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Role of a Nutraceutical Mixture in Ameliorating Liver Steatosis by Preventing Oxidative Stress and Mitochondrial Dysfunction in a NAFLD Model

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease leading to liver transplantation. There is an increase in economic and clinical burden of NAFLD in the prevention, diagnosis, and treatment of the disease. The reason behind the NAFLD progression to non-alcoholic steatohepatitis (NASH) is not completely understood. Recently, the deficiency of micronutrients (e.g., vitamins, minerals, and other elements) has been suggested as crucial in NAFLD progression, such that recent studies reported the potential hepatic antioxidant properties of micronutrients supplementation. Here we have explored the potential beneficial effects of dietary supplementation with FLINAX, a novel mixture of nutraceuticals (i.e., vitamin E, vitamin D3, olive dry-extract, cinnamon dry-extract and fish oil) in a NAFLD model characterized by oxidative stress and mitochondrial function impairment.

Methods: Steatosis was firstly induced in Wistar rats by feeding with a high-fat/high-cholesterol diet for 4 weeks, and following this the rats were divided into two groups. One group (n = 8) was treated for 2 weeks with a normal chow-diet, while a second group (n = 8) was fed with a chow-diet supplemented with 2% FLINAX. Along with the entire experiment (6 weeks), a third group of rats was fed with a chow-diet only as control.

Results: The dietary supplementation with FLINAX significantly improved hepatic steatosis and lipid accumulation compared to untreated rats. The mRNA

and protein levels analysis showed that CPT1A and CPT2 were up-regulated by FLINAX, suggesting the enhancement of fatty acids oxidation (FAO). Important lipoperoxidation markers (i.e., HNE- and MDA-protein adducts) and the quantity of total mitochondrial oxidized proteins were significantly lower in FLINAX-treated rats. Intriguingly, FLINAX restored the mitochondrial function, stimulating the activity of mitochondrial respiratory complexes (i.e., I, II, III and ATP-synthase) and counteracting the peroxide production from pyruvate/malate and succinate.

Keywords: NAFLD, micronutrients, steatosis, mitochondria, oxidative stress, electron transport chain

1. INTRODUCTION

NAFLD is an umbrella term containing a spectrum of clinicopathologic diseases like non-alcoholic fatty liver (NAFL or simple steatosis), NASH, fibrosis, cirrhosis, and their complications [1]. This disorder is characterized by excess accumulation of fat (in the form of triglycerides) in >5% of hepatocytes without alcohol consumption (<30 g per day) [2]. In a subset of individuals, NAFL combines with additional factors like hepatocyte injury (ballooning), cell death with lobular and portal inflammation, and is designated as NASH. NAFL patients carry a very low risk of adverse outcomes, while NASH increases the risks of liver and non-liver related outcomes including cirrhosis, liver failure, hepatocellular carcinoma (HCC), cardiovascular disease and malignancy [3]. In NAFLD patients, morbidity and mortality related to cardiovascular disease is the primary cause of premature death. The cardiovascular risk is recorded in NAFLD patients even with low adiposity background measured by body mass index (BMI) indicating the insulin resistance [4].

Important caveats in the diagnosis of NAFLD is the non-availability of disease specific biomarker that is simple and powerful for large scale population studies. Early stage diagnosis prevents the disease progression by applying simple approaches like diet modification and increasing physical activity [5]. Moreover, diagnosis of NASH is very important to halt its aggravation towards cirrhosis. Suspected cirrhotic patients are necessary to undergo some tests such as portal hypertension, examining the risk of HCC development. So, it is imperative to be able to differentiate between simple steatosis and steatohepatitis

to prevent advancing towards more severe liver disease [6]. Non-invasive serum markers and imaging techniques particularly magnetic resonance imaging (MRI) used for the assessment of NAFLD clinically. However, serum markers lack uniformity, standardization across the populations and yet to receive wide acceptance. Although MRI and transient elastography based methods can measure fat and liver fibrosis with fair precision and reproducibility, the limited availability of equipment and its cost is making difficult to use [7]. So, liver biopsy remains the gold standard for diagnosing NAFL, but this method is expensive, invasive, painful, risk of severe complications and also subjected to sampling errors.

NAFLD is considered as a liver expression of metabolic syndrome and the pathogenesis is still not clearly known. Insulin resistance (IR) seems to play key role in the initiation and progression of the disease from simple fatty liver to advanced forms [8]. NAFLD pathogenesis is complex and multifactorial, firstly it is described as a two-hit hypothesis, where the first hit is liver steatosis, which is due to increased hepatic lipogenesis and reduced free fatty acid (FFA) degradation due to IR as shown in Figure 1. It is followed by the second hit of oxidative stress which induce hepatocyte inflammation and cell death [9,10]. However, this simplistic theory was soon replaced by an updated theory of multiple-hit hypothesis, where many factors such as systemic and hepatic IR, intestinal microbiota, genetic predisposition, oxidative stress, act simultaneously resulting in cascade of detrimental effects like hepatic inflammation, free radical production from gut and adipose tissue, resulting mitochondrial dysfunction,

endoplasmic reticulum (ER) stress and hepatocyte apoptosis [3]. Among all contributing factors, oxidative stress plays a major role in the liver injury and disease progression in NAFLD [11].

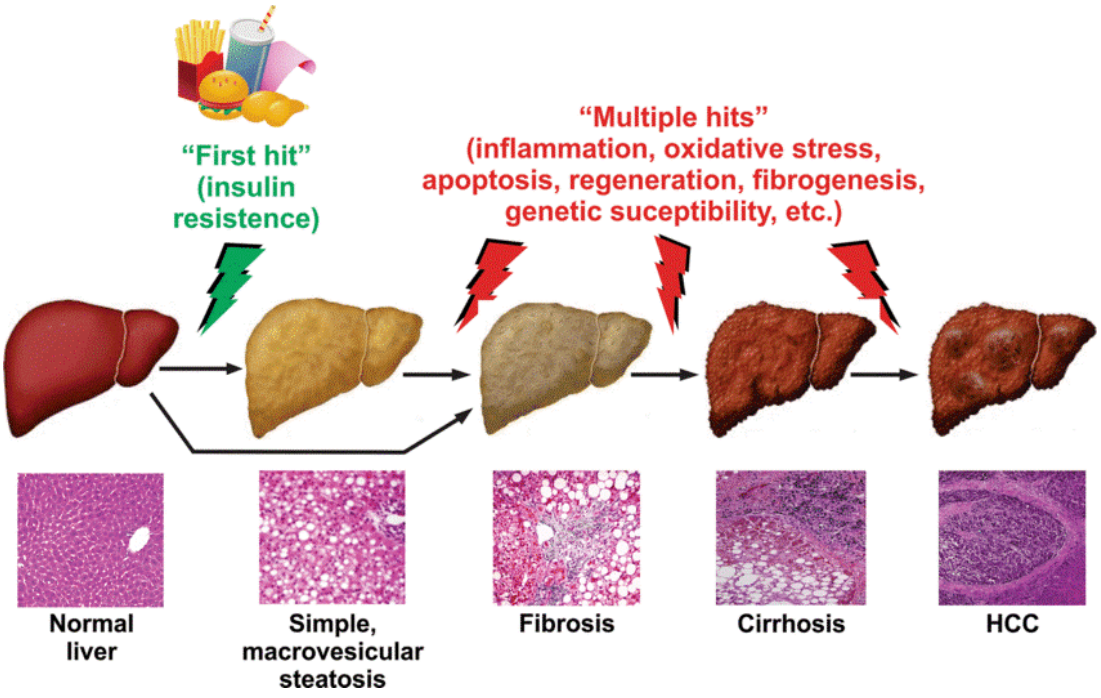

Figure 1. Multiple-hit theory of NAFLD: The insulin resistance is the initial hit leading to increased uptake and synthesis of free fatty acids stores as triglycerides leading to simple steatosis. Multiple hits act as superimposed insults involved in liver disease progression like NASH, fibrosis, cirrhosis and HCC (Bessone et al., 2018).

In physiological conditions liver does not store triglycerides (TGs), however consumption of high calorie diet and obesity cause disturbances in lipid metabolism leading to accumulation of TGs in hepatocytes [12]. Hepatic lipid accumulation leads to increased mitochondrial beta oxidation as a compensatory mechanism to culminate excess lipids. Increased beta oxidation results in

generation of excess reactive oxygen species (ROS) that induce oxidative stress [13]. These excessive free radicals were not counterbalanced by antioxidant defense system, causing liver damage [14]. In the normal physiological process ROS serve as signaling molecules in cellular homeostasis, adaption to stress, inflammatory and immune response, cellular proliferation and differentiation [15]. Whereas at high concentrations, ROS serve as biomolecular damaging molecules, oxidizing several cellular constituents such as lipids, proteins and DNA [9,10,16].

1.1. Epidemiology of NAFLD

In western countries, NAFLD is the most common cause of the abnormal liver function and according to the ultrasonography or CT imaging, the prevalence of NAFLD is between 20-50% as per the population studies [17]. Moreover, NAFLD prevalence varies among the ethnic groups and as per proton magnetic resonance spectroscopy, hepatic steatosis was 45% in Hispanic, 33% in white, and 24% in black populations. These variations could be due to the changes in lifestyle, metabolic syndrome, and genetics like polymorphism of patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene encoding a lipase mediating the triacylglycerol hydrolysis in adipocytes. Besides, NAFLD is strongly associated with metabolic disorder and fatty liver has been reported in 40-80% of type 2 diabetes mellitus (T2DM) and 30-90% of obese patients [18]. Varying degree of IR is considered to be the cellular abnormality to underlie in both NAFLD and T2DM. In this regard, a strong association exists among NAFLD and T2DM as >70% of T2DM patients have NAFLD [19]. Diabetes and

IR considered to be the risk factors for liver diseases in patients with normal serum ALT levels. Aging and NAFLD are strongly correlated and is considered as an important epidemiological factor for NASH and fibrosis. Older people are not only under risk of hepatic steatosis, but also prone to mortality and disease progression to fibrosis and HCC [5].

