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Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pelo Professor Doutor António Joaquim Matos Moreno e pela Doutora Vilma Marisa Arrojado Soares Sardão e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2020

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1. Abstract

A large amount of waste is generated within the different steps of the food value chain, representing a big loss of natural resources, plant material and economic value. Many parts of edible plants are in fact, not sold for consumption and end up as massive waste. A good example of this is Brassica by-products. Properties described in existing literature point to antioxidant and even anti-obesogenic effects from *Brassica oleracea* var italica.

A growing concern in the Western world, obesity results from incorrect lifestyles and comprises a large array of co-morbidities, including non-alcoholic fatty liver disease. Therefore, a new, natural, and economic way to prevent or mitigate obesity-related health issues is a very attractive prospect.

The objective of this work was to investigate the effect of three different extracts from *Brassica oleracea* var italica by-products (leaves, stalk and inflorescence) in models of oxidative stress and lipotoxicity on human HepG2 cells.

The protective effects of leaves, stalk and inflorescence extracts from different parts of *Brassica oleracea*, at 3 different concentrations (1, 10, 25 µg/mL) were tested against 100 µM of pro-oxidant tert-butyl hydroperoxide (tBHP) and 2 different concentrations (1 and 10 µg/mL) were tested against free fatty acid (FFA, 250, 1000 and 2000 µM)-induced cytotoxicity and accumulation. Cell metabolic viability and cell mass were measured by using the resazurin reduction and sulforhodamine B assays, respectively, for tBHP experiments. Lipid accumulation was measured using Nile Red staining assay and normalized using the sulforhodamine B assay for FFA experiments. Furthermore, metabolic flux analysis and intracellular ATP content measurements were performed against 250 µM of FFA, with extracts concentration at 10 µg/mL.

While tBHP results presented little to protection by the extracts, the FFA results showed that stalk and inflorescence extracts had varying degrees of success in preventing lipid accumulation and cell death due to lipotoxicity, especially at 10 µg/mL. Leaves extract had some success, to a lesser degree. Metabolic flux analysis showed no significant differences and inflorescence extract increased ATP intracellular content in both control and FFA.

These results clearly show biomedical value in *Brassica* by-products, and further steps should be taken to better understand this value and take advantage of it.

Key words: Lipotoxicity, free fatty acids, *Brassica* by-products, obesity, oxidative stress

Uma grande quantidade de desperdício é gerado durante os diferentes passos da cadeia alimentar, representando uma enorme perda económica, de recursos naturais e de material orgânico. Várias secções de plantas comestíveis são na verdade deitadas fora, o que acaba por ser um grande desaproveitamento. Um bom exemplo desta situação são os subprodutos de *Brassica*. Propriedades descritas na literatura existente apontam para efeitos antioxidantes e até anti-obesogénicos de *Brassica oleracea* var *italica*.

Uma crescente preocupação no mundo ocidental, a obesidade resulta de um estilo de vida incorreto e engloba um grande número de comorbidades, entre as quais fígado gordo não alcoólico. Portanto, uma nova, natural e económica forma de prevenir ou mitigar problemas de saúde relacionados com obesidade é uma perspetiva muito atraente.

O objectivo deste trabalho foi a investigação dos efeitos de três extractos de diferentes subprodutos de *Brassica oleracea* var. italica (folhas, caule e inflorescência) em modelos de stress oxidativo e de lipotoxicidade em células humanas HepG2.

Os efeitos protetores dos extractos de folhas, caule e inflorescência de diferentes partes de *Brassica oleracea*, em 3 concentrações diferentes (1, 10, 25 µg / mL) foram testados contra 100 µM de um pró-oxidante tert-butil hidroperóxido (tBHP) e 2 diferentes concentrações (1 e 10 µg / mL) foram testadas contra a acumulação e citotoxicidade induzida por ácido gordos livre (FFA, 250, 1000 e 2000 µM). A viabilidade metabólica celular e massa celular foram medidas usando os ensaios de redução da resazurina e sulforhodamina B, respectivamente, para experiências de tBHP. A acumulação de lípidos foi medida utilizando o ensaio de coloração com nile red e normalizada utilizando o ensaio sulforhodamina B para experiências de FFA. Para além disso, a análise do fluxo metabólico e as medições do conteúdo de ATP intracelular foram realizadas contra 250 µM de FFA, com concentração de extratos de 10 µg / mL.

Enquanto que os resultados de tBHP apresentaram pouca ou nenhuma proteção por parte dos extractos, os resultados de FFA mostraram que os extractos de caule e inflorescência tiveram graus variados de sucesso na prevenção da acumulação de lipídios e morte celular devido à lipotoxicidade, especialmente a 10 µg / mL. O extrato de folhas teve algum sucesso, mas em menor grau. A análise do fluxo metabólico não mostrou diferenças significativas e o extrato da inflorescência aumentou o conteúdo intracelular de ATP tanto com o controlo como com FFA.

Estes resultados mostram claramente o valor biomédico dos subprodutos de *Brassica*, e outros passos devem ser tomados para melhor entender esse valor e tirar proveito dele.

Palavras-chave: Lipotoxicidade, ácidos gordos livres, subprodutos de Brassica, obesidade, stress oxidativo

Abbreviations: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ARE: antioxidant response elements; BAT: brown adipose tissue; BBP: *Brassica* by-products; BSA: bovine serum albumin; CVD: cardiovascular diseases; DMEM: Dulbecco's Modified Eagle Medium; DMSO: dimethyl sulfoxide; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FAO: Food and Agriculture Organization of the United Nations; FBS: featl bovine serum; FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FFA: free fatty acids; FRAP: Fluorescence recovery after photobleaching; GHG: greenhouse gas; GPx: glutathione peroxidase; HCC: hepatocellular carcinoma; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-6: interleukin 6; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; Nrf2: nuclear factor erythroid-2-related factor 2; OCR: oxygen consumption rate; OS: Oxidative stress; PBS: Phosphate-

buffered saline; ROS: reactive oxygen species; SOD: superoxide dismutase; tBHP: tert-Butyl hydroperoxide; SRB: Sulforhodamine B; TCA: tricarboxylic acid cycle; TEAC: Trolox Equivalent Antioxidant Capacity; TNF-α: tumour necrosis factor-alpha; TG: triglycerides; WAT: white adipose tissue; WHO: World Health Organization;

2. Introduction

2.1. Wasted potential

The Food and Agriculture Organization of the United Nations (FAO) has estimated that about a third of the food produced for human consumption is either lost or wasted, throughout the food supply chain, from the agricultural stage to the household consumption stage. This represents a wastage of about 1.3 billion tons [1]. Furthermore, it represents wasted resources used during the production of these food items and greater greenhouse gas (GHG) emissions from the production and disposal of waste. Not accounting for emissions of GHG derived from land usage, there is an estimated emission of 3.3 Gtonnes of CO_2 equivalent, a heavy carbon footprint associated with food produced and not eaten [2]. From the production side, these emissions are increased in relation to wastage in two ways: on one side, farmers need to produce more of a certain product to achieve a certain amount of profit, thus increasing the emissions, and on the other side the disposal of such a huge amount of organic matter usually involves processes such as incineration, causing further emission of greenhouse gases [1,3]. Also worth noting are the GHG emissions generated in vain when a certain amount of agricultural produce is lost (emission per kg of product). Yet to be quantified are the certainly huge costs to society that come from loss of land, water and biodiversity and the negative impact of climate change [2]. The term food loss is used when the decrease in food mass in the supply chain occurs before retail and consumption (production, post-harvest, and processing), at which point the term food waste is employed [4]. Both terms are used in the context of edible food for human consumption. Food wastage refers to both food loss and waste.

Food loss accounts for the majority of wastage world-wide. Measures taken towards prevention and re-usage of waste would therefore have a more significant impact in this sector of the food supply chain. When we look at the different groups of commodities, vegetables and fruits are the second most-produced category for human consumption, behind cereals. However, cereals have a smaller rate of wastage over-all and a much smaller portion of this wastage is due to agriculture. The wastage of vegetables in industrialised Asia, Europe, and South East Asia constitutes a high carbon footprint, mainly due to large wastage volumes [2].

In industrialized countries, production often exceeds demand due to the need to ensure quantities of food agreed upon are delivered, even in adverse conditions such as unpredictable bad weather or pest attacks. This sometimes makes farmers over-produce, creating larger quantities than needed, even in favourable conditions. On the other hand, the standard set from supermarkets and other retailers for appealing fresh products (e.g. appearance , shape, colour) make it so large portions of crops that would otherwise be good for human consumption never leave the farms [1]. In fact, most of the food loss on the agricultural side is due to this grading system. Yet, another deciding

factor in the food loss experienced in the production side of the food chain. Some surplus crops are sold to be processed or to become animal feed, this however is often not financially profitable [1]. The prevailing attitude in these industrialized countries is that disposing is cheaper than using or re-using [1]. In the fruit and vegetable groups of commodities, for example, most of the wastage is dumped in landfills or in rivers, which creates environmental hazards [3].

Amongst the wastage generated in the agricultural stage, there is organic waste generated, including leaves and stalks, that was never meant for human consumption. This waste, resulting from harvesting and processing, is known as by-products and represents between 25% and 30% of the total organic mass produced [5]. Non-edible materials produced by the processing of fruits and vegetables such as peels, seeds and stalks can be used as sources of phytochemicals and antioxidants, as the entire tissue of fruits and vegetables is rich in bioactive compounds [6]. The use of these by-products seems to be a very good way to mitigate the environmental problems the industry causes and would provide a new avenue of profit and economic rentability for companies and farmers, and can also help bring down the price of healthy food, [1]. On this group of fruits and vegetables, one vegetable stands out: broccoli (*Brassica oleracea* L. *italica*). Broccoli beats the average 25-30% of by-products generation by a lot: about 75% of these plants are by-products, with only 25 % being used for human consumption [7]. Therefore, finding new uses for these *Brassica* by-products (BBP) could have a huge impact on return on investment, market price, and the environment, due to the sheer amount of waste generated for a small portion of usable product. Furthermore, the potential to be a source of biochemical compounds has the BBP drawing the attention of the scientific community [8].

Figure 1 - Representation of a broccoli plant. Highlighted in red is the edible part of the plant (adapted artwork from Alison Croney [9]).

2.2. Biomedical value of Broccoli

Several health-promoting compounds, such as vitamins, phenolic compounds, essential elements, glucosinolates, and related compounds, are present in broccoli. Out of these compounds, some have the potential to serve as phytochemicals protectors, which is the case of glucosinolates and its derivatives [10]. Glucosinolates are thioglucosides[11], which are secondary metabolites that have in their basic structure a β-thioglucose group, a sulfonated oxime group, and a variable aglycone side chain derived from an α-amino acid [12]. In broccoli, glucosinolates coexist with an enzyme called myrosinase which, when in contact with water during cutting, harvesting or chewing of the plant, initiates its rapid hydrolysis. The products of glucosinolates breakdown are thiocyanates, nitriles, goitrin, epithionitriles and isothiocyanates [12,13]. The latter group is known to interfere in many steps of cancer development, more specifically in the modulation of enzymes that act in phase II detoxification, acting as an antioxidant in either a direct or indirect capacity [14]. The most studied of the isothiocyanates generated is the sulforaphane [15]. This compound increases nuclear factor erythroid-2-related factor 2 (Nrf2) binding to DNA, more specifically to antioxidant response elements (ARE), responsible for the transcriptional regulation of antioxidant enzymes such as catalase, glutathione S-transferase, glutathione-peroxidase, peroxiredoxins and hemeoxygenase 1 [16]. One particularity of sulforaphane is that in hepatocytes, its antioxidant activity modulates lipid metabolism, reducing lipid levels in cases of excessive accumulation, upregulating

mitochondrial gene expression, function and mitochondrial biogenesis [17]. These properties make sulforaphane a prime candidate use against a preoccupying condition rampaging through mostly developed countries: obesity and its associated complications.

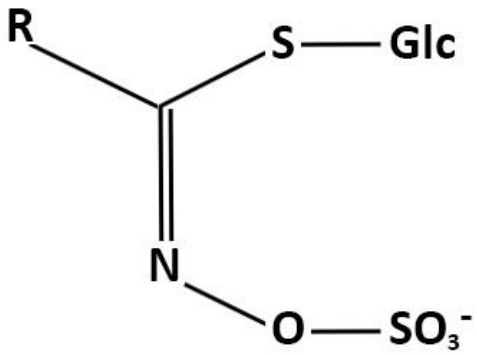


Figure 2 - Chemical structure of glucosinolates. The R represents a variable aglycone side chain derived from a α-amino acid.

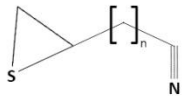
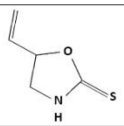
Isothiocyanate	R — NCS
Epithionitrile	
Nitrile	R — CN
Thiocyanate	R — SCN
Goitrin	

Figure 3 - Possible products of the breakdown of glucosinolate. The R represents a variable aglycone side chain derived from a α-amino acid.

Although the idea of using fruit and vegetables by-products as a source of food additives [18] or it's bioactive compounds for drug discovery in the context of human health [15,19,20] is not novel, the use of Brassica by-products in the context of obesity and its complications has only been described in some publications.

2.3. Obesity in society

According to the world health organization (WHO), in 2016 over 1,9 billion adults were considered overweight, and out of those, over 650 million were obese [21]. Making these numbers even more worrisome, obesity is still on the rise, with the incidence rate having tripled between 1975 and 2016 and it has been spreading all over the world [21]. In 2014, the impact of obesity on the world economy was estimated to be around 2.0 trillion dollars (US). In developed countries, obesity is responsible for 20% of all health care expenses. Furthermore, there is increasing evidence that these costs may be higher due to the loss of productivity of employees hindered by obesity [22]. But most of these costs are due to the many health problems caused by obesity, namely diabetes mellitus, cardiovascular diseases (CVD), metabolic syndrome, fatty liver disease and even cancer [23–27]. Besides the negative impact that these economic and health issues have in society, there is one more factor that negatively affects the lives of obese patients: the social stigma. Many of these patients are associated with negative misconceptions: that they are lazy and/or weak-willed, unsuccessful, that they lack intelligence, self-discipline and willpower and that they purposely sabotage their own weight-loss treatment [28]. These misconceptions give rise to discrimination against obese individuals in many aspects of their lives including in health care facilities, educational institutions, workplace and job interviews, their representation in the media and even in social situations [28]. Although some of these discriminatory attitudes may have a benevolent basis, as an incentive for the targeted person to become motivated to lose weight, the desired outcome may not be achieved. There is a lot of debate as to whether social stigma fails to change the subject's behaviour [29] or if social pressure will result in the modification of the patients' lifestyle [30].

2.4. What causes obesity?

Most cases of obesity is caused by an excess of energy consumption (dietary intake) relative to the expense of energy (energy loss via metabolic and physical activity). However, looking at the full spectrum of different cases, other factors, such as environmental, physiologic, psychological, genetic, economic, social, and even political can contribute to obesity development. [31]. One of these complex conjunctions of factors is the constructed environment in developed countries that promotes overeating. Affordable, very tasty, conveniently prepared and accessible in large quantities, highly caloric and fat-laden foods contribute to an elevated daily caloric intake [32], which is not offset by energy loss since at the same time, physical activity levels have been decreasing over the past decades [33]. Families stricken with economic issues in developed countries tend to consume more of these highly caloric, easily preparable and cheap meals for lack of money and/or time, due to a demanding work schedule [34,35].

During the 2020 lockdown, caused by the pandemic of COVID-19 [36], gymnasiums, parks, playgrounds, and other places of physical activity practice were forced to close down, meaning a reduction of physical activity for an extended period. Furthermore, under confinement conditions, many individuals tend to be less physically active, have increased screen times and adopt irregular sleep patterns and worse diets. The combination of these factors results in weight gain and loss of fitness. Once again, lower-income families are more affected by these stay at home rules, not only due to the factors

mentioned above, but also due to living in sub-standard homes and in more confined spaces, which makes it harder to practice physical exercise [37]. Since no studies have been conducted yet, the full scope of how COVID-19 will affect obesity rates is not yet clear. However, the outcome looks grim.

2.5. The role of oxidative stress in obesity and related health issues

Oxidative stress (OS) has been one of the factors studied to understand how obesity triggers health issues. Obesity is characterized by an excessive accumulation of fat in adipose tissue. Adipose tissue is classified as brown adipose tissue (BAT) and white adipose tissue (WAT), and although this difference has been noticed almost two decades ago, it's only in these past few years that some attention has been given to the heterogeneity of white adipose tissue [38]. Beyond being a deposit for triglycerides (TG), white adipose tissue is also an endocrine organ that secretes hormones and cytokines (adipokines or adipocytokines) with important regulatory and inflammatory functions [25,38–41].

Amongst these adipokines, the most well-known and studied are adiponectin [42], resistin [43], leptin [44], tumour necrosis factor-alpha (TNF-α) [45] and interleukin 6 (IL-6) [46]. These bioactive substances create reactive oxygen species (ROS) as a by-product of their activity, which will lead to OS, which is involved in the pathogenesis of different diseases [47].

Resistin - It is believed that resistin is a link between obesity and insulin resistance, playing a major role in the development of type 2 diabetes mellitus in obese patients [48]. A higher degree of adiposity correlates to an increased level of circulating resistin, but it does not correlate directly to the degree of resistance of insulin, although the use of anti-diabetic drugs has been shown to decrease the circulating levels of resistin [49]. Secreted by mature adipocytes, this adipokine is also a link to the pro-inflammatory environment generated in obese patients. This is due to its role in the modulation of monocytes and it's correlation with IL-6 levels [50]. Resistin increases monocyte adhesion and infiltration capability, enhancing their survival chances and stimulating them to express IL-6 [51,52].

Interleukin 6 (IL-6) - Secreted by different types of cells, including adipocytes, IL-6 possesses a wide range of functions, such as controller of energy homeostasis and pro-inflammatory factor [50]. It plays a vital role in the transition from acute to chronic inflammatory diseases, as observed in obesity. In addition, IL-6 inhibits the secretion of adiponectin and increases TG levels through the stimulation of gluconeogenesis and glycogenolysis and inhibiting glycogenesis [50].

Tumour Necrosis Factor-alpha (TNF-α) - One of the first cytokines identified, TNF-α is a pro-inflammatory factor involved in the systemic inflammatory response [49]. It has a strong connection to the development of insulin resistance, diabetes and obesity since it increases the release of free fatty acids (FFA) from adipocytes and it inhibits the production of adiponectin [49,50,53].

Leptin - When leptin was first discovered, it was believed to be the cure to obesity due to its ability to control appetite. Shortly after, it was discovered that obese patients

presented higher levels of circulating leptin than healthier individuals. It was then concluded that the lack of weight loss and appetite control were the result of a mechanism of resistance to this adipokine (hence the elevated leptin levels) [54]. Leptin is secreted mainly by adipocytes and inhibits lipogenesis and promotes lipolysis, lowering intracellular lipid levels and improving insulin sensitivity [53]. Leptin also plays a role in inflammation, promoting oxidative stress and vascular inflammation [55]. When there is a drop in weight, circulating levels of this adipokine lower and the plasma levels of obesity-associated inflammatory markers are reduced [56].

Adiponectin - Unlike other adipokines, adiponectin secretion is inhibited by increased adiposity, and lower levels are detected in patients that suffer from obesity-related diseases [49]. Its expression and secretion are exclusive to differentiated adipocytes [48]. Adiponectin acts as an anti-inflammatory factor and regulator of lipid metabolism, energy homeostasis, and glucose metabolism [57]. Loss of weight is very closely related to increased levels of this adipokine. Furthermore, adiponectin increases insulin sensitivity, decreases circulating FFA through the promotion of their oxidation on-site, inhibits the enzymes responsible for gluconeogenesis in the liver and stimulates glucose utilization [53].

The increase in adiposity will lead to increased production of adipokines, such as those mentioned above, which will create a chronic pro-inflammatory environment in the adipocytes, characteristic of obese patients [57–59]. An exception is adiponectin, which is inhibited by the abnormal increased secretion of IL-6 and TNF-α [49]. This inflammation state in the adipose tissue inhibits the expression and activity of antioxidant enzymes, like catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx), usually found in high quantities in the adipocytes, [59]. These enzymes are responsible for the elimination of ROS: Catalase catalyses the splitting of hydrogen peroxide into water and oxygen ($2\ H_2O_2 \rightarrow\ 2\ H_2O + O_2$), SOD catalyses the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen ($2\ O_2^{\bullet-} + 2H^{2+} \rightarrow H_2O_2 + O_2$) and glutathione peroxidase catalyses the reduction of hydrogen peroxide [60–62]. A pro-inflammatory environment also increases the expression of NADPH oxidase, an enzyme that generates ROS as an immune response by oxidising NADPH ($NADPH + 2\ O_2 \leftrightarrow NADP+ + 2\ O_2^{\bullet-} + H^+$). Thus, excess adipocytes will secret excess adipokines (except adiponectin), which will induce ROS formation on-site, increasing OS. Chronic inflammation state observed in the adipose tissue of obese patients raises OS levels [59]. Also, excess fatty acids, due to the patients' diet, will increase peroxisomal and mitochondrial fatty acid metabolism in the adipose tissue, which will generate further ROS and consequently OS [63].

The increased FFA in circulation is particularly problematic in the liver. Here, mitochondria switch to the β-oxidation pathway to try to deal with excess fatty acids, generating ROS in the process. The excess may lead to cell death. Mitochondria switch to the β-oxidation pathway to try to deal with excess fatty acids. Furthermore, accumulation of FFA and TG, in the form of fat droplets in the cytoplasm of hepatocytes, impairs mitochondria. Hepatocytes lose their oxidative capacity and increase the reduced state of the electron transport chain complexes, leading to an increased peroxisomal pathway of fat oxidation. These events can lead to a disease called non-alcoholic fatty liver disease (NAFLD) also referred to as just fatty liver disease [64,65].

The insulin-resistance, responsible for elevated glucose levels in the bloodstream, is characteristic of type 2 diabetes mellitus, also observed in obese people [66]. This hyperglycemia is responsible for an increased generation of ROS in β-cells, seemingly due to mitochondrial overproduction. In these cells, high levels of intracellular glucose lead to more and more glucose being metabolized and oxidized by means of the tricarboxylic acid cycle (TCA) [67,68]. This TCA "over-drive" will increase the flux of electrons originating from NADH and succinate into the mitochondrial electron transport chain, leading to the accumulation of ubiquinol, the reduced form of ubiquinone, generating ROS, in particular superoxide anion. β-cells are cells found in the pancreas and are responsible for synthesizing and secreting insulin. This dysfunction leads to glucotoxicity and lipotoxicity. To make matters worse, β-cells have naturally low expression of antioxidant enzymes, making ROS-induced damage more destructive [69,70].

CVD is a very common disease among obese patients. The risk of CVD increases when high levels of cholesterol and TG in bloodstream, a condition known as dyslipidemia, occurs. The insulin-resistance state caused by OS increases the production of TG-rich lipoproteins and the clearance of these lipoproteins is impaired. This impairment clearly indicates a strong relation between dyslipidemia and OS. These processes lead to an increase of circulating levels of TG and may result in increased generation of ROS in the endothelium, which will promote a pro-inflammatory environment, thus creating a vicious cycle [71–73].

Cancer has also been shown to have higher incidence in obese patients. Breast, pancreatic, liver, and colorectal cancers are the most common types of cancer that have been found in obese patients. Leptin, adiponectin, and inflammation of the liver, adipose and skeletal muscle tissue have been shown to play a big role in the mechanisms that connect obesity and cancer. In models of obese animals suffering from non-alcoholic steatohepatitis (NASH), the lack of adiponectin increased hepatic tumour formation and OS generation. Furthermore, ROS are also involved in the development of cancer: they lead to increased rates of mutations or increased susceptibility to mutagenic agents, which will lead to DNA modifications and/or damage during the early stages of carcinogenesis. Further, ROS also play a role in tumour proliferation, promoting invasion or metastasis of cancer cells. They facilitate the stabilization of hypoxia-inducible factor 1, a transcription factor of vascular endothelial growth factor, leading to angiogenesis of the tumour [74–76].

2.6. Objectives

The main objective of this study was to investigate the biomedical potential of *Brassica* by-products (BBP) against hepatic steatosis and hepatic oxidative damage observed in obesity. For this context, the HepG2 cell line was the chosen biological model. In the first stage, the protective capability of the BBP, against the pro-oxidant effect of tert-Butyl hydroperoxide (tBHP), was tested. The metabolization of tBHP in hepatocytes occurs in two different ways. On the one hand, during the process of tBHP detoxification, a depletion of the enzyme glutathione peroxidase occurs, [77], reducing the antioxidant potential of the cells. On the other hand, detoxification by cytochrome P450 enzymes creates peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals [78]. These are reactive oxygen species

that are excellent oxidizing agents, capable of removing hydrogen atoms from other molecules [79]. The antioxidant characteristics of *Brassica, as* mentioned in section 2.2, make this experiment a logical step. Although some experiments have been conducted *in vitro* with this plant, they are usually focused on the characterization of intrinsic properties, forgoing experiments involving cells [80,81], or using the plant in bulk with no concern for the different parts of the plant [82].

On a second stage, the ability of BBP to prevent lipid accumulation was also tested. As mentioned in section 2.2, the BBP have, in their composition, compounds that modulate lipid metabolism in hepatocytes suggesting a possible anti-hepatosteatotic effect. This course of action was chosen due to the negative effects of hepatic steatosis on NAFLD (mentioned in section 2.5) and its more severe form, NASH, which causes increased hepatic dysfunction and cirrhosis [83]. There is growing evidence that NAFLD is a considerable risk factor for the development of hepatocellular carcinoma (HCC) [84,85]. Cancer is the second leading cause of death worldwide (pre-Covid-19 era), with liver cancer having the sixth highest incidence amongst all types of cancer and the fourth highest death rate [86]. Because of the risks posed by this illness, and because the risk factor NAFLD is the most frequent chronic liver disorder around the world [87–89], an accessible and affordable way to prevent or mitigate this condition is desirable.

On a final stage, a more detailed look at the eventual effects of BBP will be made by analysing how it affects different parameters of mitochondrial respiration and ATP levels. Cellular respiration occurs in the inner membrane of the mitochondria by means of respiratory chain complexes. These complexes generate an electrochemical gradient across the membrane, with three of them pumping protons (H^+) to the mitochondrial intermembrane space and four of them transporting electrons from one complex to another until a final acceptor, the oxygen. The ATP synthase (also known as mitochondrial complex V) uses this proton-motive force to promote ADP phosphorylation, resulting in ATP production [90]. These complexes are informally known as Complex I-V, but their more scientific names are as follows: NADH:ubiquinone oxidoreductase (Complex I), Succinate:Ubiquinone oxidoreductase (Complex II), Ubiquinol:Cytochrome c oxidoreductase (Complex III), Ferrocytochrome c:Oxygen oxidoreductase (Complex IV) and ATP synthase (Complex V) [91]. Complexes I, III and IV are responsible for pumping electrons out and transporting electrons. Together they make up the electron transport chain. Complex V on the other hand is responsible por ATP synthesis and belongs to the mitochondrial phosphorylative system. The first link in this chain is the more complex and largest of all, complex I, where one molecule of NADH is oxidised and donates 2 electrons that are transferred to a ubiquinone. This process pumps 4 H^+ from the mitochondrial matrix to the intramembrane space of the mitochondria [92,93]. From the largest to the smallest link, complex II does not pump protons, however, it does provide more electrons to ubiquinone complexes (such as the one mentioned above), adding to the quinone pool. The electrons are provided by the oxidation of succinate at a specific binding site of complex II, with the help of co-enzyme FAD [94]. These ubiquinones are then oxidised at complex III, where the electrons are transferred to cytochrome c (two for each ubiquinone), a small hemeprotein that exists in association with the inner membrane of the mitochondria. This process causes 4 more H^+ to be transported to the intramembrane space [95]. In the final complex of the electron transport chain, complex IV, 2 H+ are translocated out of the mitochondrial matrix and

two cytochrome c's give away the electrons they have previously received from complex III (one each) to the final electron acceptor: oxygen. Oxygen is then quickly reduced to water [96,97]. The proton transport made by these complexes creates a deficit of positive charges on the inside of the mitochondrial matrix, allowing the creation of a gradient of protons from the intermembrane space into the mitochondrial matrix. The resulting energy created by this proton-motive force causes conformational changes in ATP synthase that will trigger the binding of ADP and inorganic phosphate. The end product is ATP, which is released after being produced [98].

2.7. Biological model

Human hepatocellular carcinoma HepG2 cells were used as a biological model for human hepatocytes. These cells are more limited in metabolic functions than primary hepatocytes cells, however they offer several advantages when performing *in vitro* studies, such as easier handling, higher availability, longer life span and a more stable phenotype that is not dependent on characteristics of the subject donating the cells [99]. In the case of HepG2, they were derived from liver biopsies from a 15-year old, Caucasian male, with differentiated hepatocellular carcinoma [100,101]. They are nontumorigenic and highly proliferative cells. Since they are the most widely used human hepatocellular carcinoma cell line in the fields of pharmacological and toxicological research, the functional characteristics of these cells have been studied comprehensively [99]. Hepatic functions such as insulin signaling, metabolism of lipoproteins, cholesterol and triglycerides, synthesis of glycogen, bile acid and plasma proteins, transport of lipoproteins and secretion of plasma proteins have been observed in HepG2 [101–104]. However, as mentioned before, these cells are not without their limitations. Some important phase I enzymes (like cytochrome P450 enzymes) are absent or are found in very minute quantities while phase II enzymes (such as glutathione S-transferase (GST)) are generally found in lower levels when compared to hepatocytes [105].

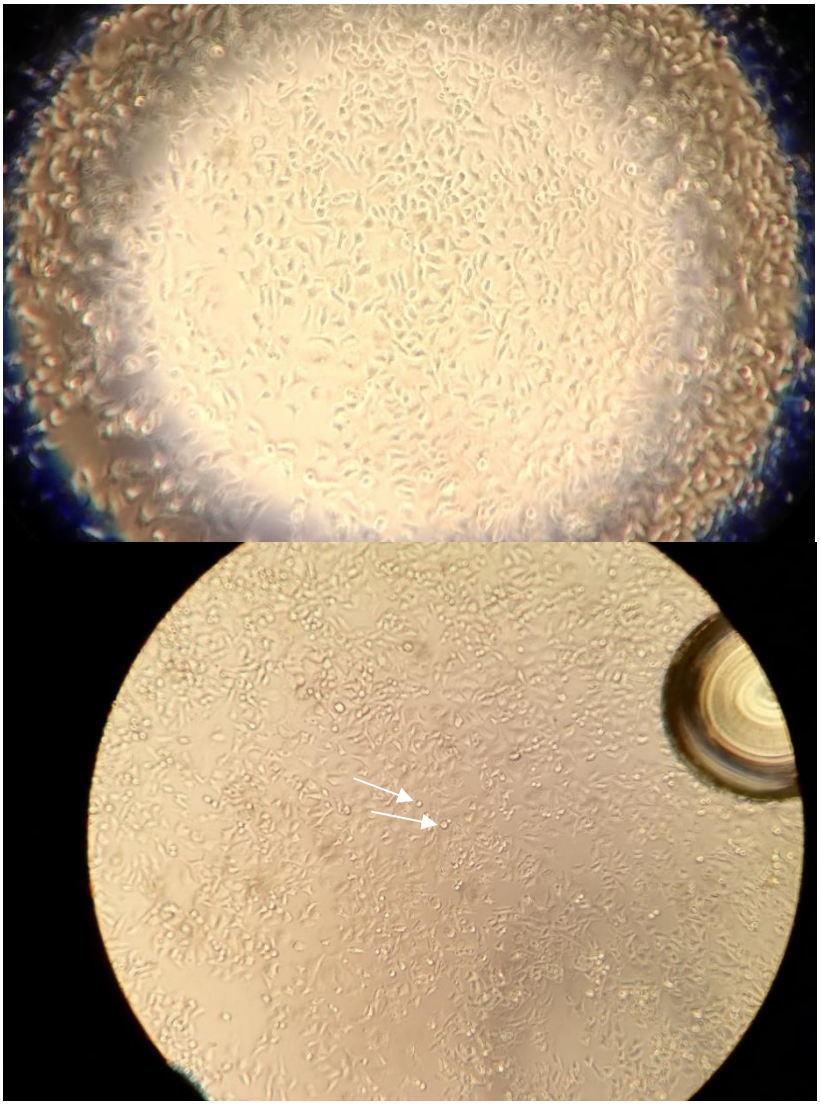


Figure 4 – Microscopic photography of HepG2 (top) and HepG2 co-incubated with 250 µM of free fatty acids (bottom). White arrows point out examples of lipid accumulations in cells. Photographs were taken on a Zeiss microscope, with a magnification of x10,

2.8. Plant extracts

Plant extracts in powder form were provided by our collaborators from the University of Trás-os-Montes and Alto Douro. The powers were suspended in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL and kept at -20°. DMSO is an extremely polar organic liquid widely used as a chemical solvent [106].

Plant powder extracts were obtained from plants collected from the producer Quinta do Celão, Unipessoal Lda, Campo Bolão-Adémia, Coimbra, from a crop field located in Quinta das Abertas, Condeixa-a-Nova, Coimbra, on October 22 of 2018, under fall season conditions. During harvest, entire plants were carefully removed from the soil including their roots and soil, as much as possible, in order to extend the post-harvest lifetime under transportation to the laboratory, and then carefully placed in individual bags. Each plant was cut and divided into inflorescences, leaves and stalks. The roots were discarded. Each part was then weighed and kept at -80°C. The intact plant material was then freeze-dried and grounded to a fine powder. This process is known as lyophilization. Bioactive compounds were then extracted using a methanol / H2O solvent (70:30, v/v) and lastly, antioxidant activity quantification was performed by the TEAC method, by two methodologies: ABTS, DPPH and by the method of iron reduction power by one methodology, FRAP.

Table 1 - Results of the determination of the antioxidant activity of broccoli by-products (mean ± SD) of the harvest of October 2018.

