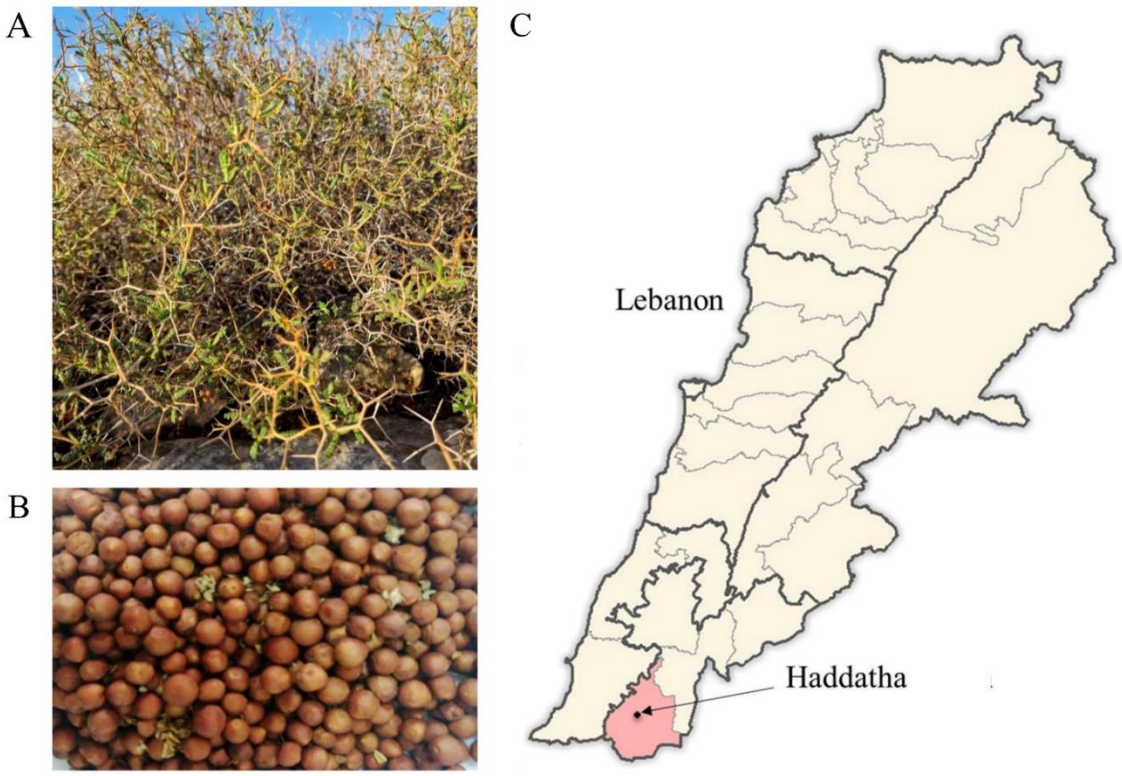


Figure 3- 2 Characteristics and distibution of Sarcopterium spinosum shrub

S. spinosum plant in its natural environment: the shrub **(A);** the fruits **(B);** the region of the fruit collection: Haddatha, South Lebanon **(C).**

The *in vitro* radical scavenging potential was tested using three different assays (Table 3-1). The ABTS assay gave the highest radical scavenging activity for SEE, with a TEAC (654.2 µg TE/mg) higher than SWE (420.94 µg TE/mg) and SBWE (373.80 µg TE/mg). Moreover, the radical scavenging activity of SEE was further assessed by the DPPH assay (549.5 µg TE/mg), the standard assay for hydrophobic compounds, whereas the radical scavenging activity of the aqueous extracts SWE and SBWE were assessed by the FRAP assay, which is appreciated for its rapid performance and high accuracy and showed similar activities for the two extrcats (312.3±20 *vs* 312.0±12 µg TE/mg, respectively). Based on these results we selected the ethanolic extract for further investigations.

S. spinosum fruit extract	TPC	TFC	ABTS	DPPH	FRAP
	mgGAE/gr	mg QE/g	µg TEAC/mg	µg TEAC/mg	µg TEAC/mg
Water (SWE)	158.44 (±33.1)	45.39 (±5.85)	420.94 (±37)	-	312.3 (±20)
Boiling Water (SBWE)	162.22 (±29.3)	41.33 (±4.24)	373.80 (±25)	-	312.0 (±12)
Ethanol (SEE)	147.85 (±36.7)	38.81 (±2.98)	654.2 (±65)	549.5 (±31)	-

Table 3- 1 Phytochemical content and antioxidant ability of S. spinosum fruit extracts

Three extracts were prepared using different extraction solvents: water (SWE), boiling water (SBWE) and ethanol (SEE). Total phenolic content (TPC), total flavonoid content (TFC) radical scavenging ability (ABTS and DPPH) and ferric reducing antioxidant power (FRAP) were reported. ABTS, DPPH and FRAP were expressed as Trolox equivalent antioxidant capacity TEAC (µg TE/mg dry extract). Values are the mean ± standard deviation (S.D.) from at least three independent experiments in triplicate (n = 3).

3.3.2 HPLC-MS/MS analysis of the ethanolic *S. spinosum* extract

The phenolome of SEE was analyzed by HPLC-MS/MS in negative ionization mode. Fruits are rich in carbohydrates and fats that may form insoluble complexes with the PCs thus interfering with HPLC detection. Therefore, we preliminarily treated the crude extract by water/methanol mixture and hexane fractionation to eliminate most of the apolar molecules. By HPLC-MS/MS analysis we detected 24 peaks (Figure 3-3A). By comparison with isolated standards and data in literature, at now, we could identify 17 major compounds (Table 3-2). Ellagitannins was the most abundant group of PCs in SEE (approximately 50.8% of the total extract). The ellagitannins identified in SEE were the following: the casuarictin isomer, the corilagin, the pedunculagin and the castalagin/vescalagin. A second abundant group of PCs was that of flavonoids, with the quercetin glucuronide and the quercetin-diglucoside being the main ones (about 7.5% of the total extract). Also triterpenes were present in high quantity in the SEE (about 11.9%). The main triterpenes identified were the tormentic acid its derivatives: 23-

hydroxytormentic acid ester glucoside [M+HCOO]-, oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO]-, gallic acid ester triterpenoid glycoside, oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO]- isomer, di-oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO], oxidized tormentic derivative, 23-hydroxytormentic acid. We wish to note that seven peaks could not be identified yet, due to lack of similarities for negative ionization segments and retention time in literature libraries.

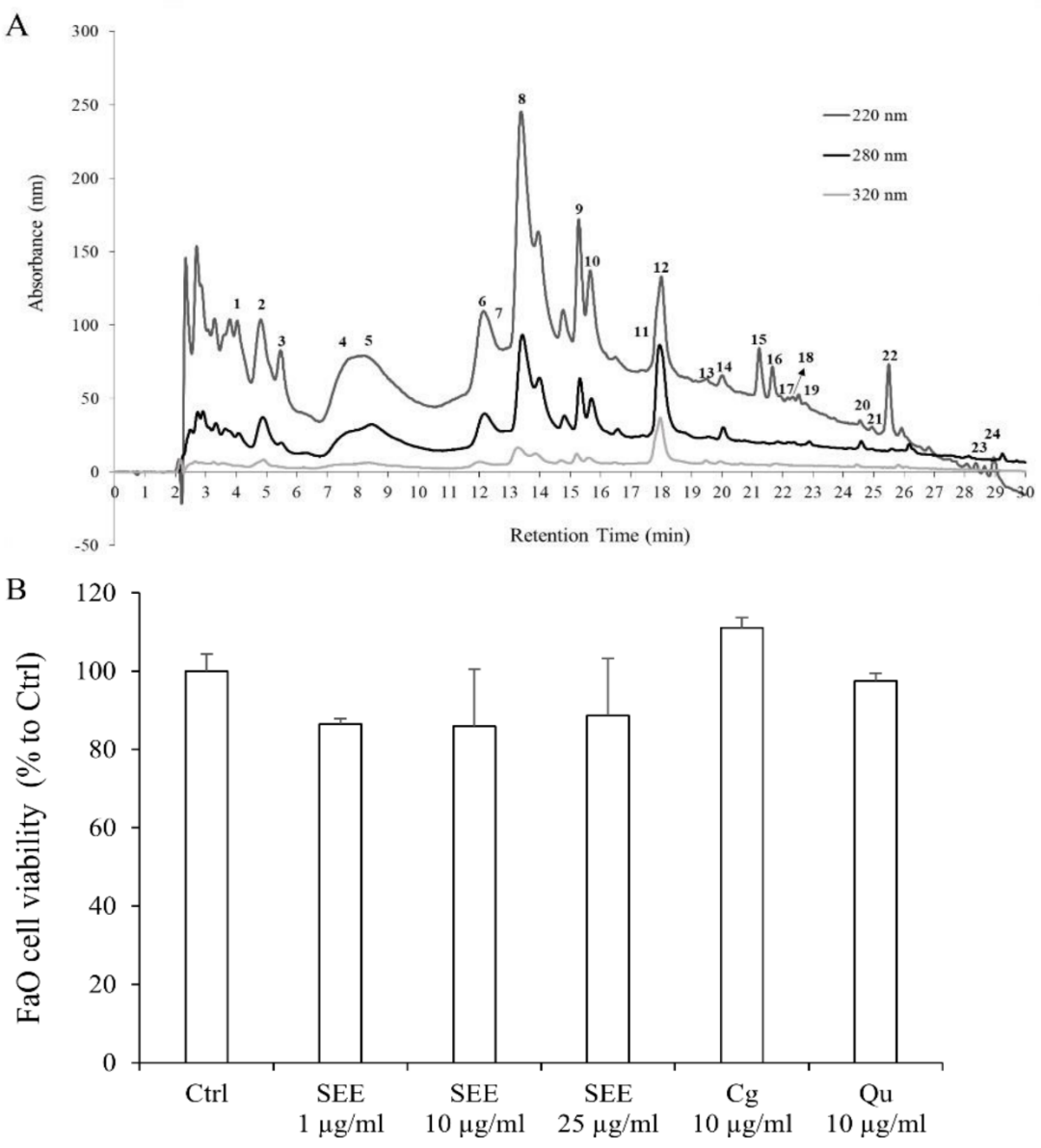


Figure 3- 3 HPLC-MS/MS analysis and cytotoxicity of the S. spinosum ethanolic extract

The HPLC chromotograms (A) of the ethanolic extract of *S. spinosum* fruits recorded at two wavelengths (220 nm and 280 nm).

The identified peaks: 3= Corilagin; 4= Pedunculagin; 6= Castalagin/Vescalagin; 8= Casuarictin isomer; 9= Casuarictin isomer; 11= Quercetin derivative/Quercetin -diglucoside; 12= Quercetin glucuronide; 15= 23-hydroxytormentic acid ester glucoside [M+HCOO]-; 16= oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO]-; 17= Gallic acid ester triterpenoid glycoside; 18= oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO]- isomer; 19= Di-oxidized 23-hydroxytormentic acid ester glucoside [M+HCOO]-; 20= Tormentic acid derivative; 21= oxidized tormentic acid derivative; 22= 23-hydroxytormentic acid; 23= Tormentic acid; 24= oxidized tormentic acid. (B) cell viability after 24 h incubation of FaO cells with SEE different concentrations, Cg and Qu. Data are the mean ± S.D. of three independent experiments.

#	RT (min)	[M-H]-	MS/MS fragments	Tentatively identified compound	Class
1	5.6	633.1	301/463	Corilagin	Ellagitannins
2	7.5	783.2	633/301	Pedunculagin	Ellagitannins
3	12.2	935	633/301	Castalagin/Vescalagin	Ellagitannins
4	13.5	935	633/301/897	Casuarictin isomer	Ellagitannins
5	15.4	935	633/301/897/783	Casuarictin isomer	Ellagitannins
6	15.8	625.3	301	Quercetin derivative/Quercetin -di-glucoside	Flavonoids
7	17.9	477.1	301	Quercetin glucuronide	Flavonoids
8	21.4	711.3	503/665	23-hydroxytormentic acid ester glucoside $[M+HCOO]^-$	Triterpenoids
9	21.8	709.3	501/663	Oxidized 23-hydroxytormentic acid ester glucoside $[M+HCOO]^-$	Triterpenoids
10	22.4	817.4	655/697	Gallic acid ester triterpenoid glycoside	Triterpenoids
11	22.6	709.3	501/663	Oxidized 23-hydroxytormentic acid ester glucoside $[M+HCOO]^-$ isomer	Triterpenoids
12	22.8	707.3	499/661	Di-oxidized 23-hydroxytormentic acid ester glucoside $[M+HCOO]^-$	Triterpenoids
13	24.6	695.3	487/647	Tormentic acid derivative	Triterpenoids
14	25.0	693.3	485/645	Oxidized Tormentic acid derivative	Triterpenoids
15	25.6	503.2	485/471/453/441	23-hydroxytormentic acid	Triterpenoids
16	28.4	487.3	468/425/443/407	Tormentic acid	Triterpenoids
17	28.8	485.3	466/423/441/405	Oxidized tormentic acid	Triterpenoids

Table 3- 2 Major components identified in S. spinosum fruit ethanol extract by using HPLC-MS/MS in the negative ionization mode.

3.3.3 The ethanolic *S. spinosum* extract counteracts lipid accumulation in hepatocytes

The biological effects of SEE were assessed on lipid-loaded FaO cells representing a reliable *in vitro* model for hepatic steatosis. First, by MTT assay we excluded any significant

cytotoxicity of SEE at all the tested concentrations (1, 10, 25 µg/mL) (Figure 3-3B). Then, the intracellular TG accumulation was quantified spectrophotometrically showing an increase (+158%, $p \leq 0.0001$) in the steatotic FaO cells (OP) compared to controls. The incubation of the steatotic cells with SEE for 24 hr resulted in a significant decrease in the TG content at all the concentrations with maximal efficacy at the intermediate concentration (SEE 10 µg/mL) (-139% vs OP ($p \leq 0.0001$) (Figure 3-4A).

For a deeper analysis, the morphometry of the cytosolic lipid droplets (LDs) was assessed using both absorption microscopy after ORO staining (Figure 3-4B), and fluorescence microscopy after BODIPY 493/503 staining (Figure 3-4C). While few and small LDs were detected in the control heaptocytes (about 9 LDs/cell, and 1.45 μm^2 average area), in the steatotic haptocytes (OP) we observed a significant ($p \leq 0.0001$) increase in both the number (18 LDs/cell) and the average size (3.22 μm^2, +83.48% vs control) of LDs (Figure 3-4D and Figure 3-4E,respectively). The exposure of the steatotic cells to SEE led to a slight reduction in the LD number, but a large reduction in the LD size. The maximal effect was observed at the intermediate concentration of SEE (10 µg/mL) which reduced both the LD number (to about 15 LDs/cell) and the average size to 1.98 μm^2 (-85.57%; $p \leq 0.0001$) compared to OP.