A subgroup of patients with NAFLD who are not obese are being recognized and considered as "lean NAFLD" according to their BMI. These non-obese lean NAFLD group are less metabolically abnormal than obese NAFLD but are more metabolically abnormal than lean subjects without NAFLD [20]. Data from Europe suggests that the prevalence of lean NAFLD in biopsy proven NAFLD cohort is about 20%, while in Asia it is reported in the range of 19 % to 23%. Both lean and obese NAFLD share common altered metabolic profile like an excess of abdominal adipose tissue and an increased prevalence of comorbidities such as T2DM and hypertension [21].

1.2. Lipid metabolism and Pathophysiology of NAFLD

Liver plays a crucial role in lipid homeostasis by balancing lipogenesis and lipid disposal. Any disturbances in lipid production and lipid degradation due to overwhelming metabolic capacity of the liver leads to steatosis [22]. FFAs accumulation in liver is mainly contributed by three sources 1) Lipolysis of adipose tissue due to IR 2) hepatic de-novo lipogenesis (DNL) and 3) Dietary fat. Isotope labelling study on excessive FFA flux into hepatocytes found that `59% of FFAs comes from adipocytes, 26% from DNL and finally 15% from Dietary fat [23]. These FFAs have different fates like β-oxidation in mitochondria

or peroxisomes for energy needs, esterified into TGs and stored in lipid droplets or packaged into lipoproteins and secreted as very low density lipoproteins (VLDL) [12].

IR is an integral component in the pathogenesis of NAFLD. Low insulin sensitivity or insulin resistance causes less glucose disposal to the peripheral tissues. In addition, IR in adipose tissue impairs suppression of lipolytic enzymes such as, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase, that leads to the breakdown of TGs to form glycerol and FFAs [24,25]. The FFAs released into blood plasma are taken up by the liver and esterified to from TGs, which is a storage form of lipids in hepatocytes [26]. Uptake of FFAs across the plasma membrane of hepatocytes is favoured by fatty acid transporter proteins (FATPs) and fatty acid translocase (CD36). The two major FATPs in liver are FATP2, FATP5 and the studies on mice with knock down of these genes demonstrated reduced uptake of fatty acids by liver [27,28]. FATP5 gene silencing in mice also reversed diet induced NAFLD [29]. Expression of CD36 is low in hepatocytes compared to other tissues like skeletal muscle and adipose but, CD36 expression is increased with obesity and high calorie diet intake [30,31]. Reports also suggest that there is direct relation between long chain fatty acid uptake and lipid accumulation in liver related to CD36 expression. Studies on rodent models and human liver biopsies from NAFLD patients has shown higher levels of CD36 compared to controlled subjects [32]. A study on subjects with high intrahepatic fat compared to normal levels of TGs in hepatocytes demonstrated higher expression of CD36 in muscle

and decreased expression in adipose tissue. Though this study doesn't mention about hepatic CD36 levels, we can presume that alterations in adipose tissue FAs uptake can involve in accumulation of FAs in hepatocytes (redirecting plasma FAs from adipose to other tissues like muscle and liver) [33].

IR also modulates hepatic lipid accumulation via synthesis of TGs through DNL. DNL is the process of generation of FFAs from excess carbohydrates and it is the second major source of hepatic FFAs. IR in skeletal muscles hampers glucose uptake, redirecting the excess glucose into the liver [3,26]. In the liver, glucose is converted into pyruvate via glycolysis and then transformed into acetyl CoA in the presence of pyruvate dehydrogenase complex (PDC). Acetyl CoA, combines with oxaloacetate (OAA) and forms citrate that is oxidised by krebs cycle/tricarboxylic acid cycle (TCA). However, in case of excess energy needs, citrate goes out of the mitochondrial matrix into cytosol and forms acetyl CoA in the presence of enzyme citrate lyase. Acetyl CoA is converted into malonyl coA catalysed by acetyl CoA carboxylase (ACC) which is the rate limiting step in DNL [34]. Malonyl-CoA act as a key substrate in the de novo synthesis of FFAs in the presence of fatty acid synthase (FASN) [35]. DNL is tightly regulated by transcription factors such as sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP). SREBP-1c is activated by insulin signalling, and promotes the expression of lipogenic enzymes (ACC, FASN, stearoyl-CoA desaturase 1 (SCD1)) for FFA synthesis, transporters required for FFA uptake (CD36) and

TG synthesis (Glycerol -3-Phoshphate acyltransferase) [36,37]. In addition, liver X receptor α (LXR α) also activates SREBP-1c through phosphorylation [36]. ChREBP is glucose mediated transcription factor, which is activated when glucose concentration is increased in the liver. Glycolysis intermediates and final products also activate ChREBP which regulates the expression of lipogenic enzymes such as ACC, FAS [38].

The third source of FFAs in the liver is dietary lipids. Regular consumption of high fat diet associates IR, dyslipidaemia, metabolic disorders can develop NAFLD. Bile acids secreted by liver are involved in digestion and absorption of dietary FAs through specific bile acid nuclear receptors such as farnesoid X receptor (FXR) FXR plays an important role in regulating the lipid metabolism. Moreover, dietary cholesterol, and saturated FAs can upregulate SREBP-1c and increase lipogenesis. Chronic fructose intake also favours steatosis by directly activating SREBP1c, ChREBP and by reducing mitochondrial β-oxidation [39,40].

1.3. Lipid disposal and lipotoxicity

Disposal of FFAs is important to maintain lipid homeostasis in the liver. Hepatic FFAs are disposed majorly in two ways by esterification to form TGs to export into the plasma as very-low-density lipoproteins (VLDLs), and β-oxidation through mitochondria or peroxisomes within the cell. Apolipoprotein B100 (apoB100) and microsomal triglyceride transfer protein (MTTP) are required for assembly and secretion of VLDL. In addition, CD36 also plays a

role in VLDL secretion along with hepatic FFA uptake [31]. Any defects in these proteins could not export lipids from the liver that can lead to accumulation of lipids in the hepatocytes causing steatosis [41]. Shortage of S-adenosylmethionine (SAM), a main human methyl donor for phosphatidyl choline synthesis in the ER, catalysed by phosphatidylethanolamine N-methyltransferase (PEMT) also impairs VLDL formation and stability. Phosphotidyl choline along with apo B100 are required in early stages of hepatic VLDL assembly in the ER lumen. Lack of phosphatidylcholine leads to degradation of nascent VLDL causing hepatic accumulation of TGs [42]. Secretion of VLDL increases with increased levels of intrahepatic fat content according to the studies conducted by Fabbrini etal. [43]. Increased production of VLDL, and increased secretion of TGs were considered as important adaptations of fatty liver to protect hepatocytes from harmful FFA lipotoxicity [3,26].

Removal of FFAs by mitochondrial oxidative metabolism involves β-oxidation, TCA cycle, ETC, ATP synthesis and ketogenesis [44]. During β-oxidation, short and medium chain FAs can cross the mitochondrial membrane passively, while long chain FAs are converted into acyl-coA molecules by specific acyl-coA synthases. Acyl CoA molecules enter mitochondrial matrix through three different steps. The first step is involved in the formation of fatty acyl carnitine catalysed by carnitine palmitoyltransferase 1 (CPT-1) (rate-limiting step). In NAFLD patients, reduced levels of L-carnitine decreases the fatty acid oxidation and causes mitochondrial impairment. Evidence suggest that

supplementation of L-carnitine ameliorated steatosis by increasing the FFA oxidation and improved mitochondrial function in diabetic mice [45]. The second step is intermembrane transport in the presence of acyl carnitine translocase (ACT) and the final step is reformation of fatty acyl CoA ester by CPT2 in the inner mitochondrial membrane. Fatty acyl CoA ester undergoes series of reactions including dehydrogenation by using acyl-CoA dehydrogenase enzymes to form acetyl CoA [22]. Acetyl CoA is involved in TCA cycle to generate reducing equivalents (NADH and FADH2), which are oxidized further in mitochondrial respiratory chain. Mitochondrial β-oxidation is increased in high-fat diet-fed rodent models during the initial stages of NAFLD, as an adaptation to remove excess FFA accumulated in hepatic cells. This reflects the effort of liver mitochondria to compensate for hepatic fat accretion [44]. However, increased mitochondrial β-oxidation results in higher production of reducing equivalents which causes excess flow of electrons into the ETC producing surplus generation of ROS leading to oxidative stress [46].

1.4. Structure and functions of mitochondria

Mitochondria are the powerhouses of the cell generating adenosine triphosphate (ATP), which are energy rich compounds driving the fundamental cellular functions. ATP is generated at ATP synthase in the inner mitochondrial membrane using ADP and phosphate ions [47]. Apart from ATP synthesis and cellular respiration, mitochondria are involved in the production of NADH, $FADH_2$, GTP in the citric acid cycle, biosynthesis of amino acids, heme groups, iron-sulfur clusters and the synthesis of phospholipids for

membrane biogenesis [48]. Mitochondria plays an important role in human health and their dysfunction causes severe diseases like Alzheimer's disease, diabetes, cancer, NAFLD etc.