		Plant parts	**ABTS (mmol Trolox g^{-1})**	**DPPH (mmol Trolox g^{-1})**	**FRAP (mmol Trolox g^{-1})**
Sampling 1	Repetition 1	Leaves	0.511 ± 0.050	5.234 ± 0.108	0.093 ± 0.003
		Stalk	0.127 ± 0.001	2.302 ± 0.037	0.040 ± 0.100
		Inflorescence	0.135 ± 0.001	2.299 ± 0.039	0.047 ± 0.016
	Repetition 2	Leaves	0.546 ± 0.006	5.526 ± 0.199	0.092 ± 0.009
		Stalk	0.148 ± 0.001	2.325 ± 0.112	0.038 ± 0.016
		Inflorescence	0.150 ± 0.001	2.208 ± 0.097	0.052 ± 0.015
	Repetition 3	Leaves	0.650 ± 0.009	5.462 ± 0.204	0.095 ± 0.020
		Stalk	0.190 ± 0.002	2.374 ± 0.031	0.034 ± 0.001
		Inflorescence	0.310 ± 0.007	2.571 ± 0.143	0.049 ± 0.006
	Repetition 4	Leaves	0.612 ± 0.013	5.272 ± 0.243	0.088 ± 0.002
		Stalk	0.391 ± 0.030	2.601 ± 0.124	0.042 ± 0.007
		Inflorescence	0.260 ± 0.020	2.558 ± 0.055	0.050 ± 0.001
	Repetition 5	Leaves	0.368 ± 0.026	4.666 ± 0.058	0.095 ± 0.009
		Stalk	0.309 ± 0.023	2.492 ± 0.037	0.038 ± 0.015
		Inflorescence	0.273 ± 0.019	2.572 ± 0.072	0.048 ± 0.017
Sampling 2	Repetition 1	Leaves	0.319 ± 0.034	4.710 ± 0.098	0.089 ± 0.013

		Stalk	0.260 ± 0.027	2.427 ± 0.089	0.040 ± 0.003
		Inflorescence	0.075 ± 0.007	2.191 ± 0.047	0.045 ± 0.017
	Repetition 2	Leaves	0.545 ± 0.034	5.291 ± 0.126	0.090 ± 0.002
		Stalk	0.246 ± 0.017	2.356 ± 0.048	0.039 ± 0.006
		Inflorescence	0.107 ± 0.010	2.268 ± 0.061	0.050 ± 0.007
	Repetition 3	Leaves	0.558 ± 0.037	5.195 ± 0.084	0.093 ± 0.005
		Stalk	0.132 ± 0.012	2.356 ± 0.055	0.035 ± 0.007
		Inflorescence	0.190 ± 0.018	2.350 ± 0.064	0.049 ± 0.001
	Repetition 4	Leaves	0.536 ± 0.045	5.118 ± 0.060	0.092 ± 0.002
		Stalk	0.111 ± 0.013	2.278 ± 0.038	0.039 ± 0.010
		Inflorescence	0.120 ± 0.010	2.415 ± 0.057	0.048 ± 0.011
	Repetition 5	Leaves	0.544 ± 0.068	5.259 ± 0.075	0.092 ± 0.004
		Stalk	0.145 ± 0.054	2.283 ± 0.034	0.041 ± 0.008
		Inflorescence	0.156 ± 0.008	2.307 ± 0.010	0.046 ± 0.002
Sampling 3	Repetition 1	Leaves	0.544 ± 0.024	5.103 ± 0.082	0.0 89 ± 0.005
		Stalk	0.143 ± 0.010	2.349 ± 0.050	0.038 ± 0.013
		Inflorescence	0.252 ± 0.019	2.452 ± 0.037	0.050 ± 0.004
	Repetition 2	Leaves	0.547 ± 0.027	5.324 ± 0.161	0.092 ± 0.002
		Stalk	0.132 ± 0.014	2.304 ± 0.043	0.037 ± 0.001
		Inflorescence	0.252 ± 0.042	2.526 ± 0.188	0.044 ± 0.007
	Repetition 3	Leaves	0.528 ± 0.011	5.201 ± 0.032	0.094 ± 0.004
		Stalk	0.148 ± 0.004	2.257 ± 0.043	0.039 ± 0.002
		Inflorescence	0.241 ± 0.012	2.173 ± 0.002	0.048 ± 0.004
	Repetition 4	Leaves	0.521 ± 0.008	5.305 ± 0.021	0.090 ± 0.010
		Stalk	0.131 ± 0.020	2.267 ± 0.002	0.036 ± 0.002
		Inflorescence	0.221 ± 0.003	2.231 ± 0.010	0.049 ± 0.007

2.9. Previous studies

Some exploratory experiments were performed on HepG2 cells, to test the cytotoxicity of several concentrations of the extracts. Extracts from leaves, stalk and inflorescence were tested at concentrations of 1, 10, 25, 50, 75 and 100 µg/mL. Cytotoxicity was measured using the methods resazurin reduction assay and sulforhodamine B colorimetric assay, described respectively in sections 3.5 and 3.6. From these results,

the concentrations of 1, 10 and 25 µg/mL were selected to be tested in HepG2 due to its low cytotoxicity.

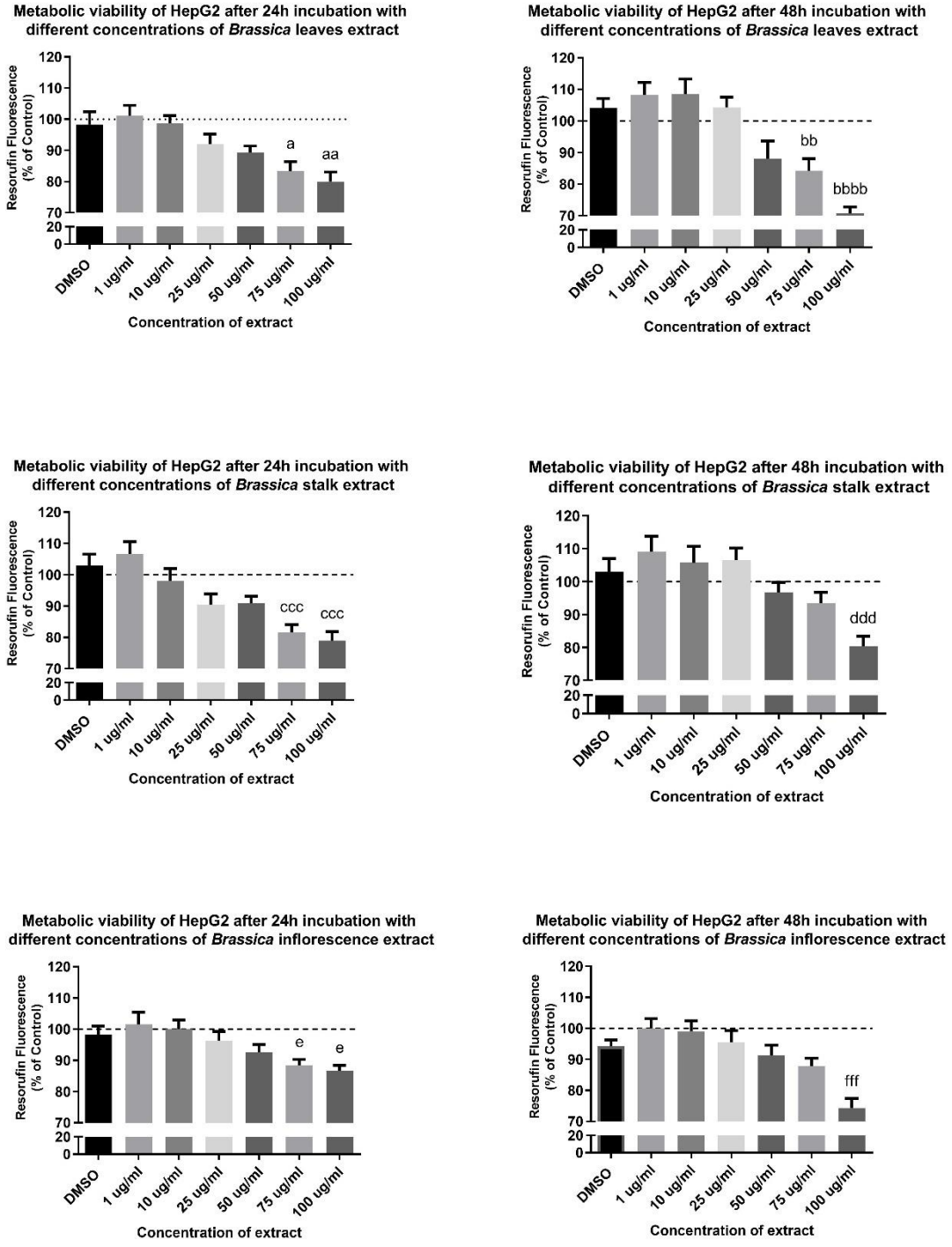


Figure 5 - HepG2 cells were incubated with broccoli leaves, stalks, or inflorescence extracts. The cells were incubated for 24 h or 48 h with the following concentrations of each extract: 1, 10, 25, 50, 75 and 100 µg/ml. The toxicity was evaluated by resazurin reducing assay. Data are expressed as mean ± SEM of three independent experiments of six replicates. $^{aa}p=0.0074$ vs DMSO; $^{a}p=0.0462$ vs DMSO; $^{bbbb}p<0.0001$ vs DMSO; $^{bb}p=0.0043$ vs DMSO; $^{ccc}p\leq0.0006$ vs DMSO; $^{ddd}p=0.0004$ vs DMSO; $^{e}p\leq0.0293$ vs DMSO; $^{fff}p=0.0008$ vs DMSO. These results were kindly provived by Sara Valente and Sara Canário.

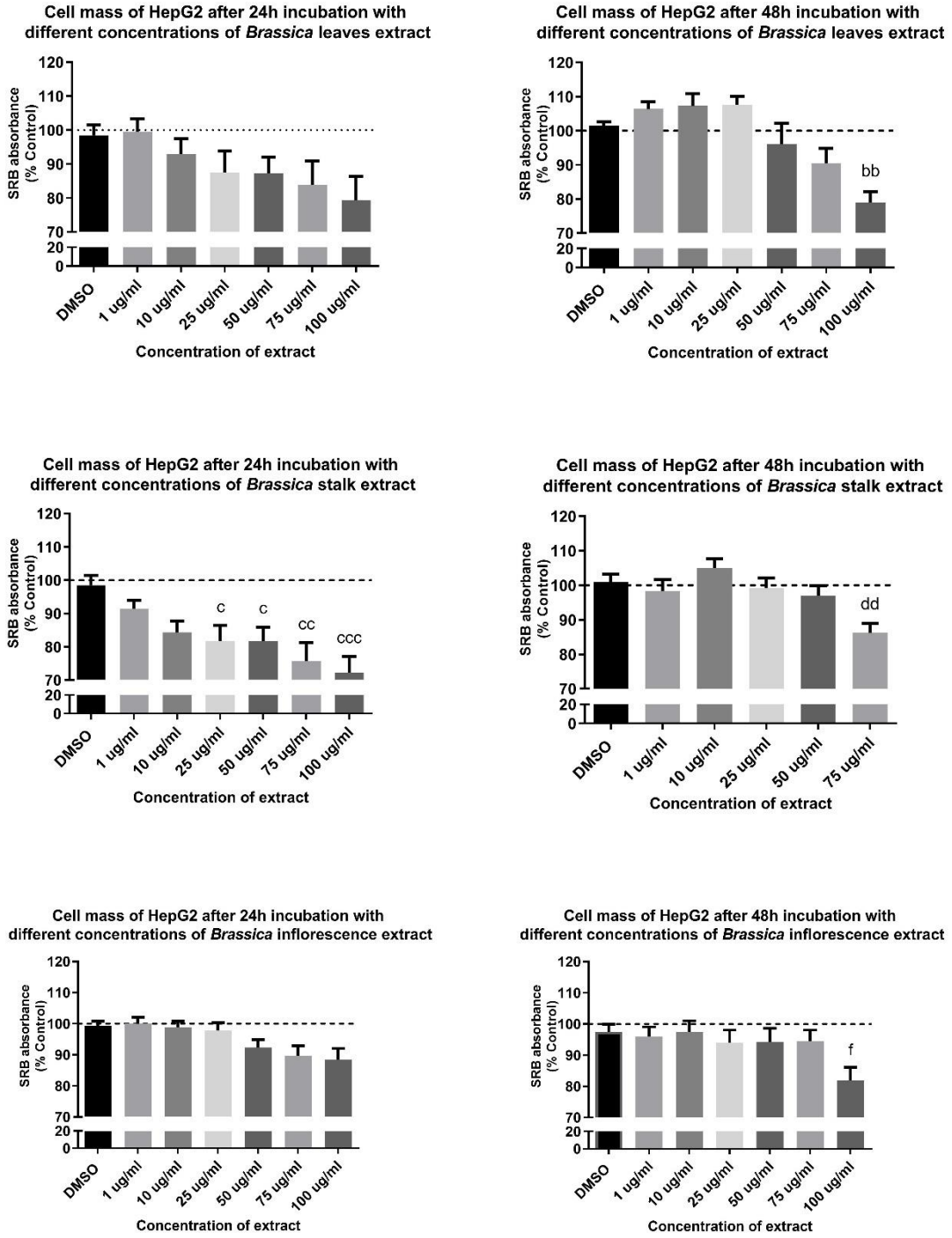


Figure 6 - HepG2 cells were incubated with broccoli leaves, stalks, or inflorescence extracts. The cells were incubated for 24 h or 48 h with the following concentrations of each extract: 1, 10, 25, 50, 75 and 100 µg/ml. The toxicity was evaluated by sulforhodamine B colorimetric assay. Data are expressed as mean ± SEM of three independent experiments of six replicates. $^{bb}p=0.0019$ vs DMSO; $^{c}p\leq0.0272$ vs DMSO; $^{cc}p=0.0026$ vs DMSO; $^{ccc}p=0.0002$ vs DMSO; $^{dd}p=0.0029$ vs DMSO; $^{f}p=0.0487$ vs DMSO. These results were kindly provived by Sara Valente and Sara Canário.

3. Materials and Methods

3.1. Cell culture

Human hepatocellular carcinoma HepG2 cells were cultivated in 96 multi-well plates at a cellular density of 20 000 cells/well in Dulbecco's Modified Eagle Medium (DMEM, Catalogue # D5030-1L, Lot # SLBW1406, Sigma), supplemented with 44 mM of sodium bicarbonate (Catalogue # S6014-1KG, Lot # SLBK2545V, Sigma-Aldrich), 4 mM of HEPES (Catalogue # H4034-1G, Lot # 100M54171V, Sigma), 6 mM of L-Glutamine (Catalogue # G3126-250G, Lot # SLBK4060V and SLBW9892, Sigma), 1 mM of sodium pyruvate (Catalogue # P5280-100G, Lot # SLBB5884V, Sigma) , 5 mM of glucose (Catalogue # G7021-1KG, Lot # SLBG2661V, Sigma) , 1% of penicillin-streptomycin 100x solution (Catalogue # 15240-062, Lot # 2051350, Gibco) and 10% fetal bovine serum (FBS, Catalogue # 10270-106, Lot # 42F9680K and 42G3193K, Gibco) except for the cases where cells were treated with FFA, in which medium was supplemented with 1% FBS rather than 10%. Cells were kept at a temperature of 37°C and a 5% CO_2 atmosphere.

DMEM is a modified version of the Minimal Essential Medium (MEM), an artificial cell culture medium first developed by Harry Eagle in 1959, constituted by glucose, salts, amino acids and vitamins [107].Renato Dulbecco later modified the MEM to have four times more vitamins and amino acids [108]. This low glucose, basic formulation was used to afford the freedom of easily making different medium formulas according to the experimental needs.

Sodium bicarbonate was used added to maintain the pH of the medium, which was set at 7,2. Due to the CO_2 rich atmosphere, the pH of the medium tends to drop and became more acidic ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$). The dissociation of sodium bicarbonate into sodium (Na+) and bicarbonate (HCO_3^-) ions shifts the chemical reaction in the direction of CO2 production [109,110].

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) also acts as buffering agent. Its zwitterionic properties make this molecule an optimal buffer between 6.8 and 8.2 values of pH [111].

Glutamine is an essential amino acid for cell growth and development, but only in its L isomer, as the D isomer is completely inactive [112]. No other amino acid is as abundant in circulation or in intracellular pools, and its unique metabolic functions include serving as a precursor for many important biomolecules such as proteins, nucleotides and even other amino acids [113].

Glucose ($C_6H_{12}O_6$) is an essential and central piece for the metabolism of animals, plants, and several microorganisms. Rich in potential energy and easily stored by cells in great quantities, it can be rapidly mobilized to match any energy demands that arise. There are many pathways that glucose can follow, but the more significant ones are: synthesis of structural polymers, storage as glycogen, starch or sucrose, oxidation through glycolysis and oxidation through the pentose phosphate pathway. During the glycolysis process, one molecule of glucose is oxidized through a series of reactions catalyzed by enzymes and yields a final product of 2 pyruvate molecules. Throughout this process free energy is released in the forms of ATP and NADH. In the less common

pathway of pentose phosphate, $NADP^+$ acts as an electron acceptor yielding NADPH and the final product is pentose ribose 5-phosphate which is used to synthesize RNA, DNA, and some coenzymes [114].

Sodium pyruvate is a salt that dissociates into one sodium ion (Na+) and one conjugate base of pyruvic acid, pyruvate (CH_3COCOO^-). Pyruvate goes through an oxidative decarboxylation in which a carbonyl group is removed in the form of CO_2 and the two remaining carbons go on to become part of newly formed acetyl-CoA, more precisely the acetyl group, under aerobic conditions. Acetyl-CoA feeds the citric acid cycle, a metabolic hub, which occurs in the mitochondria of eukaryotes. In this cycle, various compounds derived from the breakdown of proteins, carbohydrates and fats are oxidized to CO_2, and the energy resulting from these oxidations is temporarily stored in the electron carriers $FADH_2$ and NADH [114].

Penicillin and streptomycin are an antibiotic and an antimycotic respectively and were added to the medium to prevent contaminations.

FBS is extracted from the blood of bovine foetuses, and it's a mixture of factors essential for cells to attach, grow and develop, such as vitamins, growth factors, transport proteins and hormones [115]. It's more widely used than other bovine serums (from new born calves or calves) due to its low level of immunoglobulins [116].

Whenever passage of cells was required, cells were chemically detached using a Trypsin-EDTA (0.05%) solution (Catalogue # 25300-062, Lot # 2091244, Gibco). Trypsin is a pancreatic protease that belongs to the serine protease family. In a physiological environment, trypsin breaks down larger peptides and proteins into smaller peptides, acting as an endopeptidase [117].

3.2. Experimental design

Initially, for each extract, three different concentrations were tested: 1, 10 and 25 µg/mL. However, due to the large amount of conditions generated (26), and the fact that edge effect was playing a role in the final results (which made it necessary to fill the wells in the edges with PBS 1x), the experimental design of the plate became overly complex and impractical; therefore, a choice was made early on to abandon the 25 µg/mL condition. An intermediate dilution of 100 µg/mL was made in cell media out of the 50 mg/mL stock solution of extracts. Further dilutions were made in cell media, from the 100 µg/mL solution, to arrive at the desired concentrations. Extracts were pre-incubated for 0, 24 or 48 hours and co-incubated with an aggressor for 3 or 24 hours, as indicated in Table 2.

Table 2 – Pre and co-incubation periods of the extracts during the experiments with tBHP and FFA.

	Pre-Incubation (hours)	Co-Incubation (hours)
tBHP	0	3
	24	3
	48	3
FFA	0	24
	24	24

3.3. Protection against tBHP

A dose/response experiment was made to determine what concentration of tBHP that was going to be used to test the protective capabilities of the extracts. The choices were between 50, 100, 200, 400 and 800 µM. The concentration of the stock solution was 3,33 M; therefore, a series of dilutions had to be made to obtain the desired concentrations. First, 2,4 µL of tBHP (Cat# B-2633, Lot# 75H0141, Sigma) were added to cell media, performing 10 mL of a 800 µM tBHP solution, then, serial dilutions were performed to get to the remaining concentrations (5000 µL of the more concentrated solution + 5000 µL of media). As will be shown ahead in section 4.1, the chosen concentration was 100 µM, so, when the time came to add tBHP to the wells, 5 µL of 2100 µM tBHP solution was added to the experimental wells, bringing the final volume to 105 µL and tBHP concentration to 100 µM. The pro-oxidant was then left to incubate for 3 hours.

3.4. Free fatty acids accumulation prevention

A mixture of different fatty acids was prepared. Each individual fatty acid was prepared separably from the others, as 10 mM stock solution in a 25 mM potassium hydroxide (KOH, Lot# SZBD1570V, Cat# P5958-250G, Sigma-Aldrich) solution and saponified several minutes at 70 °C until dissolution. After cooling to room temperature, the fatty acid solutions were ready to be used. The fatty acids that were not used on the day were stored at -20 °C, where they could remain for several months for further use.

The fatty acid mixture contained 39 % palmitic acid (C16:0), 5.5 % stearic acid (C18:0), 50 % oleic acid (C18:1), 3.7 % linoleic acid (C18:2) and 1.8 % arachidonic acid (C20:4). This composition was used to approximate as closely as possible lipid accumulation occurring in a high caloric western diet [118]. The final volume of fatty acids mixture prepared was 10 mL. An equal volume of 20 % bovine serum albumin (BSA, fatty acid-free, Lot# SLCB8822, Cat# A6003-25G, Sigma) solution was added to the fatty acid mixture and incubated for 10 minutes at 56 °C and after cooling at room temperature, the fatty acid-BSA solution was sterile filtered. This solution could also be stored for several months at -20 °C. The 20 % BSA solution was prepared by dissolving 4 g of BSA in 20 mL DPBS and heated to 37°C to facilitate solving (temperatures above 40 °C were avoided, as BSA becomes gelatinous and insoluble at those temperatures). pH was adjusted to 7.4. The remaining BSA was used as control.

Three different concentrations of FFA were tested: 250, 1000, and 2000 µM. Since the stock solution of FFA was kept at 5000 µM, dilutions in cell media with 1% FBS were made. The same process for to solution of BSA control. To avoid extracellular interactions, the extracts and the FFA/BSA solutions were prepared apart and added one at a time to the wells of a 96 multi-well plate. First, the media already in the wells (with or without extracts) was aspirated. Then, 50 µL of extract at double the concentration desired (2 or 20 µg/mL) was added, since only half the total volume was being put in the wells. FFA and BSA were next added, also in 50 µL volumes and at double the desired concentrations (500, 2000 or 4000 µM).

3.5. Cell metabolic viability measurement – Resazurin reduction assay

Resazurin is a redox indicator that is also capable of entering cells, as these are permeable to it. The combination of these two characteristics make resazurin a great compound to use to assay the viability of cells in culture. The active metabolites of viable cells, such as NADH, reduce the resazurin, which has a blue tonality, into a pinkish fluorescent product called resorufin. Taking advantage of resorufin fluorescent properties, with the help of a microplate fluorescence reader, it is possible to correlate the intensity of the fluorescence with the amount of viable cells: the bigger the intensity, the higher the amount of viable cells [119]. In this case a Biotek Cytation 3 reader (Biotek Instruments, Winooski, VT, USA) was used. The protocol for this assay was performed as follows: a 1% (v/v) solution of previously prepared stock of 1 mg/mL resazurin was prepared in cell medium, for a final concentration of 10 µg/mL. Cell medium in the wells was aspirated and 100 µL of the recently prepared solution was added to each one. The plate was incubated, wrapped in aluminium foil, at 37°C, in a 5% CO_2 atmosphere, for 2 hours. Plates were then read on Cytation 3 at 540 nm excitation and 590 nm emission.

3.6. Cell mass measurement – Sulforhodamine B (SRB) colorimetric assay

Sulforhodamine B (SRB) is a dye with two sulfonic groups that binds to amino acids residues in cells, after they have been fixed. The dye binds under acidic conditions and dissociates under basic conditions [120]. An assay using SRB has been widely used since its inception in 1990 as method for screening for cytotoxicity *in vitro*, since it's binding is stoichiometric, meaning the amount of dye retrieved is directly proportional to the mass of stained cells [120,121]. The following protocol was used, based on the protocol of Silva, F.S.G. *et all* [122]. After treatments, cells were washed with PBS 1x and fixed in the wells of the plates using 80 µL of a solution of methanol, 1% acetic acid, and left overnight at -20°. The next day, the methanol solution was aspirated, and the wells were washed with distilled water. The plate was then left to completely dry at 37°C. For standard plates, 70 µL of 0,05% SRB in 1% acetic acid solution were added to each well and left to incubate for 30 minutes at room temperature. For seahorse specific plates (see section 3.7) 20 µL were added instead of 70. The SRB solution was then aspirated and the well were washed three times with a 1% acetic acid solution to remove the excess dye. The first of the washes was also aspirated instead of just discarded like the following two. This was done to avoid any SRB left in the walls of the wells. The plate was, once again, left to completely dry at 37°C. Afterwards, a solution of 10 mM Tris-NaOH, pH 10,5, was added to the wells: 200 µL for standard plates and for seahorse specific plates as well. The SRB was then dissolved with the help of a multichannel pipette (up and down) and 100 µL of the SRB-Tris solution of each well were transferred to a new plate. Absorbance was then read in this new plate on Cytation 3 at 510 nm and background measurements were made at 620 nm, in room temperature. This plate was discarded, while the original plate was washed with distilled water and stored, in case further SRB assays were necessary.

3.7. Cellular lipid accumulation measurement – Nile red staining assay

Nile red is a hydrophobic coloring agent of intense fluorescence, under the proper spectral conditions, capable of dyeing cytoplasmatic lipid droplets. As such nile red is not soluble in water, which quenches its fluorescence, however it does dissolve in a wide variety of organic solvents [123]. For this assay, a stock solution of 0,5 mg/mL of nile red (Cat# N3013-100MG, Lot# SLBP9326V, Sigma) in acetone (Cat# A/0600/17, Lot#

1687471, Fisher Reagents) was prepared in a dark eppendorf and kept at 4°C. Then, on the day of the assay only, a 1:200 dilution of the stock was made in the cell medium already in use. Since this was an experiment involving FFA media with only 1% FBS was used. Usually medium without serum or albumin is used because there is a possibility that they could draw out nile red from the cells [123], however keeping the cells in a starvation situation with the added stress off the FFA could negatively affect the results so a compromise was made and 1% FBS was used. Cell medium was removed from the wells, 100 µL of diluted nile red was added, the plate was wrapped in aluminum foil and incubated for two hours at 37°C and 5% CO_2 atmosphere. After the nile red solution was removed and the wells were carefully washed with PBS 1x twice. Fluorescence from nile red was then read in Cytation 3 at 520 nm excitation and 620 nm emission.

3.8. Mitochondrial respiration – Metabolic flux analysis

Several parameters of the mitochondrial function were evaluated through the oxygen consumption rate (OCR) of the cells, after exposure to some mitochondrial respiration modulators. OCR was measured using the Seahorse XF^e96 Extracellular flux analyzer (Agilent Scientific Instruments, USA) and the modulators used were oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and a rotenone/antimycin A mixture. Cells were plated as described in section 3.1 in special seahorse plates, coated with collagen to obtain a monolayer of cells, since HepG2 cells tend to clump together. The day before the experiment, a sensor cartridge was placed in a 96 well calibration plate that contained 200 µL of distilled water in each well and left to hydrate at 37°C. On the day of the experiment the water was replaced by 200 µL calibration buffer (Lot# 18318001 and Lot# 25219003, Agilent Technologies). After the exchange, 100 mL of t assay media was prepared, using a stock solution of DMEM (0.83 g/100 mL) supplemented with 5 mM glucose, 1 mM sodium pyrophyte, 6 mM L-glutamine and 4 mM HEPES. pH was set at 7,4. After heating the mitostress media to a temperature of 37°C, the seahorse plate with the cells was retrieved from the incubator, the culture media in the wells was removed and cells were rinsed with 175 µL of mitostress test assay media three times. On the third wash the assay media was left in the wells, and the plate was placed in the incubator at 37ºC, in absence of CO_2 enriched atmosphere, for approximately one hour. The seahorse cartridge was loaded 25 µL of each mitochondrial respiration modulators (one per each port). The loaded modulators are sequentially injected in the wells of the seahorse plate while the assay is running. The first to be loaded was oligomycin, at 16 µM, for a final concentration of 2 µM. Oligomycin is an antibiotic that binds to the F0 subunit of ATP synthase, thus preventing protons from going into the mitochondria, resulting in the shutdown of the proton flux responsible for ATP synthesis during oxidative phosphorylation [124]. The modulator that followed was FCCP, added at a concentration of 4,5 µM, for a final concentration of 0,5 µM. FCCP is an ionophore that transports hydrogen ions across the inner mitochondrial membrane, and dissipates the proton gradient characteristic of the oxidative phosphorylation, uncoupling the electron flow and ATP production [125]. Finally, a mix of rotenone and antimycin A was loaded at a concentration of 10 µM for a final concentration of 1 µM. Rotenone is an inhibitor that acts on the Complex I (NADH CoQ1 reductase) of the respiratory chain, blocking the reduction of ubiquinone and interrupting the flow of the mitochondrial electron transport at this step [126]. Antimycin A on the other hand, inhibits further down the electron transport chain, acting on Complex III, preventing

ubisemiquinone conversion to ubiquinone [127]. The combination of these inhibitors completely stops the electron transportation. After the loading was complete, the cartridge was placed in the flux analyzer and calibrated. Following the conclusion of the calibration, the seahorse plate containing the cells was placed in the flux analyzer. Twelve measurements of three minutes each, were taken of the OCR, in each well, at twelve different times. Every measurement was preceded by three minutes of mixing. The first three measurements were performed without any injection and defined the first of the parameters being evaluated: basal respiration. The OCR observed during basal respiration accounts for the oxygen consumed under baseline conditions to meet the cells energetic demand. The next three measurements were made after the injection of oligomycin. The differential OCR between these two sets of measurements define the oxygen consumption associated with ATP production. The drop in OCR corresponds to the oxygen consumed by the ATP synthase to synthesize ATP, that is no longer being used due to the inhibition of the complex. The second injection was FCCP and the measurements that followed offered a third parameter. The differential OCR between basal respiration and the new values defined the spare respiratory capacity, which indicates how capable the cells are to meet an energy demand. Cells reached their maximum level of OCR at this point. The final injection was the mixture of rotenone and antimycin A, taking OCR values to their lowest. The differential between the highest and lowest values of OCR defined the parameter maximal respiration, in which the respiratory chain is operating at maximum capacity due to the FCCP simulation of a physiological situation where there is a great energy demand. The lowest values of OCR revealed yet another parameter, the non-mitochondrial oxygen consumption rate. This parameter represents the oxygen consumed by other cellular processes other than mitochondrial respiration. Finally, the last parameter measure was given by the differential of OCR values after the oligomycin injection and the lowest values after the rotenone + antimycin injection: the proton leak. This parameter symbolizes a faction of the basal respiration that is not associated to ATP production, and can be an indication of mitochondrial damage. All these parameters are laid out and exemplified in figured 4. The protocol was based of the protocol in the Agilent user guide for mitostress test assay [128]. After the assay, 50 µL of trichloroacetic acid (TCA) were added to each well of the seahorse plate to fixate the cells [129]. The next day SRB assay was performed on the plate.

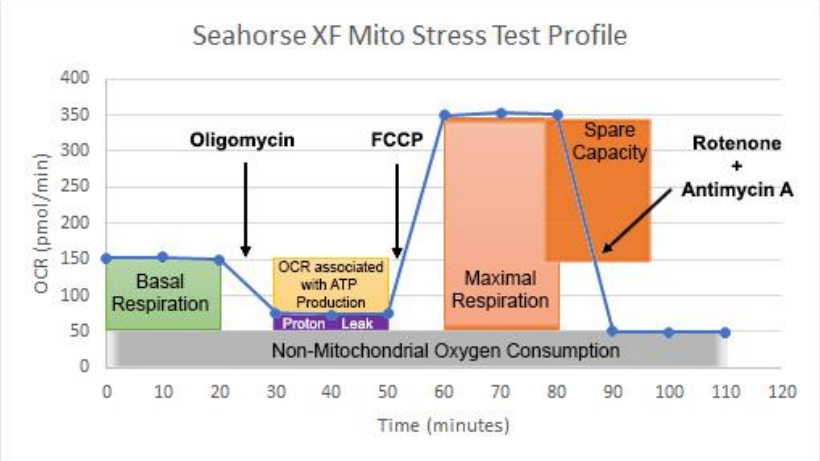


Figure 7 - Seahorse XF Cell Mito Stress profile, highlighting the different parameters measured during the metabolic flux analysis. Based on Agilent user guide [128].

3.9. Intracellular ATP content measurement – Luminescent Cell Viability Assay

This assay was performed not only to quantify ATP, but also to complement the results obtained from the metabolic flux analysis, in which one of the parameters measured was OCR associated with ATP production. The assay was performed using the CellTiter-Glo® Luminescent Cell Viability Assay kit. The kit is divided in two components: the CellTiter-Glo® substrate and the CellTiter-Glo® buffer. Together they make up the CellTiter-Glo® reagent that will be used to determine ATP content. This reagent lysates cells and generates a luminescent signal that will be proportional to the ATP content. This is possible due to the Ultra-Glo™ Recombinant Luciferase, a proprietary thermostable luciferase. The luciferin (also present in the reagent) suffers mono-oxygenation, catalysed by luciferase in presence of magnesium (Mg2+), ATP and molecular oxygen [130].

Cells were plated in an opaque 96 multi-well plate (as described in section 3.1) to prevent luminescence from neighbouring wells from affecting the readings of less luminescent wells. Some wells were left without cells, but with culture media to serve as control, to measure background luminescence. The buffer was thawed and equilibrated to room temperature up to 48 hours before the assay. The 96 multi-well plate was taken from the incubator and left to equilibrate at room temperature for thirty minutes. The flask with the substrate was taken from the freezer and equilibrated to room temperature, then buffer was added to it. After gently mixing the contents of the flask to obtain a homogeneous solution, reagent was added in the same amount of volume as the volume of media in the wells of the plate. The plate was then placed in Cytation 3 and shaken for two minutes. The plate was then left for ten minutes to allow the luminescent signal to stabilize. Finally, luminescence was read.

A standard curve was generated within each plate tested. ATP disodium salt (Lot# 58H7013, Cat# A-2338, Sigma) was used to make the curve. Four serial concentrations were prepared in cell media: 100, 10, 1 and 0,1 µM. For 0 µM, just cell media was used. This was done just before adding the reagent, to prevent endogenous ATPases in the media from consuming ATP. The protocol was based on the Promega technical bulletin for the CellTiter-Glo® Luminescent Cell Viability Assay [130].

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3.10. Statistical analysis

Statistical analysis was performed on the collected data employing the GraphPad Prism 8.4.3 program (GraphPad Software, Inc., La Jolla, CA, USA). Direct comparisons between two independent groups were made using a Student's t-test for data that followed a normal distribution and a Mann-Whitney test for non-normal distribution, multiple comparisons between groups affected by 1 factor (e.g. several concentrations of DMSO) that followed a normal distribution were made using a one-way analysis of variance followed by Tukey's multiple comparison test and multiple comparisons

between groups affected by more than 1 factor (e.g. tBHP and the extract) that followed a normal distribution were made using a two-way analysis of variance, followed by a Dunnett's multiple comparisons test. Multiple comparisons for non-normally distributed data, were performed by using Kruskal-Wallis test. Results were considered statistically significant for $p \leq 0.05$.

Data was expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all independent experiments and presenting all biological replicates. An independent experiment was defined as a 96 MW plate, whose cultivated cells came from a different origin (disk or flask) and underwent a separate cell counting, independent of cell passage. Biological replicates were deemed to be wells within the same 96 MW plate, under the same conditions (e.g. several wells treated with 1 µg/mL of stalk extract and 250 µM of FFA in the same plate). In the presented graphics, each independent experiment was assigned a colour and every symbol of the same colour represented biological replicates. This representation was chosen as a way to better illustrate the variability and the experimental reproducibility of the experiments [131]. The minimum number of independent experiments performed to assume statistical significance was three.

4. Results

4.1. The potential protection of *Brassica* by-products against a model of hepatic oxidative damage, induced by tBHP

For this part of the project metabolic viability and cell mass of HepG2 cells were measured to evaluate the protective capability of the BBP against the pro-oxidant insult induced by of tBHP. HepG2 cells were treated with the extracts and either immediately co-incubated with tBHP for 3 hours or left to incubate for 24 or 48 hours, before tBHP was added (also for 3 hours). The co-incubation was performed to see if the effect of the extracts was on the pro-oxidant itself. If a protective effect is detected, it means the extracts prevent the tBHP from acting rather than triggering a defence mechanism on the cells. The pre-incubations with the extracts were performed to test if any protective effect associated with the extracts is detected, it means the extracts, over time, have triggered a cellular defence mechanism against the pro-oxidant.