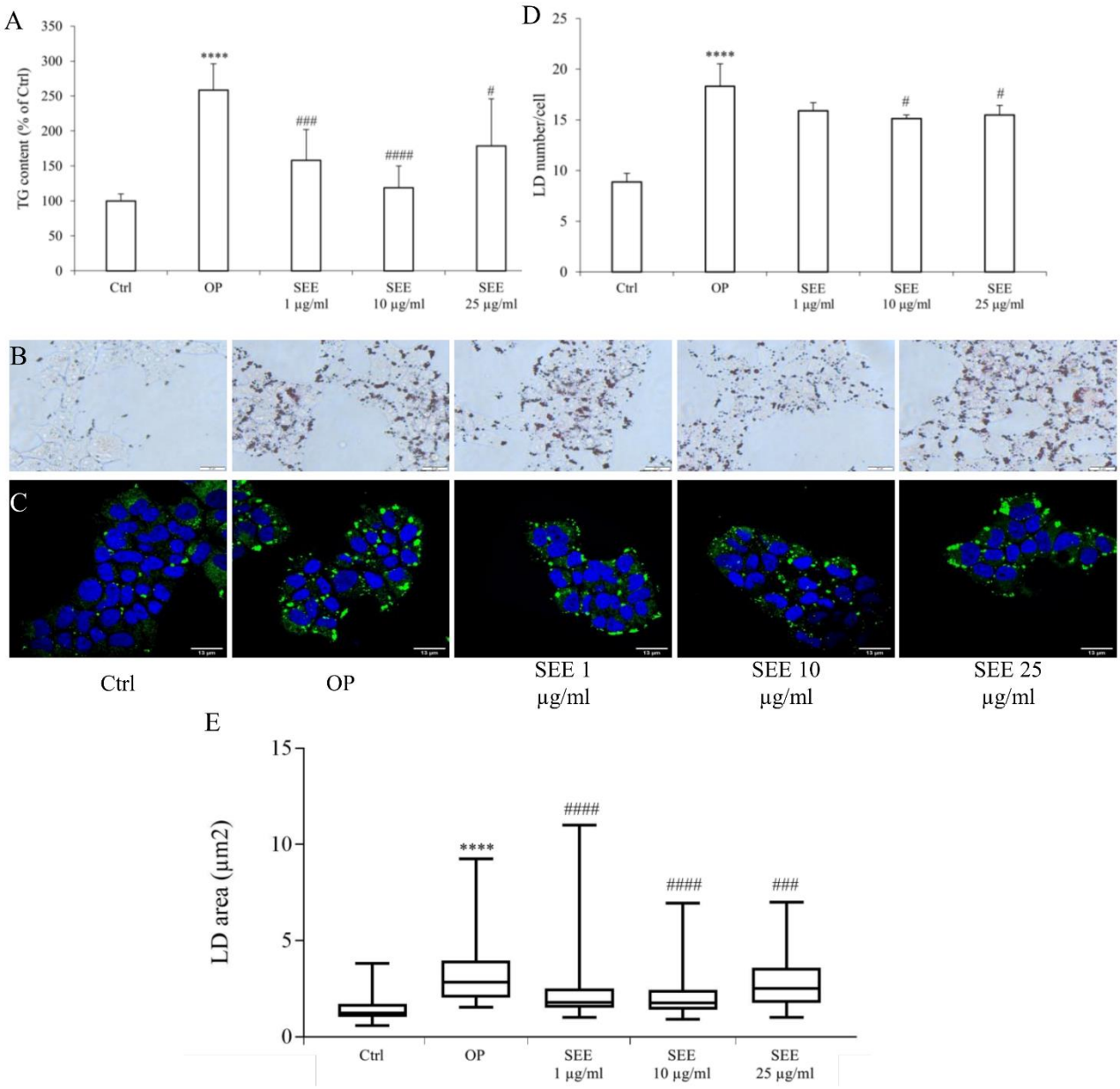


Figure 3- 4 Antisteatotic activity of the S. spinosum fruit ethanolic extract

FaO cells were incubated in the absence (Ctrl) or the presence of oleate/palmitate (OP); then the OP cells were treated with different concentrations of *S. spinosum* ethanolic extract for 24 hr. **(A)** The TG content expressed as percent relative to control after normalization for protein content. Representative images of FaO cells (B) after ORO staining and acquisition by Leica DMRB light microscope, and (C) after Hoechst/BODIPY 493/503 staining and acquisition by A1R Nikon inverted CONFOCAL microscope. Magnification 100X. ImageJ software was used for the image analysis to detect: **(D)** the number of LDs/cell and **(E)** the average size of LDs. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl vs OP cells ****p≤0.0001; OP vs all treatments #p≤ 0.05, ###p≤0.001, #### p≤0.0001.

3.3.4 The ethanolic *S. spinosum* extract ameliorates oxidative stress in hepatocytes

The ectopic TG accumulation in hepatocytes typically leads to excess ROS production that, in part, is counteracted by antioxidant enzymes, such as catalase, and in part triggers lipid peroxidation reactions leading to oxidative stress [110]. We quantified the intracellular ROS production, mainly hydrogen peroxide (H_2O_2), by *in situ* fluorometric analysis of DCF-stained cells (Figure 3-5A). The DCF signal increased in steatotic hepatocytes (OP) compared to control (+31%; $p \leq 0.05$), and it was reduced after treatment with all the concentrations of SEE. The maximal efficacy was observed at the intermediate concentration (SEE 10 µg/mL) leading to a reduction of -48% vs OP ($p \leq 0.001$).

Regarding the antioxidant enzymes, we observed an impairment in the catalase activity in steatotic hepatocytes (OP) compared to control (-28.05%; $p \leq 0.0001$) (Figure 3-5B). The catalase activity showed a recovery after treatment with the SEE both at the lower and intermediate concentrations (+26.26% and +22.65% vs OP, respectively; $p \leq 0.001$ and $p \leq 0.01$). Then we assessd the lipid peroxidation processes being triggered by the excess ROS. We quantified the MDA level by the TBARS assay (Figure 3-5C) and we observed an increase in steatotic hepatocytes (OP) compared to control (+98%; $p \leq 0.0001$), and a significant decrease upon exposure to all the SEE concentrations. Also in this case the maximal efficacy was at the intermediate concentration (10 µg/mL) leading to MDA reduction of -121% compared to OP ($p \leq 0.0001$).

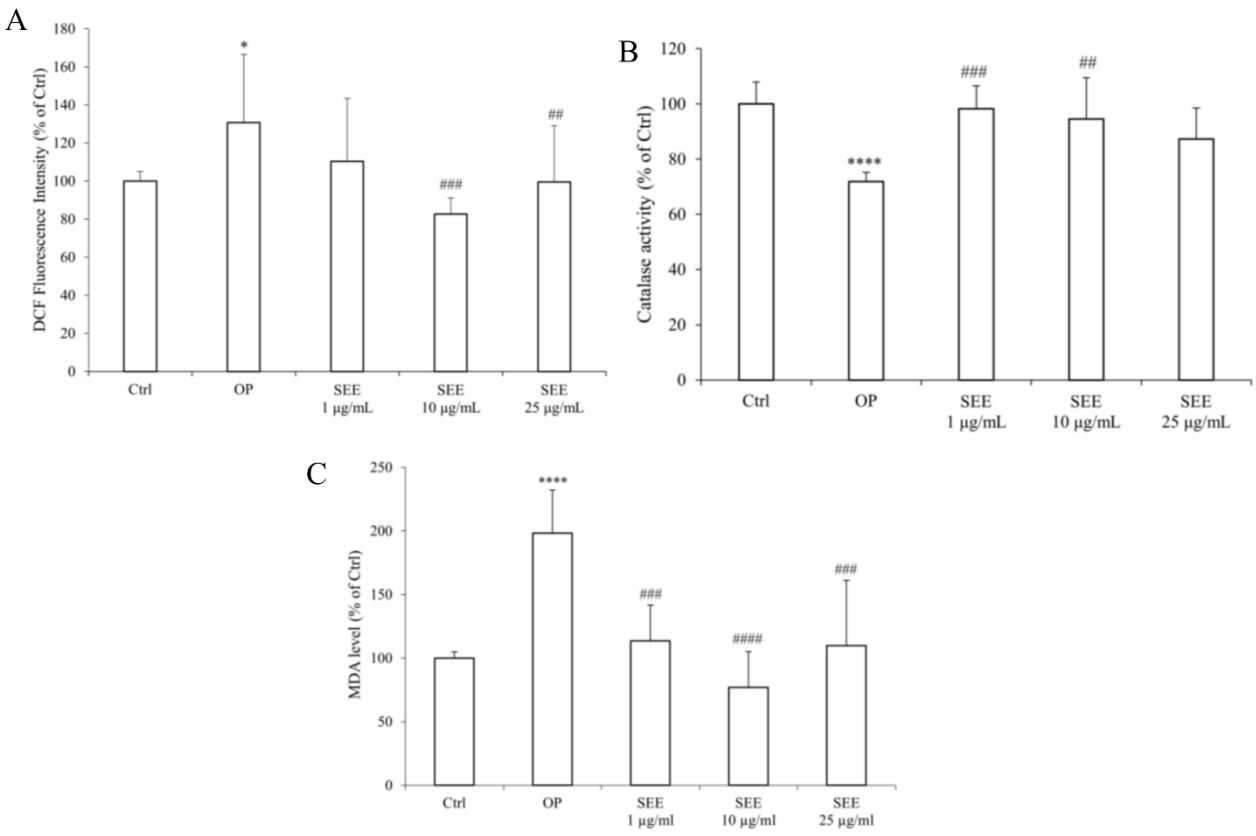


Figure 3- 5 Antioxidant activity of the S. spinosum fruit ethanolic extract

FaO cells were treated as described above. **(A)** The intracellular ROS level was quantified by spectrofluorometric assay on DCF-stained cells and expressed as percent relative to control after normalization for total proteins. **(B)** The catalase activity was evaluated by spectrophotometric assay and expressed as percent relative to control after normalization for total proteins. **(C)** The intracellular level of MDA (pmol MDA/mL×mg of sample protein) was quantified by TBARS assay and expressed as percent relative to control after normalization for total proteins. ANOVA followed by Turkey's test was used to assess the statistical significance between groups. Symbols: Control vs OP, *p≤0.05, ****p≤0.0001; OP vs all treatments, ##p≤0.01, ###p≤0.001, #### p≤0.0001.

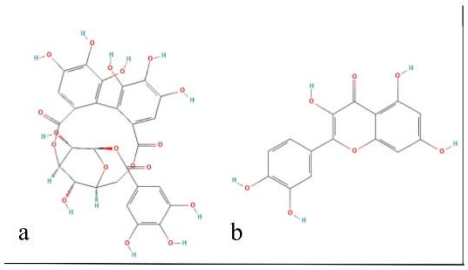
3.3.5 Biological activity of the single agents corilagin and quercetin

HPLC-MS/MS analysis showed that SEE is extremely rich in Cg and Qu, an ellegitannin and a flavonoid, respectively (Figure 3-6). Therefore, we assessed the antisteatotic and antioxidant effects of these two PCs as single agents as comparison with the SEE. Also in this case any cytotoxicity of the compounds at the tested concentration was excluded by MTT assay (Figure

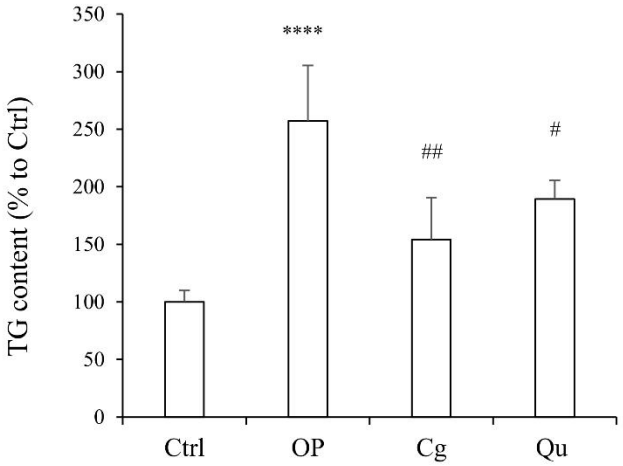
3-3). We selected the concentration of 10 µg/mL for the single agents because it resulted the most effective for all the studies on SEE.

When steatotic hepatocytes were incubated for 24 hr with either Cg or Qu (10 µg/mL) we observed a significant decrease in the intracellular TG content for both compounds, but the effect was larger for Cg (-103.19%; $p \leq 0.01$) than for Qu (-67.91%; $p \leq 0.05$) (Figure 3-6A) A significant reduction in both the number (Figure 3-6C) and the size (Figure 3-6D) of LDs was observed in ORO stained cells (Figure 3-6B), confirming that Cg was more effective than Qu also in reducing the LD number (fom 9 to about 7 LDs/cell) and the LD size (form 0.7 μm^2 to 0.48 μm^2) with respect to OP.

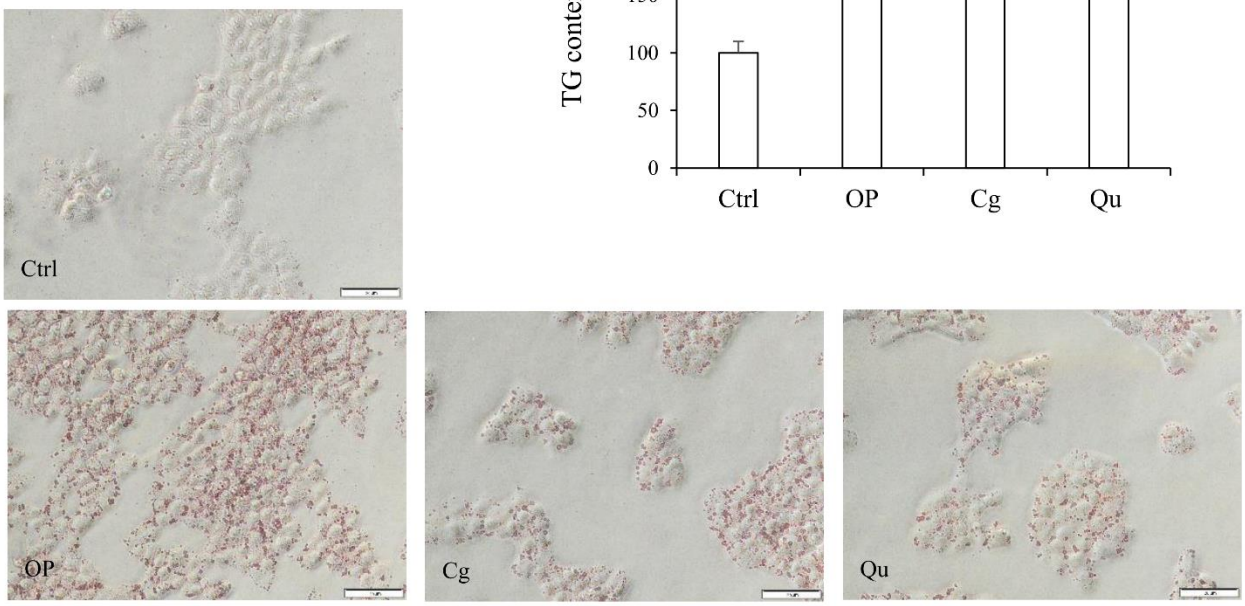
The oxidative stress was quantified by DCF staining (Figure 3-6E), and the TBARS assay (Figure 3-6F). The DCF fluorescence intensity was reduced after treatment with both compounds, and the maximal efficacy was observed for Qu that led to a reduction of -31% compared to OP ($p \leq 0.0001$). Aso the MDA level was significantly reduced by both compounds, but in this case the maximal efficacy was observed for Cg leading to a reduction of -143% compared to OP ($p \leq 0.001$).