1.4.1. Mitochondria structure

Mitochondria are the semi-autonomous organelles separated from the cytoplasm and have four distinct compartments-inner mitochondrial membrane (IMM), outer mitochondrial membrane (OMM), inter membrane space (IMS), and matrix (Figure 2) [49]. The outer membrane has porins like voltage-dependent anion channel (VDAC), which helps in free diffusion of ions, molecules into the intermembrane space. Cytochrome c is present in intermembrane space playing a major role in mitochondrial bioenergetics and apoptosis. Conversely to OMM, IMM is highly impermeable and requires transporters for the transfer of ions and molecules into the mitochondrial matrix. IMM composed of cardiolipins to form cristae and contain oxidative phosphorylation (OXPHOS) machinery [50]. Mitochondria contains its own circular genome having 16,500 base pairs containing 37 genes encoding for 13 proteins, 22 tRNAs, and 2 rRNAs. Maintenance, transcription, and packaging of mtDNA is controlled by mitochondrial transcription factor A (TFAM) [51].

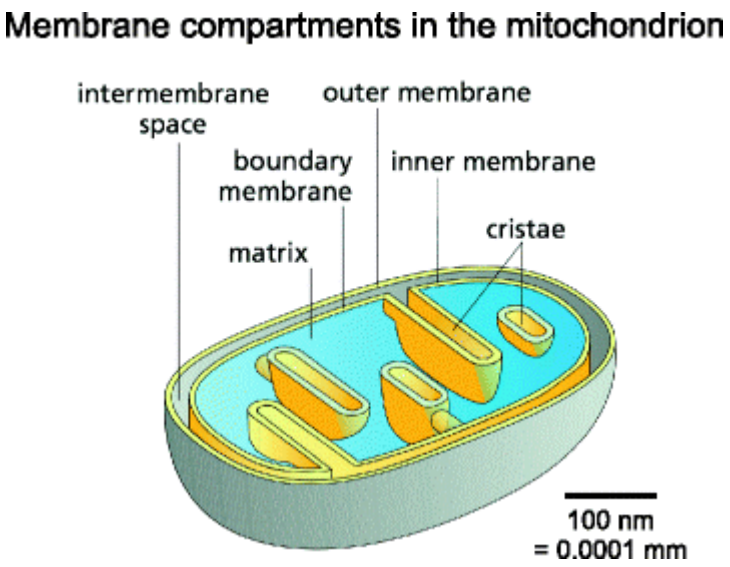

Figure 2. Structure of mitochondria (Werner Kühlbrandt., 2015)

1.4.2. Electron Transport Chain in Mitochondria

Mitochondria play a central role in generation of energy by oxidation of nutrients. Hepatocytes are rich in mitochondria and are involved in oxidation of acetyl coA into NADH and $FADH_2$ through TCA cycle. The electrons generated from NADH are donated to complex I (NADH ubiquinone oxidoreductase) and finally to coenzyme Q. During the process, 4 hydrogen ions are passed from mitochondrial matrix to intermembrane space contributing to the electrochemical gradient [52]. At complex II (succinate dehydrogenase), succinate oxidizes to fumarate and 2 electrons are accepted by FAD within complex II and then to coenzyme Q similar to complex I (Figure 3). However, no protons are translocated across membrane by complex II and therefore less ATP is produced through this pathway. Coenzyme Q also known as ubiquinone (CoQ) undergoes reduction to ubiquinol ($CoQH_2$) through Q cycle The electrons from $CoQH_2$ are transferred to complex III (cytochrome c reductase) [53]. A

cytochrome is a protein involved in electron transfer that contains heme group (between Fe^{2+} and Fe^{3+} states). As cytochrome c can accept a single electron at a time, this process occurs in two steps (Q cycle). Complex III also releases 4 protons in to the intermembrane space at the end of full Q cycle contributing to the electrochemical gradient [54]. At complex IV (cytochrome c oxidase), cytochrome c gets oxidized and transfers electrons to oxygen, the final electron carrier in aerobic cellular respiration. The cytochrome proteins a and a3, in addition to heme and copper groups in complex IV transfer the donated electrons to the bound dioxygen species, converting it into molecules of water [55,56]. Complex V (ATP synthase) uses the proton gradient generated by electron transport chain to form ATP. ATP synthase contains F0 and F1 subunits acting as rotational motor system. F0 is hydrophobic embedded in mitochondrial membrane and F1 is hydrophilic facing the mitochondrial matrix. Conformational changes in F1 subunits catalyze the formation of ATP from ADP and Pi [57].

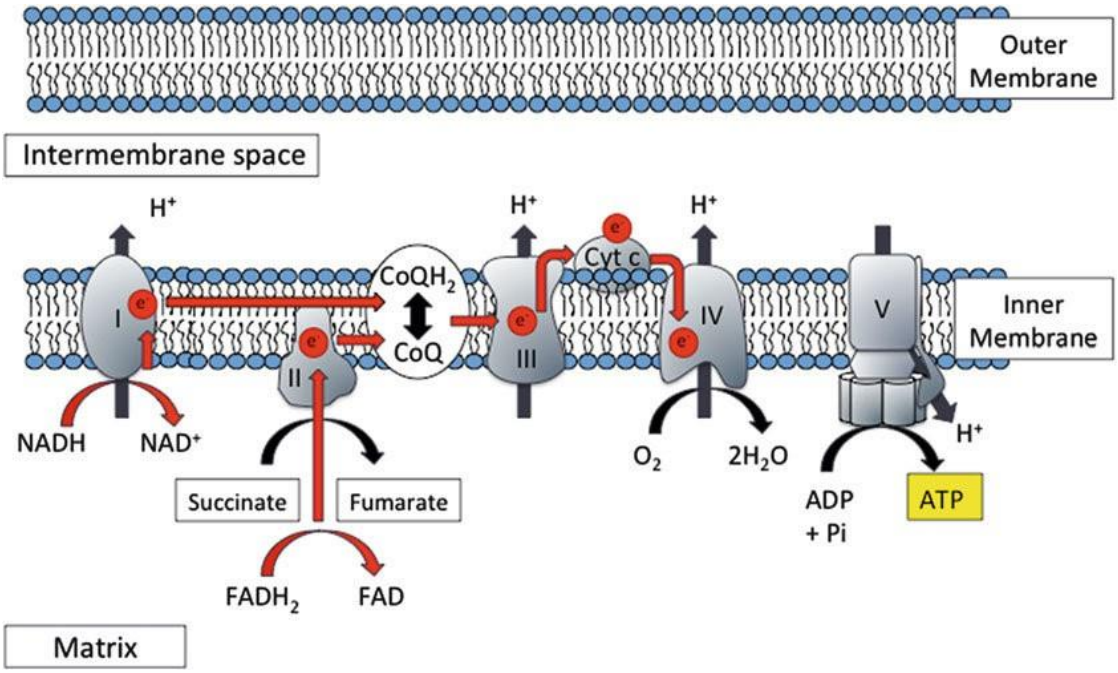

Figure 3. Mitochondrial electron transport chain: Electrons are transferred from complex I to IV and finally electrons are accepted by oxygen and results in water formation. The proton gradient generated by electron transfer provides energy needed to generate ATP at complex V (De Villiers et al., 2017)

1.4.3. Generation of ROS from ETC

It is well established that mitochondrion are the significant source of cellular ROS and the predominant route of ROS production by the ETC is the premature leak of electrons from complexes I, II and, III mediating one electron reduction of oxygen to superoxide ($O_2^{\bullet -}$) [58]. The rate of superoxide production is dependent on one-electron donor reacting with oxygen and the superoxide is dismutated to hydrogen peroxide (H_2O_2). Two sites of superoxide generation are identified in complex I – i) Flavin mononucleotide (FMN) cofactor which accepts electrons from NADH and ii) the Q binding site at which two electrons are transferred from Fe-S to Q. Electrons from NADH fully reduce the FMN centre, which reacts with oxygen to generate superoxide [59]. Thus, the

superoxide production at this centre is regulated by ratio of NADH to NAD+. At complex III, specifically at the site of $CoQH_2$ oxidation, superoxides are generated. The two electrons carried by $CoQH_2$ are transferred to the Fe-S centre and then to cytochrome c in sequence. The transfer of one electron with other remaining on CoQ favours the formation of unstable $CoQ^{\bullet -}$ species, which then react with oxygen to produce superoxides [60]. While complex II is not generally considered as a major source of ROS compared to complex I and III, several reports describe the superoxide production. Brand and colleagues demonstrated that the flavin site, at which FAD binds the active site of the enzyme is responsible for ROS production. This study also demonstrated that in isolated mitochondria, ROS generation is highly regulated by succinate concentration in a bell-shaped response in which ROS production is optimized when succinate concentration not too high or too low [60].

1.5. Oxidative stress

Oxidative stress is the discrepancy caused due to the ROS generation and antioxidant defense. This may occur due to increase in the production of pro-oxidant factors and dysfunction of antioxidant system [61]. Liver is an important organ consuming 20% to 25% of body's oxygen due to the blood supply [62]. ROS generated by mitochondrial impairment in NAFLD are responsible for oxidative stress. In clinical samples, biomarkers of oxidative stress that are frequently determined include lipid damage products like HNE, MDA, thiobarbituric acid reactive substance, and 8-isoprostane, DNA oxidation products like 8-hydroxy-2'-deoxyguanosine (8-OHdG), protein oxidation

products like protein carbonyl, nitrotyrosine [63-65]. Antioxidants commonly evaluated in clinical samples of NAFLD include enzymatic antioxidants like catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and non-enzymatic antioxidants like glutathione (GSH), α-tocopherol and ubiquinone [66].