The results obtained are develop in detail in sections 4.1.2. – 4.1.4.. For each experimental design, results are presented by the chronological order in which the assays were performed. Resazurin assay was the first, after which cells were fixated and SRB assay was performed, at earliest, the day after. Therefore, resazurin results are presented first followed by the SRB results. A summary of these results can be found in full in Tables 3 and 4, in section 4.1.5.

4.1.1. Dose/Response curve of tBHP

A series of concentrations of tBHP were tested to determine the best concentration to use as a model of oxidative stress damage in the liver. Although the use of tBHP in HepG2 cells to simulate OS in hepatocytes has been widely used, this experiment was

used to compensate for eventual differences in brand and condition of the pro-oxidant. All the concentrations tested decreased cell viability. In the end, 100 µM of tBHP was chosen for further testing.

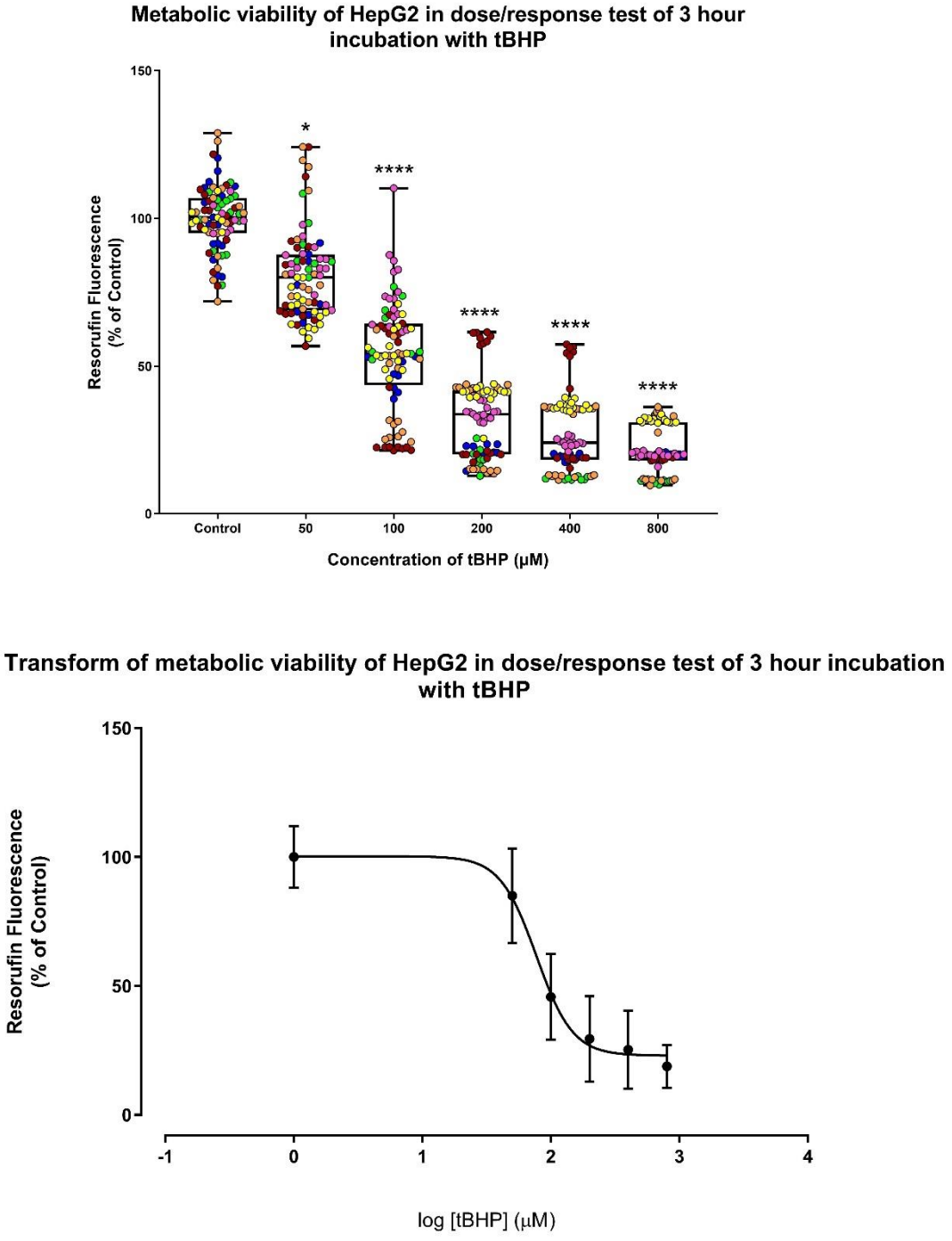


Figure 8 - Metabolic viability of HepG2 cells in dose/response test of 3 hours incubation with tBHP, after an overnight and further 24 hour growth period. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of six independent experiments of either eight or sixteen replicates for control and eight or sixteen replicates for each concentration of tBHP, where every color represents an independent experiment and every same colored symbol represents a biological replicate (top) and as a logarithmic transform (bottom). *p=0.0175 vs Control; ****p<0.0001 vs Control. **Control:** Cell culture media only.

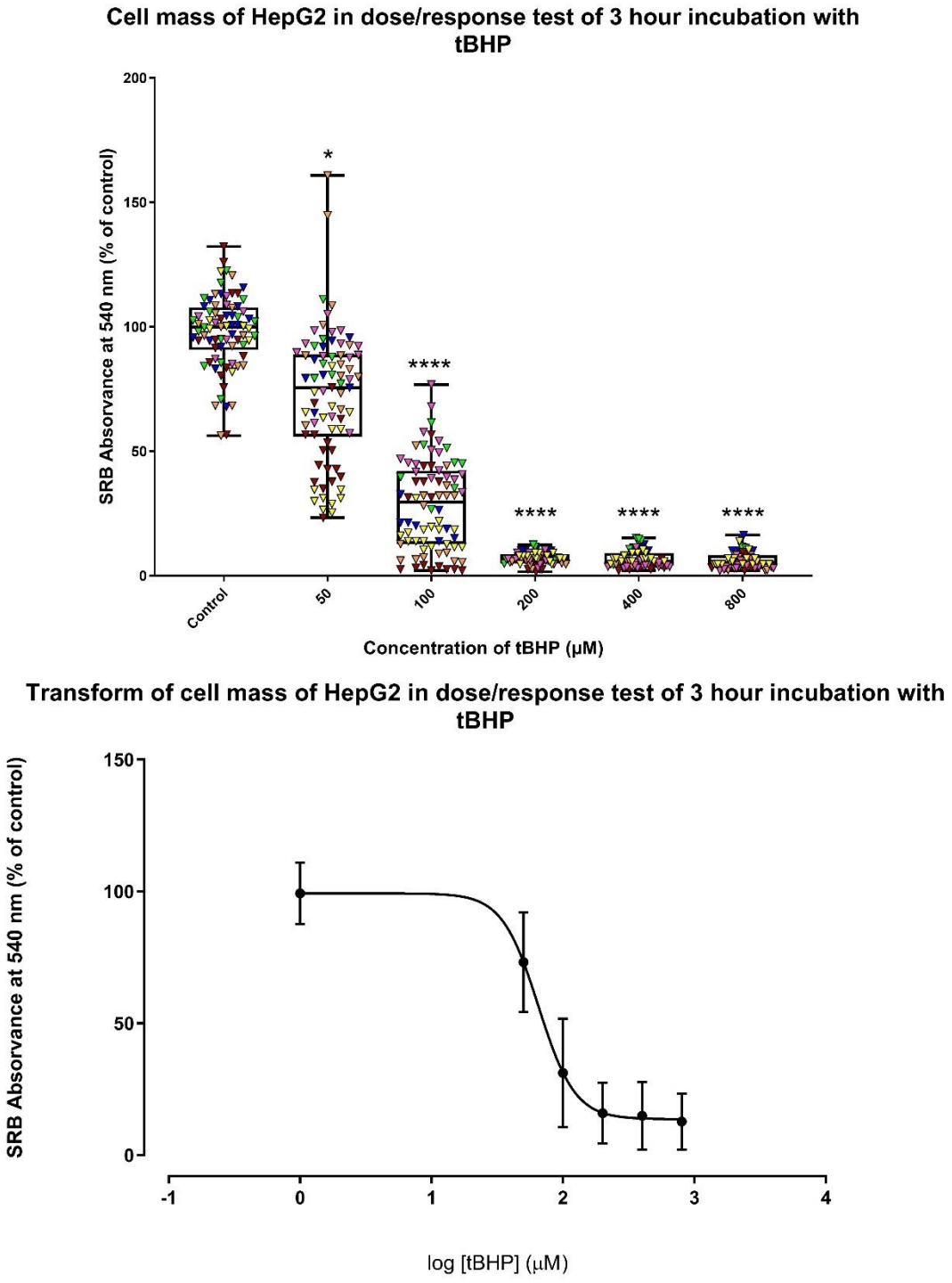


Figure 9 - Cell mass of HepG2 cells in dose/response test of 3 hours incubation with tBHP, after an overnight and further 24 hour growth period. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of six independent experiments of either eight or sixteen replicates for control and eight or sixteen replicates for each concentration of tBHP, where every color represents an independent experiment and every same colored symbol represents a biological replicate (top) and as a logarithmic transform (bottom). *p=0.0122 vs Control; ****p<0.0001 vs Control. **Control:** Cell culture medium only.

4.1.2. The protective effect of *Brassica* by-products extracts co-incubated against oxidative damage induced by tBHP in HepG2 cells for 3 hours

HepG2 cells were co-incubated with extracts and tBHP for 3 hours to test their direct effect on the pro-oxidant.

No statistically significant differences were observed between the control groups with tBHP plus DMSO and the corresponding groups with tBHP plus extracts.

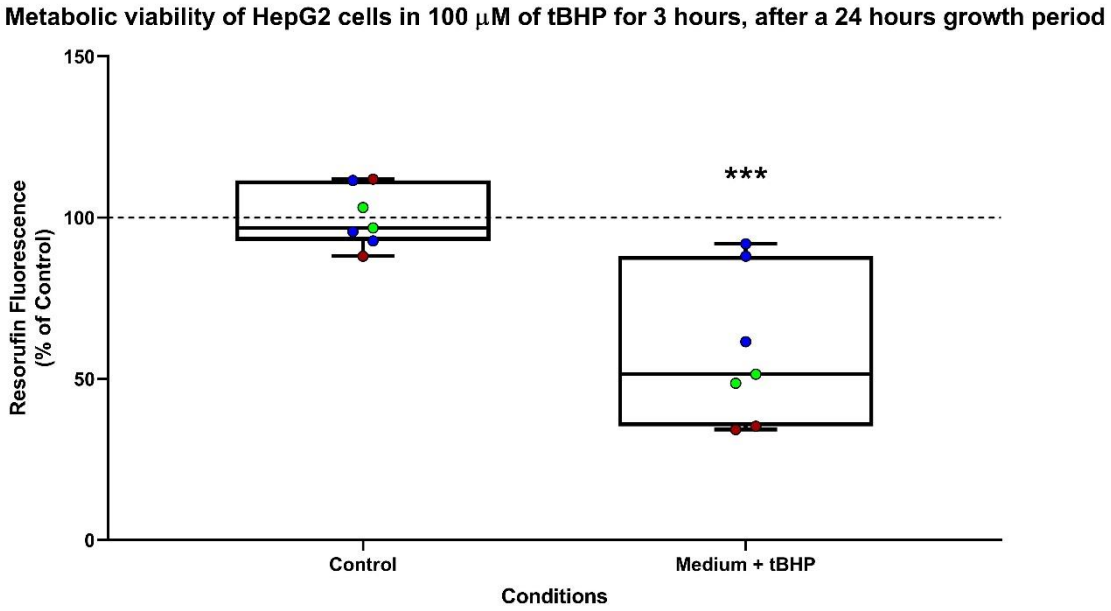


Figure 10 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ***p=0.0009 vs Control. **Control:** Cell culture medium only; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP.

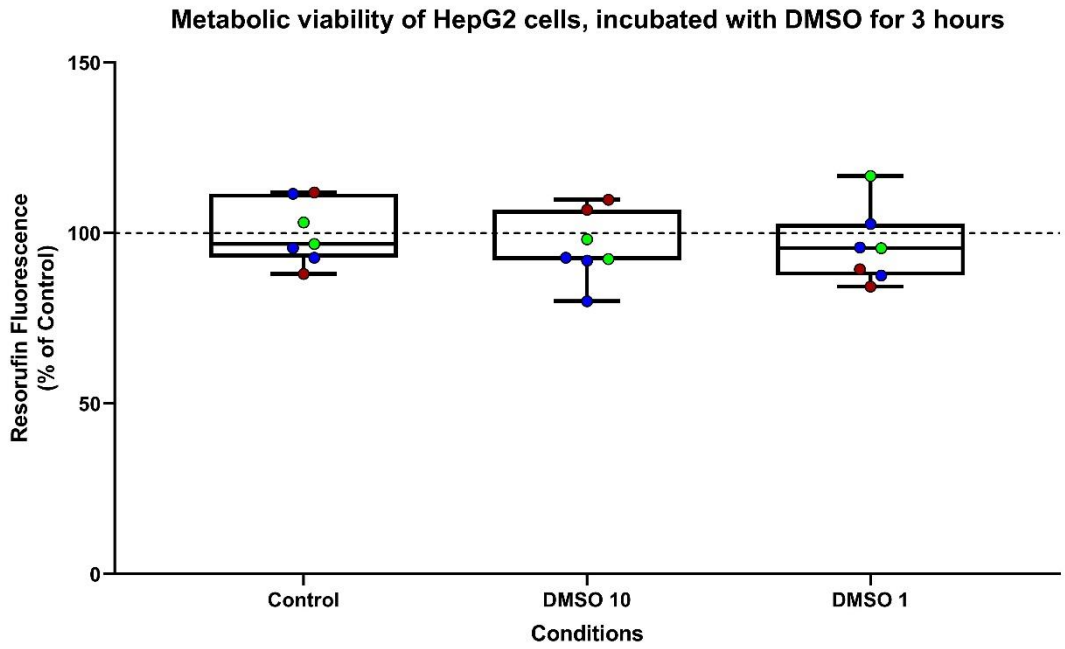


Figure 11 - Metabolic viability of HepG2 cells incubated with DMSO for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **DMSO 1:** Cell culture medium with DMSO (0,002%).

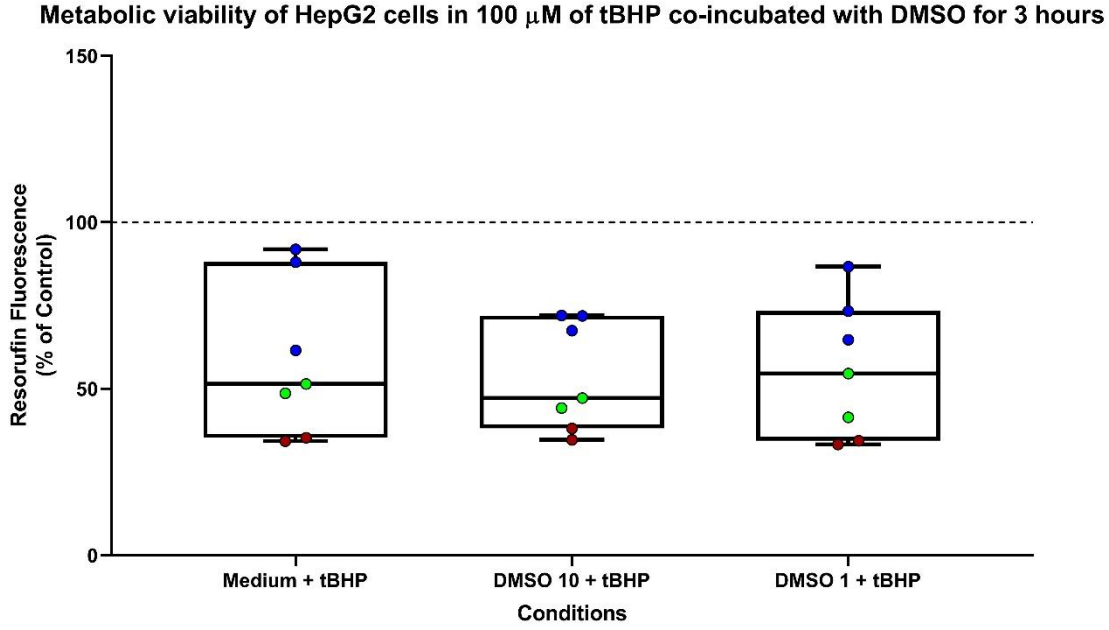


Figure 12 - Metabolic viability of HepG2 cells co-incubated with DMSO and tBHP for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP.

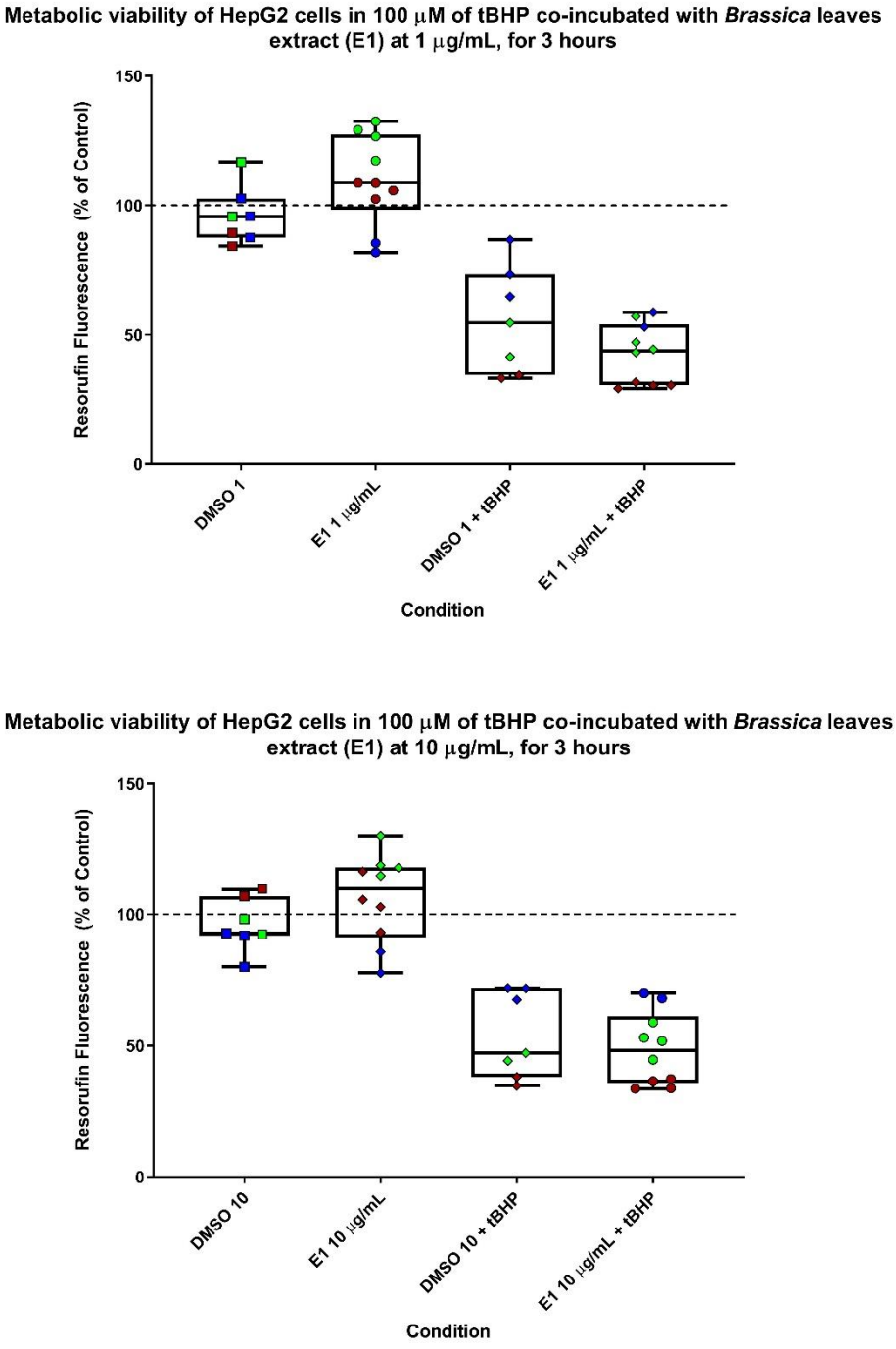
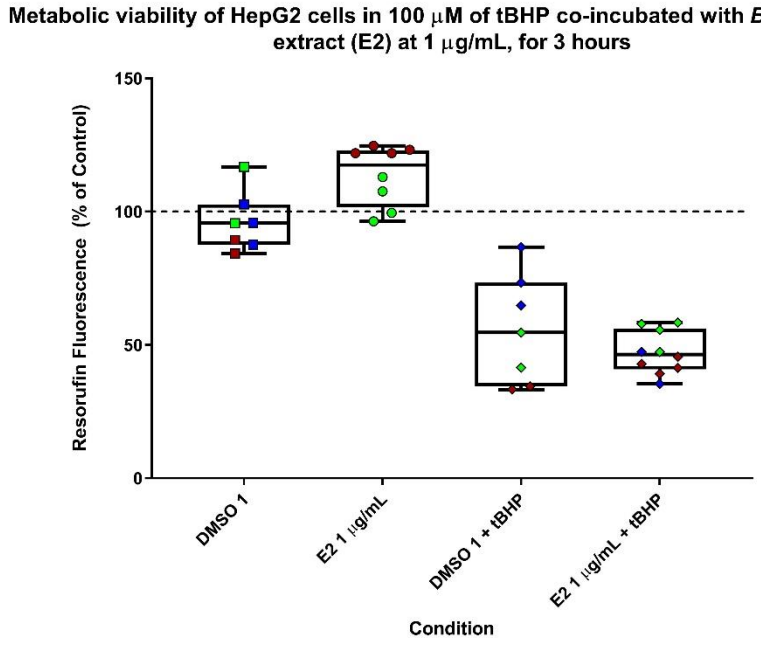


Figure 13 - Metabolic viability of HepG2 cells co-incubated with 100 µM of tBHP and leaves extract (E1) at 1 and 10 µg/mL, for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with1 µg/mL of E1; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP.



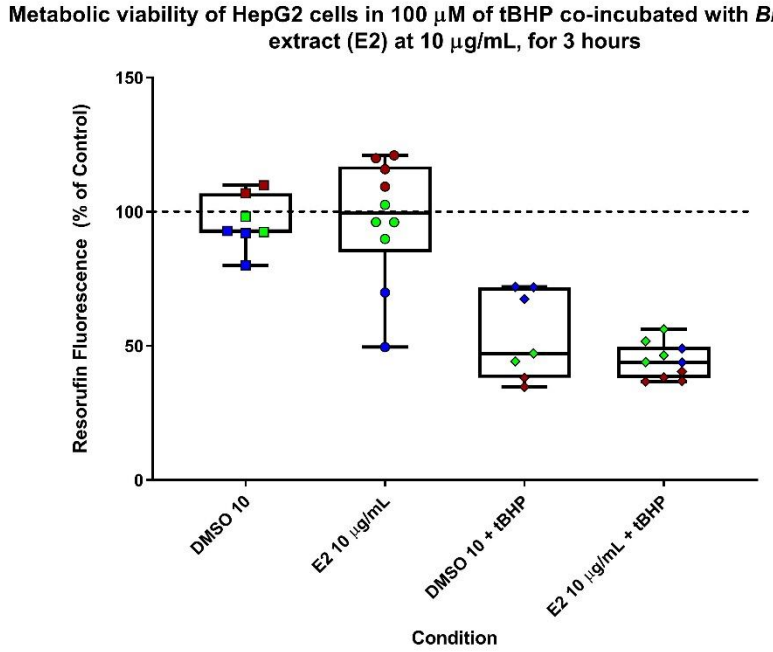


Figure 14 - Metabolic viability of HepG2 cells co-incubated with 100 µM of tBHP and stalk extract (E2) at 1 and 10 µg/mL, for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with1 µg/mL of E2; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP.

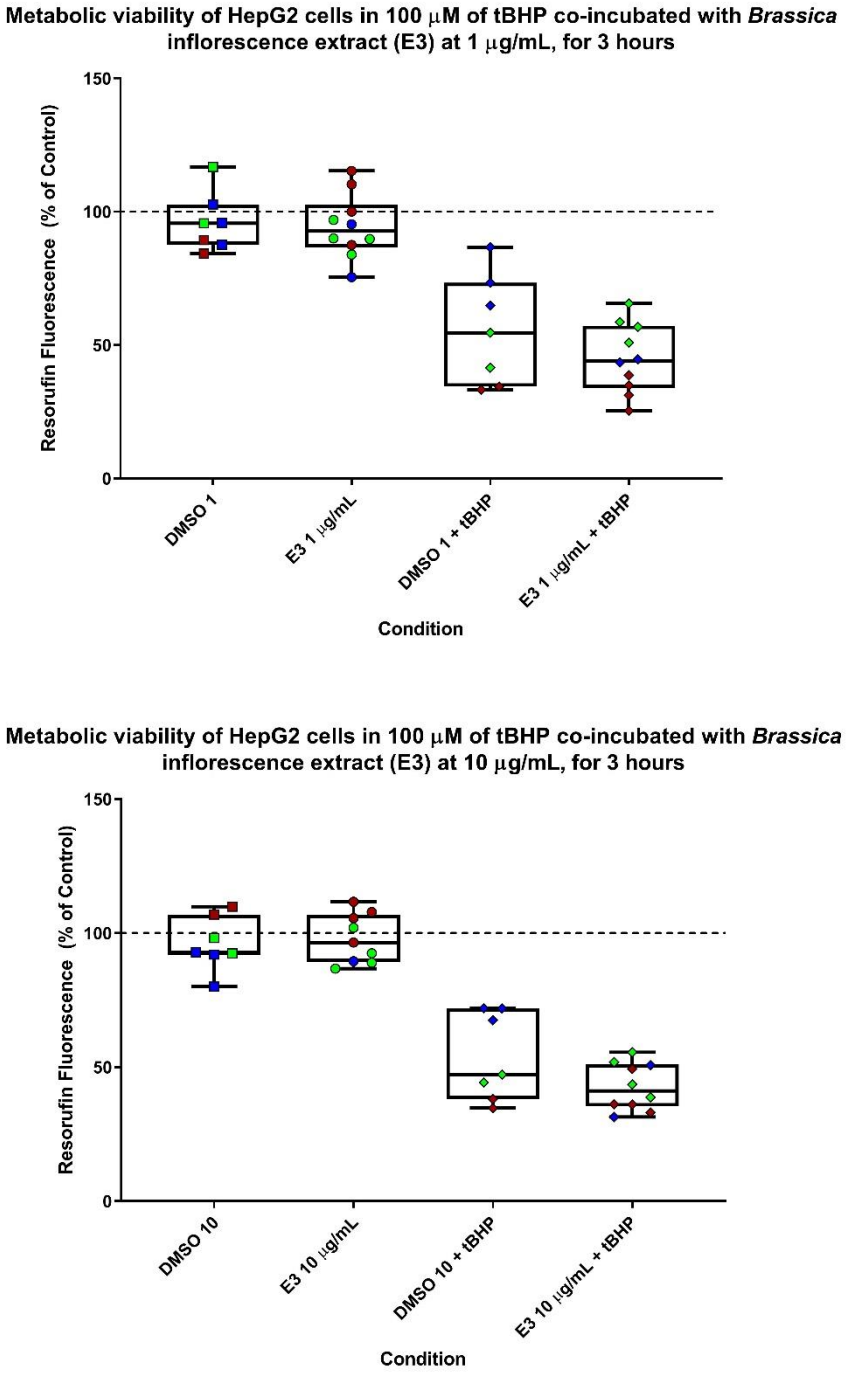


Figure 15 - Metabolic viability of HepG2 cells co-incubated with 100 µM of tBHP and inflorescence extract (E3) at 1 and 10 µg/mL, for 3 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with1 µg/mL of E3; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP.

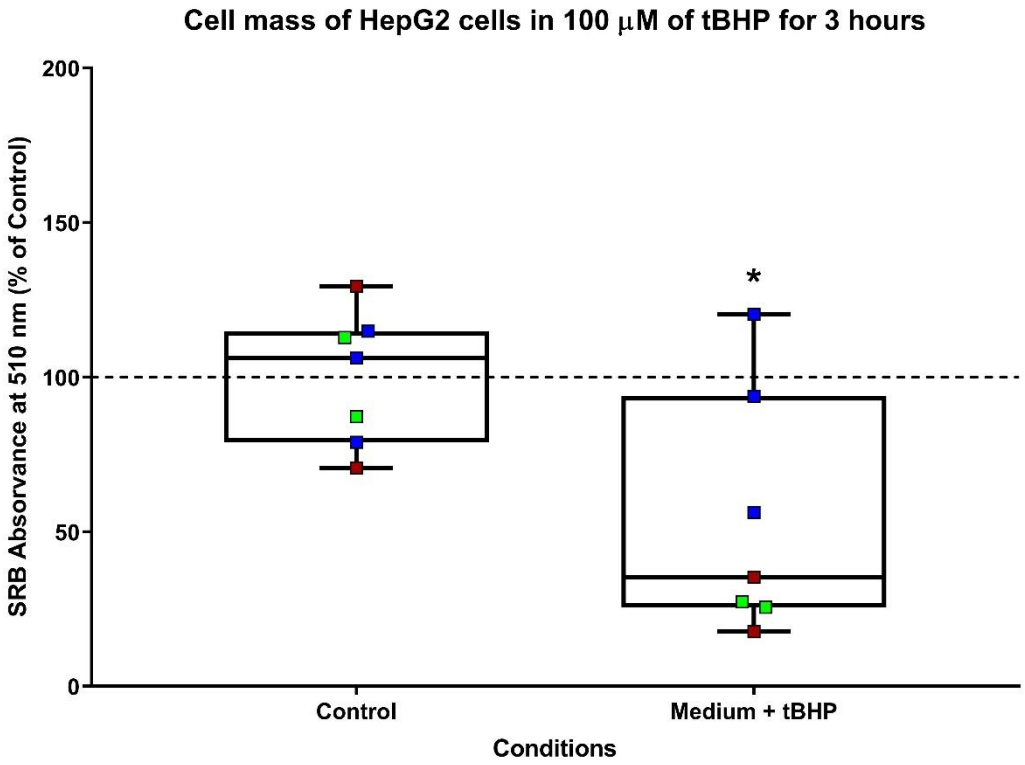


Figure 16 – Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. *p=0.0178 vs Control. **Control:** Cell culture medium only; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP.

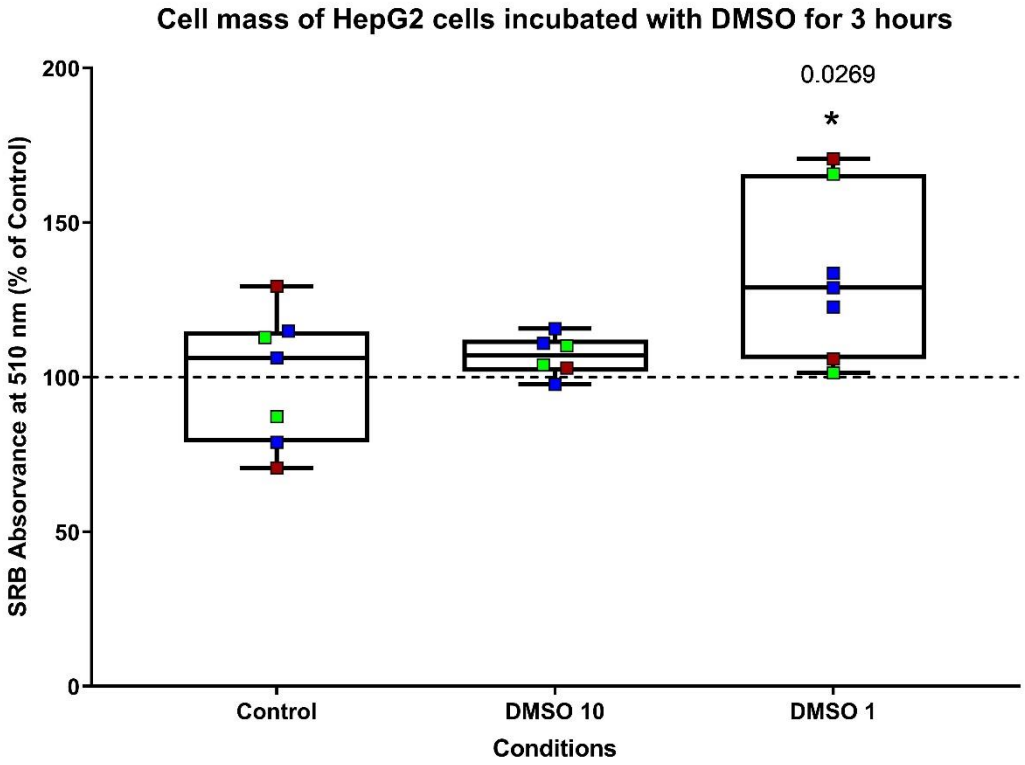


Figure 17 - Cell mass of HepG2 cells incubated with DMSO for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. *p=0.0269 vs Control. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **DMSO 1:** Cell culture medium with DMSO (0,002%).

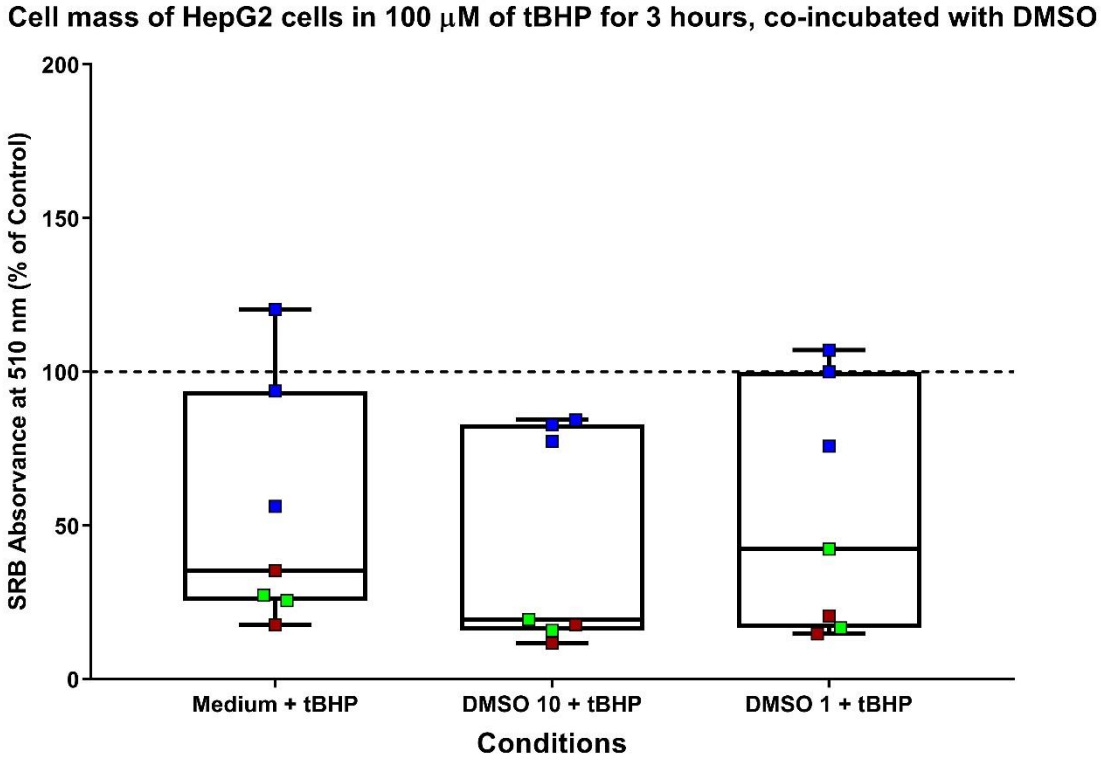


Figure 18 – Cell mass of HepG2 cells co-incubated with DMSO and tBHP for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP.