a
b

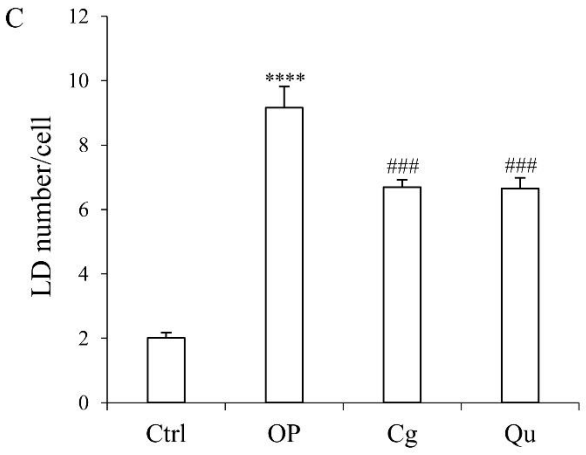
A


TG content (% to Ctrl)
350
300
250
200
150
100
50
0
****
##
#
Ctrl
OP
Cg
Qu

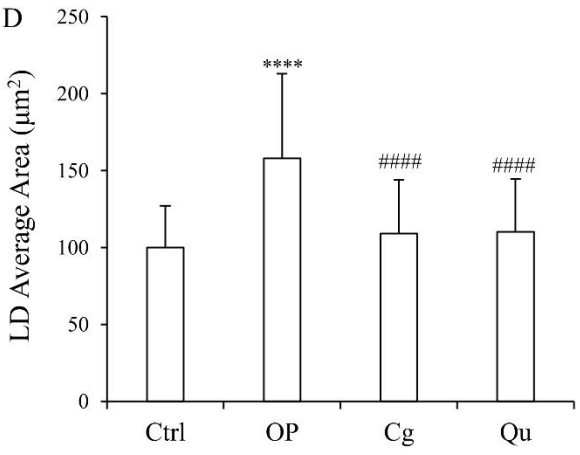
B


Ctrl
OP
Cg
Qu

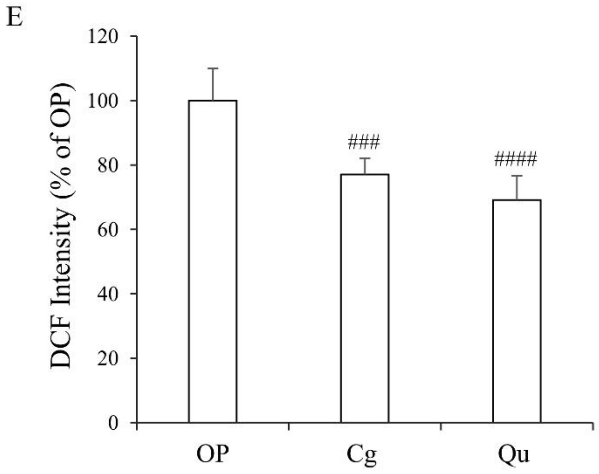
C


LD number/cell
12
10
8
6
4
2
0
****
###
###
Ctrl
OP
Cg
Qu

D


LD Average Area (μm²)
250
200
150
100
50
0
****
####
####
Ctrl
OP
Cg
Qu

E


DCF Intensity (% of OP)
120
100
80
60
40
20
0
###
####
OP
Cg
Qu

F

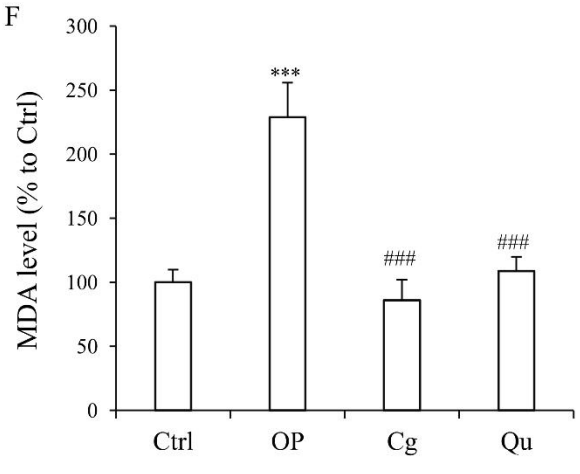

MDA level (% to Ctrl)
300
250
200
150
100
50
0
***
###
###
Ctrl
OP
Cg
Qu

Figure 3- 6 Antisteatotic and antioxidant activity of Corilagin and Quercetin

In the inset, the chemical formula of **(a)** corilagin and **(b)** quercitin (National Center for Biotechnology Information (2023). https://pubchem.ncbi.nlm.nih.gov/**).** The FaO cells treated as described above were analysed for the bioligcal activity. **(A**) The TG content was quantified by spectrophotometric assay. **(B**) Representative images of ORO stained cells were captured by Leica DMRB light microscope and analyzed by ImageJ software to detect: **(D)** the number of LDs/cell and **(E)** the average size of LDs. **(F)** The ROS level was quantified by spectrofluorometric assay on DCF-stained cells. **(G)** The intracellular level of MDA (pmol MDA/mL×mg of sample protein) was quantified by TBARS assay. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl vs OP cells ***p≤0.001, ****p≤0.0001; OP vs all treatments #p≤0.05, ##p≤0.01, ###p≤0.001, #### p≤0.0001.

3.4. Discussion

The present study reports the first characterization of the phenolome of an ethanolic extract from *S. spinosum* fruits, whereas the previous studies focused on the roots. Moroever, our findings showed for the first time the direct biological effect of *S. spinosum* fruits on hepatic cells, focusing on the lipid lowering and antioxidant activity.

In the Beduin traditional medicine, *S. spinosum* shrub is employed as antidiabetic [111] and anti-inflammatory [35] agent. The few scientific publications on *S. spinosum* focused almost exclusively on roots, which seemed to improve the insulin sensitivity and type 2 diabetes, prevented the development of hepatic steatosis in mouse models [32]–[34]. By contrast, the beneficial potential of fruits has not been elucidated yet, although edible fruits are among the best sources of dietary polyphenols [112]. At now, there is only one paper comparing the effects of fruits, leaves and roots of *S. spinosum* in adipocytes [37]. We wish to emhaisize that Lebanese people employ *S. spinosum* fruits more than roots for sustainibility reason, becuase leaves and stems were typically used for fire and cooking in the old times, and also because the preservation of the roots ensures the regrowth of the plant.

Typically fruits may be consumed after processing into functional products, including extracts, in order to increase the polyphenol content and improve the biological effects. Our study

investigated *S. spinosum* fruits collected in South Lebanon and subjected to solvent extraction using either ethanol or water, at both room and boiling temperature, to mimic the traditional way of use. We did not observe significant differences among the extracts for their phenolic and flavonoid content, but the ethanolic extract showed the highest radical scavenging ability *in vitro*. Although usually the phenolic content of an extract directly correlates with the radical scavenging potential [113], our findings showed the best radical scavenger activity for the ethanolic extract without a significantly higher phenolic content. This can depend on the different pool of polyphenols contained in the different extracts, and on the level and type of their hydroxylation [114].

Once selected the *S. spinosum* ethanolic extract for further investigations, we characterized its phytochemical profile by HPLC-MS/MS. This represents the first characterization of the phenolome of *S. spinosum* fruits. The extract results extremely rich in polyphenols, and the most abundant group are the ellagitannins. Ellagitannins are the hydrolyzable subgroup of tannins that are known for their antioxidant and anti-inflammatory activities [115]. In our extract, the main ellagitannins identified are the following: corilagin known for its antidiabetic, antihyperlipidemic and antioxidant properties [116], casuaricitin being endowed with antioxidant and antiviral potential [117], pedunculagin with anti-inflammatory effects [118], and castalagin/vescalagin with antibacterial activity [119]. Also an appreciable quantity of flavonoids was detected in SEE, in particular two quercetin derivatives which were previously detected in *S. spinosum* [23]. Of note, the quercetin is well known for its lipid lowering and antioxidant effects [120]. Moreover, the SEE is also rich in triterpenes such as the tormentic acid and its derivatives; these bioactive compounds had been detected also in *S. spinosum* root extracts [121], and are known for their hypolipidemic and hepatoprotective effects [122]. In conclusion, the phenolome of the *S. spinosum* fruit extract shows large similarity with those of other Rosaceae [123].

Recently, western diet and sedentary lifestyle habits increased the prevalence of hepatic steatosis and NAFLD worldwide. A relaible cellular model of hepatic steatosis had been developed and widely employed by our group [104], [105], [124], and adopted for the present study. Our findings show that the ethanolic extract from Lebaneese *S. spinosum* fruits is able to counteract *in vitro* the moderate hepatic steatosis resulting from exposure of hepatic cells to excess of exogenous fatty acids. The lipid-lowering activity of the extract was sustained by its action on lipid droplets whose number and size were increased markedly in steatotic cells compared to controls, and decreased significanlty upon incubation with the extract. Interestingly, our results on a cellular model of hepatic steatosis are in line with previous studies showing the biological activity of an aqueous extract of *S. spinosum* roots on both a mouse model of fatty liver disease [31], and on diabetic mice [32]. Of note, while in mice with fatty liver, the root extract was able to improve insulin resistance and to prevent hepatic steatosis [31], in diabetic mice the extract improved glucose tolerance and insulin sensitivity, but did not reduce hepatic steatosis. The novelty of our approach is the demonstration that a polyphenols-enriched ethanolic extract prepared from *S. spinosum* fruits exterts a potent and direct action on steatotic hepatic cells thus indicating that the biological activity of the extract does not depend on the secondary metabolites derived from digestion and biotransformation preocesses.

As well known, excess hepatic fat depot leads to overproduction of free radicals and oxidant species resulting in a vicious circle leading to oxidative stress and inflammation. Indeed, both oxidative stress and the consequent lipid peroxidation are elevated in NAFLD patients [125]. The oxidative stress condition was observed also in our cellular model of moderate steatosis, where both the ROS levels and the lipid peroxidation increased markedly in steatotic hepatocytes. The *in vitro* free radical scavanger ability of SSE was confirmed also *in situ* at the cellular level thus sustaining the anti-oxidant potential of the extract. Indeed, in the steatotic hepatocytes, the fat-induced oxidative stress was counteracted by SEE which led to a significant

reduction in both ROS and lipid peroxidation levels. Interestingly, catalase, the main antioxidant enzyme which decomposes the excess of H_2O_2, resulted impaired in the steatotic FaO cells, and this may be a sign of an unbalance in the anti-oxidant system due to the excees fat depot. The catalase activity was restored by the treatment with SEE. Of note, the published results on catalase activity are conflicting with some studies reporting a decrease [53] and others reporting an increase [126] depending on the models of NAFLD. Of note, our findings about the anti-oxidant and hepatoprotective ability of SEE well parallel a previous study reporting the hepatoprotective activity of a water/methanol extract from *S. spinosum* roots being able to reduce the oxidative stress in a model of hepatic damage in CCl4- insulted rats [127].

Based on the phenolome profile recorded by HPLC-MS/MS analysis and on the literature data, we focused on the ellegitannins (corilagin) and the flavonoids (quercetin) as the possible central players of the beneficial activity of the *S. spinosum* extract. Indeed, both quercetin [128] and corilagin [116] are well known for their beneficial effects. We assessed the hepatoprotective efficacy of both these PCs as single agents and compared them with the SEE. Both Cg and Qu played antisteatotic activity, but Cg was more effective than Qu in reducing the intracellular TG accumulation and in protecting the cell membrane from the lipid peroxidation. Interestingly, Qu was more effective in reducing the ROS production. Indeed, different polyphenols can influence different number of signaling pathways that have an impact on various cellular and tissue-level processes [129]. However, we want to note that the *S. spinosum* extract was more effective than both Cg and Qu in counteracting fat accuumulation and ROS production. Maybe this effects depends on synergistic or additive effects along the pool fo PCs being present in the crude plant extract [130].

In conclusion, a lot of epidemiological data provide evidence for the health benefits of diets rich in herbs, fruits and vegetables, mainly due to their bioactive constituents, including polyphenols endowed with well-documented potential to ameliorate the interconnected pathological processes of inflammation and oxidative stress [131]. The present study

demonstrates the hepatoprotective effects of an ethanolic extract from *S. spinosum* fruits being rich in PCs and suggests that the ellagitannins and flavonoids that are abundant in the SEE may sustain the beneficial activity. These findings unveil the potential of this shrub to be employed as functional food or nutraceutical supplementary to counteract/improve the metabolic disorders associated to overnutrition, oveweight and NAFLD.

Acknowledgements

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Funding

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4. Chapter Four: The anti-inflammatory potential of an ethanolic extract from Sarcopoterium spinosum fruits for protection and/or counteraction against oxidative stress in dysfunctional endothelial cells.

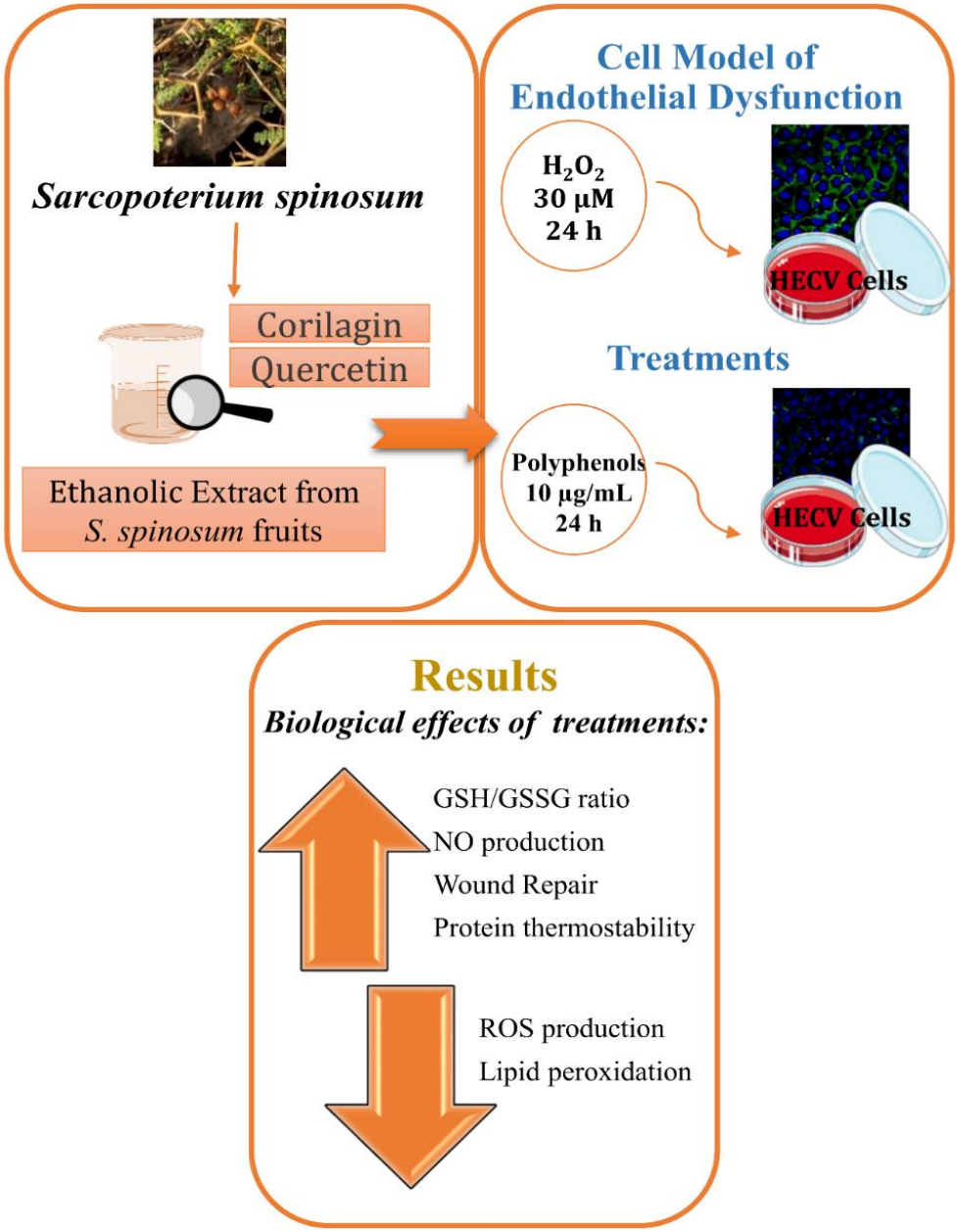

Figure 4- 1 Chapter four graphical abstract

The anti-inflammatory potential of an ethanolic extract from *Sarcopoterium spinosum* fruits for protection and/or counteraction against oxidative stress in dysfunctional endothelial cells.

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Abstract

Plants and plant extracts are a relevant source of bioactive compounds widely employed as functional food. The shrub *Sarcopoterium spinosum* is traditionally used as herbal medicine for weight loss and diabetes treatment in the Mediterranean area. Inflammation is a protective mechanism involved in the development of many pathological conditions, including cardiovascular disease (CVD). The present study aimed at investigating in vitro the antioxidant and cytoprotective properties of an ethanolic extract from S. spinosum fruits (SEE) in a cellular model of endothelium dysfunction. Corilagin and quercetin are two polyphenols abundant in SEE and tested for comparison. Exposure of HECV cells for 24 h to 30 µM H2O2 lead to an oxidative stress condition. When HECV cells were treated with 10 µg/mL of SEE, or single compounds, after or before the oxidative insult, the results showed their ability to: *(i)* decrease the ROS production measured by fluorometric analysis, and the lipid peroxidation measured by the spectrophotometric assay; *(ii)* to rescue both the GSH/GSSG and NO impair and the tissue protein denaturation; *(iii)* to accelerate the wound repair measured by T-scratch assay. Taken together, our findings indicate that the ethanolic extract from S. spinosum fruits could be a potential candidate for nutraceutical application.