The activities of these antioxidants decreased in NAFLD patients, while some reported an increase in their activities. Researchers showing lower antioxidant activity explain their observation is due to loss of protective capacities owing to higher oxidative stress, while high antioxidant activity research papers explain that it is an adaptive mechanism to detoxify the harmful metabolites of oxidative stress [67]. Along with mitochondria, CYP2E1, a microsomal oxidising enzyme act as a main source of ROS induced by endo and xenobiotics like FFAs and ethanol. FFA oxidation induced by CYP2E1 releases electrons into the cytosol and produce highly reactive carbonyl free radicals. CYP2E1 protein is highly expressed in NASH patients compared to normal one and steatosis patients and its activity is positively correlated with the degree of steatosis. CYP2E1 expression can be inhibited by insulin, thus IR may aggravate induction of CYP2E1 and contributes to NASH progression [68,69].

Excessive Iron in the liver can act as pro-oxidant metal and contribute to the initial development of macro vesicular steatosis and NASH progression [70]. Since liver is the major storage organ of iron, it can accumulate at mild levels in both hepatocytes and kupffer cells). However, in NAFLD down regulation of an iron export transporter ferroportin-1 and iron sensing protein hemojuvelin can

result in excessive accumulation of iron [71]. Iron exhibits its role in ROS production through Fenton reaction [15].

1.6. Role of micronutrients in NAFLD

Micronutrients are defined as nutrients that are needed in microgram or milligram quantities for physiologic functions as defined by the World Health Organization [72]. Liver plays an important role in micronutrient metabolism and this metabolism is impaired in chronic liver diseases like NAFLD [73]. Micronutrients include electrolytes, minerals, vitamins, and carotenoids that are required for enzymatic activity and cellular metabolism [74]. Electrolytes like sodium chloride, potassium and minerals like calcium, zinc, phosphorous are the inorganic compounds that are required for the tissue structure, pH regulation, neuronal signalling, muscle contraction and enzymatic activities [75]. Minerals are the inorganic compounds that share basic functions of the electrolytes. Major minerals include calcium, phosphorous, and magnesium, while minor minerals include zinc, copper, iron, and iodine [76]. Vitamins are the organic compounds that regulate cellular growth and metabolism and their solubility in lipids or water determines the mechanism by which they are absorbed, transported, stored, and excreted. Lipid soluble vitamins like low serum levels of vitamin A, have been linked to NAFLD. Treatment with vitamin E decreased the transaminase levels, liver lobular inflammation and improved liver fibrosis and reduced steatosis. Vitamin E supplementation is a common practice in NAFLD treatment as the patients are subjected to increased oxidative stress. Fat-soluble

vitamins like A, D, E, and K are transported through lymphatic system via chylomicrons and stored in liver and adipose tissue. While water-soluble vitamins like thiamine, riboflavin, vitamin B6, niacin, folate, vitamin B12, biotin, pantothenic acid, and vitamin c enter bloodstream [76].

Carotenoids are class of phytochemicals with anti-inflammatory and anti-oxidant properties and it includes carotenes like α-carotene, β-carotene, lycopene and xanthophylls like lutein, zeaxanthin, β-cryptoxanthin, and astaxanthin [77]. They are mainly present in fruits, vegetables and a smaller concentration in poultry. Carotenoid metabolism in indirectly linked to NAFLD pathogenesis in obese individuals because several carotenoids can be converted into vitamin A and this conversion occurs in intestine, which is impaired in obese patients [76,78].

Liver is involved in transport and storage of micronutrients and majority of body's vitamin A is stored in stellate cells. Besides, liver is a major site of protein synthesis and it produces binding, transport, and regulatory proteins required for micronutrient homeostasis. For instance, as zinc and other micronutrients bind to albumin, zinc deficiency is linked to hypoalbuminemia. Thus, targeting micronutrients through supplementation in NAFLD could help in improving the pathology [76,79].

2. AIMS

To investigate the therapeutic effects of a novel nutraceutical mixture FLINAX containing Vitamin E, Vitamin D3, olive-dry extract, cinnamon dry extract, and fish oil (DHA/EPA) in a rat model of NAFLD by characterizing:

- Hepatic steatosis and oxidative stress
- Mitochondrial respiratory activity

3. Material and Methods

3.1. Animals and Experimental Design

All animals received care in compliance with national and local law, including ethical approval. Male, 8-week-oldWistar rats (n = 22) (Harlan Laboratories, San Pietro al Natisone, Italy) were maintained in individual cages with a 12 h light/12 h dark cycle. All the rats were fed with a high-fat and high-cholesterol diet (HF-HC; 60% cocoa butter + 1.25% cholesterol) ad libitum for 4 weeks in order to induce steatohepatitis. After 4 weeks, rats were divided into two dietary groups: one group (n = 8) was fed with chow-diet (HFHCD+CD), while the test group (n = 8) was treated with chow-diet supplemented with 2% Flinax (containing vitamin D3, Vitamin E, dry extract of olive, cinnamon dry extract, fish oil) (HFHCD+CD&Flinax) (Table 1) for 2 weeks.

Table 1. Flinax composition (1g). DHA: docosahexanoic acid, EPA: eicosapentanoic acid

Vitamin D3	25 mcg
Vitamin E	60 mg
Olive dry extract (Olea Europaea) titrated in 20% hydroxytyrosol	15 mg
Cinnamon dry extract (Cinnamomum cassia Presi cortex) titrated in 1% flavanoids	14 mg
Fish oil 56% DHA/EPA	830 mg

Throughout the whole experimentation time, a group of rats (n = 6) was fed with chow-diet only (6 weeks) as the control group (referred to as CD) (Figure 4). Diets were prepared by Mucedola Srl (Settimo Milanese, Italy)

according to the levels of components previously reported [14]. Rats were weekly weighted, and the amount of food consumed was daily monitored. At the end of the study (6 weeks), after 8 h fasting, animals were anesthetized with Tiletamine/Zolazepam and then sacrificed for the collection of liver samples.

All experimental procedures were approved by local *Organismo preposto al benessere degli animali (OPBA)* and *national Ministero della Salute* in accordance with European legislation.

Figure 4. Experimental design. Wistar rats were fed with a high-fat, high-cholesterol diet for 4 weeks in order to induce steatosis. Following this, one group of rats was treated with chow-diet for 2 weeks, while another group was fed with chow-diet supplemented with 2% Flinax. During the entire experiment, a third group of rats was fed with chow-diet only for 6 weeks, serving as the control. HF-HC: high fat high cholesterol.

3.1.1. Histology

Sections of formalin-fixed, paraffin-embedded liver samples were stained with hematoxylin/eosin and blind-analyzed by a pathologist in order to quantify hepatic steatosis. Microscopic analysis of at least 5 randomly chosen high-power magnification fields was used to calculate the percentages of hepatocytes with

macrosteatosis and with microsteatosis. The score was elaborated as follows: (% of cells with macrosteatosiX2) + % of cells with microsteatosis.

3.1.2. Triglyceride analysis

Hepatic triglyceride content was analyzed as previously described [80]. Briefly, frozen liver tissue was homogenized in PBS, adjusting the volume to the weight, and incubated for 30 min at 95 °C. Centrifugation at 12,000× g followed, and the triglycerides were measured by absorbance in supernatant incubated with an appropriate reagent (Roche, Switzerland). Fatty-free BSA-coated vials were used to contain the triglycerides suspensions (Sigma, St. Louis, MO, USA).

3.1.3. Isolation of mitochondria

Fresh liver tissue was chilled on ice and washed in a medium containing 0.25 M sucrose, 5 mM K-EDTA pH 7.4, 10 mM Tris-HCl pH 7.4 and 0.2% fatty acid-free BSA to remove lipids, blood and connective tissue, and was processed for the preparation of mitochondria as previously reported [81]. Briefly, the tissue was minced by scissors and washed 3 times in the prechilled glass beaker, using the same buffer. Centrifugation steps followed.

3.1.4. Mitochondrial complex activity

For the OXPHOS complexes I, II, III, IV and V, enzymatic activity was measured in isolated mitochondria. A total of 40 µg of mitochondrial protein was used to determine the activity of each complex. The assays were performed at 37 °C in 1 mL of medium as previously reported [82,83]. The volume of mitochondrial extract (40 µg) was previously determined by Bradford assay.

3.1.4.1. Complex I Assay

In total, 40 μg of each enzyme solution was mixed with complex I buffer (25 mM KH2PO4 (pH 7.4), 130 μM β-Nicotinamide adenine dinucleotide (NADH), 240 μM potassium cyanide (KCN), 10 μM antimycin A, and 0.1% BSA). The reaction was started by the addition of 50 μM 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (DB). The change in absorbance at 340 nm was recorded for 3 min. Reference was measured in the presence of 2.5 μM rotenone (dissolved in ethanol). Enzyme activity was calculated with molar extinction coefficient (ε) for the NADH (6.22 mM−1 cm−1).

3.1.4.2. Complex II Assay

In total, 40 μg of each enzyme solution was incubated with complex II buffer (25 mM KH_2PO4 (pH 7.8), 2 mM EDTA, 1 mg/mL BSA, 10 mM succinate, 1 mM KCN, 4 μM rotenone, and 10 μM antimycin A) for 10 min. After the addition of 50 μM 2,6-Dichlorophenolindophenol (DCPIP), the change in absorbance at 600 nm was recorded for 2 min for reference. The addition of 10 mM malonate inhibits the oxidation of succinate. Enzyme activity was calculated with ε for the DCPIP (19.1 mM−1 cm−1).