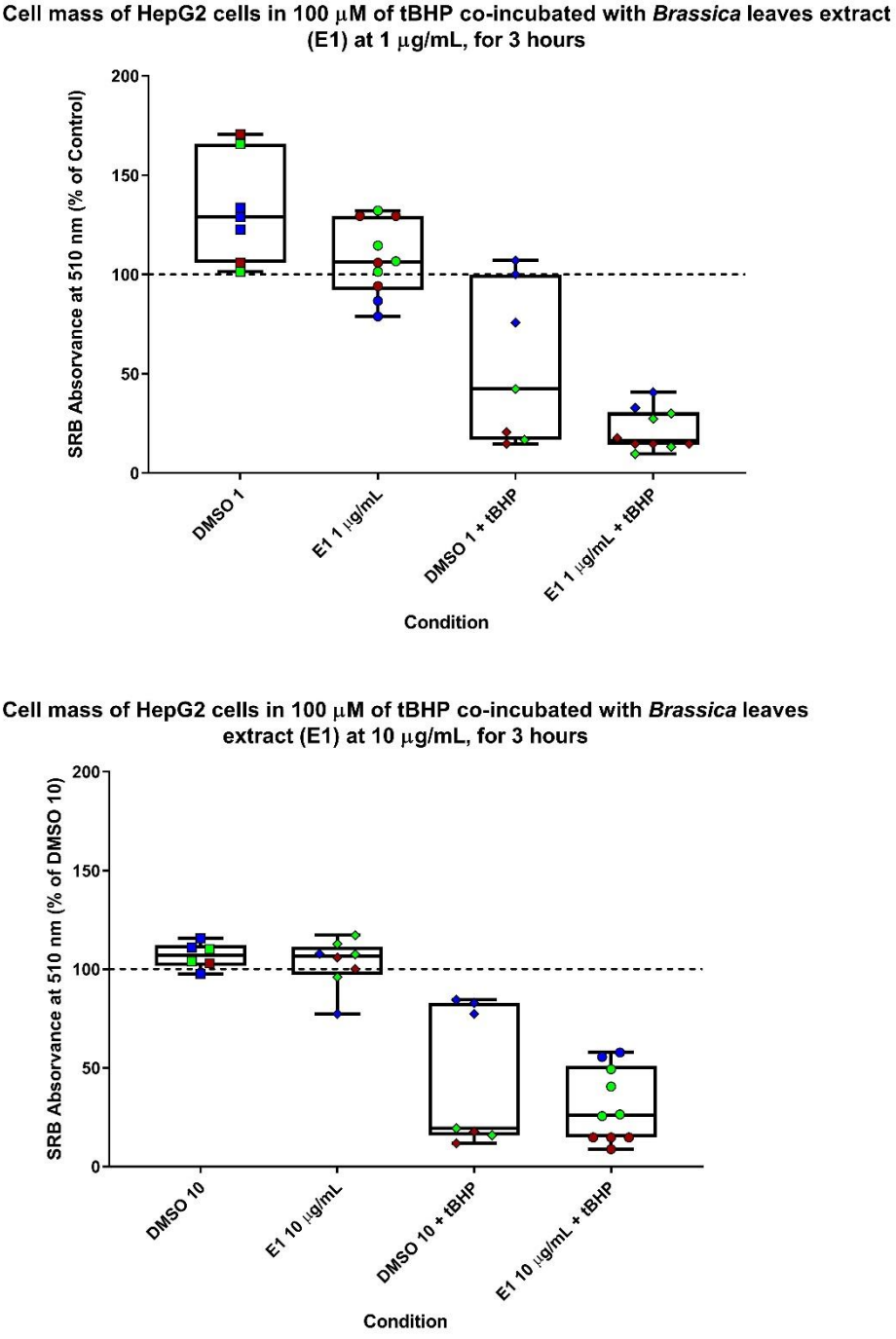


Figure 19 – Cell mass of HepG2 cells co-incubated with 100 µM of tBHP and leaves extract (E1) at 1 and 10 µg/mL, for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with1 µg/mL of E1; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP.

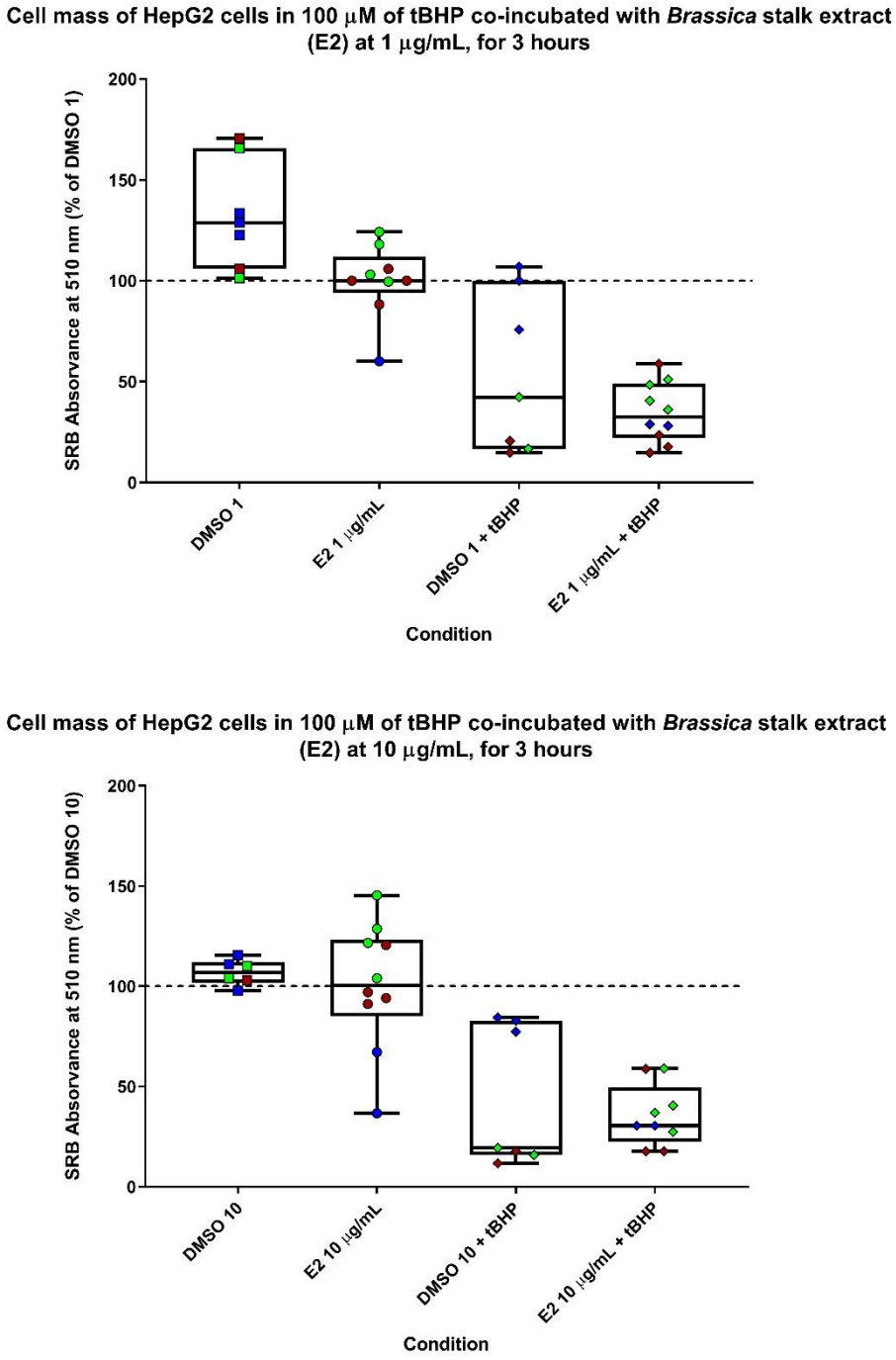


Figure 20 – Cell mass of HepG2 cells co-incubated with 100 µM of tBHP and stalk extract (E2) at 1 and 10 µg/mL, for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with1 µg/mL of E2; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP.

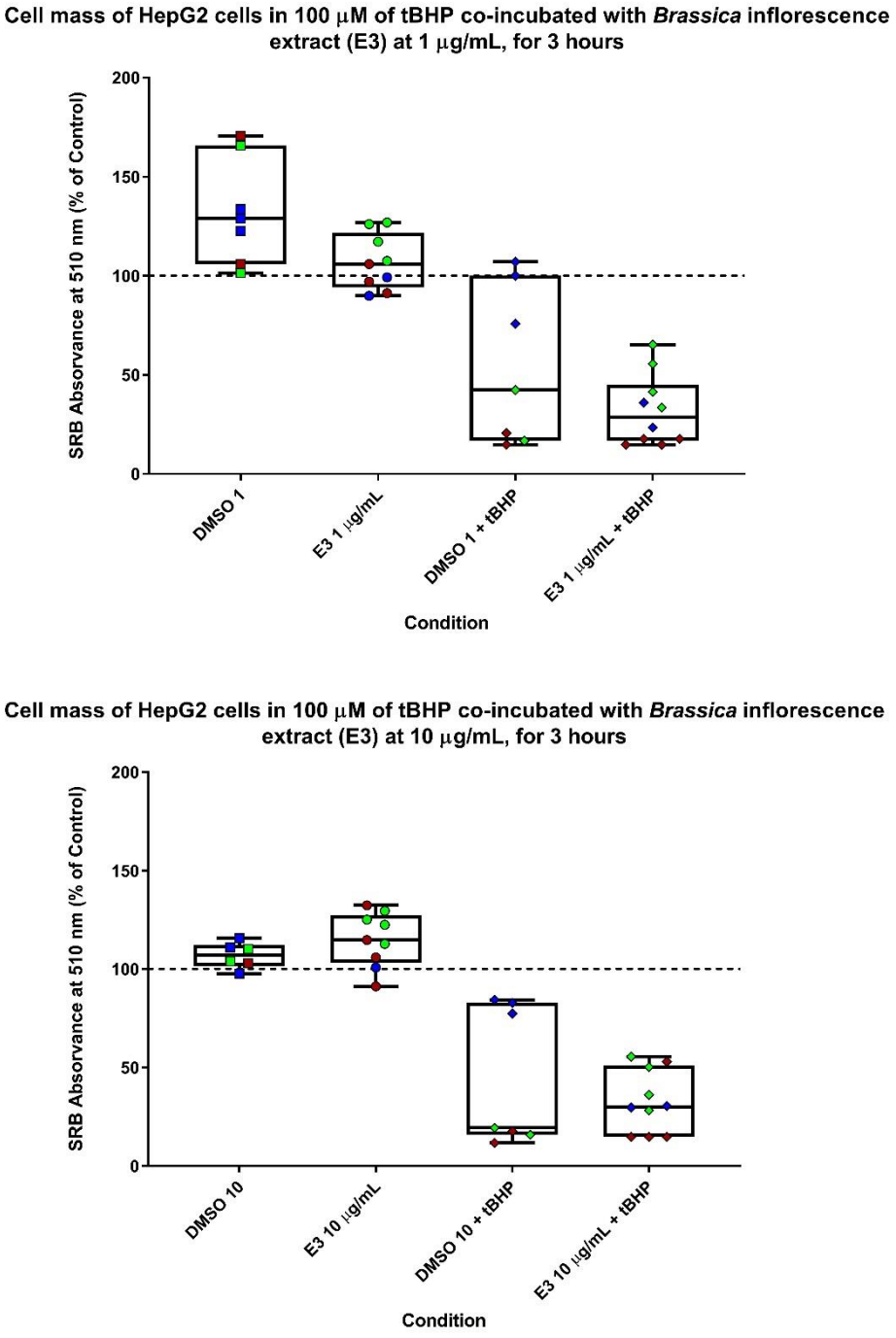


Figure 21 – Cell mass of HepG2 cells co-incubated with 100 µM of tBHP and inflorescence extract (E3) at 1 and 10 µg/mL, for 3 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either two or three replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with1 µg/mL of E3; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP.

4.1.3. The protective effect of *Brassica* by-products extracts against oxidative damage induced by tBHP in HepG2 cells for 3 hours, after a pre-incubation period with the extracts for 24 hours

HepG2 cells were pre-incubated with the extracts for 24 hours and then tBHP was added. This approach was taken to determine if any protective effect detected was due to an indirect antioxidant activity: the triggering of cellular defence mechanisms by the extracts against the pro-oxidant effect of tBHP.

In the presence of tBHP, at the concentration of 1 µg/mL, both leaves and inflorescence extract statistically significantly decreased metabolic viability and cell mass, and 1 µg/mL of stalk extract statistically significantly decreased cell mass when co-incubated with HepG2, compared to the respective control group with DMSO and tBHP. At the concentration of 25 µg/mL leaves extract also led to a statistically significant decrease in cell mass. No further statistically significant differences were found in relation to the extracts.

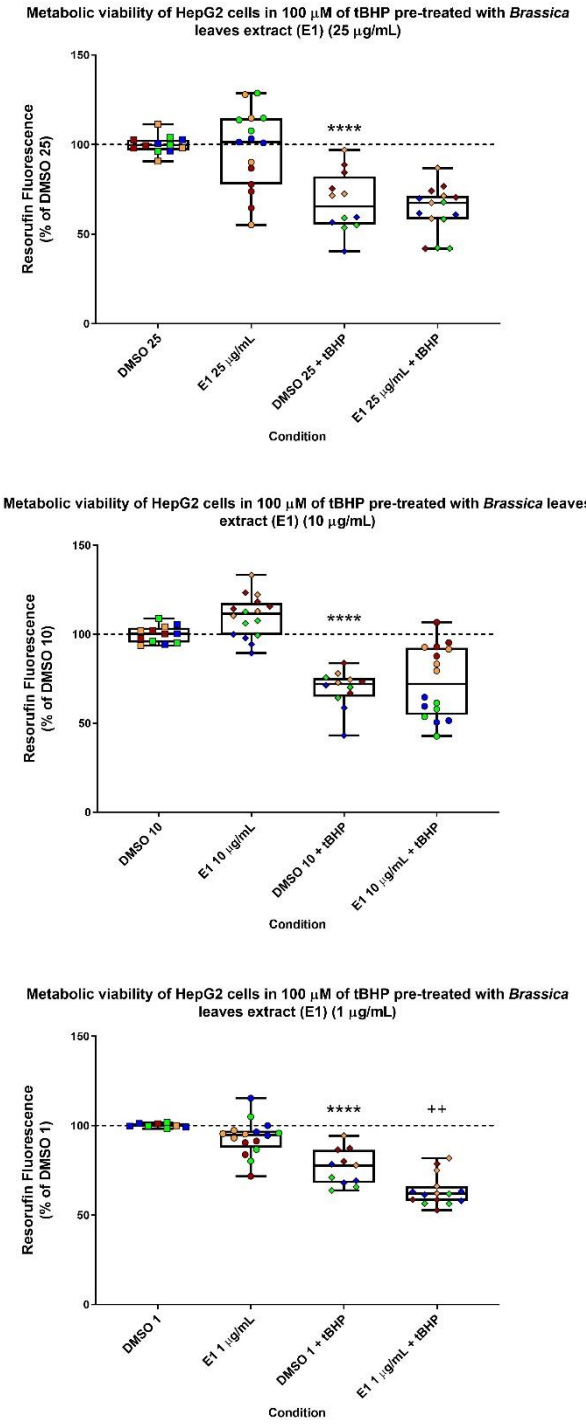


Figure 22 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 1, 10 and 25 µg/mL, for 24 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ****p<0.0001 vs DMSO 25, DMSO 10 and DMSO1; ++p=0.0028 vs DMSO1 +tBHP. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E1 25 µg/mL:** Cell culture medium with 25 µg/mL of E1; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E1 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E1 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with1 µg/mL of E1; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP.

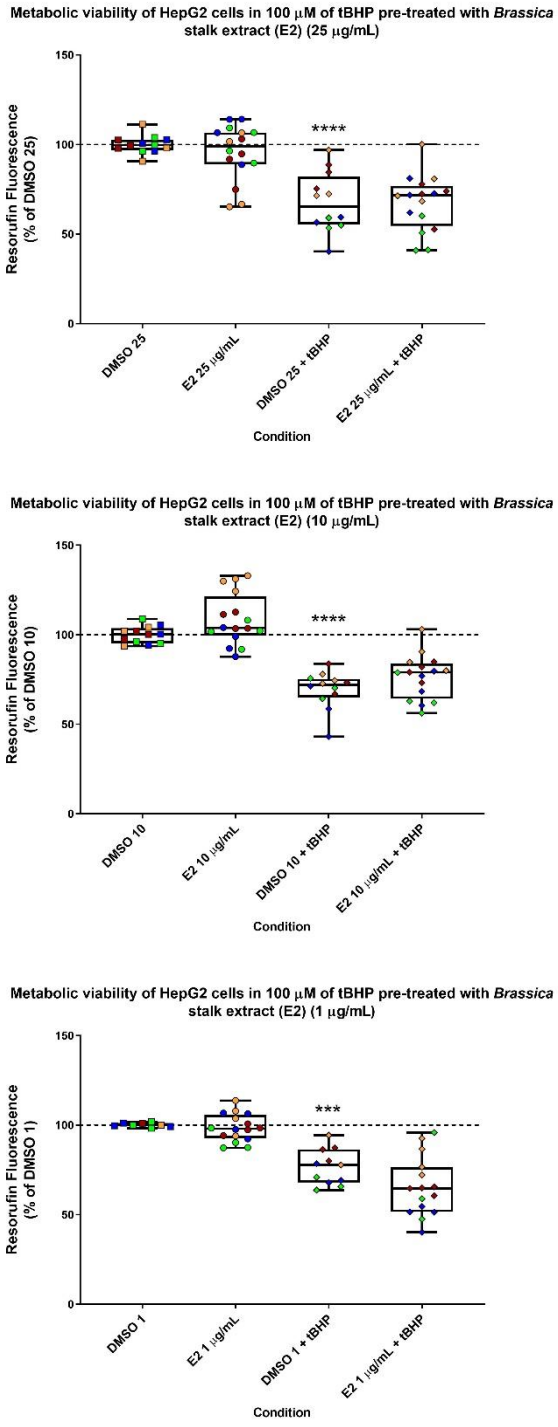


Figure 23 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 1, 10 and 25 µg/mL, for 24 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a mediumn line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ****p<0.0001 vs DMSO 25 and DMSO 10; ***p=0.0002 vs DMSO1. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E2 25 µg/mL:** Cell culture medium with 25 µg/mL of E2; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E2 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E2 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with1 µg/mL of E2; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP.

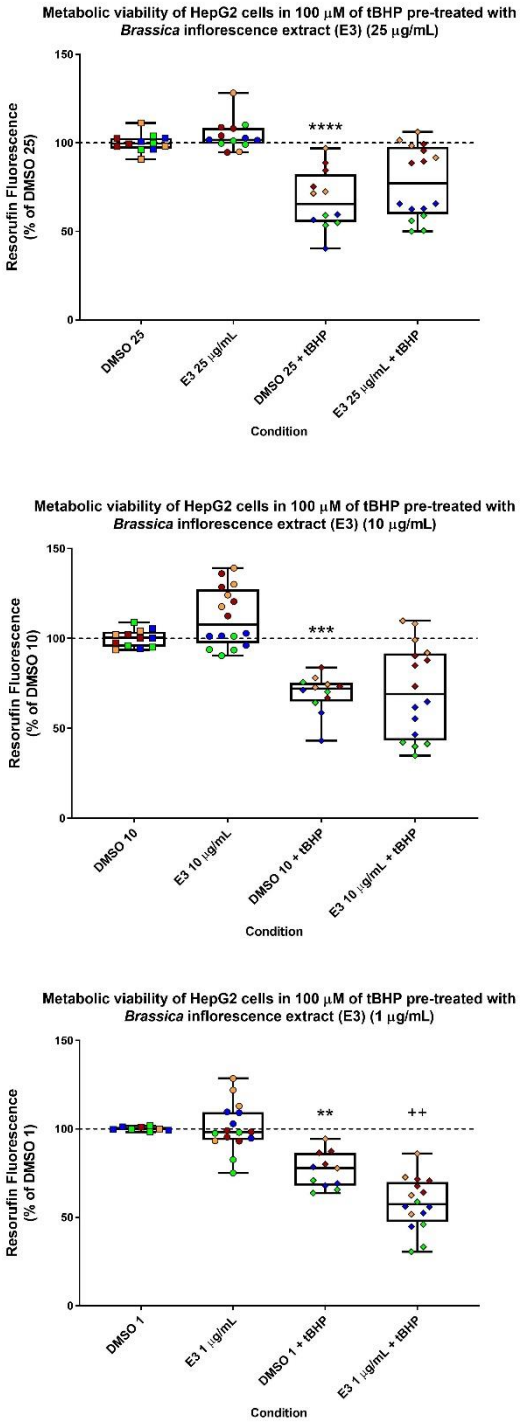


Figure 24 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with inflorescence extract (E3) at 1, 10 and 25 µg/mL, for 24 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ****p<0.0001 vs DMSO 25; ***p=0.0004 vs DMSO 10; **p=0.0007 vs DMSO 1; ++p=0.0015 vs DMSO 1 + tBHP. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E3 25 µg/mL:** Cell culture medium with 25 µg/mL of E3; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E3 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E3 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with1 µg/mL of E3; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP.

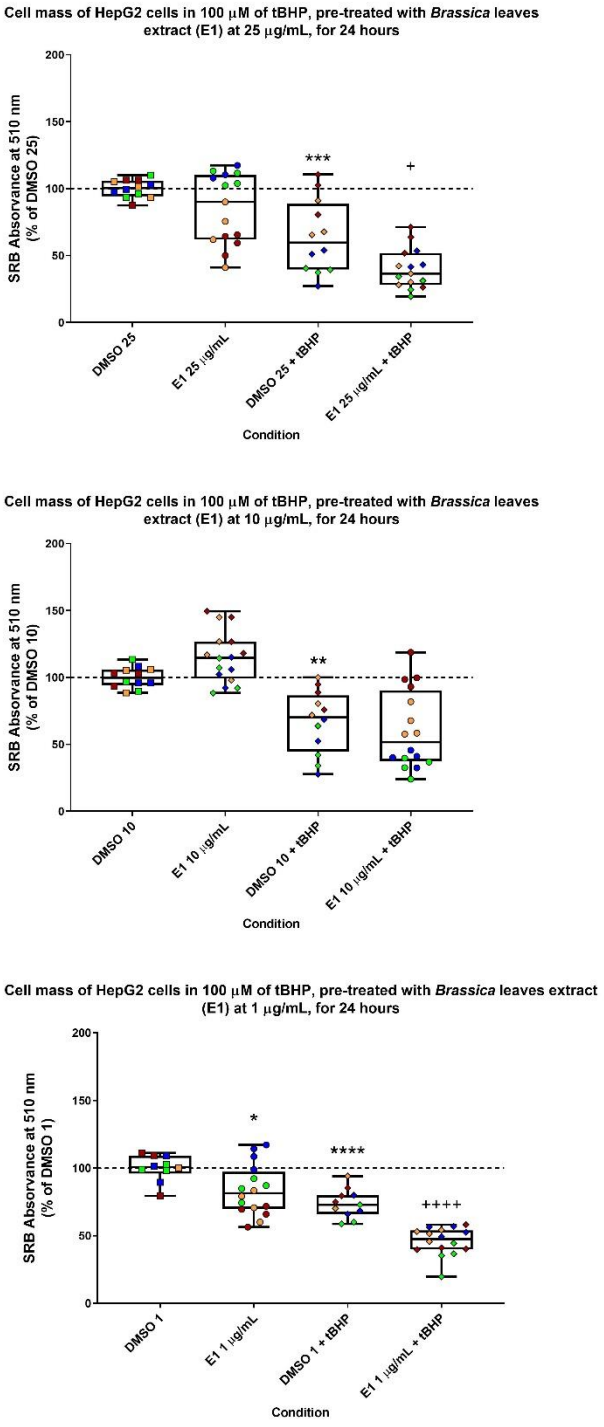


Figure 25 – Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 1, 10 and 25 µg/mL, for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ****p=0.0002 vs DMSO 1; ***p=0.0004 vs DMSO 25; **p=0.0020 vs DMSO 10; *p=0.0179 vs DMSO 1; ++++p<0.0001 vs DMSO 1 + tBHP; +p=0.0205 vs DMSO 25 + tBHP. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E1 25 µg/mL:** Cell culture medium with 25 µg/mL of E1; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E1 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E1 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with1 µg/mL of E1; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP.

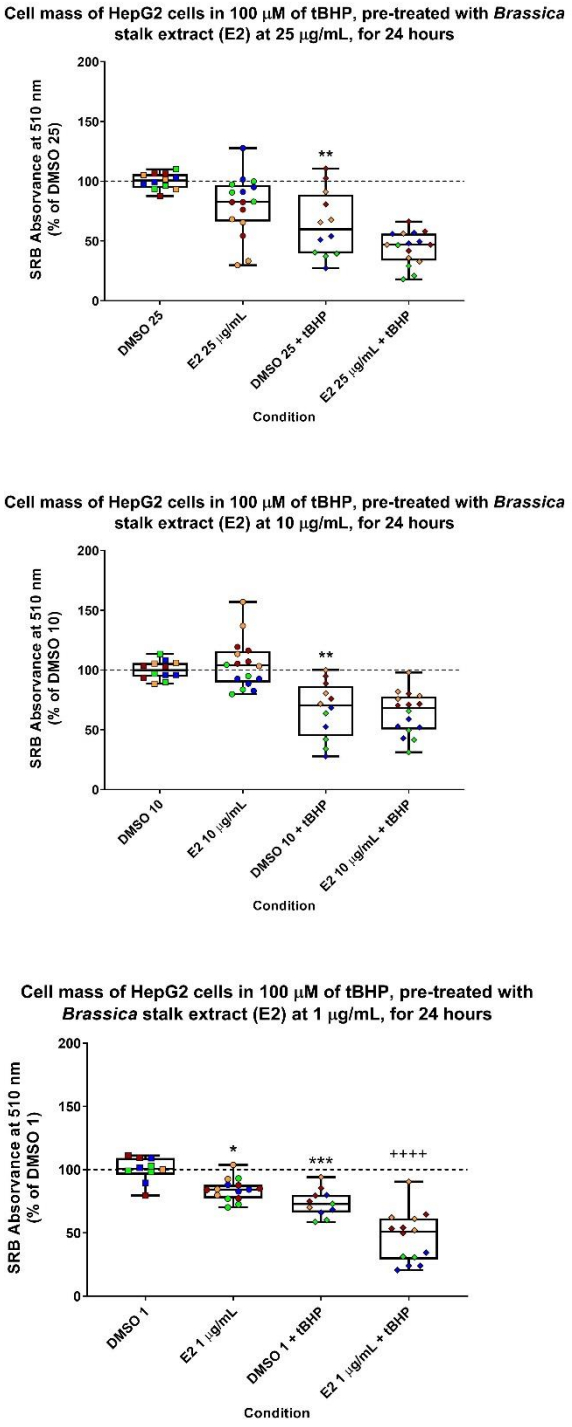


Figure 26 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 1, 10 and 25 µg/mL, for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ***p=0.0002 vs DMSO 1; **p=0.0003 vs DMSO 25 and DMSO 10; *p=0.0274 vs DMSO 1; ++++p<0.0001 vs DMSO 1 + tBHP. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E2 25 µg/mL:** Cell culture medium with 25 µg/mL of E2; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E2 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E2 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with1 µg/mL of E2; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP.

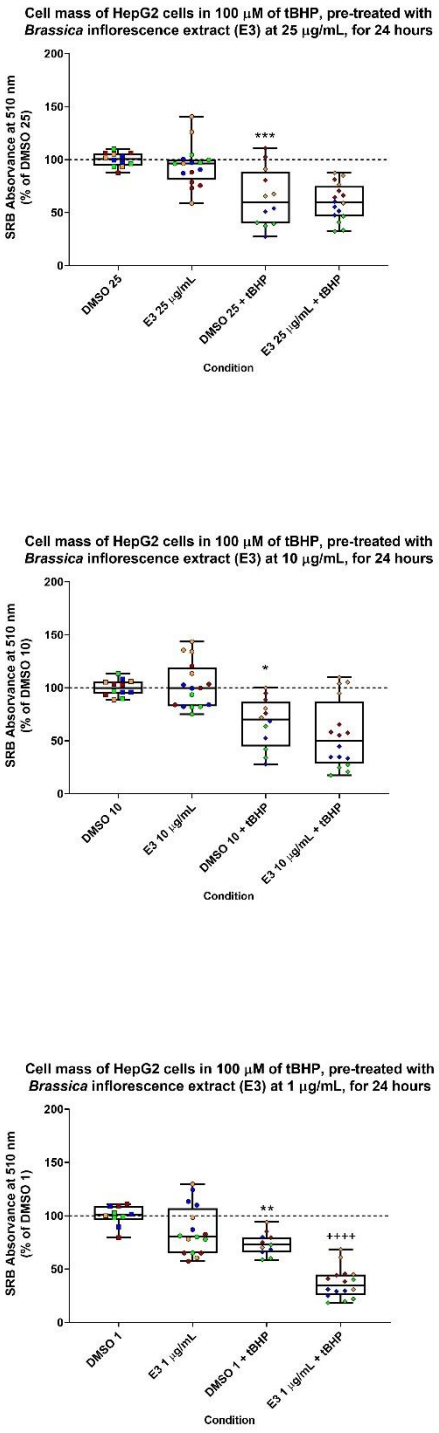


Figure 27 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with inflorescence extract (E3) at 1, 10 and 25 µg/mL, for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of three replicates for DMSO conditions and four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ***p=0.0001 vs DMSO 25; **p=0.0028 vs DMSO 1; *p=0.0274 vs DMSO 1; *p=0.0179 vs DMSO 1; ++++p<0.0001 vs DMSO 1 + tBHP. **DMSO 25:** Cell culture medium with DMSO (0,05%); **E3 25 µg/mL:** Cell culture medium with 25 µg/mL of E3; **DMSO 25 + tBHP:** Cell culture medium with DMSO (0,05%) and 100 µM of tBHP; **E3 25 µg/mL + tBHP:** Cell culture medium with 25 µg/mL of E3 and 100 µM of tBHP. **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP. **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with1 µg/mL of E3; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP.

4.1.4. The protective effect of *Brassica* by-products extracts against oxidative damage induced by tBHP in HepG2 cells for 3 hours, after a pre-incubation period with the extracts for 48 hours

HepG2 cells were pre-incubated with the extracts for 48 hours and then tBHP was added. This approach was taken to determine if any protective effect detected was due to an indirect antioxidant activity: the triggering of cellular defence mechanisms by the extracts against the pro-oxidant effect of tBHP.

Although no statistically significant differences were found between the control groups with tBHP and DMSO and the groups with extracts and tBHP, there were significant differences between the control groups with tBHP but without DMSO and some of the groups with extracts and tBHP: 10 µg/mL of stalk extract decreased metabolic viability loss and 1 and 10 µg/mL of stalk extract and 1 µg/mL of inflorescence extract decreased cell mass loss of HepG2, due to tBHP.

The stalk extract at 1 and 10 µg/mL and the inflorescence extract at 1 µg/mL significantly increased the metabolic viability of HepG2 cells when compared to control groups with and without DMSO. The leaves extract at 10 µg/mL significantly increased the metabolic viability of the cells when compared to control group with DMSO.

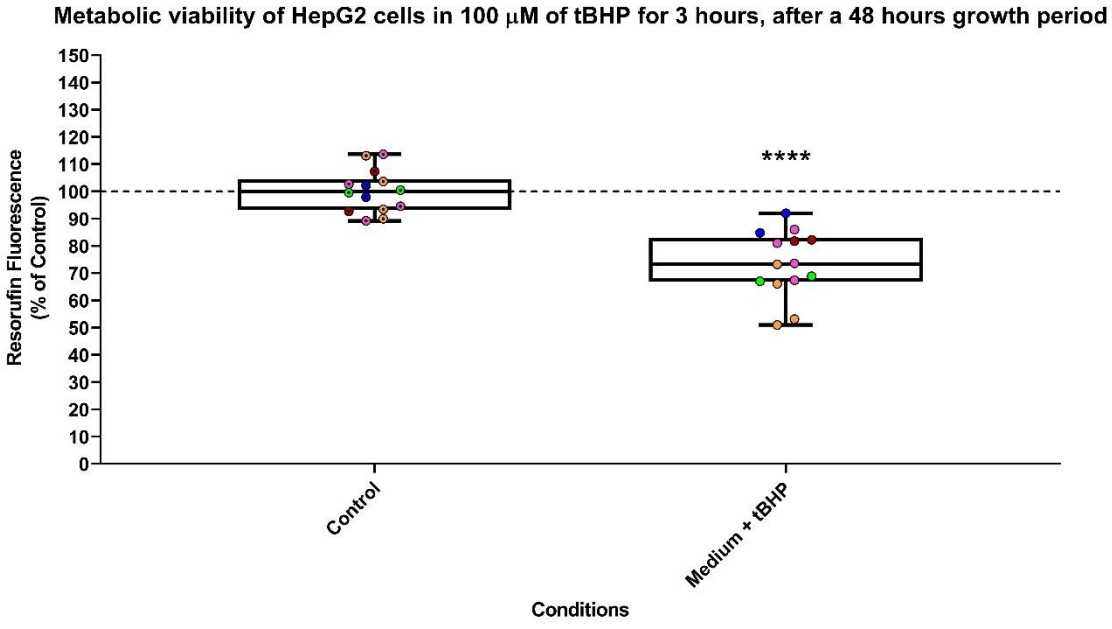


Figure 28 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours, after a 48 hour growth period. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium only; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP.