Key words:

Sarcopoterium spinosum fruit extracts; corilagin; quercetin; antioxidant activity; anti-inflammatory activity; wound healing.

4.1. Introduction

Inflammation is a protective mechanism of the organism in response to external and internal stimuli caused by mechanical, chemical or biological stresses [132]. Oxidative stress plays a crucial role in the development and perpetuation of inflammation and contributes to the pathophysiology of a number of diseases such as cardiovascular diseases, diabetes, metabolic syndrome, degenerative processes and cancer [133]. The oxidative stress refers to the unbalance between generation of pro-oxidant species and antioxidant defences, which may occur for both increasing production of oxidant molecules and/or impair of the antioxidant system [110]. A physiological production of reactive oxygen species (ROS) and free radicals results from many cellular processes such as mitochondrial respiration and metabolism, however, when the production overpasses the antioxidant defence this may lead to oxidative stress and tissue damages [134]. The denaturation of tissue proteins is well-documented in inflammatory diseases and it is considered a marker for inflammation [135].

The vascular endothelium is the largest organ in the body where it plays anti-inflammatory and anticoagulant actions, maintains vascular tones, and acts as physiological barrier to prevent blood cell adhesion [136]. Endothelial dysfunction is characterized by decreased production and/or local bioavailability of nitric oxide (NO) and excess production of ROS [137]; in turn, endothelium dysfunction may trigger a lot of pathological pathways such as CVD, being typically associated to diabetes and obesity [138]–[140]

To control inflammation, both steroidal and non-steroidal anti-inflammatory drugs (NSAID) can be used, but they may result in undesirable side effects. This has led to increasing interest in the research of bioactive compounds with anti-inflammatory activity extracted from natural sources, as they can offer certain advantages compared with synthetic drugs, such as the low incidence of adverse effects in the patient.

Polyphenols (PPs) are phenyl propanoids synthetized by plants as secondary metabolites, and are found largely in the fruits, vegetables and cereals [92]. More than 8000 phenolic molecules have been identified, and polyphenols must contain at least one aromatic nucleus and one or more -OH groups. The Mediterranean diet is characterized by high consumption of foods rich in polyphenols, and it is associated with health promotion [141]. In particular polyphenols are potent antioxidant, anti-inflammatory, antimicrobial and cardio-protective agents [142] . Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polyphenols protect against metabolic disorders and cardiovascular diseases (CVD) [143], [144].

Sarcopoterium spinosum, Bilan in Arabic, is a chamaephyte of the Rosaceae family, which is largely employed as a medicinal plant in the Mediterranean region. Ethnopharmacological studies reported the traditional usage of *S. spinosum* extracts for the treatment of several disorders, especially diabetes [2], [28], pain relief [27], asthma, kidney stones and poisoning [30]. The majority of surveys focused on the aqueous extract from *S. spinosum* roots [32], [33], whereas the biological effects of the fruits were less investigated. In a recent study of our group, different extracts from *S. spinosum* fruits were compared in terms of their phytochemical activity and lipid-lowering effect in steatotic hepatocytes [145]. As a last consideration, the folk habits in using the medicinal plants, *S. spinosum* in particular, lead the Lebanese population to assume them as infusion/tea as therapy for specific illnesses, or to drink them daily as a healthy habit for disease prevention. Therefore, in our study we tried to mimic both these modalities of their intake.

The aim of the present study was to investigate the antioxidant, anti-inflammatory and cytoprotective potential of the ethanolic extract from *S. spinosum* fruits (SEE) on a cellular model of endothelial dysfunction consisting of human endothelial exposed to an oxidative insult. Many oxidative stress markers such as the cytosolic production of ROS, the release of NO, the level of membrane lipid peroxidation, the alteration of the GSH/GSSG ratio were

determined. Moreover, the protective ability of SEE was tested in both cell-free assays as protection against protein denaturation and in cell models as promotion of the wound healing. Our findings seem to indicate that the ethanol extract, due to its main polyphenols corilagin and quercetin, is endowed with significant anti-inflammatory effects on dysfunctional endothelial cells thus representing a good nutraceutical candidate.

4.2. Materials and methods

4.2.1. Chemicals

Unless otherwise indicated, the reagents employed were supplied by Sigma-Aldrich Corp. (Milan, Italy).

4.2.2. Plant collection and extraction

Sarcopoterium spinosum (L.) Spach fruits (Figure 4-2) were collected in summer from the wild in South Lebanon, Hadatha (Latitude: 33° 09' 60.00" N, Longitude: 35° 22' 59.99"). They were identified according to Prof. George Tohme, a taxonomist and president of Lebanese CNR. A voucher specimen (R5.36) was placed in the Lebanon National Herbarium at the Lebanese University, Faculty of Sciences.

The fruits were dried for three weeks in a shaded, temperate environment. To prepare the ethanolic extract, a liter of 99% ethanol was mixed with 50g of ground material. After 3 h, the mixture was filtered and then ethanol was evaporated and the dry pellet was collected at 42°C in a rotary evaporator (Heidolph Instruments, Schwabach, Germany). The MS characterization of the SEE characterized the polyphenolic profile of the extract (Table 4-1)

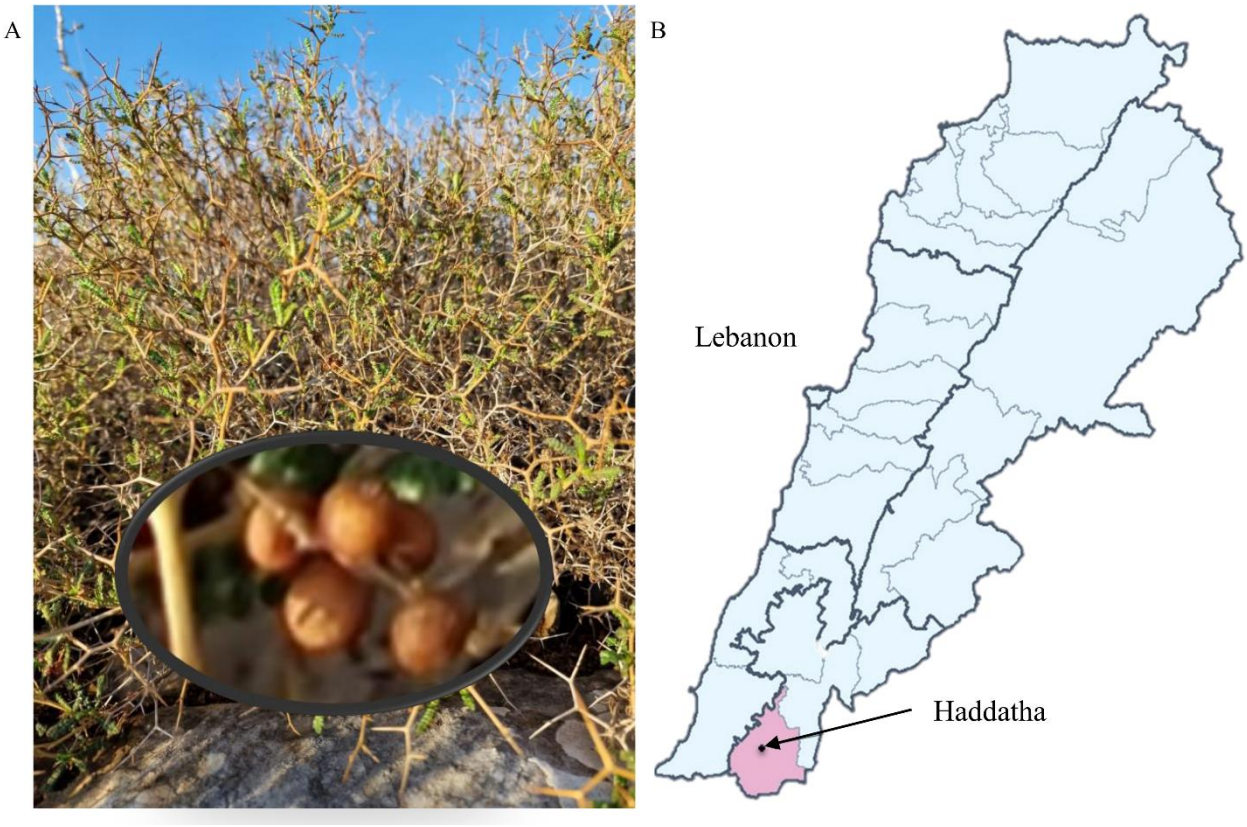

Figure 4- 2 S. spinosum characteristics and distribution

S. spinosum plant in its natural environment: **(A)** the shrub and fruits **(B)** the region of the fruit collection: Haddatha, South Lebanon **.**

4.2.3. Protein Quantification

The protein content of the samples was quantified by Bradford assay using bovine serum albumin (BSA) as a standard [98].

4.2.4. Cell culture and treatments

Human endothelial cells the HECV cells (Cell Bank and Culture-GMPIST, Genoa, Italy) were cultured in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with 2mM Glutamine and 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. To induce oxidative cells, HECV were insulted for 24 h with 30 µM hydrogen peroxide (H_2O_2). For treatrment, cells were incubated for 24 h with either 10 µg/mL of SEE or the single PPs corilagin (Cg) and querectin (Qu). The treatments with polyphenols were performed both before the H_2O_2

insult (to assess the protective activity) or after (to assess the counteraction activity). Each experiment was performed at least in quadruplicate.

4.2.5. Cell viability assessment

The antiproliferative and/or cytotoxic activity of SEE and its main components was determined using the MTT assay. Cell viability was tested by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [103]. Briefly, 2000 cell/well were seeded (200µL volume) in a 96 well plate, and incubated at 37˚C in humidified air with 5% CO2 for 24 h. After that, cells were treated with 10 µg/mL of either SEE, Cg, or Qu. After 24 h 20 µL of MTT solution (5mg/mL) were added to each well and incubated for 3 h. At the end, medium was removed, formazan was dissolved by 200 µL of isopropanol and the absorbance was read at 570 nm.

4.2.6. ROS production

Oxidant species prodution were quantified *in situ* using the oxidation of the cell-permeant 2′-7′dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2′-7′dichlorofluorescein (DCF) [107]. Briefly, cells were treated, collected and then were incubated with 1 µM DCF-DA in PBS prepared from stock 10 mM stock prepared in DMSO for 30 min at 37°C in the dark. After that, cells were centrifuged, suspended in 2 mL PBS and the fluorescence was read at 25°C using a water-thermostated cuvette holder (λ_{ex}=495 nm; λ_{em}=525 nm) with a LS50B fluorimeter (Perkin Elmer, USA). Protein quantification was used for normalization. Results are expressed as percent flourescence intensity relative to control. The results represent the average of at least three independent experiments in triplicate.

For fluorescence microscopy, the oxidant species were stained with 1 µM DCF-DA in PBS, and DNA with 2 µg/mL Hoechst 33342 (Thermofiher Scientific, Italy). Images were acquired at 20X magnification by optical and/or phase contrast microscopy using an inverted Olympus IX53 microscope (Olympus, Milano, Italy) equipped with a CCD UC30 camera and a digital

image acquisition software (cellSens Entry). All images were processed by using ImageJ software.

4.2.7. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) assay [108] quantify spectrophotometrically lipid peroxidation level which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA). Briefly, 250 uL of cell solution was incubated for 45 min at 95°C with 500 µL of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 750 µL of N-butanol was added and the organic phase was read at (λ_{ex}=532nm) in a UV-VIS spectrophotometer at 25°C using Peltierthermostated cuvette holder.

4.2.8. Nitrite/Nitrate levels

The nitric oxide level produced by HECV cells was determined by the Griess reagent following the previously described method [146]. The NO production using spectrophotometric analysis of the end products, nitrites and nitrates. Following treatments, nitrite accumulation was measured by incubating 500 mL media with 500 mL of griess ragent and incubated in dark for 10 min, absorbance was recorded at λ540 nm. $NaNO_2$ standard curve was used as reference. Results were normalized with protein quatification and were expressed as (µmol NaNO2/mg sample protein).

4.2.9. GSH/GSSG Ratio

The ratio between the reduced (GSH) and oxidized (GSSG) glutathione is a frequently used indicator of oxidative stress in cells and tissues. In our cellular samples, the levels of GSH and GSSG were measured using the glutathione colorimetric assay Kit (ZX-44100-96, Zelix, Germany). Briefly, cells seeded in 100 mm^2 plates were treated and then harvested in 1 mL of cold PBS, homogenized and deproteinized by 5% 5-sulfo-salicylic acid. Total glutathione (GSH+GSSG) and GSSG levels were evaluated in the supernatant according to the manufacturer's protocols. The absorbance of each sample was read in a UV-VIS

spectrophotometer at 25°C (λex = 405 nm) using plate reader. Then the GSH level and then GSH/GSSG ratio were calculated as stated below:

$$GSH = Total\ glutathione - GSSG$$

$$GSH/GSSG = \frac{GSH}{\mathrm{GSSG}}$$

4.2.10. Wound healing assay

For the wound healing assay [147], HECV cells were seeded on 35 × 10 mm^2 tissue culture dishes and incubated until 100% confluency, then by using p100 pipet tip the cell monolayer was scraped making two crossing straight lines to create a ‘‘scratch’’. Then, three views on the scratch were photographed by an inverted Olympus IX53 microscope (Olympus, Milan, Italy) and representative images were captured with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). After scratching, medium was replaced with fresh medium in the presence of each plant extract and the polyphenols or H_2O_2. Set of images were acquired at 0 and 24 h. To determine the migration of HECV, the images were analysed using ImageJ free software (http:// imagej.nih.gov/ij/). Percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells. Data are means ± S.D. of at least three independent experiments

4.2.11. Protein denaturation assay

The protein denaturation assay was performed according to a classical method [148] with minor modifications. Breifly, either 2 mL (10 μg/mL) of the extract or of the NSAID diclofenac sodium (2-[(2,6 dichlorophenyl)amino] benzene acetic acid sodium salt) were mixed with 2.8 mL 1M phosphate buffer (pH 6.4) and 0.2 mL 1% BSA and incubated at 37°C for 15 min; then, the temperature was increased to 70°C for 5 min and, after cooling. the absorbance of the

sample was read at 660 nm. The control (100% protein denaturated sample) was prepared as 4.8 mL phosphate buffer with 0.2 mL 1% BSA. The percentage inhibition of BSA denaturation was calculated as stated below:

$$\%\ BSA\ inhibition = 100 * (\frac{Ab\ control - Ab\ sample}{Ab\ control})$$

4.2.12. Statistical analysis

Data are means ± S.D. of at least three independent experiments. Statistical analysis was performed using ANOVA with Tukey's post-test (GraphPad Software, Inc., San Diego, CA, USA).

4.3. Results

4.3.1. *Cytoprotective activity of SEE on enothelial cells*

As previously described [145], the ethanolic extract from *S. spinosum* fruits is rich in polyphenols (PPs). The ellagitannin family was found to be the most abundant group of polyphenols in the extract (approximately 50.8%). Triterpenes and flavonoids were also present in significant amounts (about 11.9% and 7% of the extract, respectively) (Table 4-1). As the preliminary step we assessed if SEE, single PPs and H_2O_2 affect viability and/or proliferation of HECV cells in order to identify the concentrations at which they could be employed. Any cytotoxicty on HECV cells after 24 h was excluded for both the oxidative insult (30 µM H_2O_2) as well as for the SEE and the single polyphenols corilagin (Cg) and quercetin (Qu) at the dose of 10 µg/mL (Figure 4-3A). Interestingly, the exposure of HECV cells to corilagin was able to slightly stimulate the cell proliferation (+7%, $p \leq 0.05$ compared to Ctrl) .