3.1.4.3. Complex III Assay

In total, 40 μg of each enzyme solution was mixed with complex III buffer (25 mM KH2PO4 (pH 7.8), EDTA 2mM, and 1 mg/mL BSA) with 80 μM decylubiquinol, 240 μM KCN, 4 μM rotenone, 200 μM ATP and 30 μM cytochrome c. The change in absorbance at 550 nm was recorded for 3 min.

Cytochrome c was fully reduced at the end of the measurement with dithionite. The reference was measured without enzyme solution. Enzyme activity was calculated with ε for the reduced cytochrome c (19.6 mM−1 cm−1).

3.1.4.4. Complex IV Assay

Reduced cytochrome c was prepared using sodium dithionite. In total, 40 µg of each enzyme solution was mixed with complex IV buffer (10 mM KH_2PO4, 0.25 M sucrose, and 1 mg/mL BSA). The change in absorbance at 550 nm was recorded for 2 min. Following this, 240 µM KCN was added to fully oxidized cytochrome c at the end of the measurement. The reference was measured without enzyme solution. Enzyme activity was calculated with ε for the reduced cytochrome c (19.6 mM−1 cm−1).

3.1.4.5. Complex V Assay

Complex V buffer (50 mM Tris-Hcl (pH 8.0), 5 mg/mL BSA, 20 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 10 mM phosphoenolpyruvate, 5 µM antimycin A, 15 µM Carbonylcyanide3-chlorophenylhydrazone), 4 units of lactate dehydrogenase, and pyruvate kinase were incubated with 2.5 mM ATP for 2 min. After this, 40 µg of each enzyme solution was added to the above mixture, and the change in absorbance at 340 nm was recorded for 5 min. The reference was measured in the presence of 3 µM oligomycin for 5 min. Enzyme activity was calculated with ε for the NADH (6.22 mM−1 cm−1).

3.1.5. Western blot analysis

In total, 40 μg samples of proteins from liver homogenates were loaded in a 10% SDS-PAGE and transferred to a nitrocellulose membrane, blocked for 1.5 h using 5% non-fat dry milk in TBS-t and incubated over night at 4 °C with primary UCP2 antibody (goat polyclonal UCP2 purchased from Santa cruz Biotechnologies, Santa Cruz, CA, USA). Then, the membrane was incubated for 1.5 h with a rabbit HRP-conjugated anti-mouse (Bio-Rad Laboratories Inc., Segrate (MI), Italy). Bands were detected by the ClarityTM Western ECL Blotting Substrate using a ChemiDoc MP system (Bio-Rad Laboratories Inc., Segrate (MI), Italy), and quantified by the Image LabTM Software.

3.1.6. Gene expression analysis by RT-PCR

RNA was extracted from liver tissue using pure link RNA kit (Ambion) according to the manufacturer's protocol. Equal amounts of RNA were reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed, using Sso Advanced universal SYBR green supermix on a Bio-Rad CFX96 Real-Time system as previously reported [19]. The cycle threshold (Ct) was determined, and the relative gene expression was calculated with the ΔΔCT method. The following primers were used in qPCR assays (**Table 2**):

Table 2. Primers list

S.No.	Gene	Forward primer	Reverse primer
1	**GAPDH**	TCAAGGCTGAGAATGGGAAG	ATGGTGGTGAAGATGCCAGT
2	**SCD1**	TGTTCGTCAGCACCTTCTTG	TCTTGTCGTAGGGGCGATAC
3	**SREBP1**	ATCTGTGAGAAGGCCAGTG	GCGGGCCACAAGAAGTAGA
4	**CPT1**	TTCAAGGTCTGGCTCTACCA	TCCCTCGTGCAAAATAGGTC
5	**CPT2**	GCCTCTCTTGGATGACAGC	CTGGTGTGCTTATTCTGCT
6	**UCP2**	CTTTGAAGAACGGGACAC	TCCTGCTACCTCCCAGAA
7	**UCP3**	ATGAGTTTTGCCTCCATTCG	AATCGGACCTTCACCACATC

3.1.7. HNE and MDA adducts in liver tissue

4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) fluorescent adducts formed with mitochondrial proteins were monitored by spectrofluorimetry as previously reported [84,85]. Briefly, 100 mg of liver tissue is homogenized with 100 µL of 1.15% KCl buffer, treated with 500 µL of 10% TCA and washed with 6 mL Ethanol/ether (3:1) three times. The pellet obtained after centrifugation at 6000 RPM for 5 min is dried and suspended in distilled water. Fluorescent emission was at 460 nm, and excitation was at 390 nm for MDA-adducts and 355 nm for HNE-adducts.

3.1.8. SOD and catalase activity

Commercial kits were used to measure superoxide dismutase activity (706002, Cayman Chemical, Ann Arbor, MI, USA) and catalase activity (707002, Cayman Chemical, Ann Arbor, MI, USA) in freshly prepared liver mitochondria according to the manufacturer's protocols.

3.1.9. Measurement of mitochondrial H_2O_2 production

The rate of peroxide production was determined in isolated liver mitochondria as previously reported [81]. Briefly, mitochondrial H_2O_2 production was measured at 37 °C following the oxidation of Amplex Red by horseradish peroxidase in isolated rat liver mitochondria using 5 mM pyruvate plus 1 mM malate or 5 mM succinate as respiratory substrates. The fluorescence of supernatants was measured using 530 nm as excitation wavelength and 590 nm as emission wavelength. The rate of peroxide production was calculated using a standard curve of H_2O_2.

3.1.10. Western blot analysis of oxidized proteins

The analysis of oxidized proteins was performed by western blot in liver mitochondria using an Oxyblot kit (Millipore Bioscience Research Reagents, Temecula, CA, USA) [81]. The same amounts of mitochondrial proteins (35 µg) were reacted with dinitrophenyl hydrazine (DNPH) for 20 min, followed by neutralization with a solution containing glycerol and 2-mercaptoethanol, resolved in 12% SDS-polyacrylamide gel electrophoresis. After the transfer to a nitrocellulose membrane, a blocking step with non-fat milk and incubation with

a rabbit anti-DNPH antibody (1: 150) at 4 °C overnight followed. After washing, the membrane was incubated with the secondary antibody (1:300) conjugated to horseradish peroxidase and detected by a chemiluminescence detection kit (Cell Signaling Technology Inc., Danvers, MA, USA). Reactive bands were visualized by the enhanced chemiluminescence method on a VersaDoc Image System (Bio-Rad Laboratories, Hercules, CA, USA). Band density was determined with TotalLab software. The test provides a qualitative analysis of the total proteins' oxidation state change.

3.1.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8. One-way analysis of variance, followed by post-hoc Bonferroni test, was used to analyze differences between three groups and multiple comparison. Student's t test was used for differences between two groups. Data are shown as mean ± SEM. Statistical significance was considered with $p < 0.05$.

4. Results

4.1. Flinax reduced hepatic steatosis and oxidative stress

Flinax contains micronutrients and fish oil that plays an important role in combating chronic liver diseases. The aberration in energy and nutrient homeostasis are sufficient to cause NAFLD. So, we investigated the potential beneficial effects of supplementation with Flinax in an established model of NAFLD. To do this, wild type rats were fed for 4 weeks with an HF-HC diet, as we have previously demonstrated that this model is very efficient in inducing fat accumulation and oxidative stress in the liver [83]. Following this, a chow-diet supplemented with 2% of Flinax was administered as the therapeutic for 2 weeks. The daily monitoring of food consumption highlighted that each rat consumed about 0.3 g of Flinax daily. Very interestingly, the Flinax-supplemented rats presented a significantly lower hepatic fat accumulation compared to non-supplemented rats, as shown by histology and triglycerides content (Figure 5-A,B).

In contrast, compared to control CD-fed rats, all HF-HC-fed rats showed a higher production of CPT1A and CPT2 (Figure 5-C), two enzymes crucial in FAO, as they are involved in acylcarnitine and acyl-CoA formation, respectively [86]. However, the increases in mRNA and the protein levels of CPT1A and CPT2 were more significant in Flinax-treated rats (Figure 5-C,D). Therefore, we might assert that the excessive fat introduced with the HF-HC diet was contrasted by FAO, and Flinax boosted this adaptive mechanism.