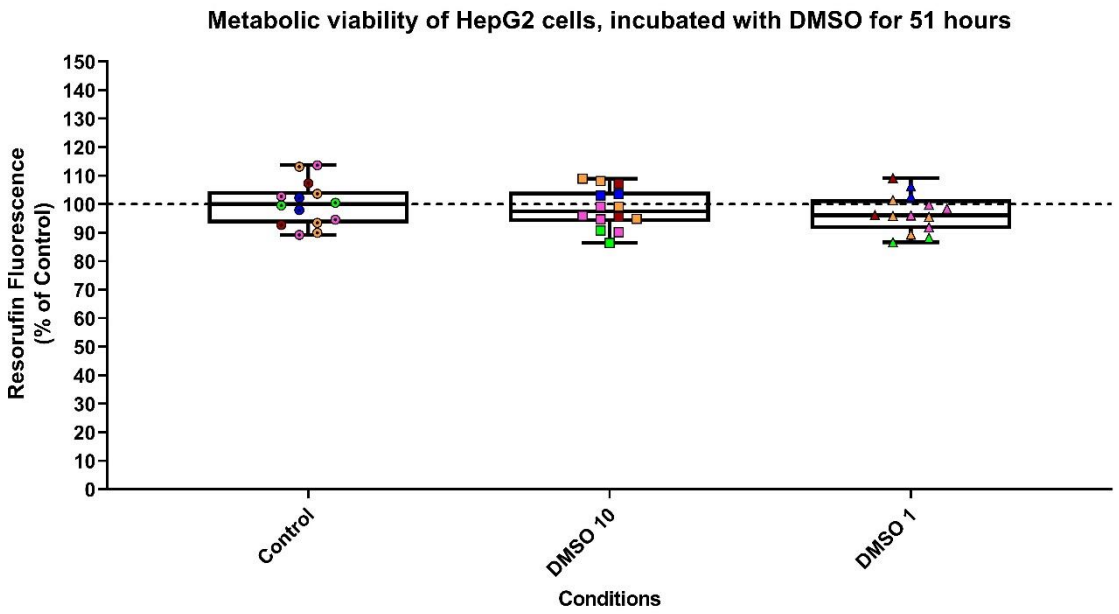


Figure 29 - Metabolic viability of HepG2 cells, incubated with DMSO for 51 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%) **DMSO 1:** Cell culture medium with DMSO (0,002%).

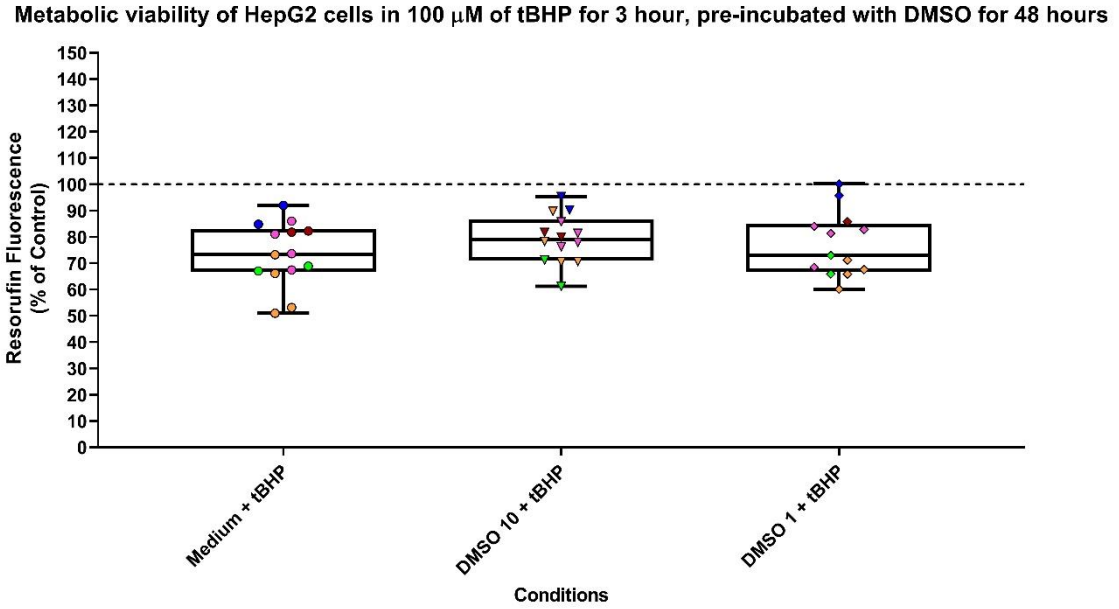


Figure 30 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours, pre-incubated with DMSO for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP.

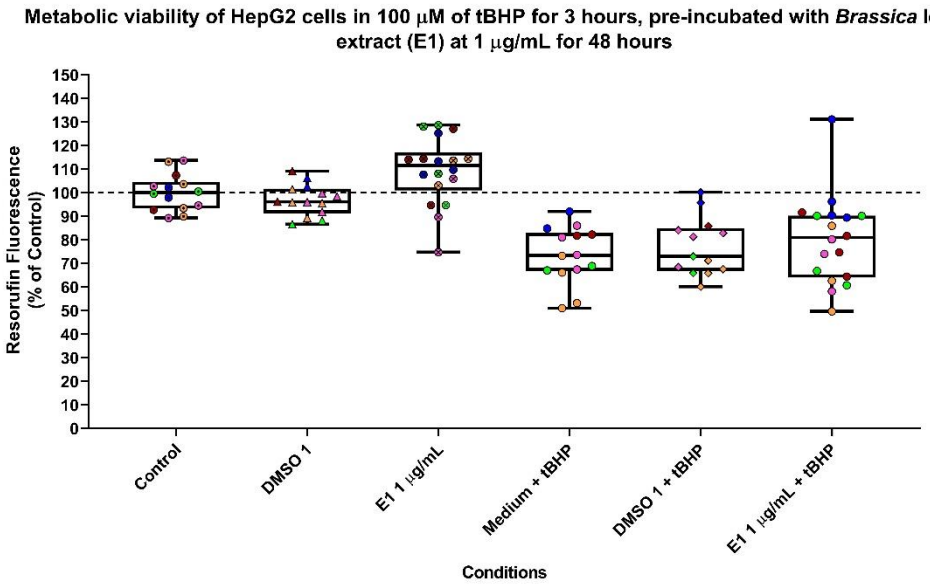


Figure 31 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 1 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with 1 µg/mL of E1; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP.

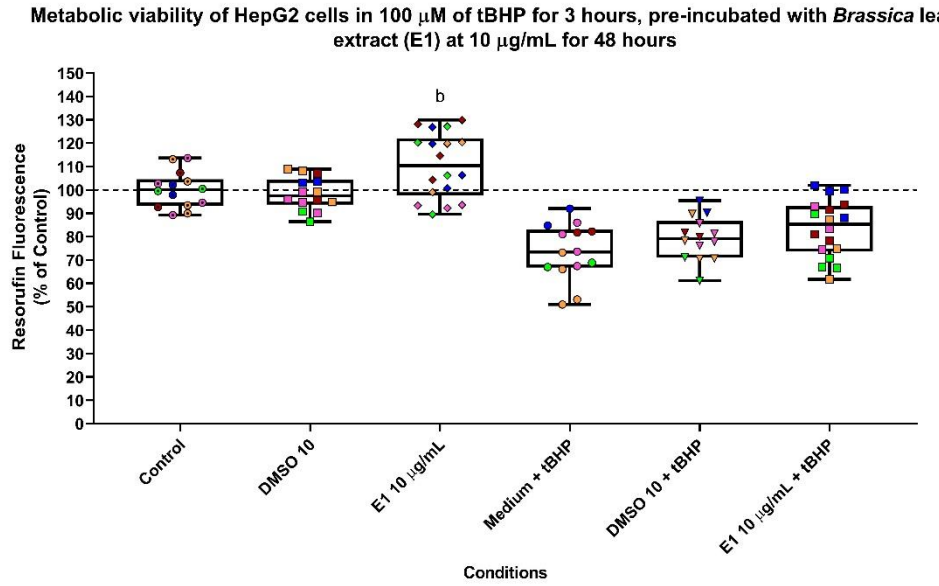


Figure 32 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 10 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. [b]p=0.0257 vs DMSO 10. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP.

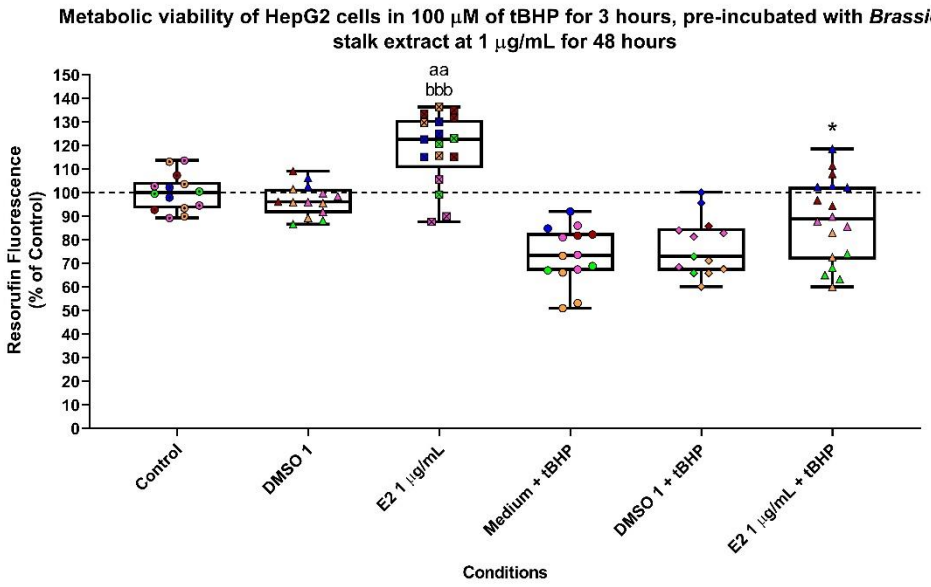


Figure 33 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 1 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. [aa]p=0.0021 vs Control; [bbb]p=0.002 vs DMSO 1; *p=0.0254 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with 1 µg/mL of E2; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP.

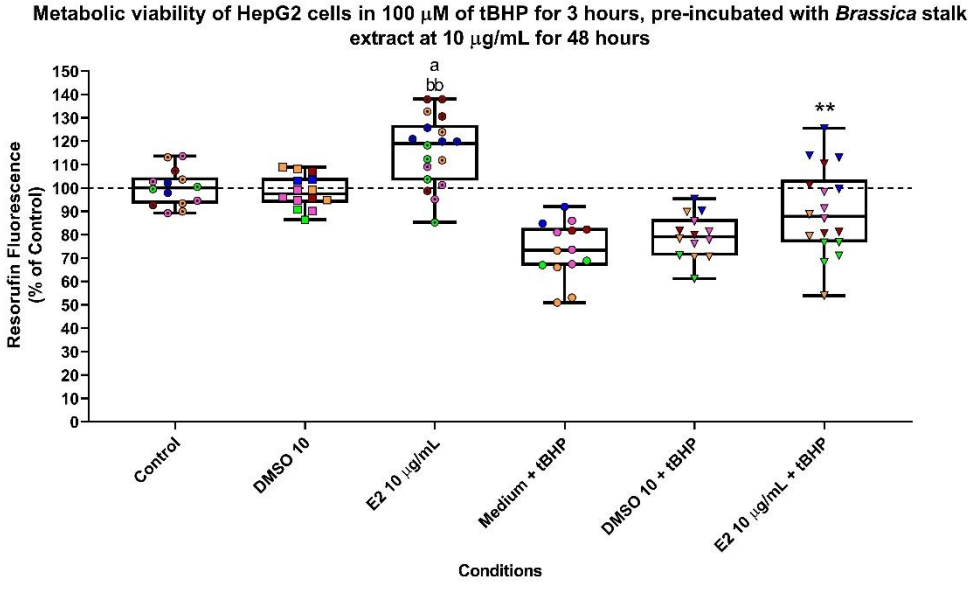


Figure 34 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 10 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. [a]p=0.0102 vs Control; [bb]p=0.0032 vs DMSO 1; **p=0.0071 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP.

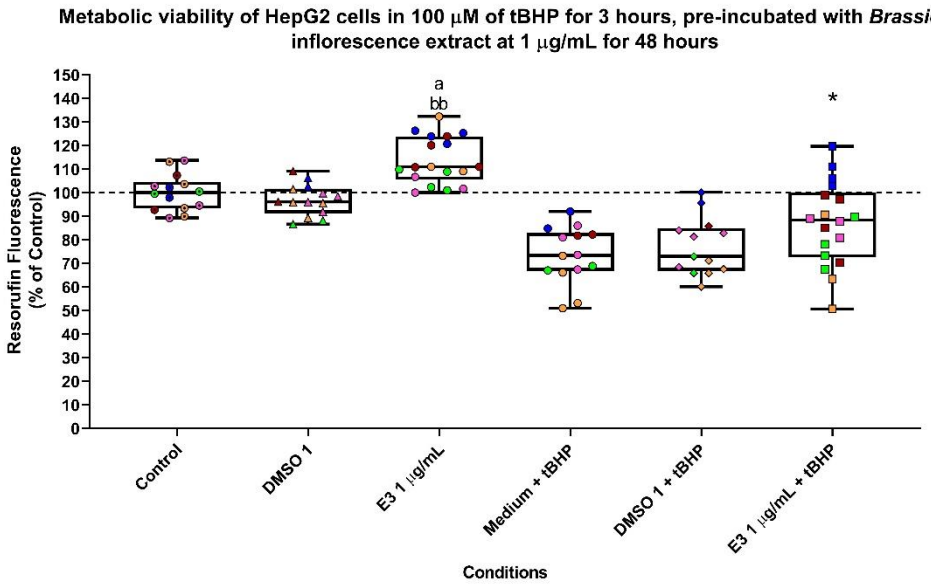


Figure 35 - - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 1 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. [a]p=0.0239 vs Control; [bb]p=0.0024 vs DMSO 1; *p=0.0278 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with 1 µg/mL of E3; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP.

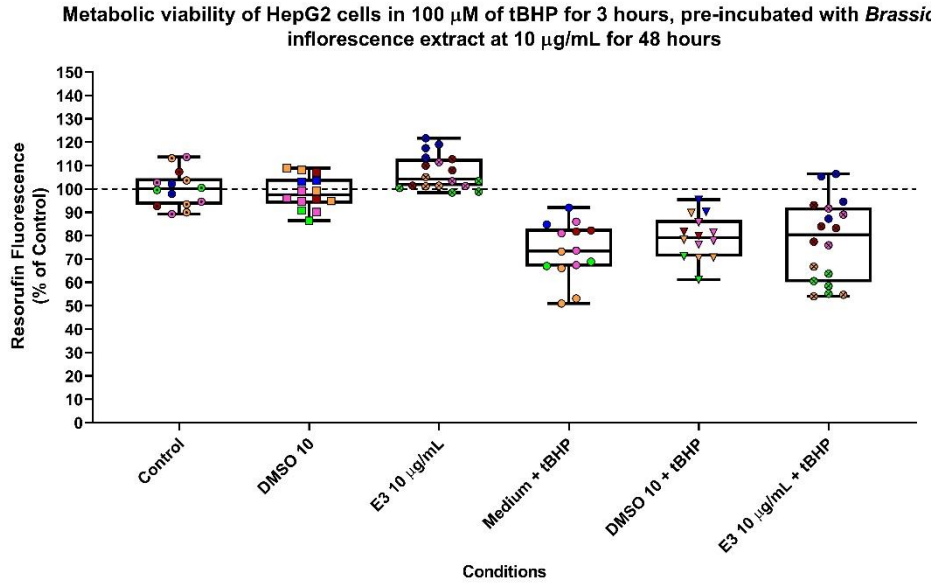


Figure 36 - Metabolic viability of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 10 µg/mL, for 48 hours. Metabolic viability was measured using the resazurin reduction assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP.

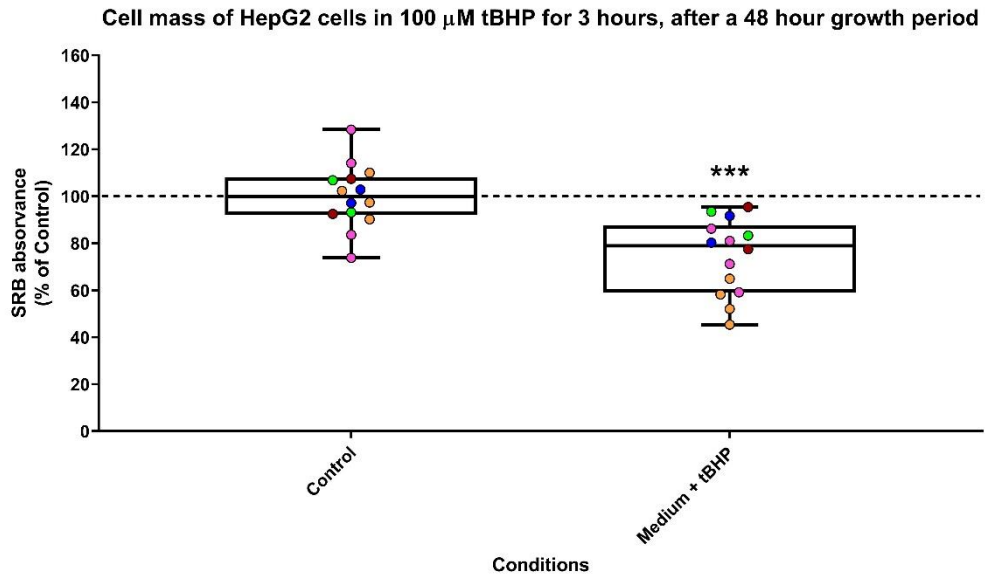


Figure 37 – Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours, after a 48 hour growth period. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. ***p=0.0001 vs Control. **Control:** Cell culture medium only; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP.

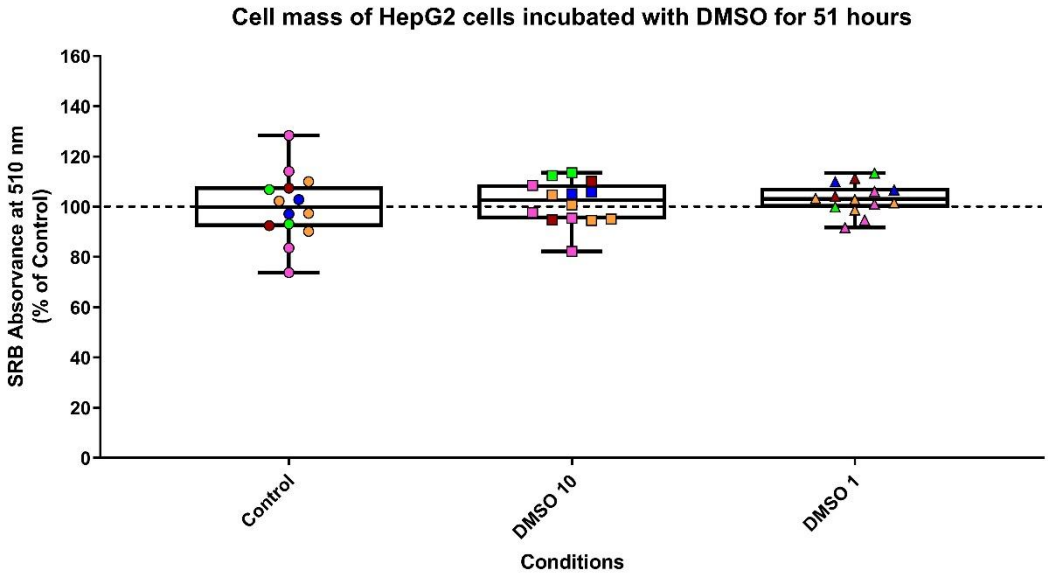


Figure 38 - Cell mass of HepG2 cells, incubated with DMSO for 51 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%) **DMSO 1:** Cell culture medium with DMSO (0,002%).

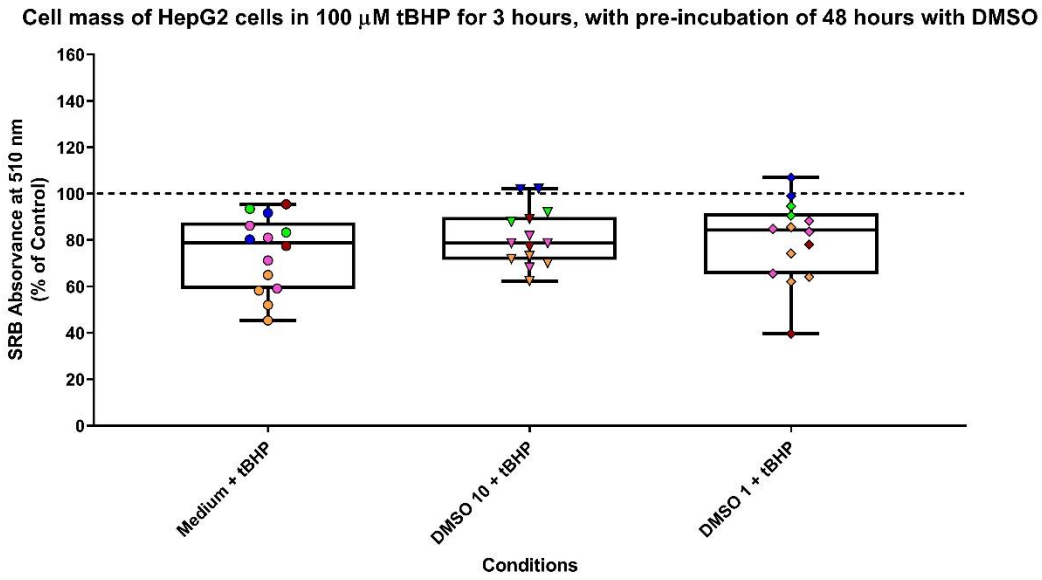


Figure 39 – Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours, pre-incubated with DMSO for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP.

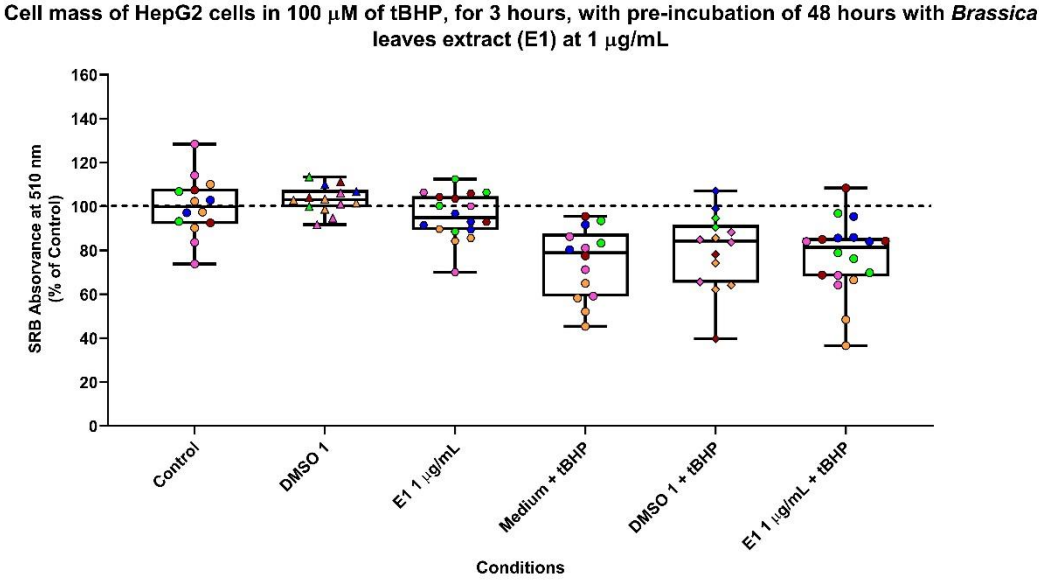


Figure 40 – Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 1 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E1 1 µg/mL:** Cell culture medium with 1 µg/mL of E1; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E1 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E1 and 100 µM of tBHP.

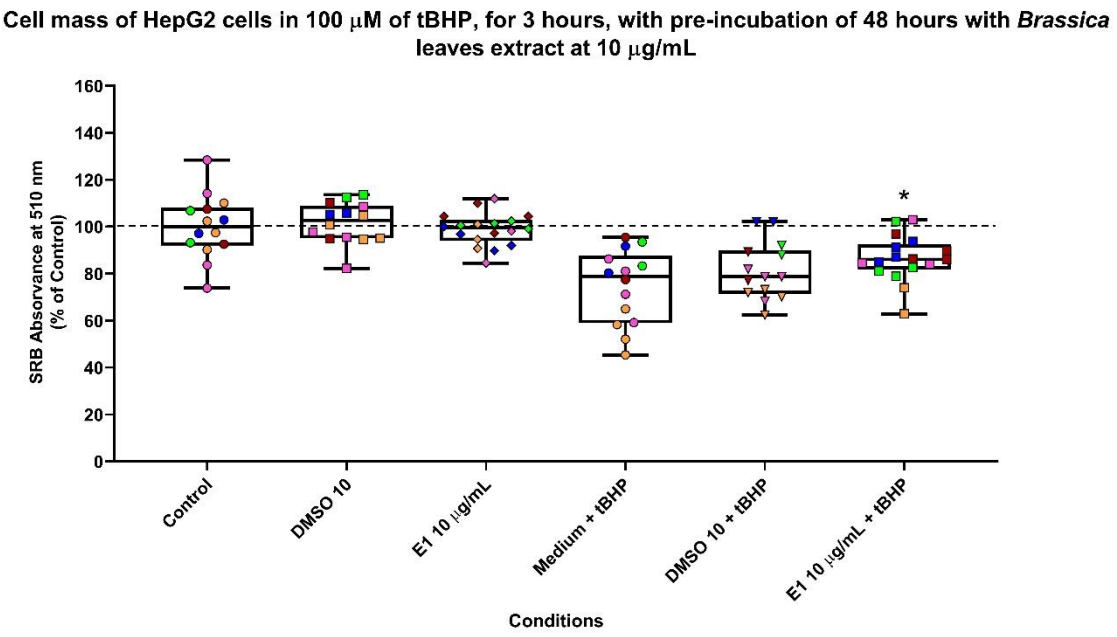


Figure 41 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with leaves extract (E1) at 10 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. *p=0.0456 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E1 10 µg/mL:** Cell culture medium with 10 µg/mL of E1; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E1 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E1 and 100 µM of tBHP.

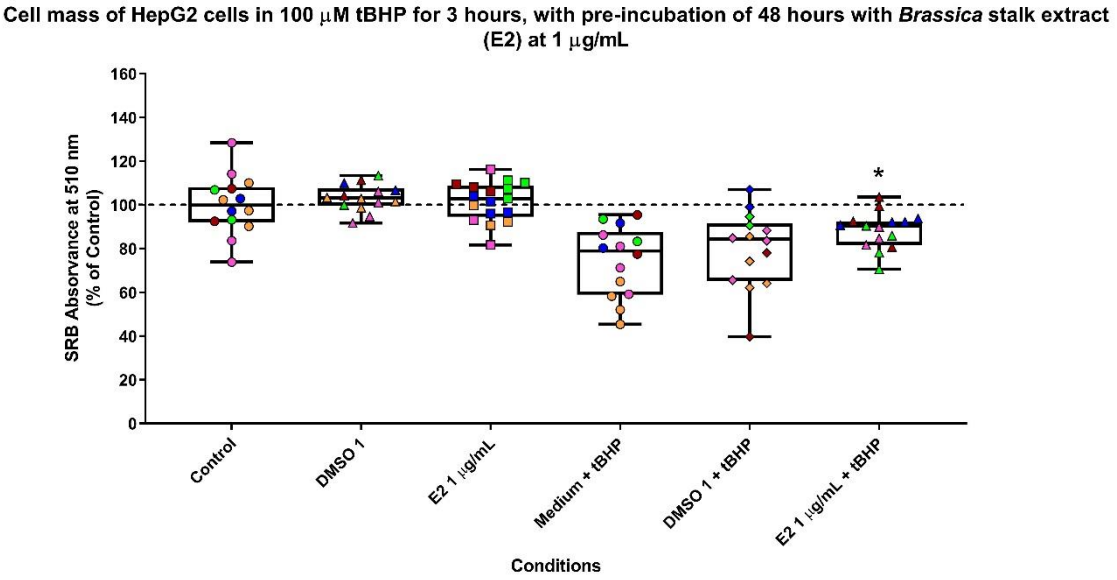


Figure 42 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 1 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. *p=0.0322 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E2 1 µg/mL:** Cell culture medium with 1 µg/mL of E2; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E2 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E2 and 100 µM of tBHP.

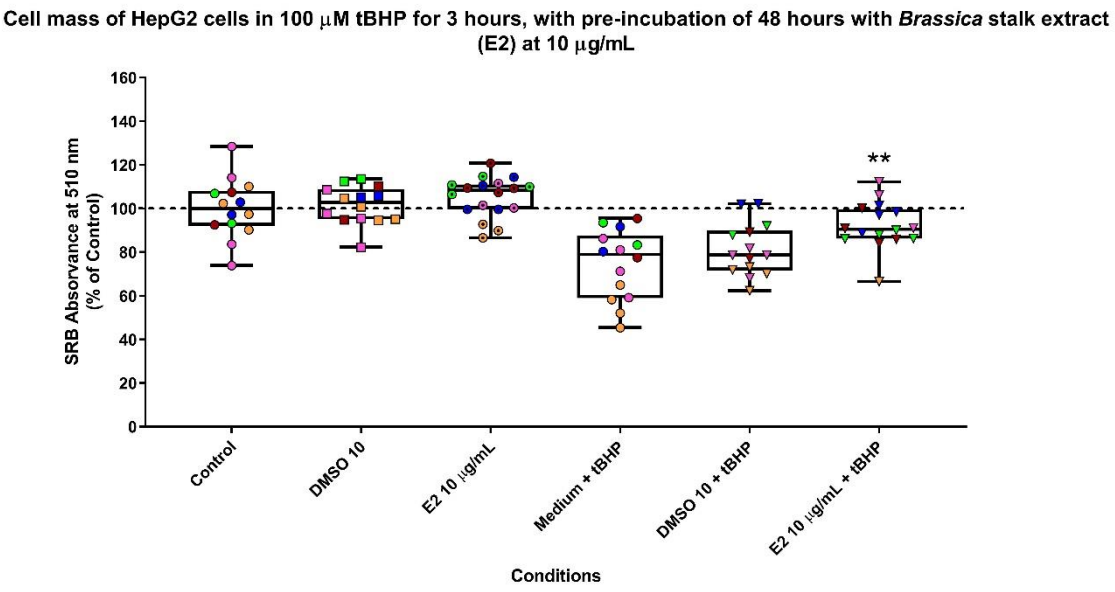


Figure 43 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with stalk extract (E2) at 10 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **p=0.0013 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E2 10 µg/mL:** Cell culture medium with 10 µg/mL of E2; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E2 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E2 and 100 µM of tBHP.

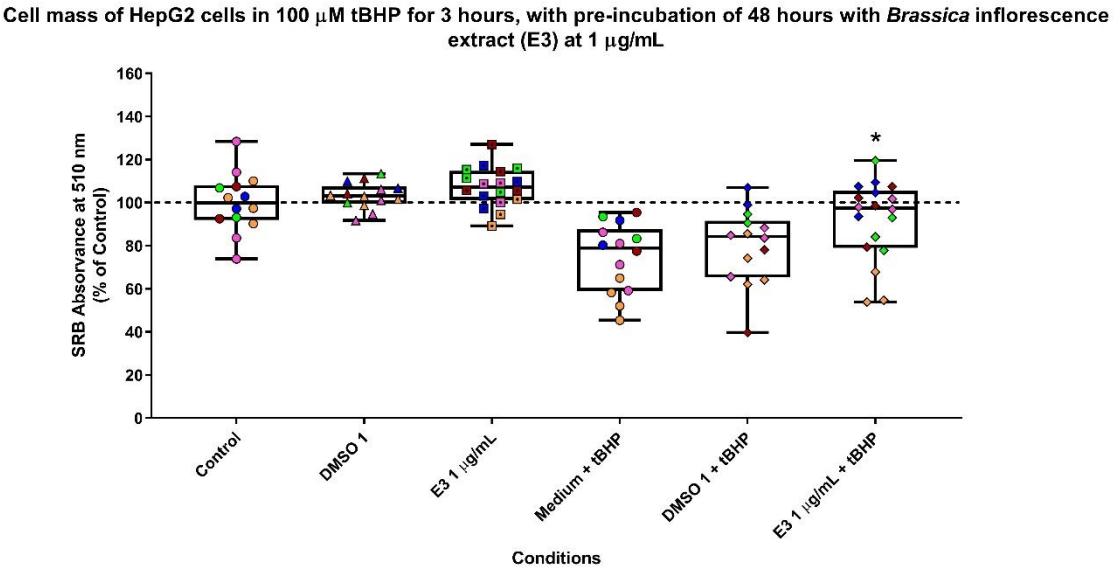


Figure 44 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with inflorescence extract (E3) at 1 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. *p=0.0119 vs Medium + tBHP. **Control:** Cell culture medium only; **DMSO 1:** Cell culture medium with DMSO (0,002%); **E3 1 µg/mL:** Cell culture medium with 1 µg/mL of E3; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 1 + tBHP:** Cell culture medium with DMSO (0,002%) and 100 µM of tBHP; **E3 1 µg/mL + tBHP:** Cell culture medium with 1 µg/mL of E3 and 100 µM of tBHP.

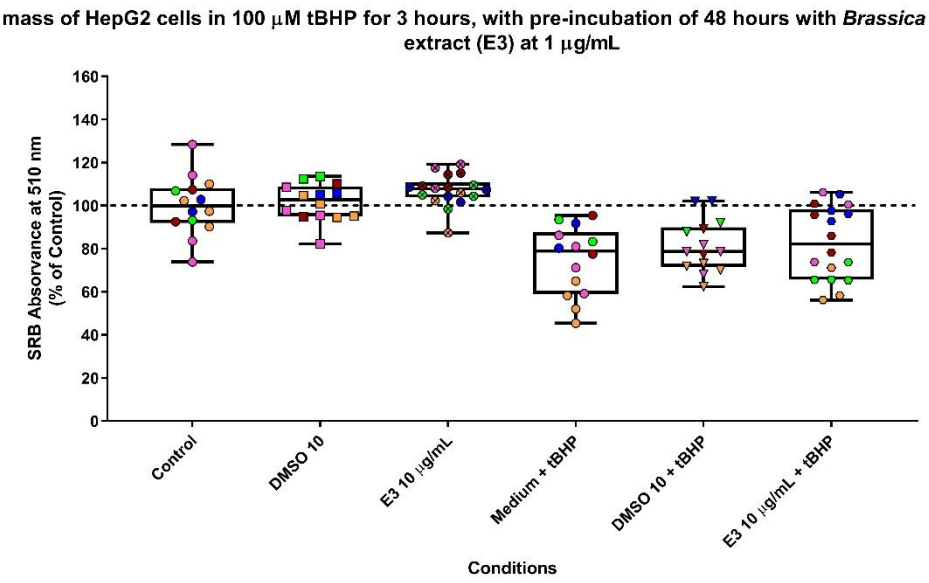


Figure 45 - Cell mass of HepG2 cells in 100 µM of tBHP for 3 hours and pre-incubated with inflorescence extract (E3) at 10 µg/mL, for 48 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for DMSO conditions and three or four replicates for the extract conditions, where every color represents an independent experiment and every same colored symbol represents a biological replicate. **Control:** Cell culture medium only; ; **DMSO 10:** Cell culture medium with DMSO (0,02%); **E3 10 µg/mL:** Cell culture medium with 10 µg/mL of E3; **Medium + tBHP:** Cell culture medium with 100 µM of tBHP; **DMSO 10 + tBHP:** Cell culture medium with DMSO (0,02%) and 100 µM of tBHP; **E3 10 µg/mL + tBHP:** Cell culture medium with 10 µg/mL of E3 and 100 µM of tBHP.