RT (min)	[M-H]-	MS/MS fragments	SEE major compounds	Classification	%
13.5	935	633/301/897	Casuarictin isomer	Ellagitannins	15.0
15.4	935	633/301/897/783	Casuarictin isomer	Ellagitannins	8.9
17.9	477.1	301	Quercetin glucuronide	Flavonoids	7.0
12.2	935	633/301	Castalagin/Vescalagin	Ellagitannins	6.7
7.5	783.2	633/301	Pedunculagin	Ellagitannins	4.8
5.6	633.1	301/463	Corilagin	Ellagitannins	3.3
22.6	709.3	501/663	23-hydroxytormentic acid ester glucoside [M+HCOO]- isomer	Triterpenoids	2.2
22.8	707.3	499/661	Di-reduced 23-hydroxytormentic acid ester glucoside [M+HCOO]-	Triterpenoids	1.8
25.6	503.2	485/471/453/441	23-hydroxytormentic acid	Triterpenoids	1.2

Table 4- 1 Major components identified in the ethanolic extract from S. spinosum fruit by using HPLC-MS/MS in the negative ionization mode and their respective abundance

4.3.2. Anti-oxidant activity of SEE on dysfunctional endothelial cells

The exposure of cells to H_2O_2 represents an oxidant insult leading to over-production of ROS and free radicals that, in part, may be neutralized by the antioxidant enzymes, and in part may trigger lipid peroxidation reactions which are the general marker for oxidative stress. The intracellular ROS production was assessed by *in situ* fluorometric analysis of DCF-stained cells (Figure 4-3B). As expected, the exposure to 30 µM H_2O_2 led to significant increase in ROS production (+20% vs untreated control; $p \leq 0.05$). A significant decrease in the ROS level was observed when SEE was added after (counteracting activity) or together (preventing activity) the H_2O_2 insult (-22% and -24% vs control, respectively; $p \leq 0.01$ and $p \leq 0.0001$). A similar effect was observed for corilagin alone which significanlty reduced the ROS production of -33% ($p \leq 0.0001$) in the counteraction mode, and of -24%, $p \leq 0.01$ in the protection mode. By contrast, quercetin alone was not able to decrease the ROS production in protection mode, whereas it was effective in counteraction mode (-22%; $p \leq 0.001$). The changes in ROS production as a

function of the treatments were visualized in parallel by fluorescence microscopy (Figure 4-3C).

As the ROS over-production may trigger lipid peroxidation of the cell membranes, by TBARS spectrophotometric assay we quantified the levels of the byproduct MDA (Figure 4-3D). The MDA level markedly increased in H_2O_2-treated cells compared to control (+63%, $p \leq 0.001$ for protection condition, and +97%, $p \leq 0.0001$ for counteraction condition). The lipid peroxidation was significantly reduced by all treatments. For the counteraction condition all agents exerted a similar antioxidant effect by reducing the MDA increase of -110% (SEE), -109% (Cg) and of -111% (Qu) ($p \leq 0.0001$) compared to H_2O_2-treated cells. Also in the protection condition we observed an antioxidant effect with a reduction in the MDA levels by -70% (SEE), -72% (Cg) and -64% (Qu) ($p \leq 0.01$) compared to only H_2O_2 treated cells.

In the cytosol, the reduced glutathione (GSH) can be oxidized to glutathione disulfide (GSSG) to balance the redox status. Figure 4-3E shows the changes in the GSH/GSSG ratio as a function of the treatments. We observed a signifcant reduction ($p \leq 0.05$) of the GSH/GSSG ratio when HECV cells were exposed to H_2O_2 (from 9.5 ± 0.3 in control cells to 6.3 ± 0.7 in H_2O_2-treated cell in counteraction conditions, and 6.6 ± 0.6 in protection conditions). The GSH/GSSG ratio was significantly increased by the treatments with polyphenols. In the protection condition, all polyphenos rescued the H_2O_2-induced decrease in the GSH/GSSG ratio (11.3 ± 0.5 for SEE, 10.7 ± 1.3 for Cg, and 12.3 ± 1.6 for Qu) compared to H_2O_2-treated cells ($p \leq 0.01$ and $p \leq 0.001$, respectively). For the counteraction condition, only SEE and Cg were able to significantly ($p \leq 0.01$) rescue the decrease in GSH/GSSG (10.5 ± 2 for SEE and 11.1 ± 1.1 for Cg).

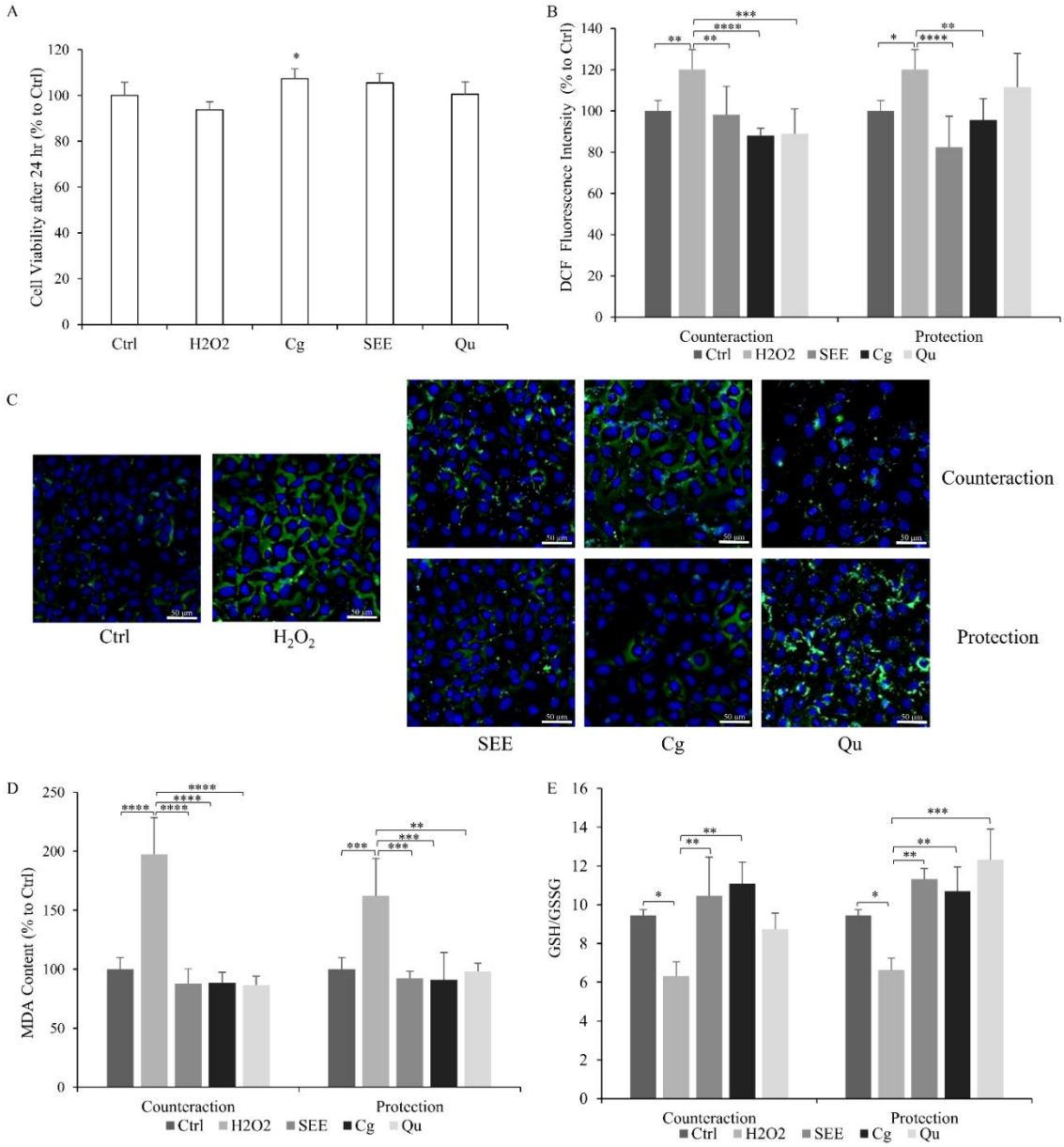

Figure 4- 3 Antioxidant activity of the S. spinosum fruit ethanolic extract

Effects of 24 h treatment with either 10 µg/mL of extract (SEE), or corilagin (Cg) or quercetin (Qu) on H_2O_2-insulted HECV cells evaluated in terms of: **(A)** cell viability evaluated by MTT assay; **(B)** intracellular ROS level quantified by spectrofluorometric assay on DCF-stained cells and expressed as percent relative to control after normalization for total proteins; **(C)** fluorescence microscopy of DCF/Hoechst stained cells; **(D)** intracellular level of MDA (pmol MDA/mL×mg of sample protein) quantified by TBARS assay and expressed as percent relative to control after normalization for total proteins. **(E)** GSH:GSSG ratio quantified by colorimetric kit. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: *p≤ 0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001

4.3.3. Anti-inflammatory activity of SEE on dysfunctional endothelial cells

The anti-inflammatory potential of SEE was evaluated *in vitro* by a cell-free assay measuring the inhibition of the thermal denaturation of albumin. Protein denaturation refers to a process in which the proteins loose their 3D structure due to exposure to external stress which generally leads to a loss of their biological functions. The inhibitory effect on protein denaturation was measured for all compounds at 10 µg/mL concentration. Diclofenac reported the highest ability to protect protein from thermal denaturation (inhibition of 60%; $p \leq 0.0001$). Among polyphenols, the strongest effect was reported for querectin and SEE (inhibition of 53% and 49% vs BSA alone, respectively; $p \leq 0.0001$) followed by corilagin (inhibition of 38%; $p \leq 0.0001$) (Figure 4-4A).

The anti-inflammatory potential was also evaluated *in vivo* by measuring the NO release in H_2O_2-stimulated HECV cells. Our data showed a decrease in NO release after stimulating the cells with H_2O_2, (-41% in protection condition and -22% in counteraction conditions compared to control; $p \leq 0.05$). Both the single polyphenols and the SEE rescued the H_2O_2-induced NO decrease. For the protection condition, the NO release increased significantly of +57%, (SEE), +65% (Cg) and of +67%, (Qu) compared to H_2O_2-treated cells. Also in the counteraction condition we observed a significant increase in the NO levels by +82%, (SEE), +65%, (Cg) and +74% (Qu) compared to only H_2O_2-treated cells (Figure 4-4B).

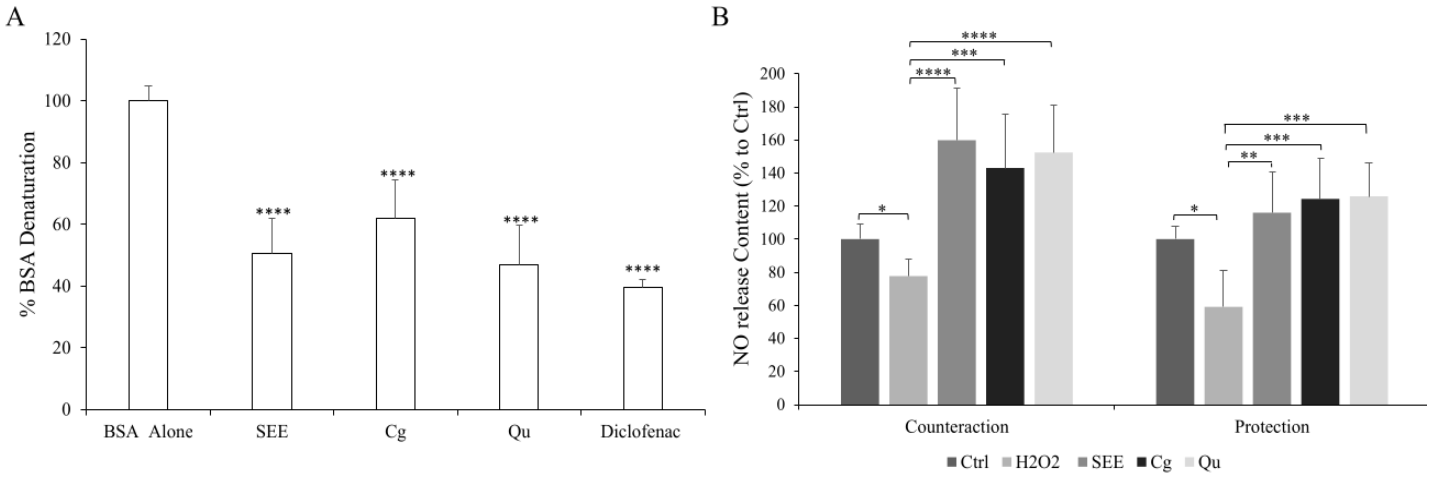

Figure 4- 4 Anti-inflammatory activity of the S. spinosum fruit ethanolic extract

Influence of 10 µg/mL of either extract (SEE), or corilagin (Cg) or quercetin (Qu) as antiinflammatory agent. We asessed: **(A)** *in vitro* cell-free inhibition of heat-induced BSA denaturation at 75˚C, using diclofenac sodium as a reference; **(B)** NO production in H_2O_2-stimulated HECV cells quantified in the medium as µmol NaNO2/mg sample protein by Griess reaction. All values are expressed as % of control. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: *p≤ 0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001

4.3.4. Promoting effect of SEE on the wound repair of endothelial cells

The T-scratch assay is typically utilized to quantify cellular migration on two-dimensional (2D) surfaces over time upon treatments. Figure 4-5 reports the images and the histograms for all the samples with the wound width at t24 expressed as % respect to the original wound width at t0 from the scratch. At t24, control HECV cells showed a reduction in the wound width of -54%, while in the H_2O_2-treated cells the wound width was reduced only of -25% (conteraction condition) and of -36% (protection condition). This indicates a slowdown in the healing process due to the H_2O_2 insult around -25% (p≤0.01) in both counteraction and protection conditions compared to normal cells. The treatment with PPs resulted in an average acceleration of the wound repair compared to positive and negative controls. Of note, a significant acceleration in the wound healing process was observed only when SEE and Cg were added before or after the H_2O_2 insult, while quercetin did not affcet significantly the wound repair. In counteraction

condition, both SEE and corilagin reduced the wound width of a similar extent (-60% and -62%, respectively) Similar results were observed in the protection condition, with SEE and corilagin reducing the wound width of a similar extent (-64% and -68%, respectively). These data indicate that both SEE and corilagin trigger a significant acceleration of the wound repair in both counteraction condition (+46 % and +45%, respectively $p \leq 0.0001$) and in the protection conditio (+50 % and +59 %; $p \leq 0.0001$) compared to H_2O_2-insulted cells.