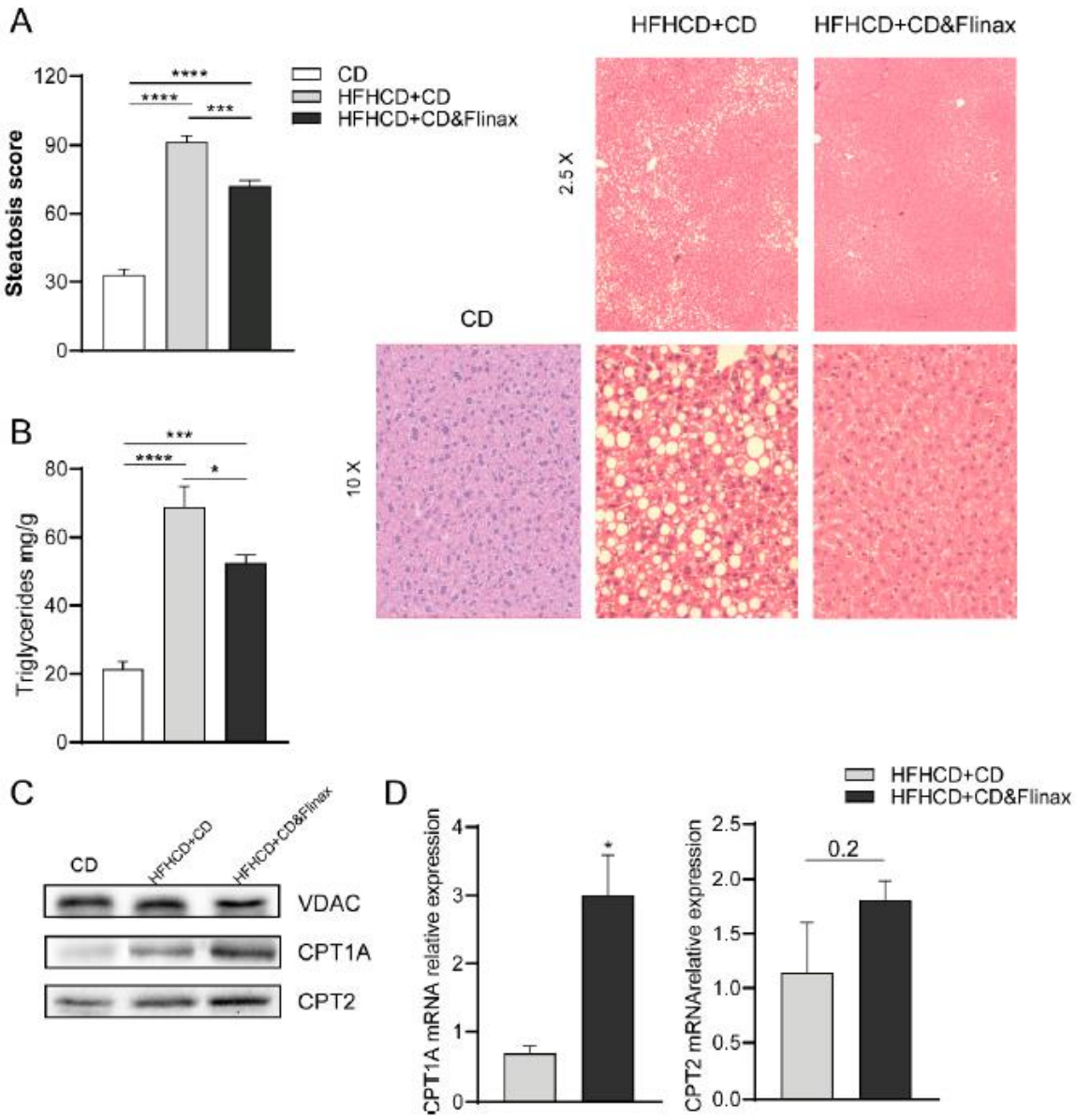

Figure 5. Dietary supplementation with Flinax reduces hepatic steatosis. (A) Histological determination of hepatic steatosis with representative pictures of H&E staining (CD = n5; HFHCD+CD = n8; HFHCD+CD&Flinax = n8). (B) Liver Triglycerides content (CD = n6; HFHCD+CD = n8; HFHCD+CD&Flinax = n8); (C) Protein levels of CPT1A and CPT2 determined by western blot analysis (CD = n4; HFHCD+CD = n8; HFHCD+CD&Flinax = n8); (D) CPT1A and CPT2 mRNA expression fold over HFHCD+CD (HFHCD+CD = n6; HFHCD+CD&Flinax = n6). Data are expressed in mean ± SEM; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ according to Student's t test for comparison between two groups, or one-way ANOVA followed by post-hoc analysis with Bonferroni test for comparison between more groups; CD, chow diet; HFHCD, high-fat, high-cholesterol diet; CPT1A, Carnitine Palmitoyltransferase 1A; CPT2, Carnitine Palmitoyltransferase 2; VDAC, voltage-dependent anion channels.

The expression studies did not show differences of the important genes involved in FAs synthesis when compared to control and Flinax treated groups (i.e., SCD-1, SREBP1c, FASN) (Figure 6).

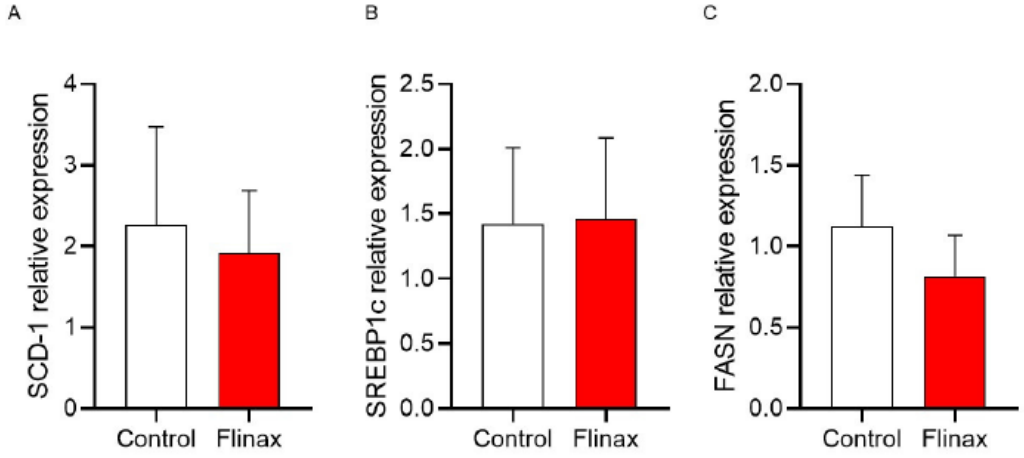

Figure 6. Expression of genes involved in fatty acids synthesis. (A–C) mRNA levels of SCD-1, SREBP1c and FASN fold over control (Control = n6; Flinax = n6). Data are expressed in mean ± SEM; SCD-1, stearoyl-CoA-desaturase-1; SREBP1c, Sterol regulatory element-binding transcription factor 1-c; FASN, fatty acids synthetase. Control: rats treated with chowdiet; Flinax: rats treated with chow-diet+Flinax.

In a second step we analyzed the oxidative burden. Notably, the treatment with Flinax significantly reduced the amounts of MDA- and HNE-protein adducts, important lipoperoxidation markers, both measured in isolated mitochondria and whole tissue homogenate (Figure 7-A,B). Accordingly, the Oxyblot (Millipore Bioscience Research Reagents) revealed that the total quantity of oxidized proteins in the mitochondria was significantly higher in the untreated rats (Figure 7-C).

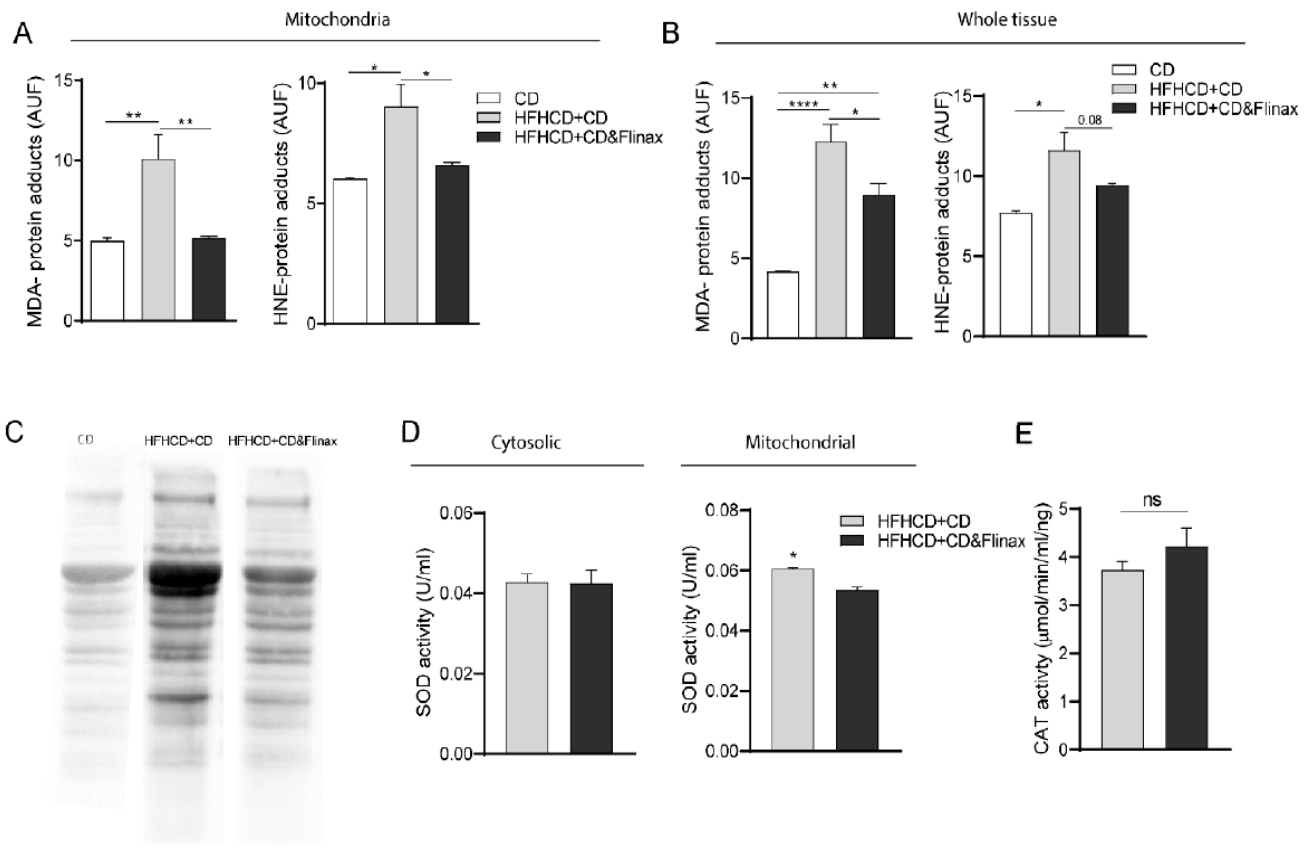

Figure 7. Dietary supplementation with Flinax reduces hepatic oxidative stress. (A) Mitochondrial levels of MDA- and HNE-protein adducts (CD = n4–6; HFHCD+CD = n6; HFHCD+CD&Flinax = n6); (B) Levels of MDA- and HNE-protein adducts in whole tissue homogenate (CD = n4–6; HFHCD+CD = n6; HFHCD+CD&Flinax = n6); (C) Representative picture of the quantity of mitochondrial oxidized proteins detected with Oxyblot (Millipore Bioscience Research Reagents); (D) Activity levels of cytosolic and mitochondrial SOD (HFHCD+CD = n8; HFHCD+CD&Flinax = n8); (E) Activity level of CAT (HFHCD+CD = n8; HFHCD+CD&Flinax = n8). Data are expressed in mean ± SEM; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, according to Student's t test for comparison between two groups, or one-way ANOVA followed by post-hoc analysis with Bonferroni test for comparison between more groups; CD, chow diet; HFHCD, high-fat, high-cholesterol diet, Malondialdehyde; HNE, 4-Hydroxynonenal; SOD, superoxide dismutase; CAT, catalase.