4.1.5. Summary results of the potential protection of *Brassica* by-products against a model of hepatic oxidative damage, induced by tBHP

Table 3 - Summary results of metabolic viability assays in HepG2 cells, with extracts and tBHP. **E1.1:** Cell culture medium with1 µg/mL of leaves extract; **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E1.25:** Cell culture medium with 25 µg/mL of leaves extract; **E2.1:** Cell culture medium with1 µg/mL of stalk extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E2.25:** Cell culture medium with 25 µg/mL of stalk extract; **E3.1:** Cell culture medium with1 µg/mL of inflorescence extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **E3.25:** Cell culture medium with 25 µg/mL of inflorescence extract; **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Metabolic Viability						
	Co-Incubation		24 h Pre-Incubation		48 h Pre-Incubation	
	vs tBHP + DMSO	vs tBHP	vs tBHP + DMSO	vs tBHP	vs tBHP + DMSO	vs tBHP
E1.1	ns	ns	<	-	ns	ns
E1.10	ns	ns	ns	-	ns	ns
E1.25	-	-	ns	-	-	-
E2.1	ns	ns	ns	-	ns	>
E2.10	ns	ns	ns	-	ns	>
E2.25	-	-	ns	-	-	-
E3.1	ns	ns	<	-	ns	>
E3.10	ns	ns	ns	-	ns	ns
E3.25	-	-	ns	-	-	-

Table 4 - Summary results of cell mass assays in HepG2 cells, with extracts and tBHP. **E1.1:** Cell culture medium with1 µg/mL of leaves extract; **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E1.25:** Cell culture medium with 25 µg/mL of leaves extract; **E2.1:** Cell culture medium with1 µg/mL of stalk extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E2.25:** Cell culture medium with 25 µg/mL of stalk extract; **E3.1:** Cell culture medium with1 µg/mL of inflorescence extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **E3.25:** Cell culture medium with 25 µg/mL of inflorescence extract; **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Cell Mass						
	Co-Incubation		24 h Pre-Incubation		48 h Pre-Incubation	
	vs tBHP + DMSO	vs tBHP	vs tBHP + DMSO	vs tBHP	vs tBHP + DMSO	vs tBHP
E1.1	ns	ns	<	-	ns	ns
E1.10	ns	ns	ns	-	ns	ns
E1.25	-	-	<	-	-	-
E2.1	ns	ns	<	-	ns	>
E2.10	ns	ns	ns	-	ns	>
E2.25	-	-	ns	-	-	-
E3.1	ns	ns	<	-	ns	ns
E3.10	ns	ns	ns	-	ns	>
E3.25	-	-	ns	-	-	-

4.2. The potential prevention capacity of *Brassica* by-products in a model of hepatic accumulation of lipids and protection against a model of hepatic lipotoxicity damage, induced by free fatty acids

Lipid accumulation was measured using the nile red assay which was then normalized by measurement of cell mass. Cell mass was also used to assess lipotoxicity. Since the accumulation of lipids is a more time-consuming process than the effects of tBHP, a short-timed co-incubation was not an option. Therefore, the co-incubation approach had to be extended to 24 hours. In the pre-incubation experiments, cells were pre-incubated with the extracts and then a new batch of extracts was added in conjunction with the FFA. These different approaches were taken less to see a direct vs indirect effect, but to see how long it would take for the extracts to trigger a cellular response to the accumulation of FFA.

Three different concentrations of FFA were used: 250 µM to simulate moderate lipid accumulation, 1000 µM to simulate a considerable lipid accumulation and 2000 µM to simulate cell death due to lipotoxicity.

Over-all, stalk, and inflorescence extracts show the most promise in lipid accumulation reduction and prevention of lipotoxicity.

Metabolic flux analysis was performed to see how FFA affected oxygen consumption rate in different parameters of the mitochondrial respiration and if the extracts could prevent those alterations. ATP intracellular content measurements were performed as a complement to one of the parameters of mitochondrial respiration: the oxygen consumption rate associated with ATP production. This will allow both specific and wider look at how FFA affects ATP production. These two experiments were only made for the 250 µM FFA concentration.

The results obtained are developed in more detail in sections 4.2.1 – 4.2.10. The more preliminary assays (nile red and SRB) are presented by experimental design in the chronological order in which they were performed. Nile red assay was the first, after which cells were fixated and SRB assay was performed, at earliest, the day after. Therefore, nile red results are presented first followed by the SRB results and concluding with the SRB normalized results of nile red. Metabolic flux analysis assays are presented next (for both co-incubation and pre-incubation approaches) followed by the ATP content results (for both co-incubation and pre-incubation approaches). A summary of all these results can be found in Tables 5 - 9, in section 4.2.11.

4.2.1. Co-incubation of extracts and 250 µM of free fatty acids for 24 hours

HepG2 cells were co-incubated with the extracts and 250 µM of FFA for 24 hours. This approach was taken to test the extracts capability of triggering a cell response to deal with a moderate accumulation of lipids.

Although no statistically significant differences were found between cell mass of control group and cells co-incubated with 250 µM of FFA, and despite the fact that no statistically significant differences were found between cell mass of cells co-incubated with FFA and cells co-incubated with FFA and DMSO, stalk extract at 10 µg/mL increased cell mass of HepG2 cells in FFA when compared to the respective control group.

Co-incubation of FFA with the stalk or inflorescence extracts at 10 µg/mL showed a statistically significant decrease in SRB normalized FFA accumulation on HepG2 cells when compared to their respective control group.

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

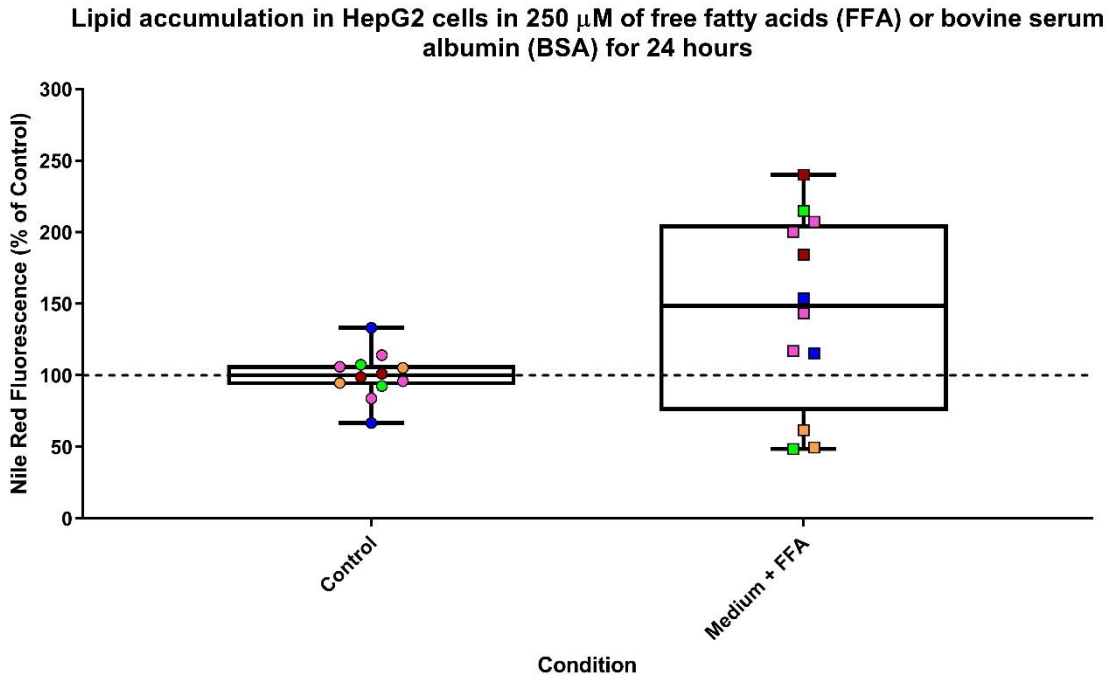


Figure 46 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA) or BSA (0,5%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

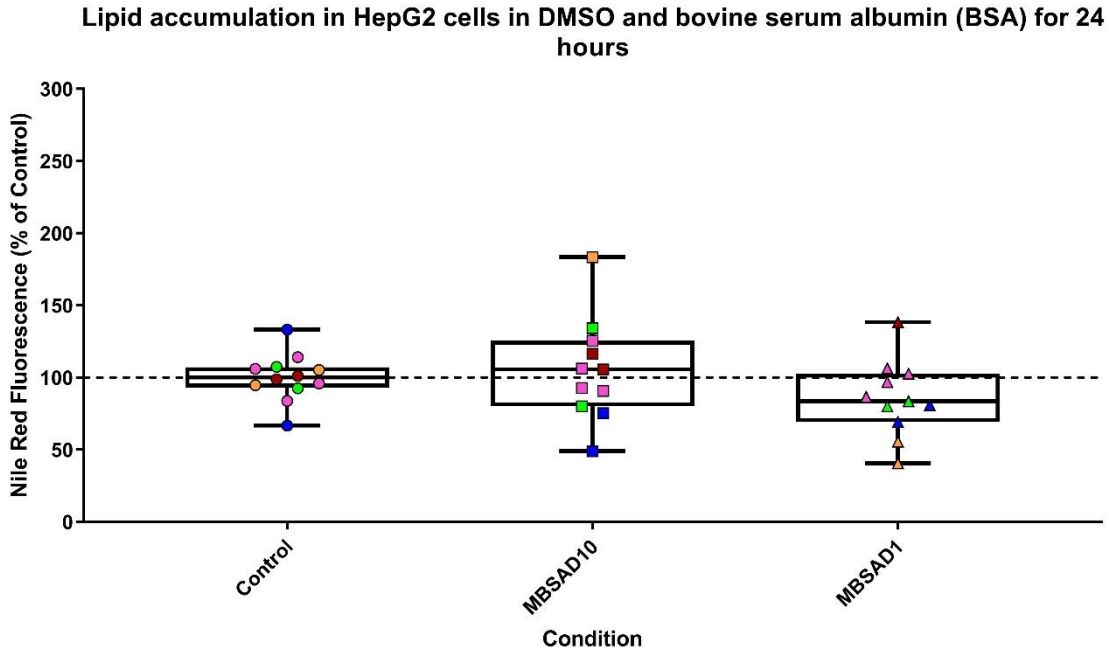


Figure 47 - Lipid accumulation in HepG2 cells in DMSO and BSA (0,5%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

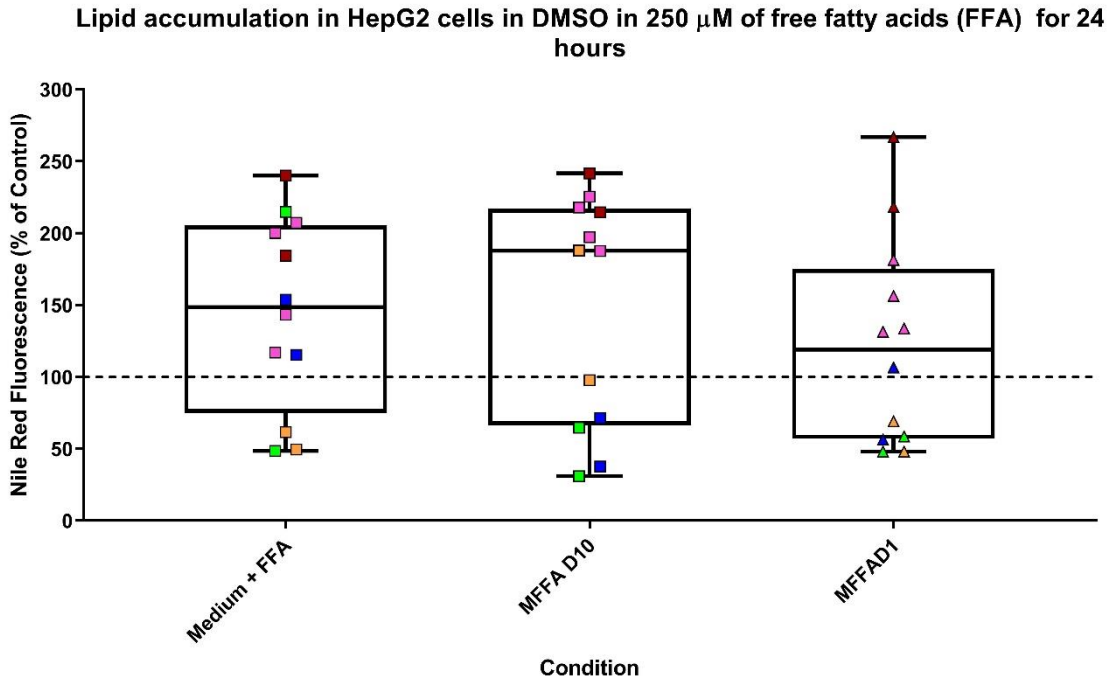


Figure 48 - Lipid accumulation in HepG2 cells in DMSO and 250 µM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 250 µM of FFA; **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).

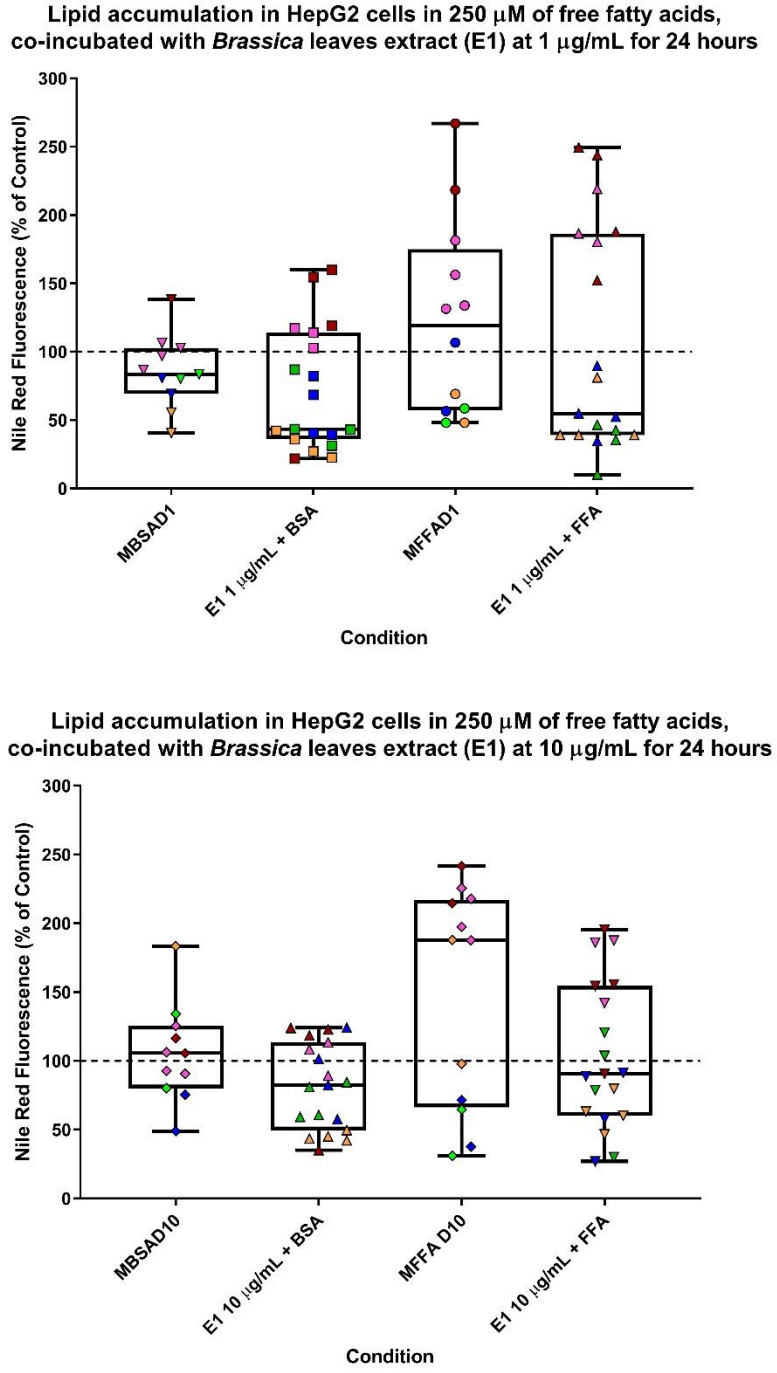


Figure 49 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

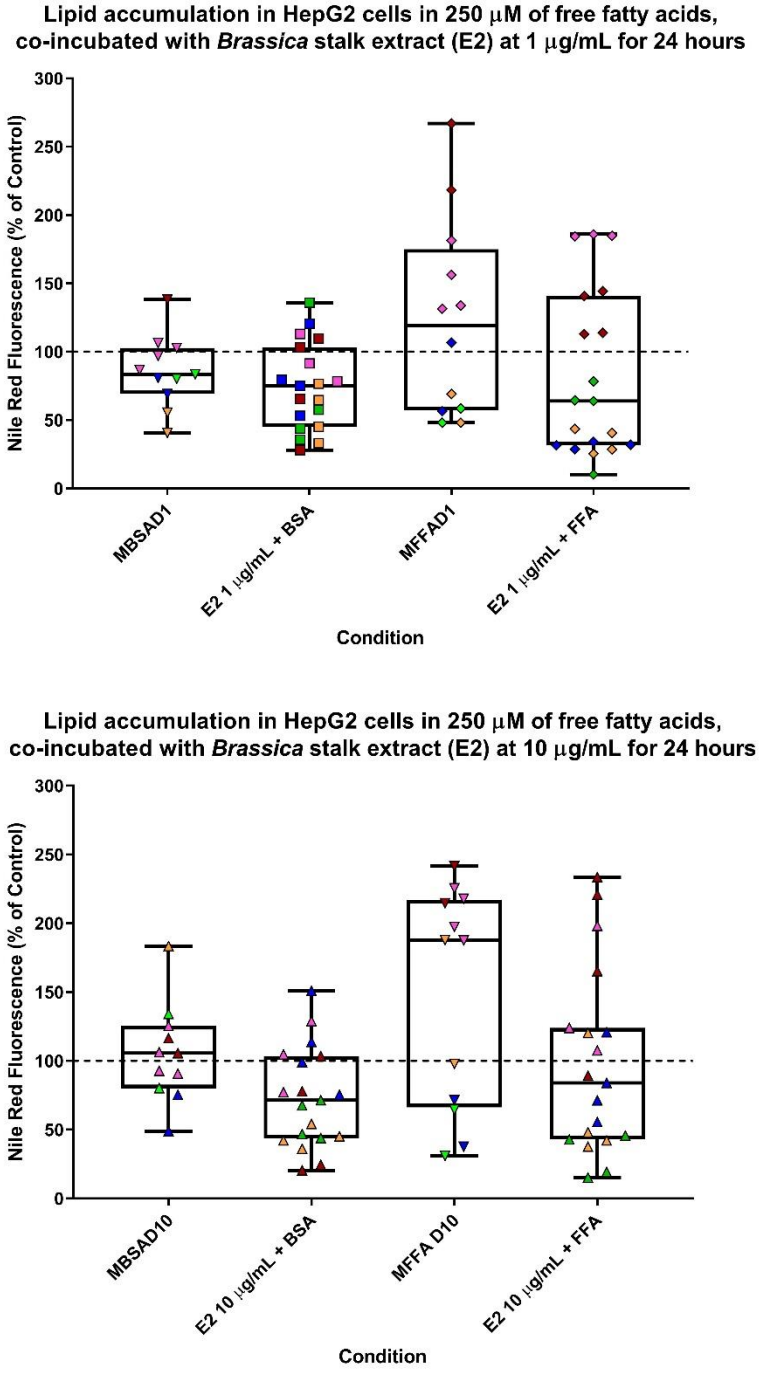


Figure 50 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

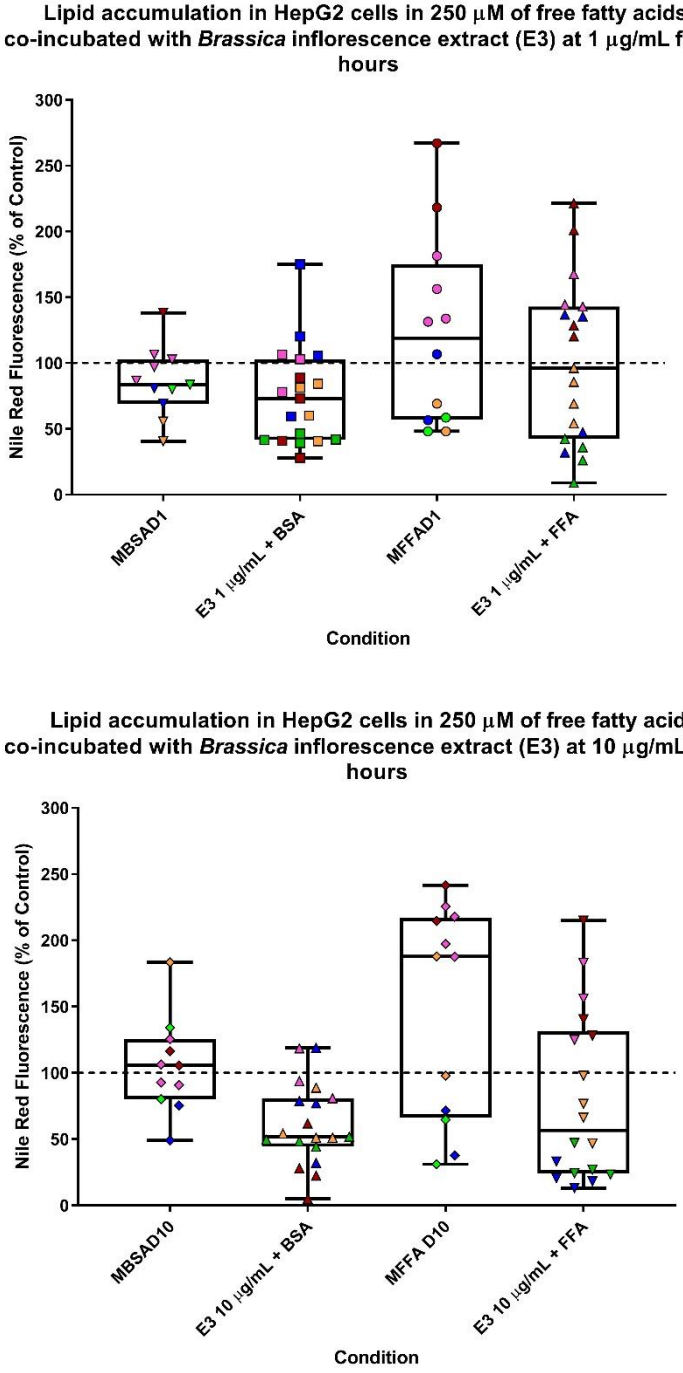


Figure 51 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

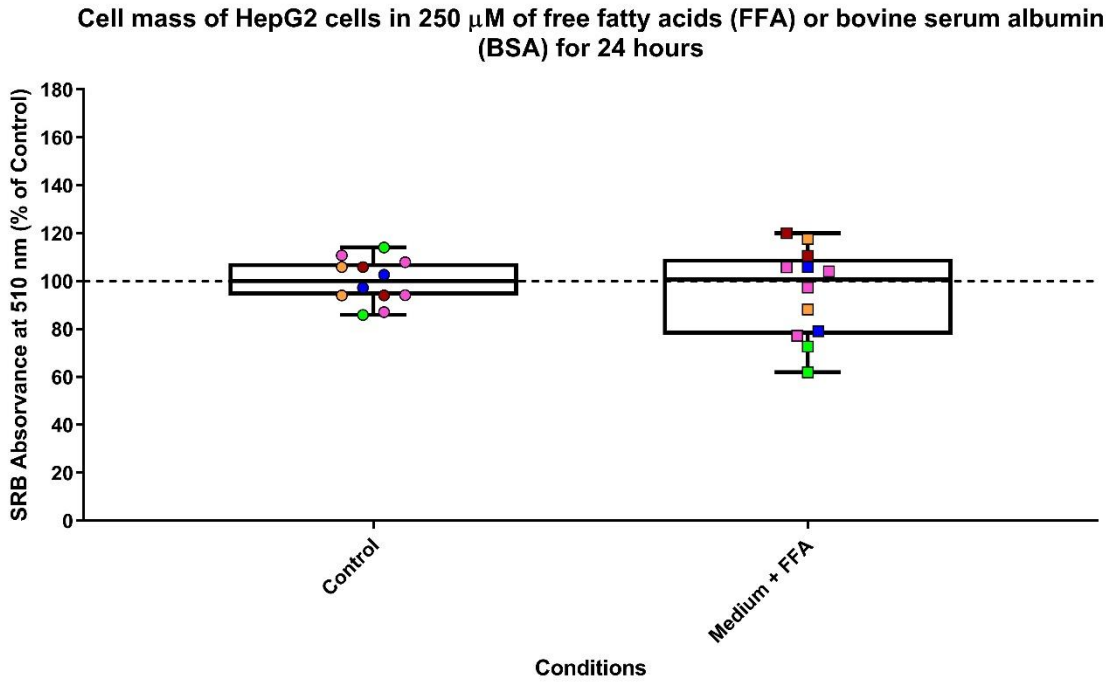


Figure 52 – Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA) or BSA (0,5%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

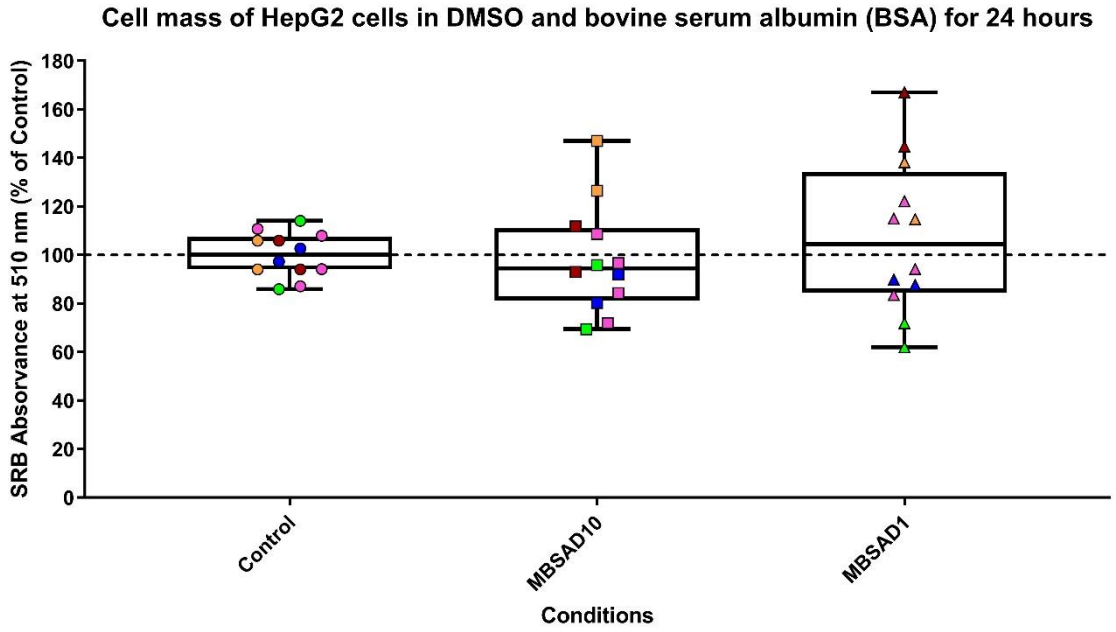


Figure 53 – Cell mass of HepG2 cells in DMSO and BSA (0,5%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

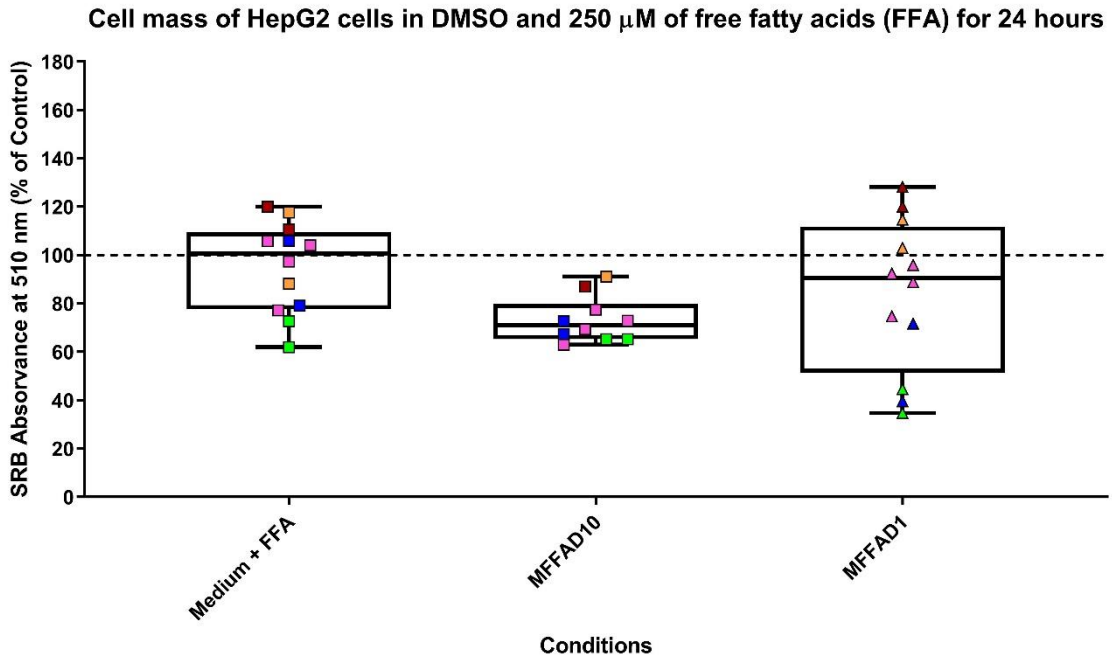


Figure 54 - Cell mass of HepG2 cells in DMSO and 250 µM of free fatty acids (FFA) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 250 µM of FFA; **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).

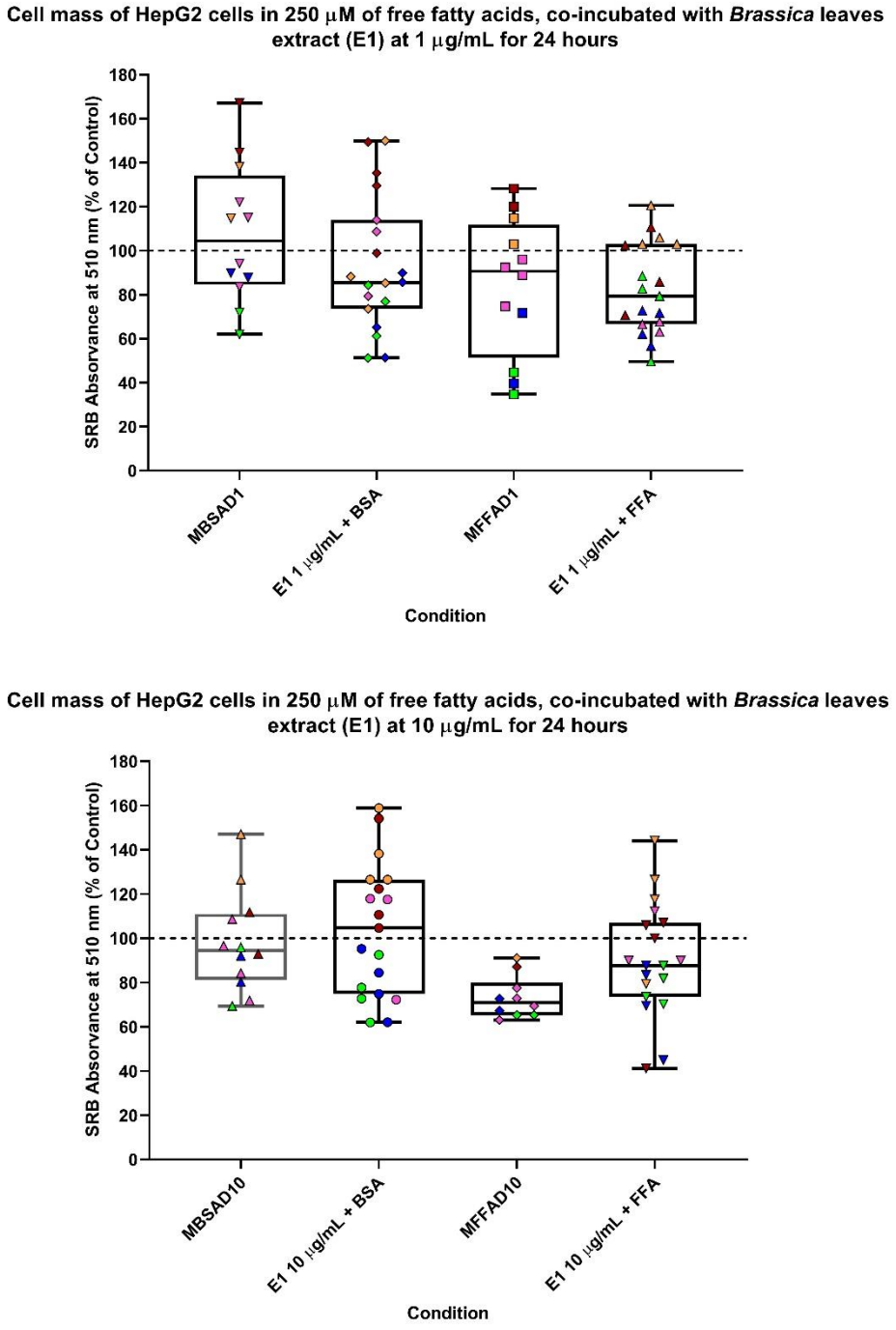


Figure 55 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

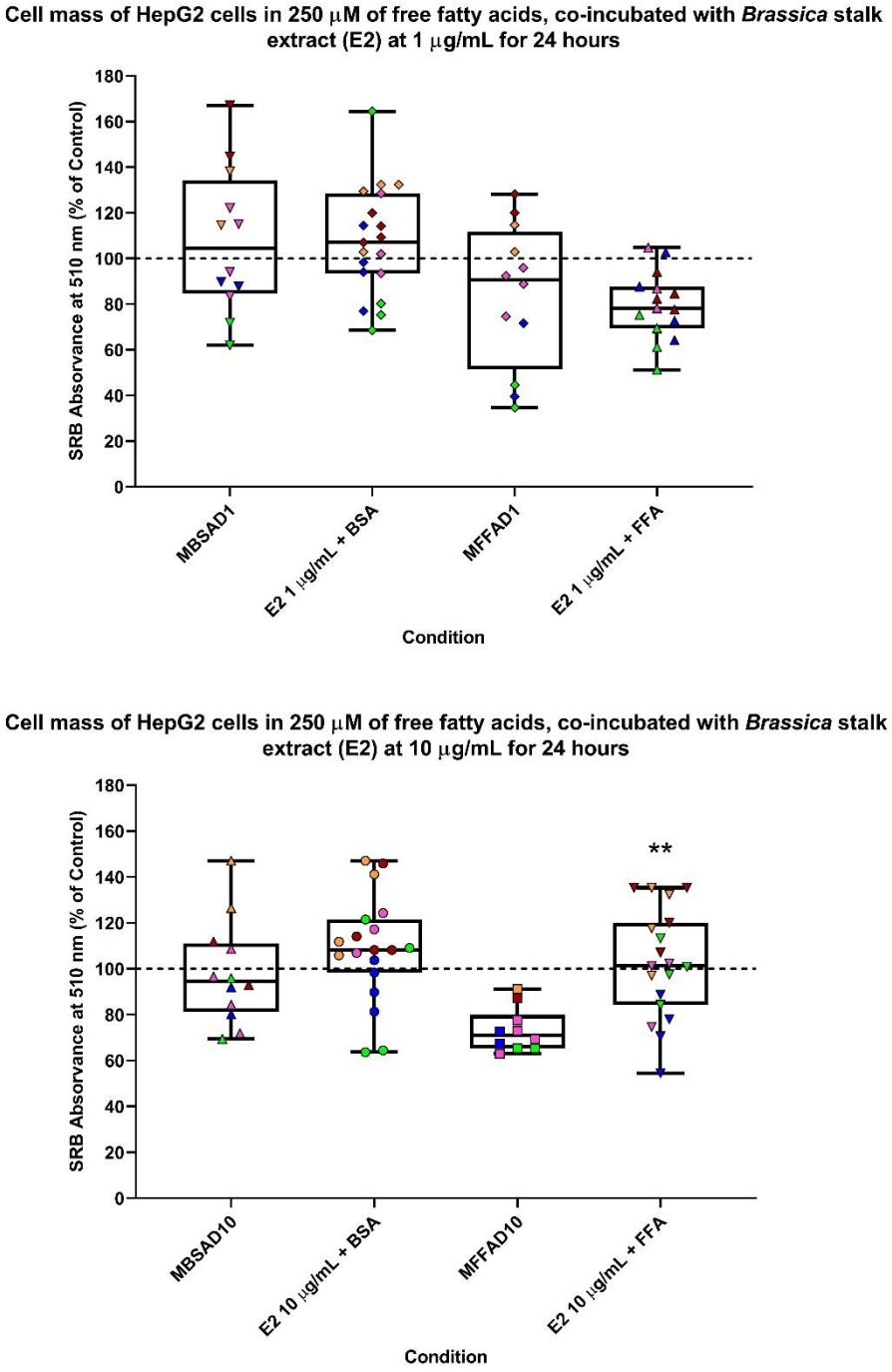


Figure 56 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0055 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