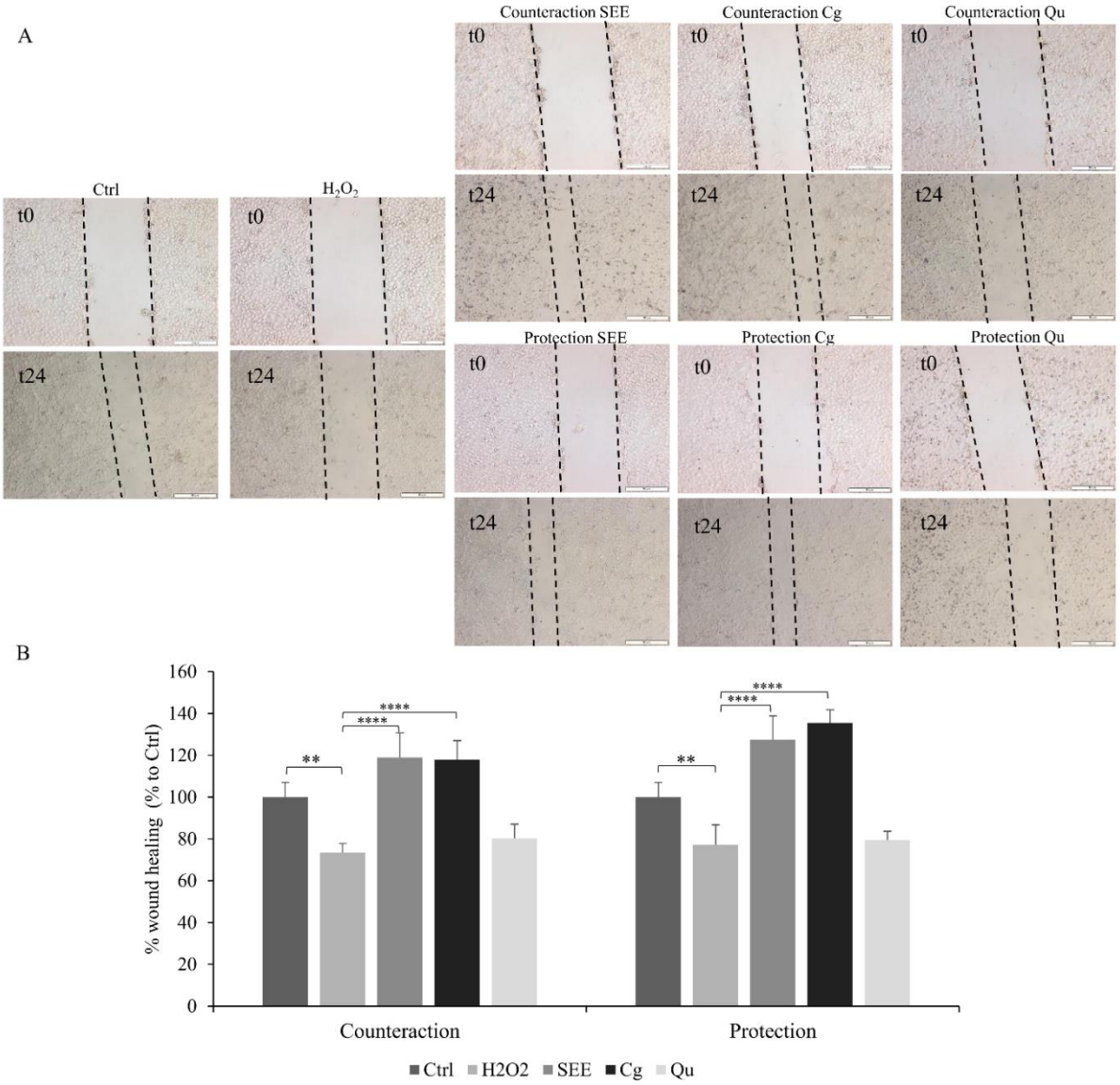

Figure 4- 5 Wound repair promoting effect of the S. spinosum fruit ethanolic extract

Influence of 10 µg/mL of either extract (SEE), or corilagin (Cg) or quercetin (Qu) on cell migration and wound repair measured using the T-schratch assay on H_2O_2-stimulated HECV cells. **(A)** The wound width was marked by dotted lines in representative images acquired by an inverted Olympus IX53 microscope (Olympus, Milan, Italy) and captured with a CCD UC30 camera and a digital image

acquisition software (cellSens Entry); **(B)** Histogrmas representing the percentage of the wound healing after 24 h of inducing the wound (% to Control)**.** Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: **p≤0.01, ****p≤0.0001

4.4. Discussion

Reinforcing the defenses of the vascular endothelium against oxidative stress and inflammation has been proposed as a viable option for reducing the onset and progression of cardiovascular disease, and other pathologies such as diabetes, metabolic syndrome, and degenerative processes. The main finding of the present study is that the ethanolic extract from *S. spinosum* fruits, being rich in polyphenols such as corilagin and quercetin, is able to protect endothelial cells against oxidative stress generated by a strong oxidant insult. The antioxidant defense exerted by the *S. spinosum* extract seems to be sustained by the scavenging of the ROS excess with consequent reduction in lipid peroxidation, by a rescue of both the GSH/GSSG and NO impair, and by protection against the tissue protein denaturation. Besides, this cytoprotective action of the SEE is accompanied by an acceleration of the wound healing process.

Polyphenols are well known for their antioxidant potential and they could be employed in slowing the progression of cardiovascular disorders [149]. *S. spinosum* is a medicinal plant traditionally used as an anti-diabetic remedy in Lebanon [2]. Although the anti-diabetic activity of *S. spinosum* root extracts is reported in different studies [32], [33] the beneficial effects of aerial parts, especially fruits, had been poorly elucidated in the past. Recently Hudec *et al.* characterized an ethanol extract from *S. spinosum* leaves and thorns showing their abundance in flavonoids (rutin), alkaloids (stachydrine) and ammonium compounds (benzalkonium chloride) sustaining its cytotoxic effects on cancerous cells [38]. Regarding the fruits, in a recent paper published by or group [145], the ethanolic extract from *S. spinosum* fruits has been

extensively investigated in terms of phenolome profile and biological activity using a model of steatotic hepatocytes. For this reason, we considered SEE a promising candidate for protecting vascular endothelium from oxidative stress and inflammatory events. Indeed, the *S. spinosum* ethanolic extract is rich in ellagitannins (such as corilagin), flavonoids (such as quercetin) and triterpenoids [145]. Ellagitannins were found to be the most prevalent subgroup, constituting more than 50% of the overall extract. In addition, triterpenes and flavonoids were abundant in the extract, especially quercetin and its derivatives, were also present in significant amounts within the ethanolic extract, making up about 11% of the total content.

Free radicals are products of the normal cellular metabolism and participate as signaling molecules in the regulation of physiological functions and in the redox mechanisms in order to protect cells against oxidative stress [150]. However, excessive production of free radicals causes oxidative damage to biomolecules (DNA, lipids, proteins) and is associated with the development of different pathological conditions of the body [151]. Excessive ROS levels trigger cellular oxidative stress leading to cell injury, endothelium dysfunction and tissue damage, thus contributing to the development and progression of many vascular diseases [152].

Endothelium dysfunction can be defined as an alteration of the endothelium physiology towards a pro-inflammatory and pro-thrombotic state. This condition is linked to cardiovascular diseases, diabetes, autoimmune diseases, bacterial and viral infections [138], [140]. Endothelium dysfunction is characterized by inflammation, altered NO production and bioavailability, ROS over-production and lipid peroxidation [153].

To mimic *in vitro* the endothelium dysfunction occurring during CVD, HECV cells were exposed to hydrogen peroxide which triggers oxidative stress inside the cells [154], [155]. To test the beneficial potential of the SEE we used two experimental conditions. In the first condition (the counteraction condition), we stressed the cells with H_2O_2 and then treated them with SEE, or single compounds (corilagin and quercetin). In the second condition (the protection condition), we treated the cells with SEE, or single compounds, then we insulted

them with H_2O_2. These two conditions try to mimic the folk habits in using the medicinal plants. Indeed, the Lebanese population may assume infusion/tea of *S. spinosum* parts as therapy for pain or specific illnesses (such as diabetes) or may drink them daily as a healthy habit for disease prevention.

We observed that, as consequence of H_2O_2 insult, the endothelial cells developed an oxidative stress, which was counteracted/prevented by SEE, or single compounds. In both the experimental conditions, a significant decrease in intracellular ROS production was observed *in situ* by fluorescence microscopy and fluorimetric analysis. Also, the ROS-related lipid peroxidation was significantly decreased by SEE and single compounds, compared to H_2O_2-treated cells. The efficacy of the SEE extract and its single compounds corilagin and quercetin probably depends on their direct action as scavenger of the hydrogen peroxide due to the presence of phenolic groups that are known to be able to transform H_2O_2 in water by donating electrons [156]. Of note, both the SEE and corilagin and quercetin were able to counteract ROS production and lipid peroxidation in steatotic hepatocytes [145].

Reduced glutathione (GSH) is the main non enzymatic intracellular antioxidant and it is a marker for oxidative stress [157]. GSH acts by reducing peroxides (hydrogen and lipid peroxides) through its oxidation to GSSG by acting as co-substrate of glutathione peroxidase [158]. In normal cells the concentration of GSH typically ranges between 1-10 mM, and in different oxidative models the GSH/GSSG ratio markedly decreases [159]. Our results showed a decrease in the physiological GSH/GSSG ratio in dysfunctional endothelial cells, and all the treatments, except quercetin, were able to rescue the impair in the GSH/GSSG ratio in both experimental conditions (protection and counteraction).

Endothelial derived NO is a versatile molecule with large impact on many physiological functions. In tissue inflammation, an increased production of NO by macrophages has been reported [160], whereas in vascular endothelium a decreased production of NO occurs [132]. Our results show a decrease in NO release in H_2O_2-stimulated cells. The primary process that

decreases the bioavailability of vascular NO is associated with the NO breakdown through oxidation by ROS, mainly superoxide [161]. Of note, NO regulates the permeability of the endothelial barriers acting as anti-inflammatory agent [162]. Our results proved the ability of the SEE, and the single phenolic compounds, to restore NO bioavailability in line with previous studies [163].

Metabolic disorders typically lead to increased protein denaturation in many tissues that results in the generation of autoantigens and amplification of inflammation [164]. Therefore, agents that can prevent protein denaturation could be of great interest in anti-inflammatory strategies. Several non-steroidal anti-inflammatory agents have been reported to preserve/safeguard albumin form thermal denaturation [135]. Our findings show that both the SEE, and the single polyphenols, were effective in protecting albumin from thermal denaturation, thus suggesting that the extract might be a stabilizing agent with anti- inflammatory potential.

Cell migration holds significant role in a broad spectrum of physiological and pathological events [165], and it encompasses essential aspects such as development, angiogenesis, inflammatory responses, wound healing, and tumor invasion [166]. Wound healing is a complex event, where a variety of cell types interact playing various functions. The endothelial cells have a special role as they undergo a series of morphological and functional alterations during wound healing [167]. When the SSE was tested for its potential in the wound repair, we observed that both the extract as well as the corilagin accelerate significantly the wound closure when compared to the positive and negative control. As quercetin was not effective in accelerating the wound repair, we can hypothesize that the extract might act mainly depending on its high content in corilagin. Indeed, we observed that corilagin increased cell proliferation in the MTT assay. Interestingly, our findings are in line with results reported for different species of the Rosaceae family. In particular, both a methanolic extract from *Rubus imperialis* was able to promote wound healing in artificially wounded L929 fibroblasts [168], and a methanol bark extract from *Prunus africana* demonstrated wound healing activity in a mouse

model [169]. According to the general idea, the wound healing effects of our extract and corilagin could be related to their ability to control oxidative stress [170].

In conclusion, substituting synthetic antioxidants with natural alternatives, due to the potential benefits for human health, is proved beneficial. The beneficial effects of the *S. spinosum* fruit ethanol extract, which is rich in polyphenols, clearly indicate that this fruit possesses a potential antioxidant ability, which explains its traditional use. In general, the extract demonstrated similar behavior and, consequently, similar anti-inflammatory capability compared with corilagin and quercetin. Moreover, both habits of taking the herbal remedies and supplements proved their efficacy in counteracting/preventing the progression of the disease. Therefore, we suggest that this plant may have a good application as nutraceutical supplement in the prevention and treatment of metabolic and chronic diseases.

Acknowledgements

We would like to thank Elaria Faraone and Francesca Torre for their help during the experimental assays.

Funding

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5. Chapter Five: Development of human models of insulin resistance in hepatoma HepG2 cells

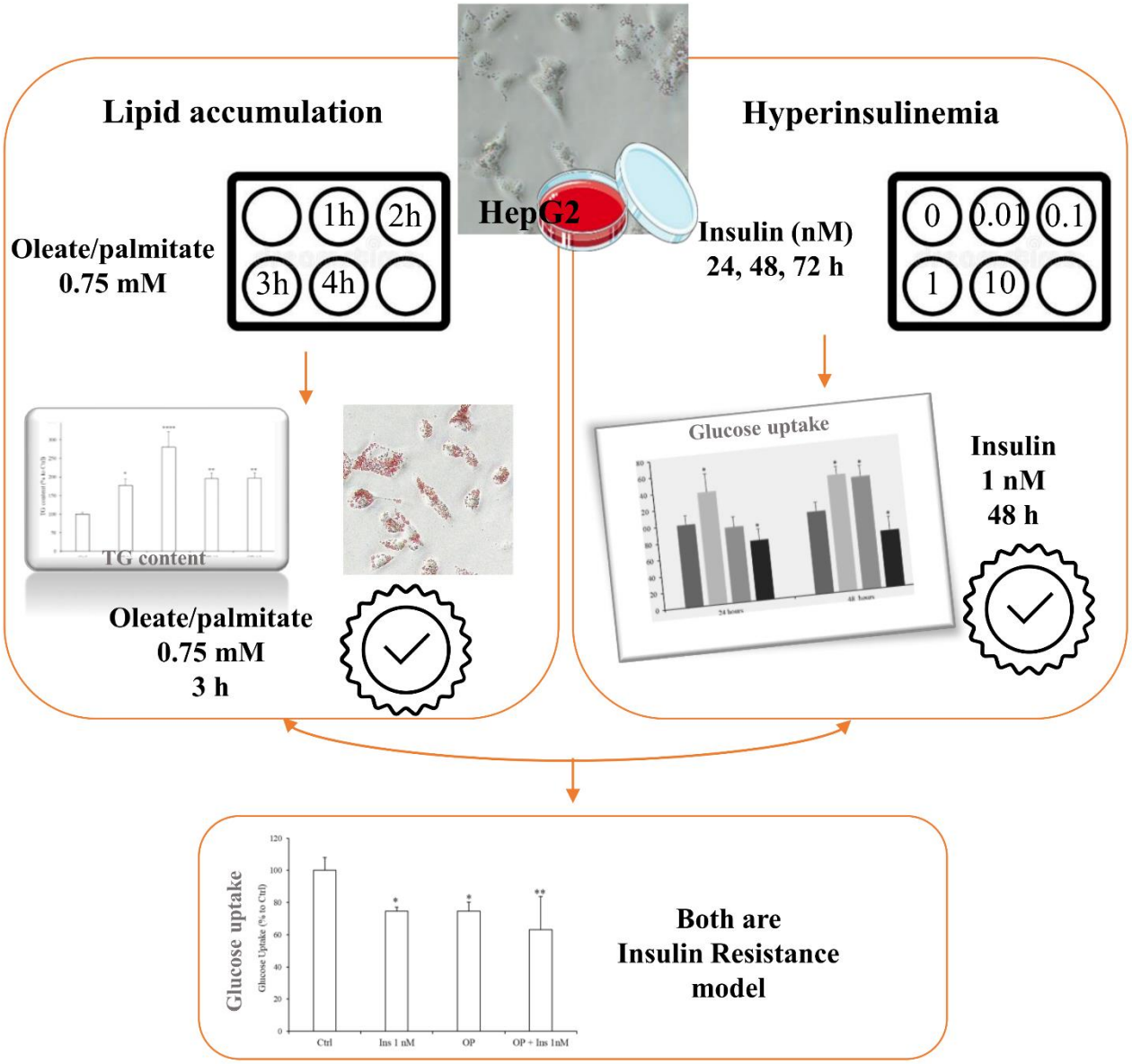

Figure 5- 1 Chapter five graphical abstract

Development of human models of insulin resistance in hepatoma HepG2 cells

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Abstract

Insulin resistance (IR) is a key factor in metabolic syndrome and type 2 diabetes, with increasing prevalence globally. This study aimed to establish an experimental model using human hepatoma cells (HepG2) to investigate hepatocellular steatosis and IR. Two pathways, hyperinsulinemia, and lipid accumulation, were explored for IR induction. The optimization of oleate/palmitate (OP) mixture (0.75 mM for 3 h) exposure revealed a significant increase in intracellular triglyceride accumulation, confirming the development of steatosis. Additionally, insulin treatment (1 nM for 48 h) demonstrated a reduction in glucose uptake, defining an IR model. Comparative analyses showed both hyperinsulinemia and lipid accumulation induced IR demonstrated by decreased glucose uptake in both conditions. This model provides a valuable platform for studying natural products' effects on IR.

Key words:

HepG2 cells, lipid accumulation, steatosis, insulin resistance, hyperinsulinemia

5.1. Introduction

Insulin resistance (IR) defines an impaired biologic response to insulin stimulation of target tissues, primarily liver, muscle, and adipose tissue. Therefore, the IR condition in the body impairs glucose disposal resulting in a compensatory increase in pancreatic insulin production and consequent hyperinsulinemia [171]. The metabolic consequences of IR can be hyperglycemia, hypertension, dyslipidemia, hyperuricemia, elevated inflammatory markers, endothelial dysfunction etc. IR is primarily an acquired condition related to excess body fat (obesity), though genetic causes are also identified [172]. Progression of IR can lead to metabolic syndrome (MetS) [173], nonalcoholic fatty liver disease (NAFLD) [174], type 2 diabetes mellitus (T2D) [175], and cardiovascular disease (CVD) [85]. Of note, IR typically precedes the development of T2D by 10 to 15 years [176]. In the last decades, the prevalence of IR and IR-associated disorders is increasing, especially in developing countries and among younger age groups, with prevalence estimates ranging from 20% to 40% across various populations [177].