The activity of important ROS scavengers such as catalase (CAT) and cytosolic superoxide dismutase (SOD) was not different between the treated and untreated rats (Figure 7-D,E). Moreover, in Flinax-supplemented rats the enzymatic activity of mitochondrial SOD was lower (Figure 7-D), underlying

the fact that in treated rats the induction of scavenging systems was not required. Overall, these results suggested that the administration of Flinax protected the liver, reducing steatosis and oxidative stress.

4.2. Flinax enhanced the mitochondrial respiratory activity

The imbalance of electron transport chain (ETC) activity in NAFLD has been widely described as a driving force both in humans and in animal models [9]. Therefore, in order to explore the impact of Flinax on mitochondrial metabolism, we analyzed the ETC enzymatic activity and the hydrogen peroxide production rate. Intriguingly, the analysis revealed that the HF-HC diet significantly reduced the activity of complex I and complex III, which was restored by Flinax supplementation (Figure 8-A, C). In contrast, the complex II activity was significantly enhanced by the HF-HC diet, and was even more pronounced in Flinax-treated rats (Figure 8-B). As the impairment of mitochondrial ATP synthesis has also been described in NASH, we measured the complex V (ATP-synthase) activity and the hepatic ATP content, which were significantly reduced in HF-HCD+CD rats, while Flinax-supplementation restored the normal levels (Figure 8-E,F). No difference was found in complex IV activity (Figure 8-D).

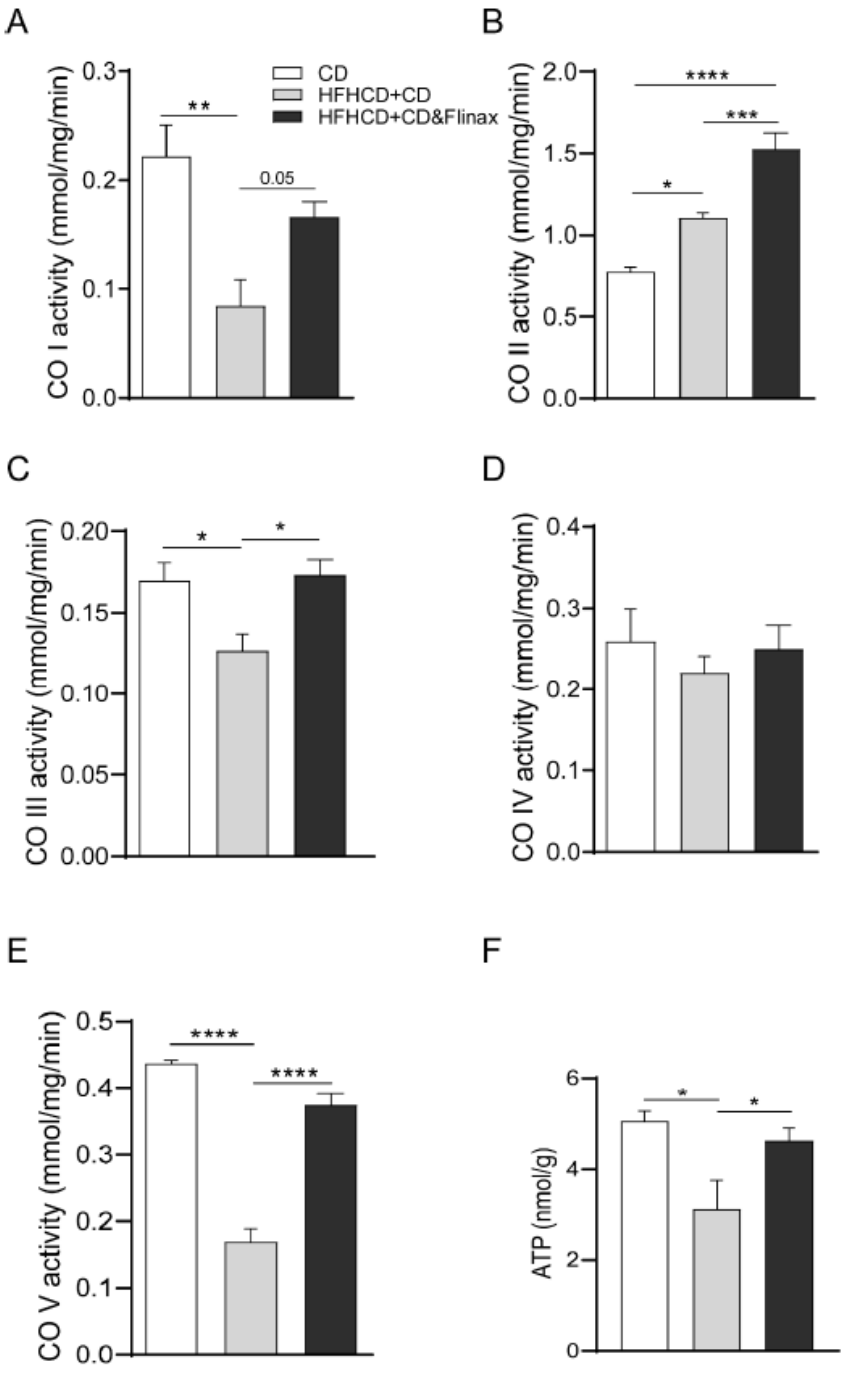

Figure 8. Flinax enhances respiratory chain complexes activity. (A) Complex I enzymatic activity; (B) Complex II enzymatic activity; (C) Complex III enzymatic activity; (D) Complex IV enzymatic activity; (E) ATP-synthase (Complex V) activity. (CD = n4–6; HFHCD+CD = n8; HFHCD+CD&Flinax = n8). (F) ATP content in liver tissue (CD = n4; HFHCD+CD = n6; HFHCD+CD&Flinax = n6). Data are expressed in mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ according to Student's t test for comparison between two groups, or one-way ANOVA followed by

post-hoc analysis with Bonferroni test for comparison between more groups; CD, chow diet; HFHCD, high-fat, high-cholesterol diet; CO, complex.

In our previous work we also showed that the HF-HC diet impaired mitochondrial activity, with the consequent production of radicals [83]. To measure this, we quantified the H_2O_2 production rate by using pyruvate/malate (complex I) and succinate (complex II) as mitochondrial substrates. Notably, the Flinax significantly reduced the peroxides levels derived from complex I and complex II activity when the analysis was conducted either on whole tissue or in mitochondria (Figure 9-A,B).

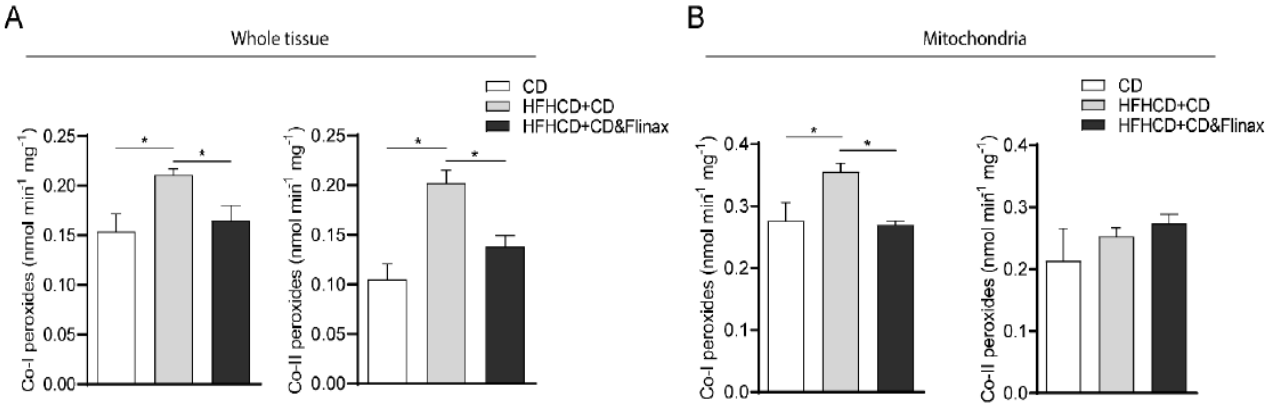

Figure 9. Flinax inhibits peroxide production. (A) Peroxide production from pyruvate/malate (complex I) and succinate (complex II), performed in whole tissue homogenate (CD = n4–6; HFHCD+CD = n8; HFHCD+CD&Flinax = n8); (B) Peroxide production from pyruvate/malate (complex I) and succinate (complex II), performed in isolated mitochondria (CD = n4–6; HFHCD+CD = n8; HFHCD+CD&Flinax = n8); Data are expressed in mean ± SEM; * $p < 0.05$ according to Student's t test for comparison between two groups, or one-way ANOVA followed by post-hoc analysis with Bonferroni test for comparison between more groups; CD, chow diet; HFHCD, high fat high cholesterol diet; Co-I, complex I; Co-II, complex II.