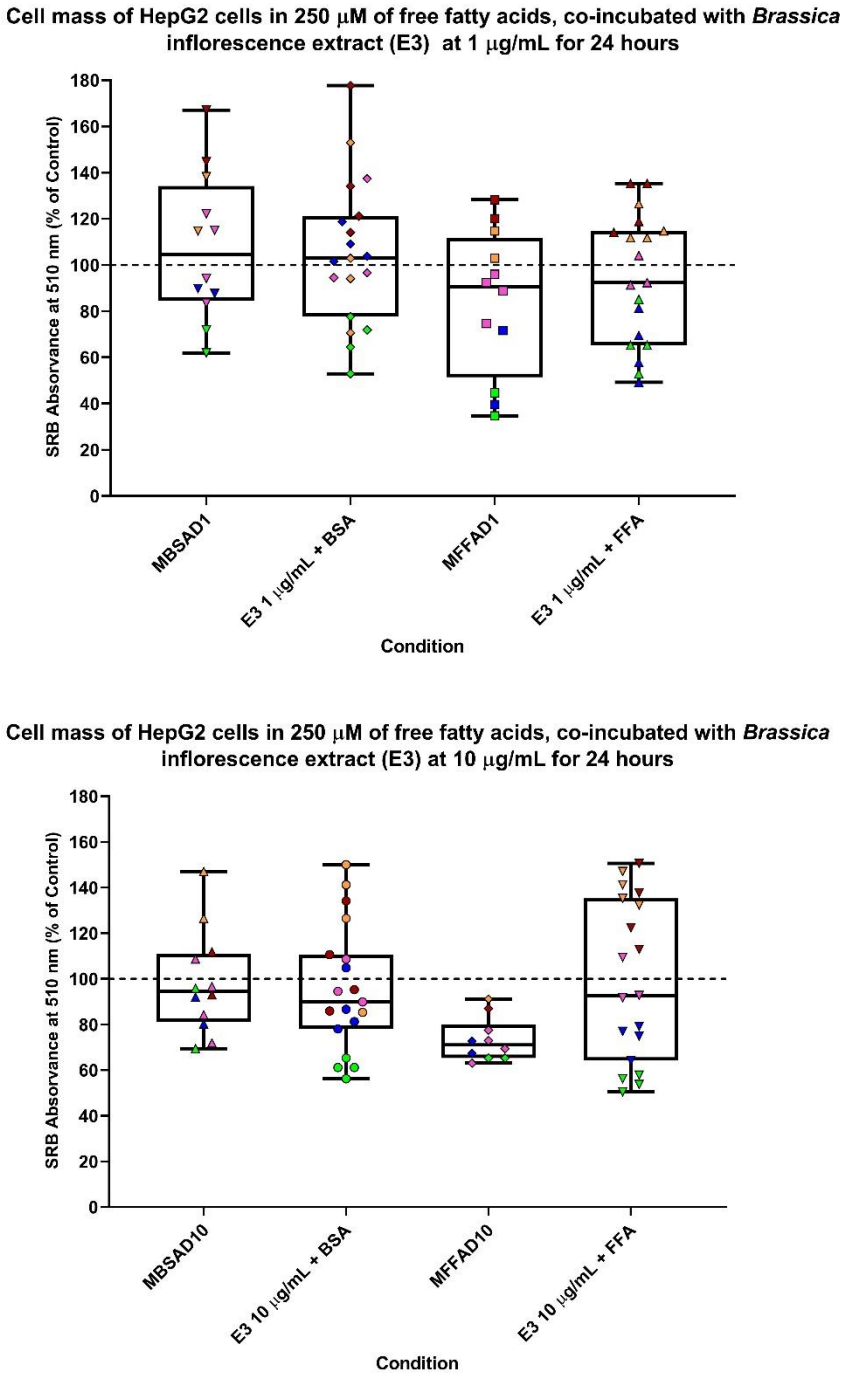


Figure 57 – Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

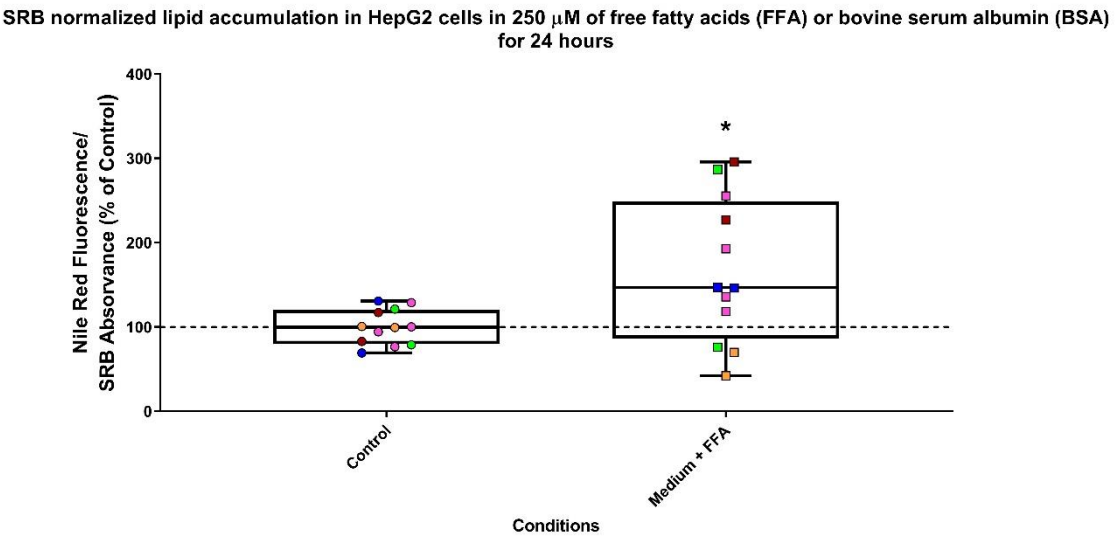


Figure 58 – SRB normalized lipid accumulation in HepG2 cells in 250 μM of free fatty acids (FFA) or BSA (0,5%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0229 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 μM of FFA.

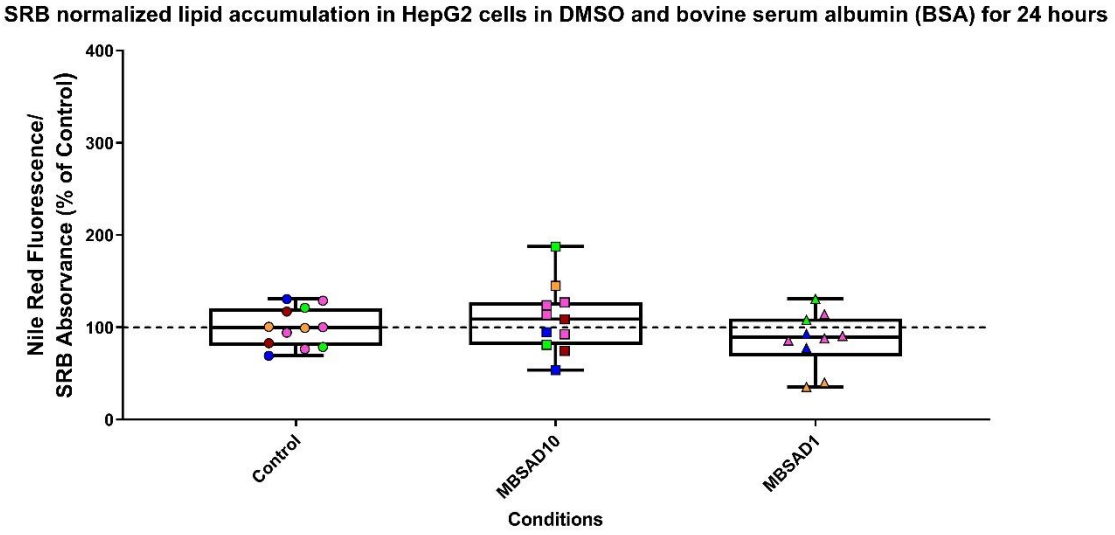


Figure 59 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (0,5%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

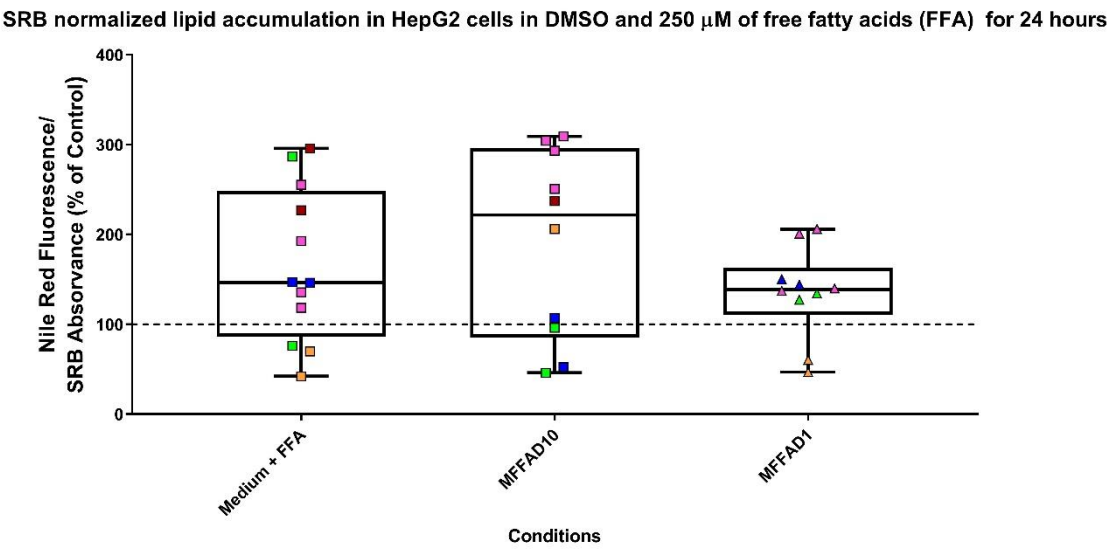


Figure 60 - SRB normalized lipid accumulation in HepG2 cells in DMSO and 250 µM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 250 µM of FFA; **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).

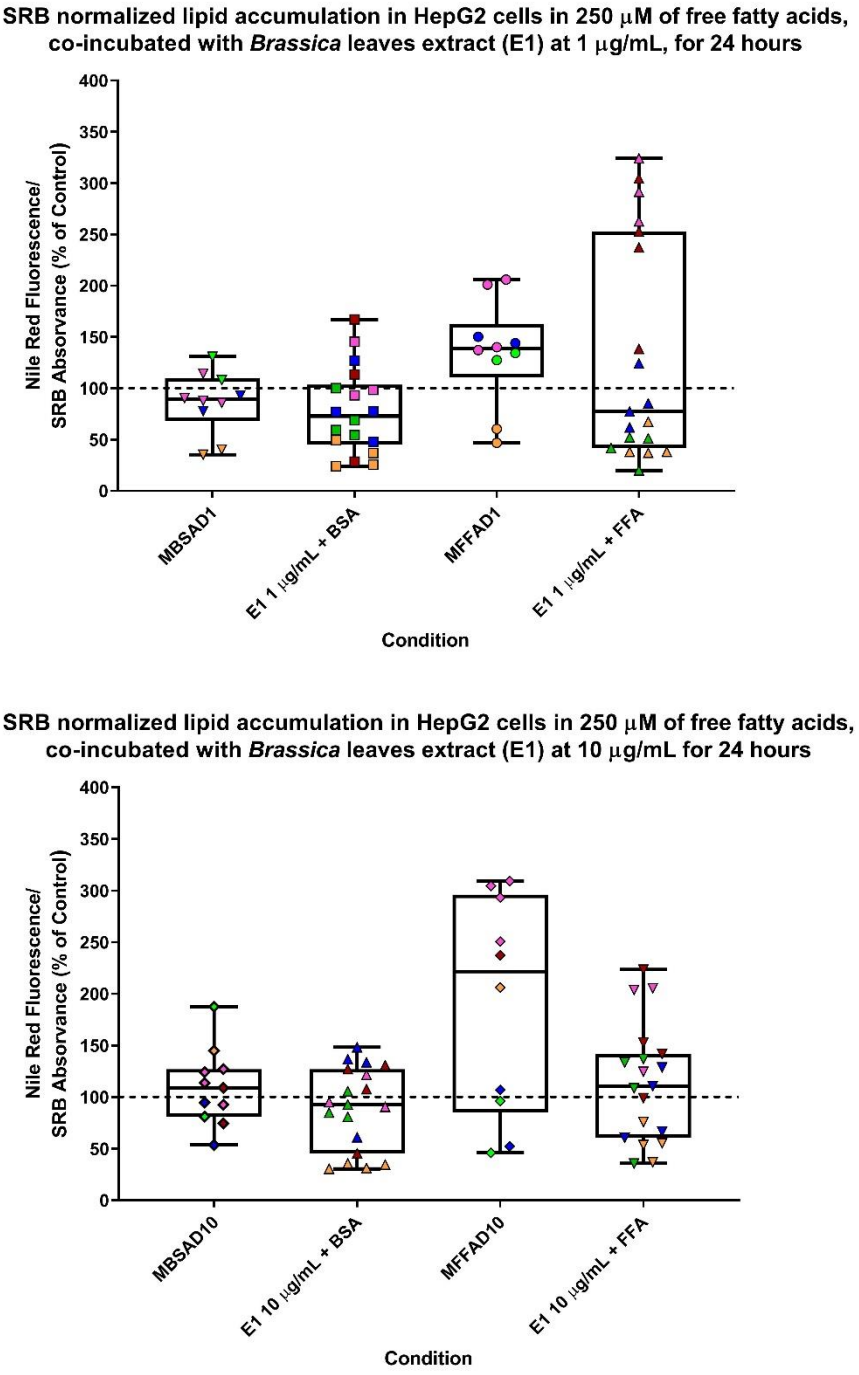


Figure 61 – SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

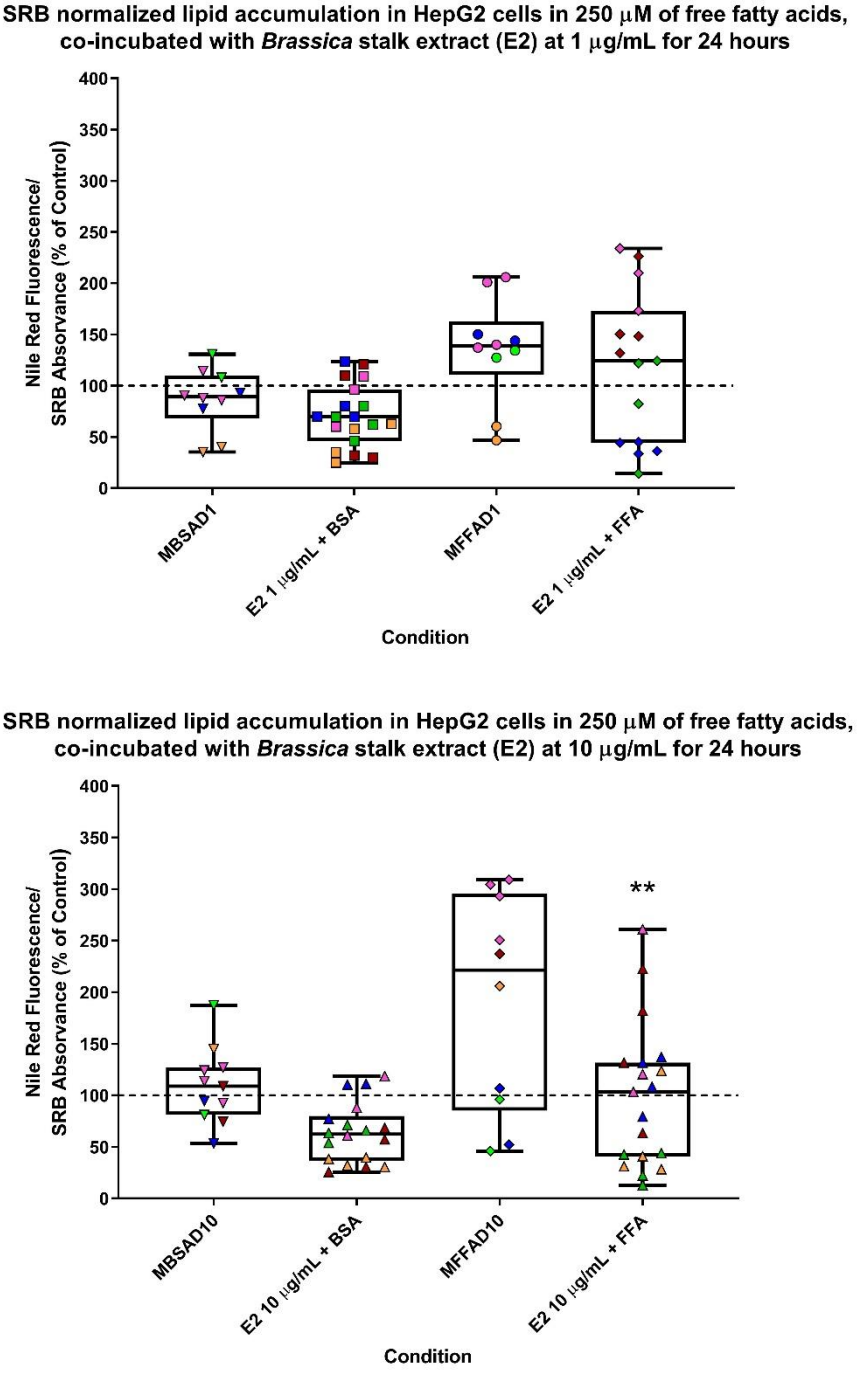


Figure 62 – SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0029 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

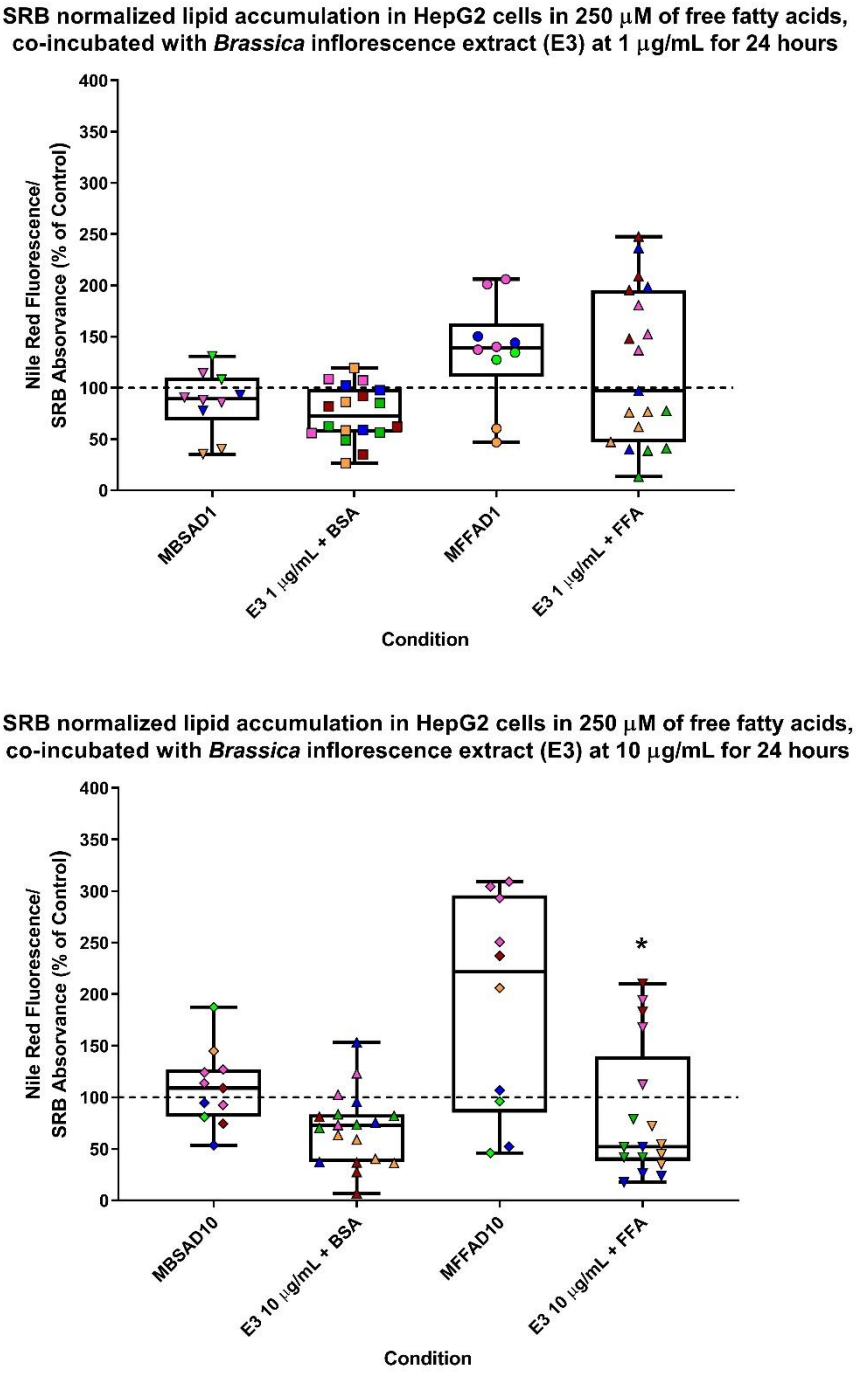


Figure 63 - SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0134 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.2. Co-incubation of extracts and 1000 µM of free fatty acids for 24 hours

HepG2 cells were co-incubated with the extracts and 1000 µM of FFA for 24 hours. This approach was taken to test the extracts capability of triggering a cell response to deal with an elevated accumulation of lipids.

No statistically significant differences were observed between the control groups with 1 mM of FFA plus DMSO and the corresponding groups with 1 mM of FFA plus extracts. However, 1 µg/mL of stalk extract showed an increase in HepG2 cell mass in control groups (with BSA instead of FFA).

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

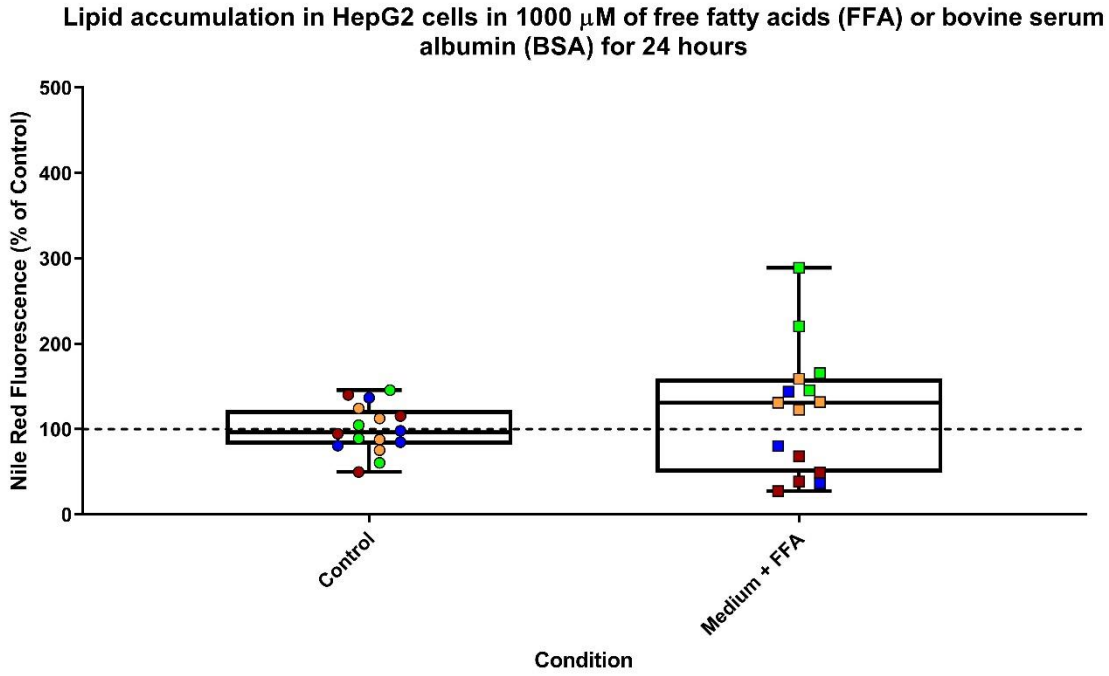


Figure 64 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **Medium + FFA:** Cell culture medium with 1000 µM of FFA.

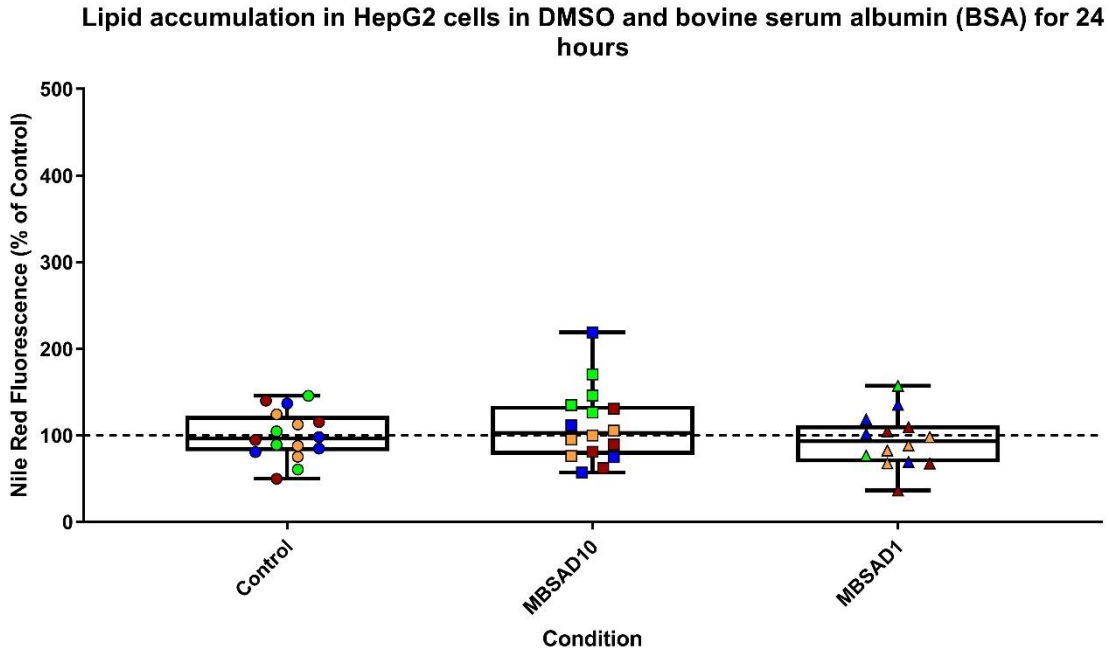


Figure 65 - Lipid accumulation in HepG2 cells in DMSO and BSA (2%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

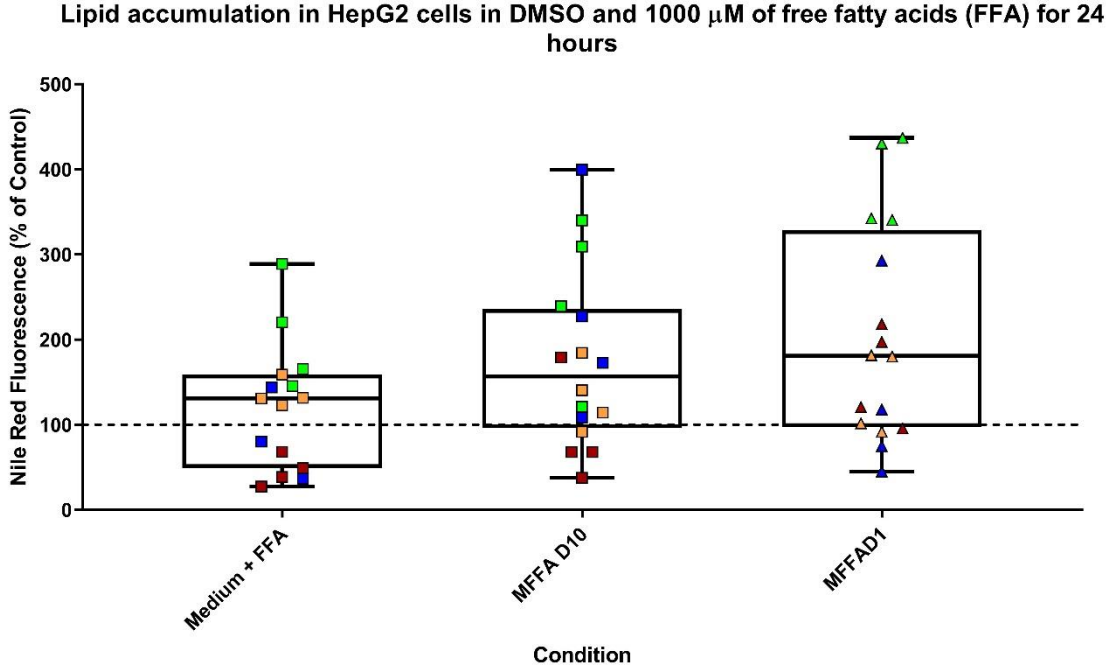


Figure 66 - Lipid accumulation in HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

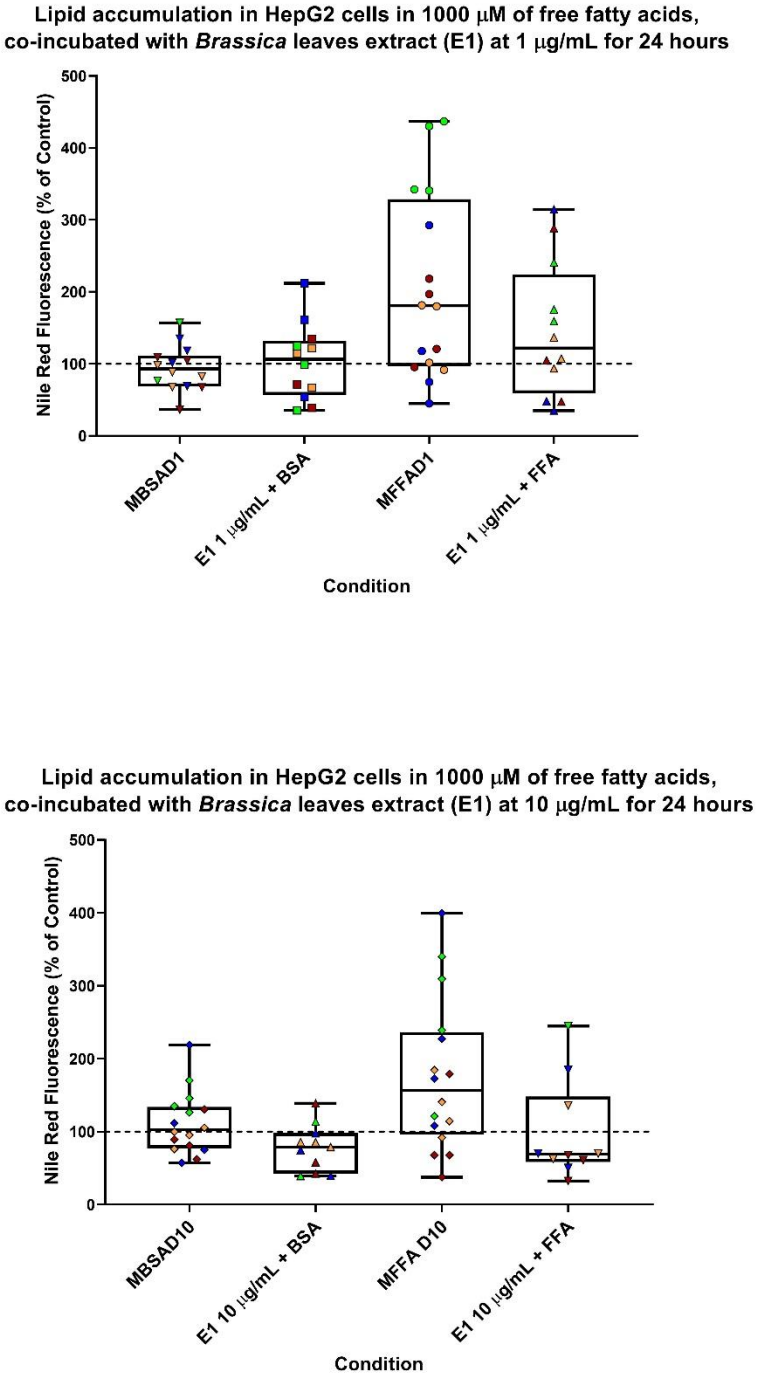


Figure 67 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.