The three primary sites of insulin resistance are the skeletal muscle, liver, and adipose tissue. Skeletal muscle is a large reservoir for circulating glucose, accounting for up to 80% of glucose disposal, and IR results in a decreased glucose uptake by muscle tissue [178]. Glucose is shunted from muscle to the liver, where de novo lipogenesis (DNL) occurs. The liver is the main site of the energy substrate processing by acting on synthesis, packaging, recirculation of fatty acids and synthesis and store of glucose [179]. When circulating glucose is high, the IR in the liver triggers stimulation of DNL and increase in plasma triglyceride content contributing to ectopic lipid deposition in and around visceral organs [180]. The adipose tissue is the physiological store of fat. In IR condition, the failure of insulin to suppress lipolysis in adipocytes increases the circulating free fatty acids (FFAs) that, in turn, directly affect both liver and muscle metabolism, further exacerbating insulin resistance condition and contributing to lipotoxicity-induced dysfunction of pancreatic beta-cell [181].

The growing occurrence of metabolic diseases has sparked a corresponding interest in comprehending the mechanisms through which the environment and nutrition may impact cell metabolism [75]. The experimental approach aiming to investigate the molecular mechanisms of human diseases relies on replicating some facet of its pathogenesis within a controlled system. Animal models have been largely employed to this aim, but they are imperfect proxies for several human dysfunction and/or toxicity. Cell models have become a new way to investigate human diseases [182].

The present study was designed to develop and test an experimental model of insulin resistance in human cells that can be used for further biological investigation of natural products and drugs.

5.2. Material and methods

5.2.1. Chemicals

Unless otherwise indicated, the reagents employed were supplied by Sigma-Aldrich Corp. (Milan, Italy).

5.2.2. Cell culture and cell model of hepatosteatosis

HepG2 is a human hepatoma cell line supplied by the cell bank American Type Culture Collection (ATCC, USA, HB-8065). This cell line is largely used in drug metabolism and hepatotoxicity studies. HepG2 are nontumorigenic cells with high proliferation rates and epithelial-like morphology that maintian many hepatic functions. HepG2 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 2mM Glutamine and 10% FBS at 37°C in a humidified atmosphere with 5% CO_2.

5.2.3. Cell model of hepatic steatosis

HepG2 cells were seeded in cell culture plates untili reaching 80% confluency.Then, cells were exposed for increasing time periods (1, 2, 3 and 4 h) to a mixture of oleate/ palmitate (0.75

mM, 2:1 molar ratio) in D-MEM with high-glucose (4.5 g/L) supplemented with 0.4% FBS, and 0.25% BSA to mimic *in vitro* the effect of high-fat diet. At the end cells were either fixed for ORO staining or collected for further investigation.

5.2.4. Cell model of IR

The first step has been to determine the optimal dose of insulin and treatment duration to establish an IR condition in HepG2 cells. HepG2 cells were seeded in 6-well plates (with $3x10^5$ cells/well) E-MEM complete medium until 80% confluency. To starve the cells, mediun was replaced with D-MEM with a low glucose concentration (1 g/mL) supplemented with 0.4% FBS for 6 h. Then the medium was changed with DMEM with High gluose concentartion (4 g/mL) supplemented with 0.4% FBS and insulin at various concentrations (0.01, 0.1, 1, 10 nM) for 24, 48 and 72 h to mimic heperinsulinemia.

5.2.5. Protein quantification

The protein content of the samples was quantified by Bradford assay using bovine serum albumin (BSA) as a standard [98].

5.2.6. Quantification of the intracellular triglyceride content

At the end of each treatment, cells were scraped and lysed by passing cell suspension through a 25 gauge needle. Lipids were extracted using the chloroform/methanol method. The TG content was quantified by adding 500 µL of Triglycerides liquid kit reagent (Sentinel Diagnostics, Milan, Italy) for 15 min at 37˚C. Absorbance (λ546) was read with a UV-VIS spectrophotometer against a blank. Glycerol (0.007–0.2 µg/mL) was used as reference. The TG content was normalized to protein content and expressed as percent TG content relative to control. For visualisation, Oil-RedO (ORO) staining was employed, and intracellular lipid droplets were stained with 0.3% ORO solution. Images are acquired by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

5.2.7. Glucose consumption determination

The amount of glucose consumption (GC) by cells in different conditions was quantified according to the glucose oxidase method [183]. For this method media without phenol red was used because the end product of this enzymatic experiment is red in color and phenol red may interfere with the results, cells were seeded in 6 well-plate (with $3x10^5$ cells/well) with one well kept cell free (as it is needed for calculation), after 80% confluency cells were treated as mentioned above. At the end of treatment 25 µL of medium was collected in 5 mL eppendorf, and 0.1 M phosphate buffer (pH 7) was added to reach a final volume of 3 mL. Then, directly in cuvette, 1 mL of the diluted media was mixed with 500 µL enzyme reagent (1500 U glucose oxidase + 100 U of glucose peroxidase + 18g 4-aminoantipyrisine + 36 mg phenol all prepared in 100 mL 0.1 M PB). In parallel, 1 mL PB and 500 µL enzyme reagent were used as blank. After incubation for 15 min at room temperature, the absorbance was measured at 520 nm by UV-VIS spectrophotometer against the blank. A standard curve of increasing concentration of glucose (0–30 µg/mL) was prepared. The results for the mediam were expressed in µg/mL of glucose. The glucose content of each sample was normalized to the protein content determined by the Bradford assay. Glucose consumption (GC) was calculated from the glucose concentrations of the cell containing wells subtracted from the cell-free blank wells.

5.2.8. Quantification of the intracellular glycogen content

The intracellular glycogen content was evaluated according to Teng *et al.* [184] with some modifications. At the end of treatments cells were collected and centrifuged. The pellet was lyzed in 400 µL 30% KOH for 90 min at 100°C. Ater cooling for 10 min, glycogen was precipitated by addition of 1.2 mL of absolute ethanol for 30 min at 4˚C. After centrifugation at 13000 rpm for 30 min the supernatant was removed and any remaining ethanol was air dried. The glycogen pellet was resuspended in 100 µL of 1 M HCL and incubated for 30 min at 95˚C. After cooling, 100 µL NaOH and 300 µL ddH_2O were added to each sample, and the sample was centifuged at 13000 rpm for 10 min. Supernatant was collected and equal volume of 5% phenol solution was added. Also, 500 mL Glucose solution of different concentrations (0–500

µg/mL) to used as standard were mixed with 500 mL of 5% phenol. After 15 min incubation at room temperature, 60 µL of each sample were transferred to 96-multiwell plates in triplicates, followed by the addition 150 µL 96% H_2SO_4. As blank we prepared 60 µL of 5% phenol and 150 µL 96% H_2SO_4. After 5 min incubation, the glycogen content was determined by the absorbance at 490 nm by UV-VIS spectrophotometer against the blank. Results were normalized to the protein content determined by the Bradford assay.

5.2.9. Cell viability assessment

The possible anti-proliferative and/or cytotoxic activity of first oleate/palmitate mixture at different exposure time and second of the insulin at different concentrations and incubation times was determined using the MTT assay. The cell viability was tested by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [103]. Briefly, 10000 cell/well were seeded (200µL volume) in a 96 well plate, and incubated at 37˚C in humidified air with 5% CO2 for 24 h. After that, cells were treated with 0.75 mM of OP for (1, 2, 3 and 4 h) or with insulin at increasing concentrations (0.01, 0.1, 1, and 10 nM) for different times (24, 48 and 72 h). After the end of each treatment, 20 µL of MTT solution (5mg/mL) were added to each well and incubated for 3 h. Then, medium was replaced by 200 µL of isopropanol to dissolve formazan salt and the absorbance was read at 570 nm.

5.2.10. Statistical analysis

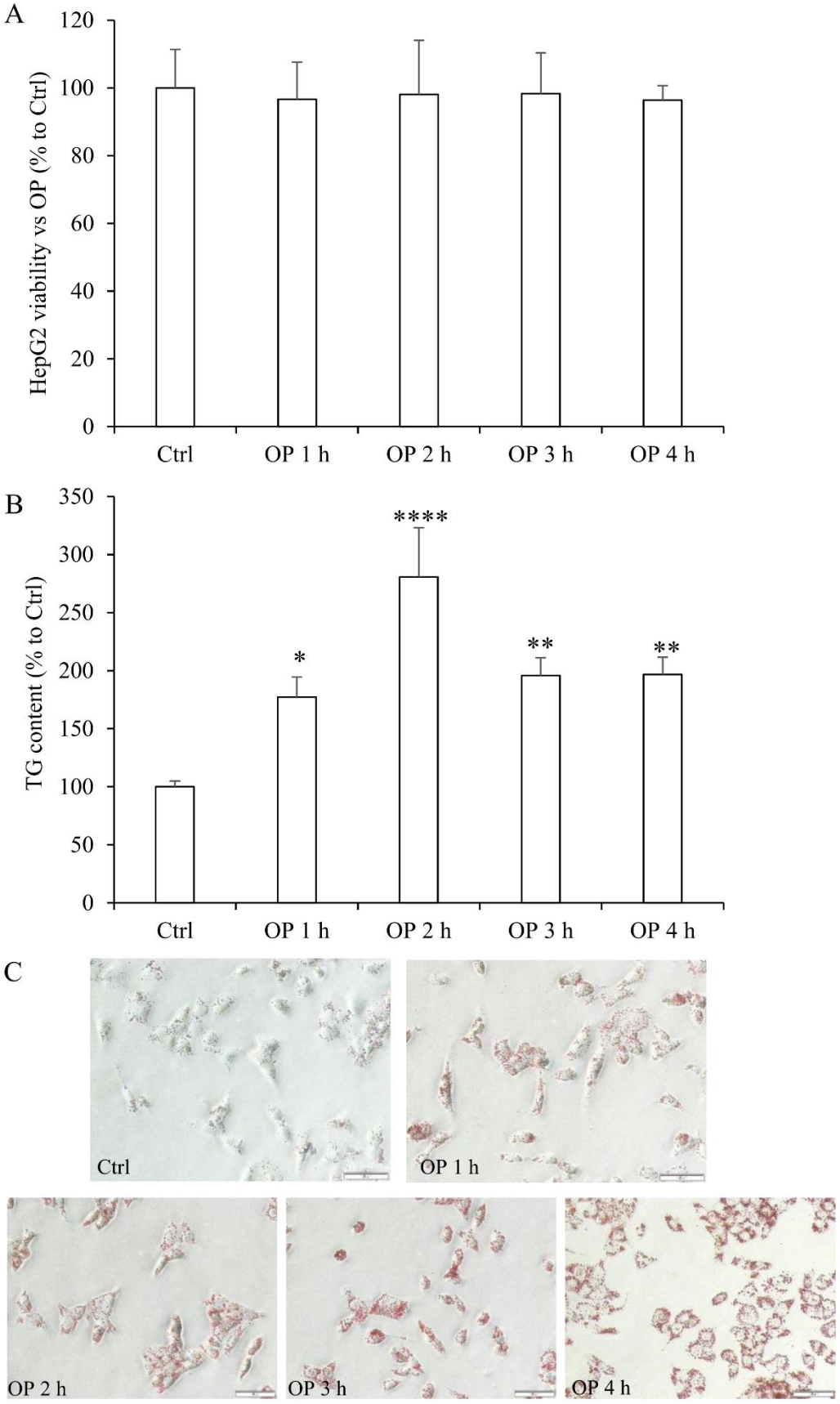
Data are means ± S.D. of at least three independent experiments. The differences among the groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post-test (version 8.0, GraphPad Software, Inc., USA).

5.3. Results

5.3.1. Model of human hepatic steatosis

In order to develop a reliable *in vitro* model for human hepatic steatosis we tested the lipid accumualtion in HepG2 cells when the cells were exposed to the same concentration of OP for increasing time periods (1, 2, 3 and 4 h). First, by MTT assay we excluded any significant cytotoxicity of OP at all the tested times (Figure 5-2A). The intracellular TG content was quantified spectrophotometrically, and the results showed a significant increase in the TG accumulation for all the incubation times compared to controls. The maximal TG accumulation in HepG2 cells was observed for 2 h incubation (+181%; $p \leq 0.0001$), followed by 3 h (+96%; $p \leq 0.01$) compared to Ctrl (Figure 5-2B).

For a deeper analysis, the morphometry of the cytosolic lipid droplets (LDs) was assessed using absorption microscopy after ORO staining (Figure 5-2C). While few and small LDs were detected in the control heaptocytes in the steatotic haptocytes (OP) we observed an increase in both the number and the size of LDs.


A
HepG2 viability vs OP (% to Ctrl)
120
100
80
60
40
20
0
Ctrl
OP 1 h
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B
TG content (% to Ctrl)
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Figure 5- 2 Steatotic activity of Oleate/Palmitate on HepG2 cells

HepG2 cells were incubated in the absence (Ctrl) or the presence of oleate/palmitate (OP) for 1, 2,3 and 4 h. **(A)** Cell viability after incubation of HepG2 cells with OP at different time. **(B)** The TG content expressed as percent relative to control after normalization for protein content. **(C)** Representative images of HepG2 cells after ORO staining and acquisition by Leica DMRB light microscope.Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl vs OP cells *p≤ 0.05, **p≤0.01, ****p≤0.0001

5.3.2. Model of human IR

The effects of different insulin concentrations (0.01, 0.1, 1 nM) and different time incubations (24, 48 and 72 h) were assessed in HepG2 cells in order to identify a reliable *in vitro* model for Insulin Resistance.

First, by MTT assay (Figure 5-3A) we evaluated the possible cytotoxicity of insulin at all the tested concentrations and times. All the insulin concentrations showed a signficant cytotoxicity at 72 h with a decrease cell viability compared to Ctrl of -16%, (p≤0.001), and -18% (p≤0.0001) for 0.01 nM 0.1 nM, respectively, and of -21% (p≤0.0001 for both the highest concentrations of 1 and 10 nM. On the other hand, the highest insulin concentration (10 nM) showed a significant decrease in cell viability compared to Ctrl also at early times of incubation: -14% at both 24 h (p≤0.001) and 48 h(p≤0.05). Therefore, based on these results we excluded this concentration for the further investigations.

The glucose uptake was quantified as a marker of IR. The results showed the maximal reduction in the glucose uptake for the insulin dose of 1 nM at both 24 h (-27% vs Ctrl; p≤0.05) and 48 h (-31% vs Ctrl; p≤0.05) of incubation (Figure 5-3B). In contrast, the lower insulin concentrations (0.01 and 0.1 nM) promoted an increase in glucose uptake at 48 h (+41% and +37% vs Ctrl; p≤0.05 respectively) triggering the normal response of insulin on cells. Therefore, in HepG2

cells we identified the tretament for 48 h with an insulin concentration of 1 nM as the best model to mimic *in vitro* an IR condition.