Along these lines, Flinax recovered an impaired mitochondrial function as shown by the enhancement of respiratory chain activity and mitochondrial ATP production with a lower formation of peroxides.

On the other hand, analyzing the expression of uncoupling proteins, we found that the production of uncoupling protein 2 (UCP-2) was higher in the Flinax group, as demonstrated by gene expression and protein levels (Figure 10-A, B). UCP-3 also showed a trend towards up-regulation in the Flinax-supplemented rats (Figure 10-C). Therefore, despite the positive impact of Flinax on mitochondrial energy homeostasis, we cannot exclude that a certain uncoupling exists, although this did not affect OXPHOS balance.

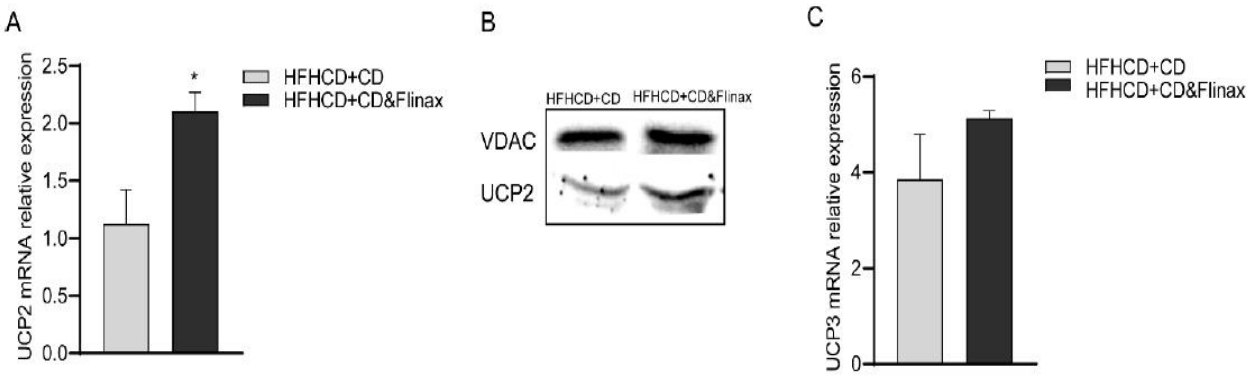

Figure 10. Flinax up-regulates uncoupling proteins. (A) mRNA levels of UCP2 fold over HFHCD+CD (HFHCD+CD = n7; HFHCD+CD&Flinax = n6); (B) Representative picture of western blot analysis of UCP-2; (C) mRNA levels of UCP three-fold over Ctrl (HFHCD+CD=n6; HFHCD+CD&Flinax = n6). Data are expressed in mean ± SEM; * $p < 0.05$ according to the Student's t test. CD, chow diet; HFHCD, high-fat, high-cholesterol diet; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3.

5. Discussion

Although considerable scientific attention is currently addressed to the study of NAFLD, several pathogenic aspects of its development and evolution are still elusive. Therefore, the incomplete mechanistic knowledge and the increasing global incidence make this disease a real health care burden. The complexity of NAFLD pathogenesis has been well described by the multiple hit hypothesis, which highlights the synergistic action of several factors [87]. However, despite the fact that multiple therapeutic approaches have been studied, scarce results have been obtained at the translational level. Recent evidence reported the oxidative imbalance as a cornerstone in the plethora of mechanisms involved in NAFLD [88]. In fact, oxidative stress is already present in simple steatosis, but also behaves as a promoter of inflammatory activation in NASH [89]. Therefore, we used a nutritional model of NAFLD constituted by the administration of an HF-HC diet, as we have recently demonstrated that cholesterol supplementation to the HF diet facilitates mitochondrial disfunction and NAFLD progression [83].

Here, we investigated the potential beneficial effects of a new mixture of micronutrients, named Flinax, containing important molecules that demonstrated antioxidant effects and promising preliminary results in NAFLD patients [76,90,91]. To do this, we used a therapeutic model, whereby NAFLD was induced with HF-HC diet feeding for 4 weeks, followed by 2 weeks of treatment with a chow-diet supplemented with 2% Flinax. This model was used to mimic the clinical condition whereby the patient with NAFLD changes his lifestyle, modifying dietary habits and assuming nutraceuticals. For the study of

nutraceuticals, this model would overcome the limitations of classical animal models wherein treatment is administered simultaneously with the high-fat diet. Therefore, the role of supplementation with Flinax was studied and compared to diet change only. Interestingly, the treatment significantly ameliorated steatosis and oxidative stress.

Recently, Mosca A. et al. demonstrated that the administration of Vit.E+hydroxytyrosol in children with NAFLD improved steatosis and reduced plasma levels of 4-HNE and 8-OHdG [91]. Here, we found that Flinax significantly reduced the hepatic levels of 4-HNE- and MDA-adducts, and oxidized proteins and peroxides. Therefore, in a second step we investigated whether Flinax impacted the hepatic mitochondrial metabolism by analyzing the activity of ETC complexes. Several studies showed that in the typical lipid-rich condition, the FA overload facilitates the production of oxidants species, which inactivates complex I and III and produces proton leakage [92-96]. Accordingly, we found a reduction in complex I and III activity in untreated rats, while Flinax restored the normal levels. Moreover, as already described in a previous work, the HF-HC diet dampened ATP-synthase (complex V) activity and ATP production in non-supplemented rats. This effect was efficiently contrasted by Flinax administration. Therefore, we may assert that dietary supplementation with Flinax fueled the respiratory chain and contrasted ROS formation, ameliorating cellular energy metabolism an efficiency.

Moreover, CPT1A and CPT2, two rate-limiting enzymes for mitochondrial FAO, were up-regulated in untreated rats, and even more so in Flinax-treated

rats. Several reports described the increase of FAO in NAFLD as an adaptation to a lipid-rich condition [34]. FAO yields electrons to ETC by mitochondrial complex II, but in NAFLD, some of these electrons leak from complexes I and III to generate ROS [97]. Accordingly, non-supplemented rats showed high FAO (CPT1A and CPT2) and the enhancement of complex II activity, while complex I and III activity decreased. Intriguingly, the supplementation with Flinax fuelled even greater FAO and complex II activity, while restoring the high activity of complexes I, III and V. Therefore, the bioenergetics efficiency recovered by Flinax facilitated cellular adaptation mechanisms and avoided ROS formation. However, the increase in UCP proteins underlined that a certain uncoupling probably occurred in Flinax-treated rats. Accordingly, some studies proposed a mild uncoupling as a strategy to prevent mitochondrial ROS formation, as a moderate proton leak would both stimulate oxygen consumption and counteract the reduction of ROS-generating sites [98].

Further studies are needed to indicate the mechanisms behind Flinax-induced mitochondrial adaptation and resistance from external injury, as we might only speculate the role of scavenging activity in preventing ROS formation and the damage of mitochondrial respiratory chain complexes. Moreover, we acknowledge that potential adverse effects have not been investigated in this study. In conclusion, dietary supplementation with FLINAX reprogrammed the cellular energy homeostasis by restoring the efficiency of the mitochondrial function, with the consequent improvement of steatosis.

ABBREVIATIONS

8-OHdG	8-hydroxy-2' -deoxyguanosine
ACC	Acetyl CoA carboxylase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CAT	Catalase
CD	Chow diet
CD36	Fatty acid translocase
ChREBP	Carbohydrate response element binding protein
CoQ	Coenzyme Q/Ubiquinone
$CoQH_2$	Ubiquinol
CPT1A	Carnitine palmitoyltransferase 1A
CPT2	Carnitine palmitoyltransferase 2
DB	2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone
DCIPIP	2,6-Dichlorophenolindophenol
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNL	De-novo lipogenesis
DNPH	Dinitrophenyl hydrazine
EDTA	Ethylenediamine tetraacetic acid

EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
$FADH_2$	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FATP	Fatty acid transporter proteins
FFA	Free fatty acid
FMN	Flavin mononucleotide
FXR	Farnesoid X receptor
GPX	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HFHCD	High fat high cholesterol diet
HNE	4-hydroxy-2-nonenal
HSL	Hormone-sensitive lipase
IMM	Inner mitochondrial membrane
IMS	Inter membrane space
IR	Insulin resistance
KCl	Potassium chloride
KCN	Potassium cyanide
LXR α	Liver X receptor α
MDA	Malondialdehyde

MRI	Magnetic resonance imaging
NADH	Nicotinamide adenine dinucleotide
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OAA	Oxaloacetate
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PDC	Pyruvate dehydrogenase complex
PNPLA3	Patatin-like phospholipase domain-containing 3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time reverse transcription-polymerase chain reaction
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element binding protein 1c
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TFAM	Transcription factor A, mitochondrial
TG	Triglycerides
UCP2	Uncoupling protein 2
UCP3	Uncoupling protein 3

VDAC	Voltage-dependent anion channel
VLDL	Very low density lipoproteins

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