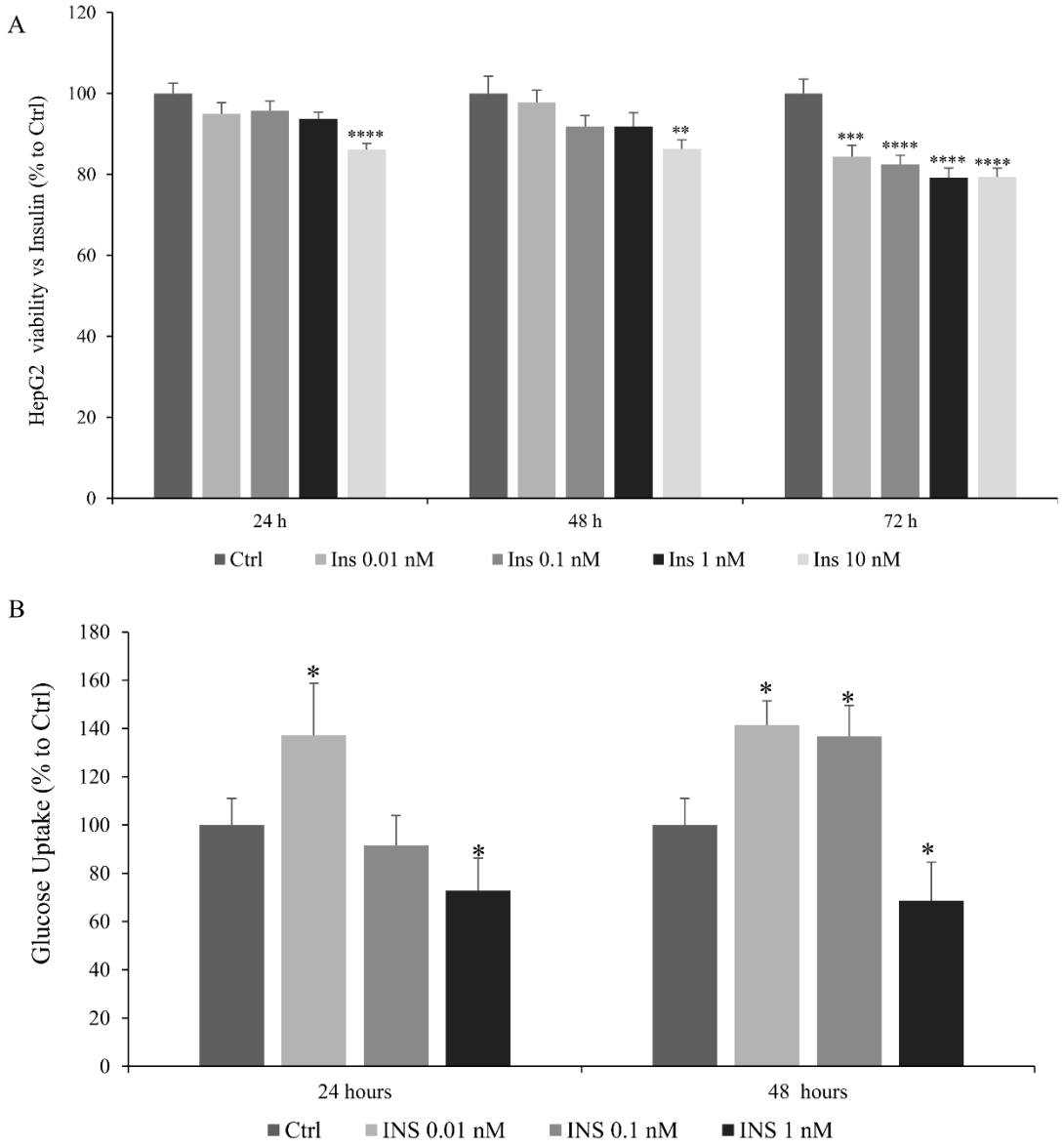

Figure 5- 3 Insulin effects with different concentrations and time exposure on HepG2 cells

HepG2 cells were incubated in the absence (Ctrl) or the presence of Insulin (INS). **(A)** Cell viability after incubation of HepG2 cells with INS (0.01, 0.1, 1 and 10 nM) for (24, 48 and 72 h). **(B)** The glucose uptake after incubation with INS (0.01, 0.1, and 1 nM) for 24 and 48 h) expressed as percent relative to control after normalization for protein content.Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl vs INS cells *p≤ 0.05, **p≤0.01, ****p≤0.0001

5.3.3. Effects of hyperinsulinemia and fatty acid excess on developing IR HepG2 cells

When the glucose uptake was assessed in HepG2 cells exposed to insulin (1 nM) for 48 h or OP for 3 h (after 3 hr the media was changed to DMEM supplemented with 0.4% FBS and collected after 48 h) or OP for 3 h followed by insulin (1 nM) for 48 h (Figure 5-4A) we observed OP or insulin exposure alone triggered a similar reduction in glucose uptake (-25%; $p \leq 0.05$), however OP followed by insulin treatment triggered the maximal efficacy in glucose uptake reduction (-37%; $p \leq 0.01$).

When we evaluated the intracellular glycogen content (Figure 5-4B) , we could appreciate as the exposure to excess FFAs didn't change glycogen content, whereas the insulin 1nM was able to significantly decrease it (-17%, $p \leq 0.05$).

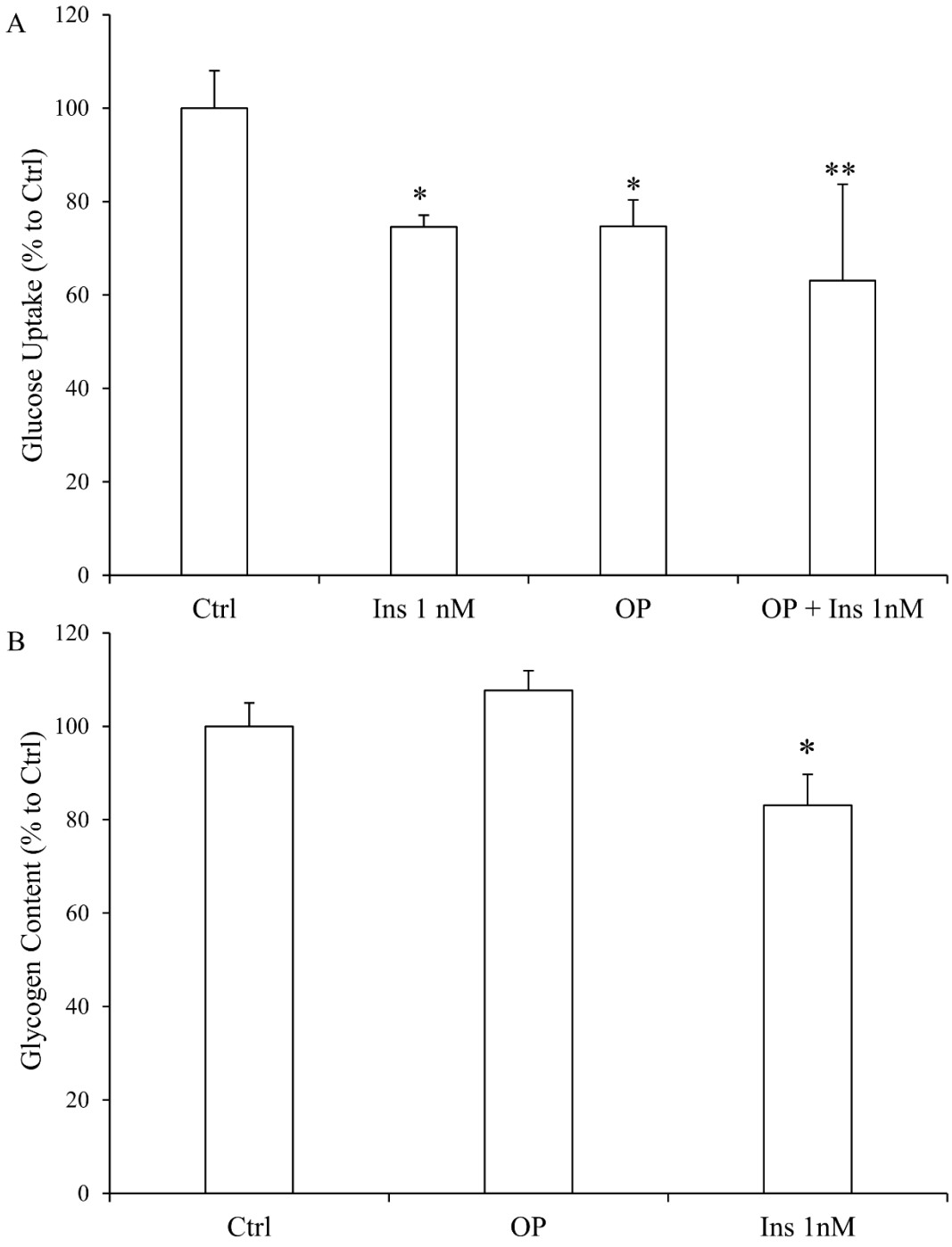

Figure 5- 4 Comparative analyses between hyperinsulinemia and lipid accumulation to induced insulin resistance

HepG2 cells were incubated in the absence (Ctrl) or the presence of oleate/palmitate (OP) for 3 h or with 1 nM insulin for 48 h. **(A)** The glucose uptake expressed as percent relative to control after normalization for protein content. **(B)** The intracellular glycogen content expressed as percent relative to control after normalization for protein content.Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups was assessed by ANOVA followed by Tukey's test. Symbols: Ctrl vs OP or INS cells *p≤ 0.05

5.4. Discussion

Metabolic disorders such as obesity, diabetes mellitus, insulin resistance, steatosis and cardiovascular disease pose a significant health risk in today's world. However, the advancement of understanding their root causes and developing effective treatments is constrained due to the absence of suitable human model systems [185]. Drawing from the shortcomings of animal models, an array of human cell and tissue models has emerged. These encompass 2D immortalized cell lines, patient-derived xenografts, stem cell-derived cell types, and organoids. Each model offers unique advantages and disadvantages in its application [186]. The present study reports our design to develop and define insulin resistance model using human hepatoma cells (HepG2) to be used in our lab for further invesitgations. Moroever, our findings showed that insulin resistance can be developed either by hyperinsulinemia or lipid accumulation which in the future can both be used depending on the purpose of the study to be done.

In our lab a set of different models were designed and developed using different cell lines. Mice 3T3-L1 fibroblasts were used to develop adipocytes hypertrophy [187], rat hepatoma FaO cells were used to develop liver steatosis [188] and human endotheilial cells were used to develop endothelial dysfunctional cells [124]. Taking into account that insulin resistance can lead to various metabolic issues such as hyperglycemia, hypertension, dyslipidemia, hyperuricemia, higher levels of inflammatory markers, endothelial dysfunction, and a tendency toward blood clot formation (prothrombotic state) [86], [171], [189], [190], it was of our intereset to study and investigate insulin resistance and possible treatment or prevention by natural products derived from plant extracts.

Hyperinsulinemia, marked by elevated insulin levels, is influenced by a complex interplay of genetic factors, demonstrated through heritability and family clustering [191]. Also, environmental contributors, such as excessive nutrient consumption and exposure to endocrine-disrupting chemicals, contribute to this condition [192], and through homologous

desensitization hyperinsulinemia develops IR [193]. IR is known to inhibit glucose uptake by cells from blood resulting in high levels of circulating glucose [85]. In our study insulin resistant HepG2 cells were defined and established using 1 nM insulin for 48 h, this successful establishment was illustrated by a significant reduction in glucose uptake a major response to insulin by cells. Indeed development of IR through hyperinsulinemia likely occurs through various mechanisms, including the downregulation of the insulin receptor [193], increased expression or activity of protein tyrosine phosphatases that can deactivate the receptor or its substrates [194], or the activation of phosphoinositide phosphatases like PTEN, SHIP2, or myotubularin, which lower the levels of signaling phosphoinositide's [195] that can be of interest to be studied during investigations for a suitable treatments.

In addition to hyperinsulinemia it was reported that palmitate induces insulin resistance in HepG2 cells [196]. Hepatic steatosis is widly validated and used in our lab using rat hepatoma FaO cells, so we tried to develop steatosis in HepG2 cells since they have human source using the same oleate and palmitate mixture with the same concentrations as we use for FaO cells [145] (0.75 mM with molar ratio 2:1) . It was validated that steatosis can be developed after 2 and 3 hours of incubation as it was proved by significant increase in intracellular triglyceride accumulation, however we chosed 3 h for our model to be in parallel with our FaO model. Moreover, the same time and concentration of OP incubation proved to develop insulin resistant HepG2 cells as demonstrated by significant reduce in glucose uptake.

Lasltly by comparing the two conditions that led to insulin resistance both lipid accumulation and hyperinsulinemia were able to induce insulin resistance in the same manner and efficacy however combining the two conditions triggered more potent reaponse. Glycogen synthesis plays a significant role in the response to insulin, and impaired glycogen synthesis is a substantial factor in insulin resistance [197]. In this study we compared the glycogen content in both models hyperinsulinbemia and lipid accumulation. The results indicated that indeed hyperinsulinemia develop insulin reistance by decreasing glycogen content inside the cells

which is expected since glycogen synthetase activity in decreased during insulin resistance [198]. By contrast lipid accumulation didn't show any significant difference in glycogen content, in litrature it was reported that lipid accumulation decrease glycogen accumulation [199], our results can be due to the fact that our assay lacks specificity since it is not enzymatic and lipids mainly triglycerides reacts with potassioum hydroxide, ethanol and sulfuric acids producing glycerol and glycerols products that can interfere with the results.

In conclusion, the developed model using HepG2 cells offers a versatile platform for studying the effects of natural products, particularly polyphenols from medicinal plants, on insulin resistance.

Acknowledgements

We would like to thank Elaria Faraone and Francesca Torre for their help during the experimental assays.

Funding

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Conclusions

The primary research interest of my PhD activity was focused on investigating the possible beneficial effects and therapeutic potential of both the extracts and the main bioactive compounds prepared from the fruits of the medicinal plant *Sarcopoterium spinosum (L.).* This small, thorny shrub belonging to the Rosaceae family is diffuse in various countries surrounding the Mediterranean Sea, including parts of Europe, North Africa, and the Middle East. My research investigated the potential of bioactive compounds contained in the *S. spinosum* fruits in the treatment and/or prevention of obesity-related disorders, especially the hepatic steatosis and endothelium dysfunction.

The journey began with the collection of *S. spinosum* fruits from their natural habitat in Lebanon, followed by the preparation of different extracts using different solvents – water, boiling water, and ethanol- and their characterization in terms of polyphenol, flavonoid and carbohydrate content. Among the extracts, the ethanol extract stood out with remarkable radical scavenging activity, prompting further investigation through HPLC/MS techniques. The phenolome of the ethanolic extract revealed a rich and complex profile being prominently characterized by ellagitannins, triterpenes, and flavonoids; corilagin and quercetin were identified as the most significant components.

The *S. spinosum* ethanol extract demonstrated a direct lipid-lowering activity on a cellular model of hepatic steatosis consisting of lipid-loaded FaO cells. The extract was able to significantly reduce the intracellular triglyceride accumulation as well as the size and number of lipid droplets. Additionally, the extract showcased its capacity to mitigate the fat-dependent oxidative stress by decreasing the ROS production and the lipid peroxidation, and restoring the catalase activity.

On the other hand, the *S. spinosum* ethanol extract was able to improve the endothelial dysfunction in a cellular model consisting of HECV cells exposed to hydrogen peroxide. Both

the ethanol extract, as well as corilagin and quercetin, the main polyphenols contained in the extract, exhibited substantial protective and counteractive effects. These effects included a reduction in ROS production and lipid peroxidation, as well as the restoration of nitric oxide bioavailability and the GSH/GSSG ratio. Moreover, both the extract and the two polyphenols showed a notable ability in accelerating the wound healing process.

Ongoing efforts involved the development of an insulin resistance cellular model using human HepG2 cells. Preliminary findings indicate the successful induction of insulin resistance, and this cellular model holds promise for unraveling the involved mechanisms of insulin resistance, offering a potential avenue for targeted studies on interventions using natural products. This model is now employed to assess the ability of the *S. spinosum* extract in improving the insulin resistance thus enlarging its potential therapeutic intervention.

In conclusion, this comprehensive exploration of *S. spinosum* fruits, marks the first and initial characterization of the entire phenolome within its ethanol extract. The analysis reveals a rich abundance of polyphenols, with ellagitannins emerging as the predominant group, trailed by triterpenes and flavonoids. Additionally, the extract and its individual polyphenolic constituents, namely corilagin and quercetin, substantiated their *in vitro* efficacy in reducing lipid levels, demonstrating anti-inflammatory properties, showcasing potent antioxidant capabilities, and confirming cyto-protective abilities.

The findings of this thesis sheds light on understanding the direct action of the bioactive compounds enrichening the *S. spinosum* fruits taking advantage of simplified *in vitro* models of some important human metabolic disorders, such as fatty liver and cardiovascular diseases, and insulin resistance. Moreover, the results set the stage for potential *in vivo* applications of *S. spinosum* fruit extracts as nutraceutical agents for treatment and/or prevention of metabolic disorders in humans. Ultimately, I am satisfied of my thesis as I think it supplies a significant scientific contribution to the field of natural remedies and phototherapies for treatment of

human metabolic disorders, offering a foundation for continued exploration and potential applications in clinical settings.

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