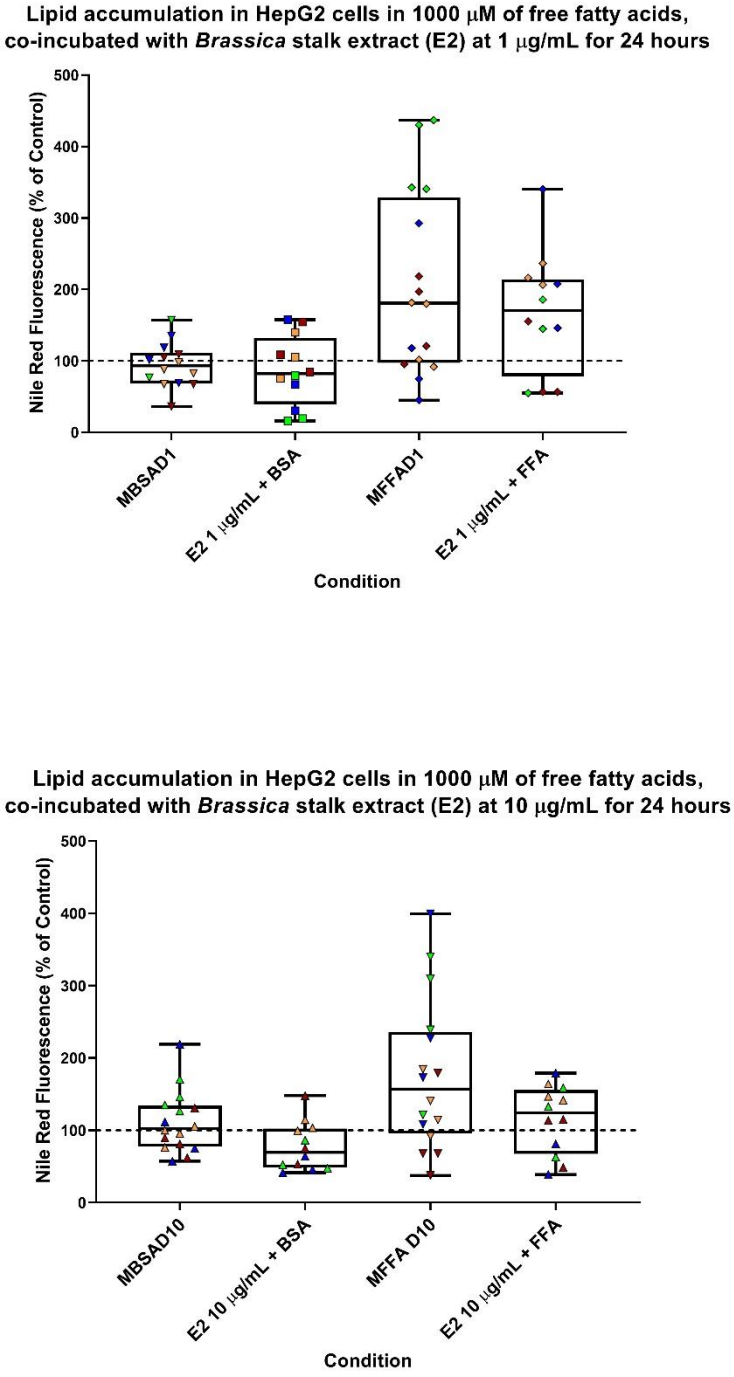
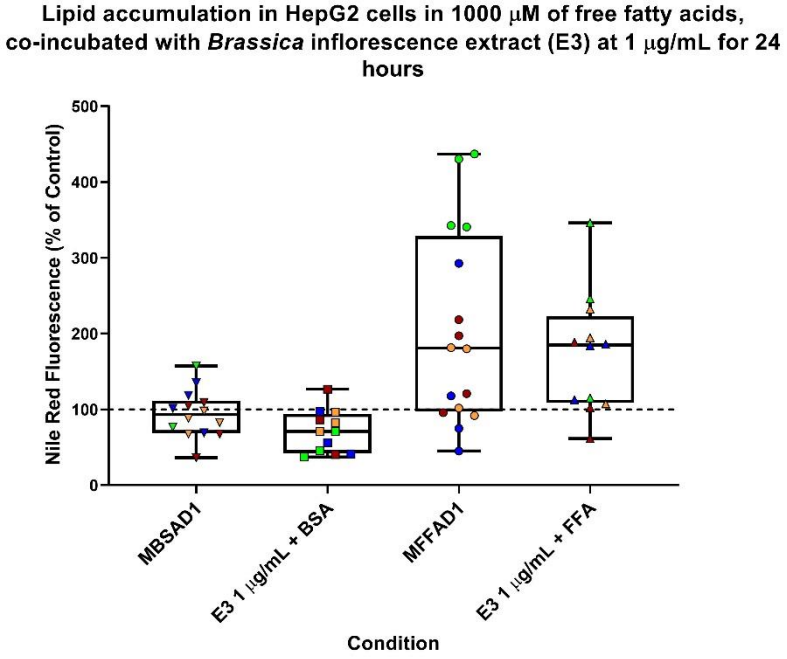


Figure 68 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.



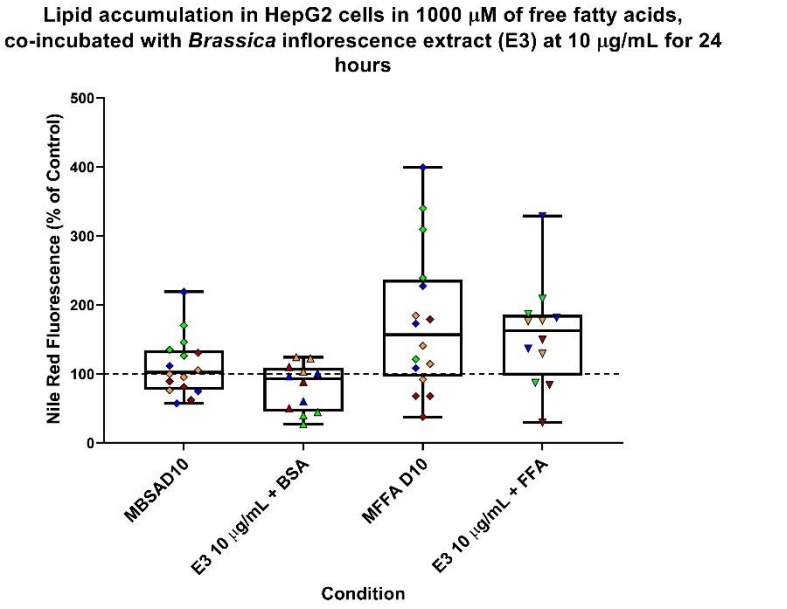


Figure 69 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

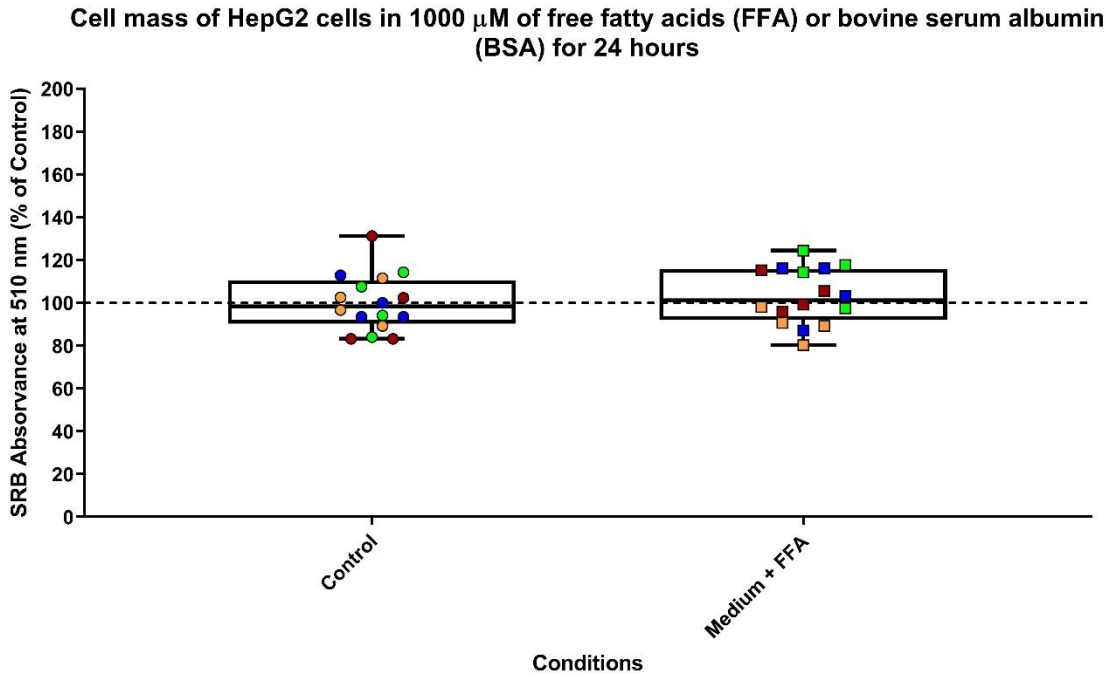


Figure 70 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

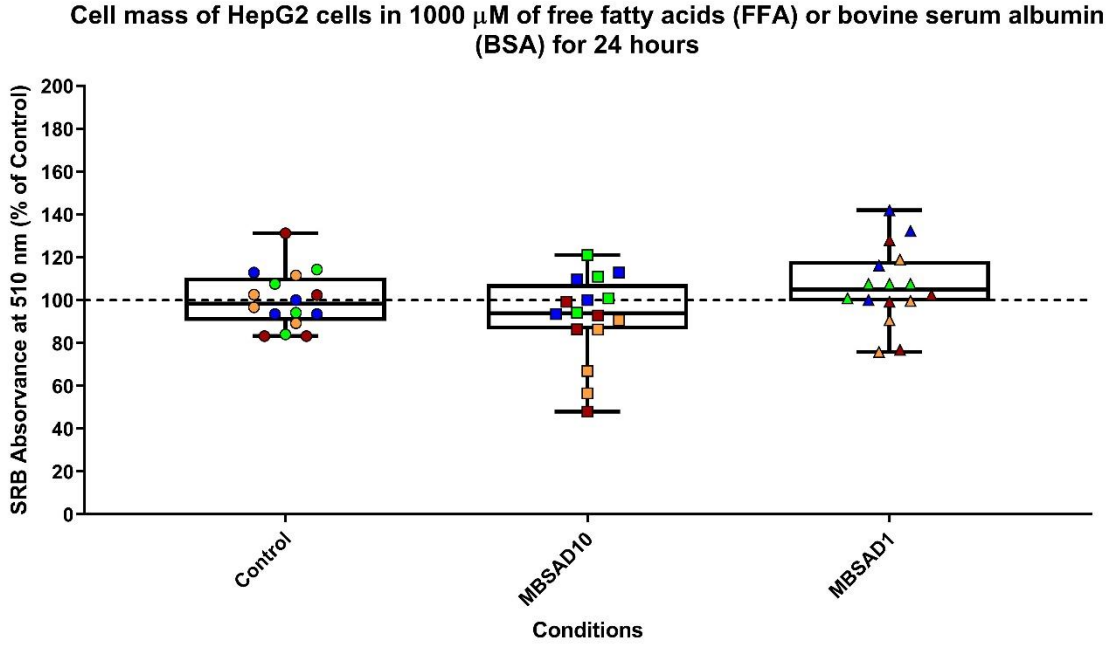


Figure 71 – Cell mass of HepG2 cells in DMSO and BSA (2%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

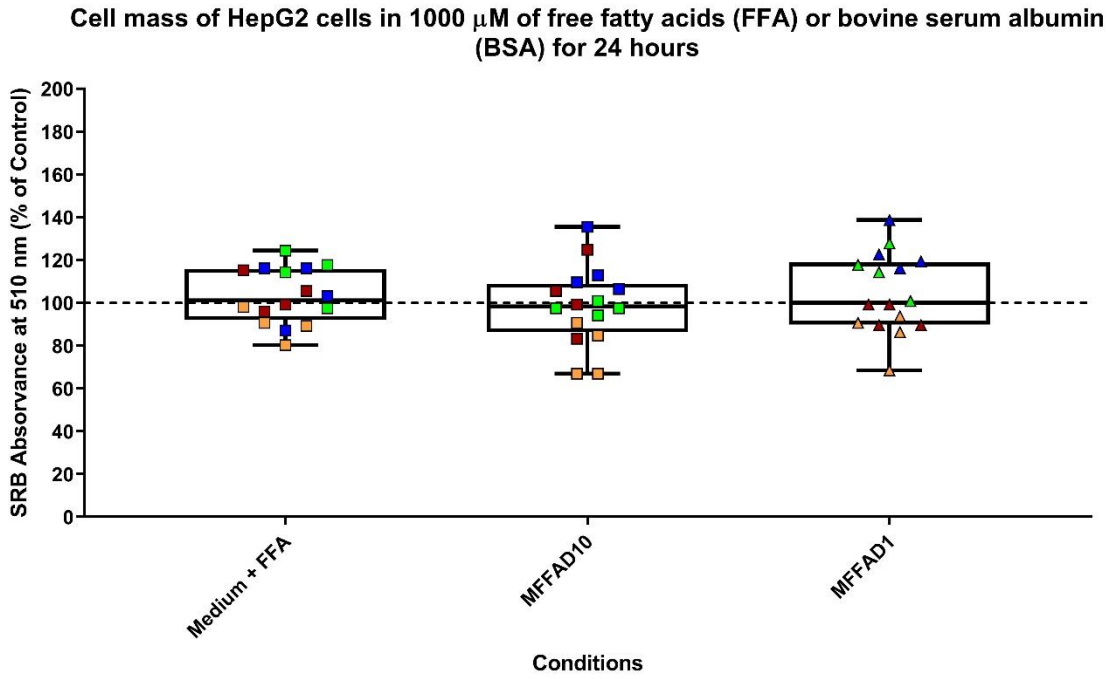


Figure 72 - Cell mass of HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture media with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

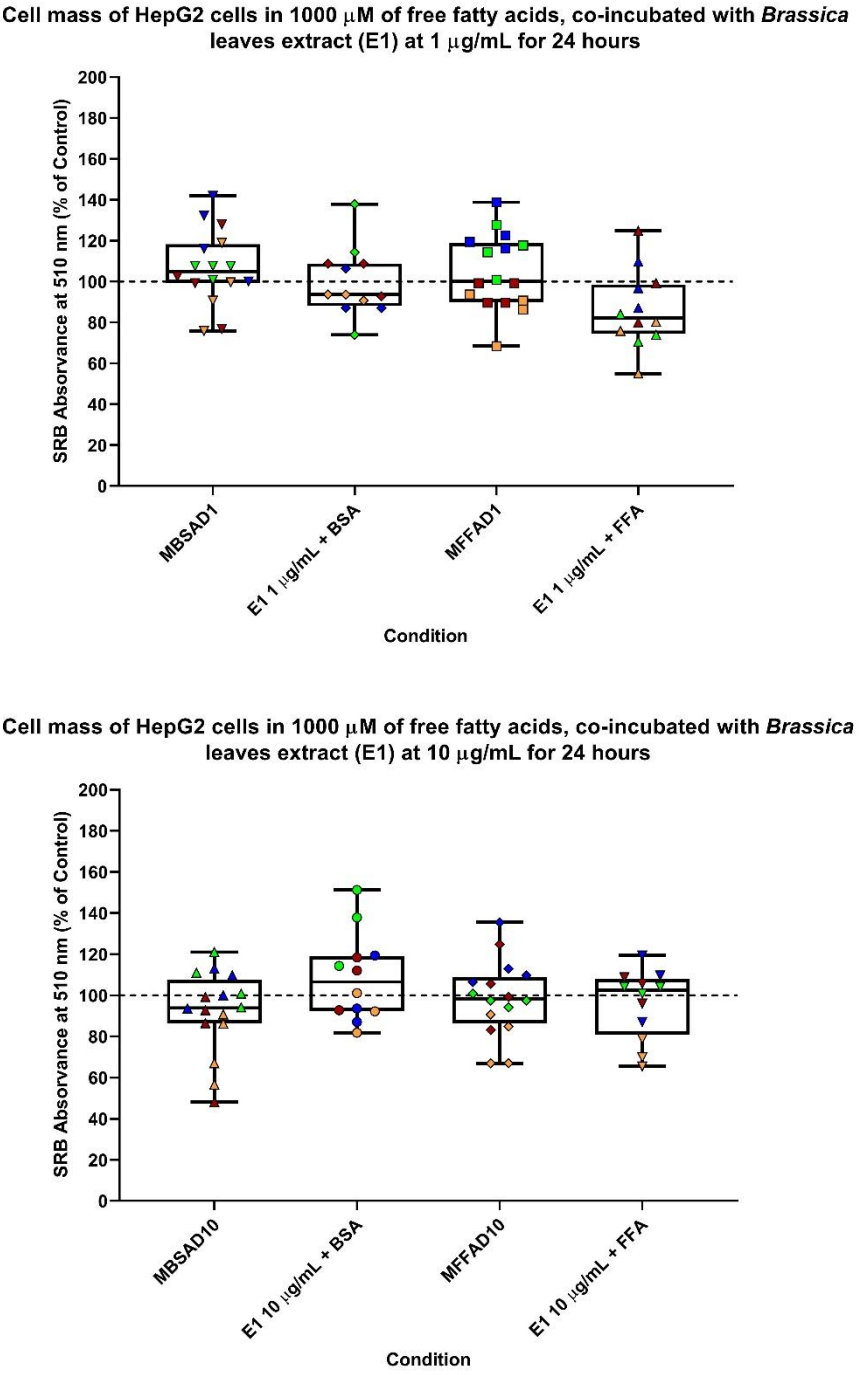


Figure 73 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.

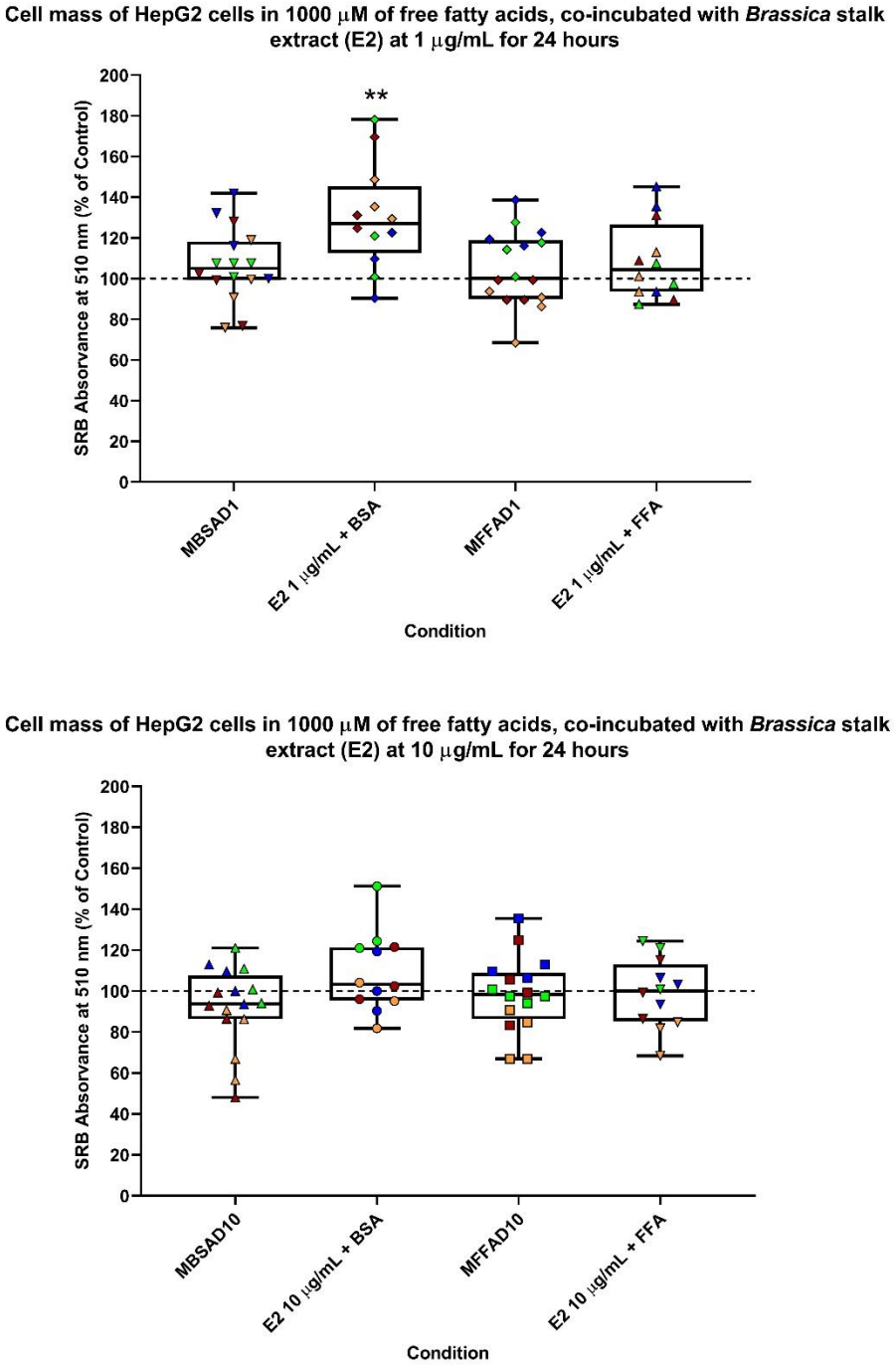


Figure 74 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0092 vs MBSAD1. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.

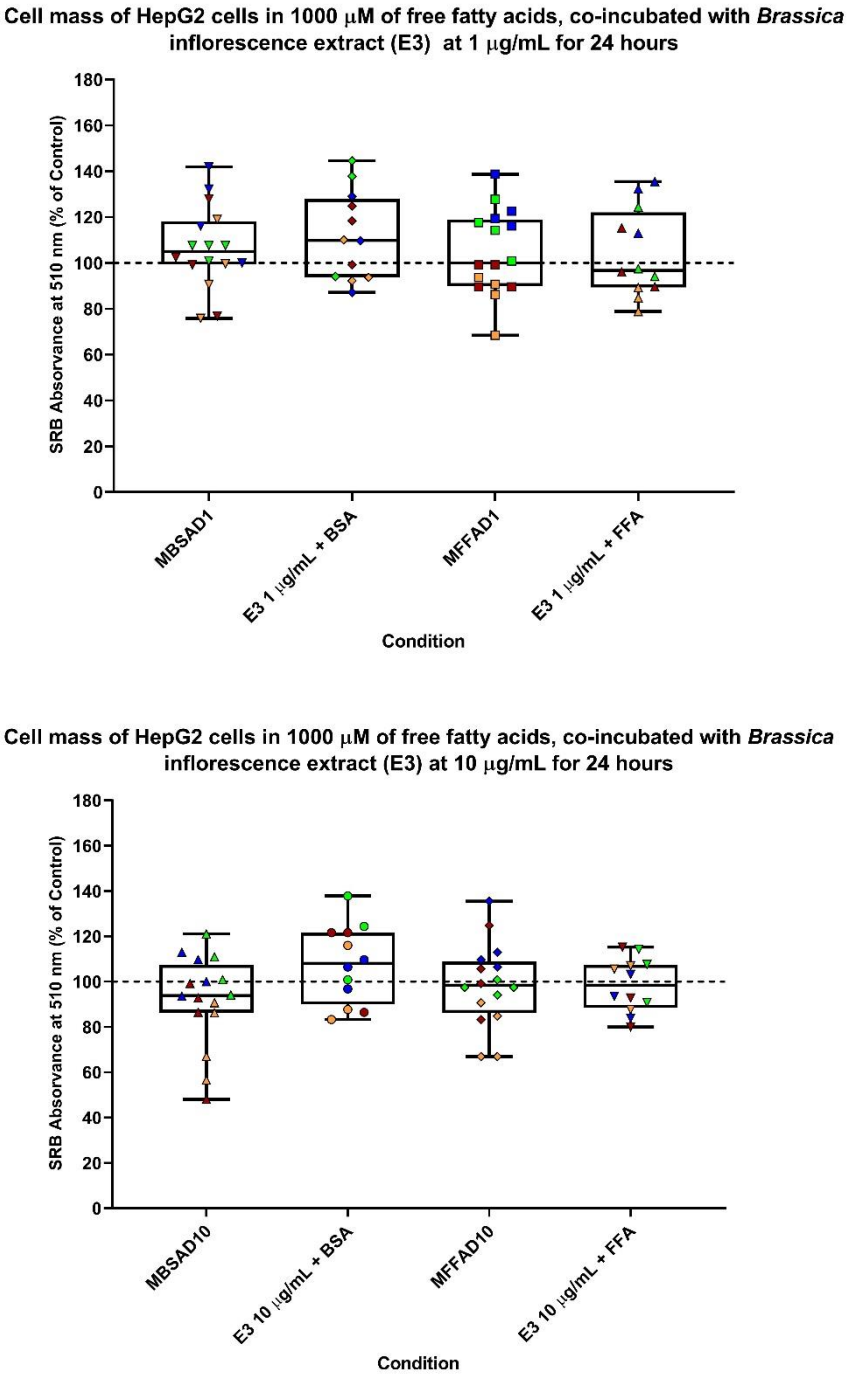


Figure 75 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

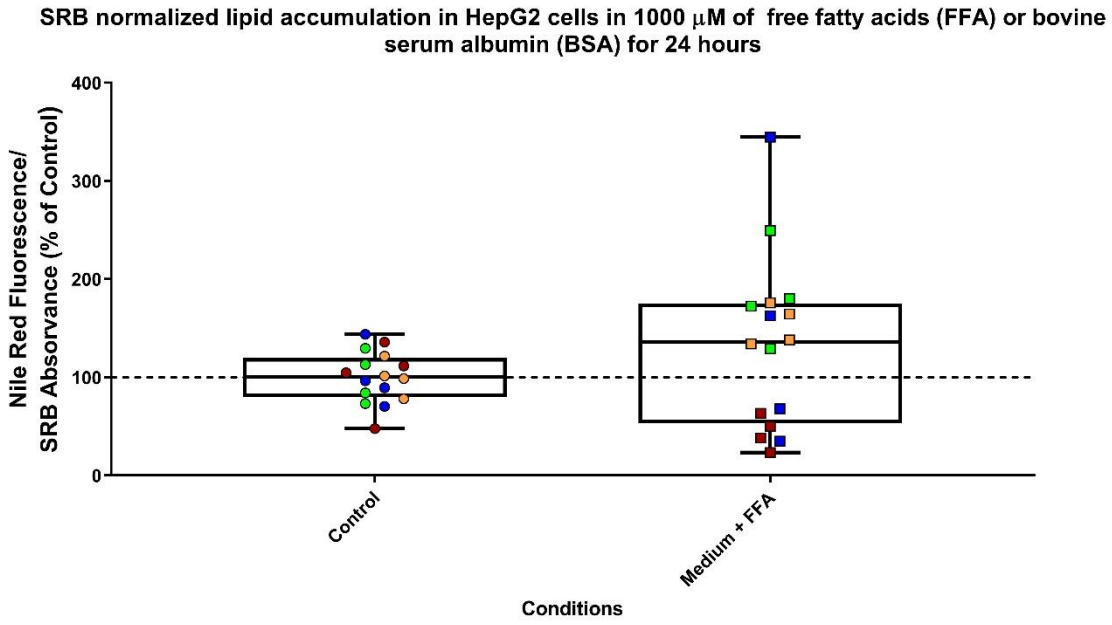


Figure 76 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **Medium + FFA:** Cell culture medium with 1000 µM of FFA.

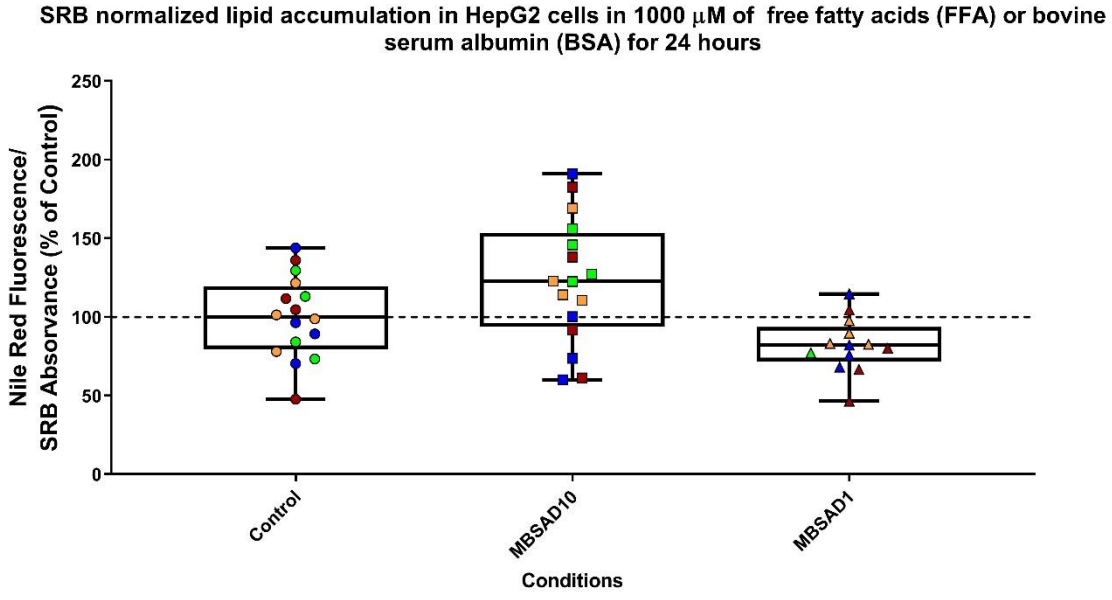


Figure 77 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (2%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

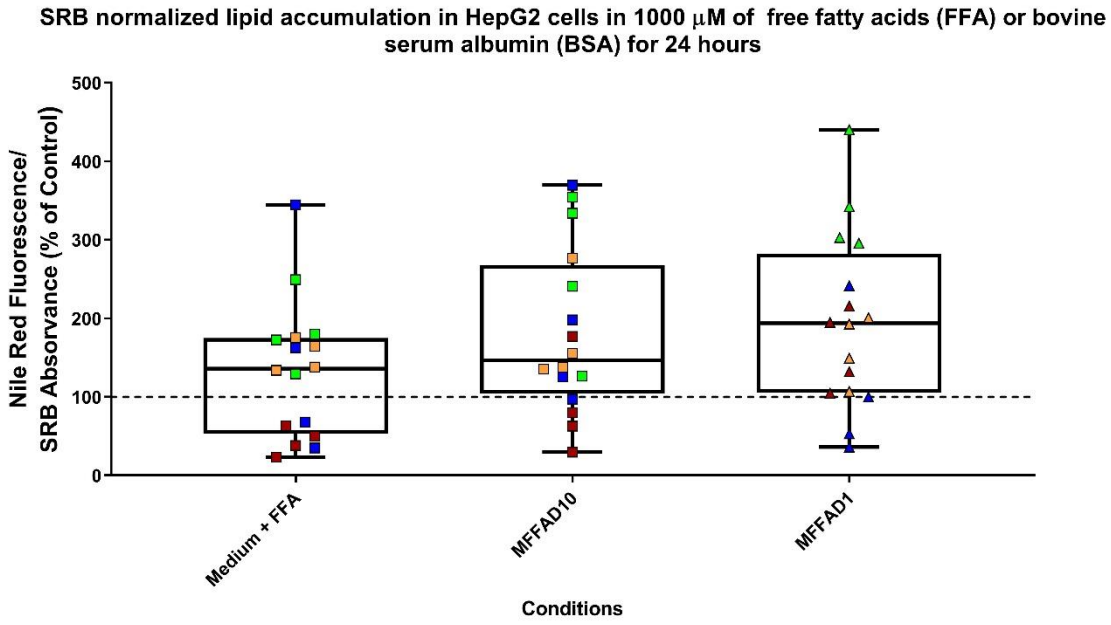


Figure 78 - SRB normalized lipid accumulation in HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

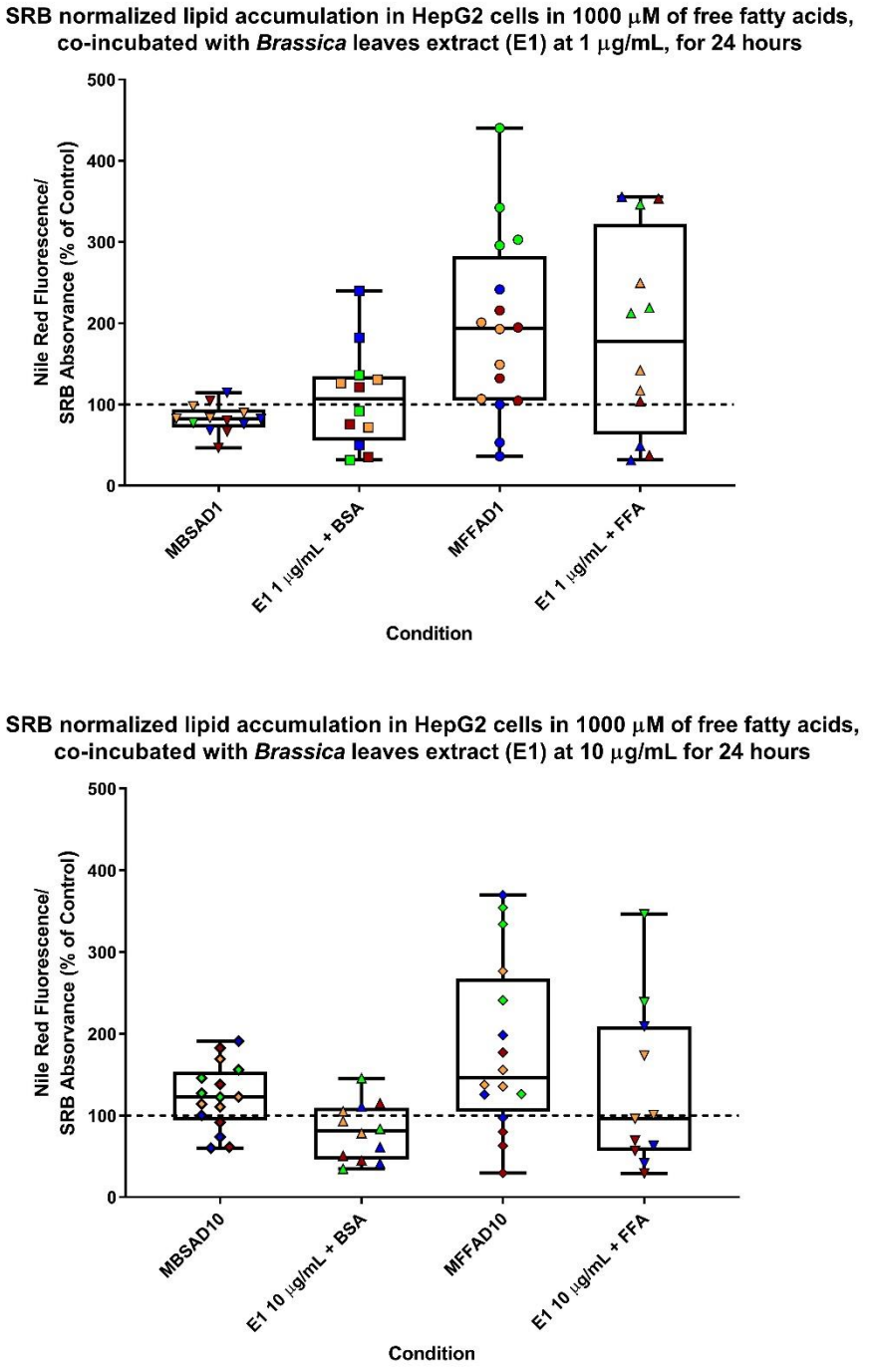


Figure 79 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.

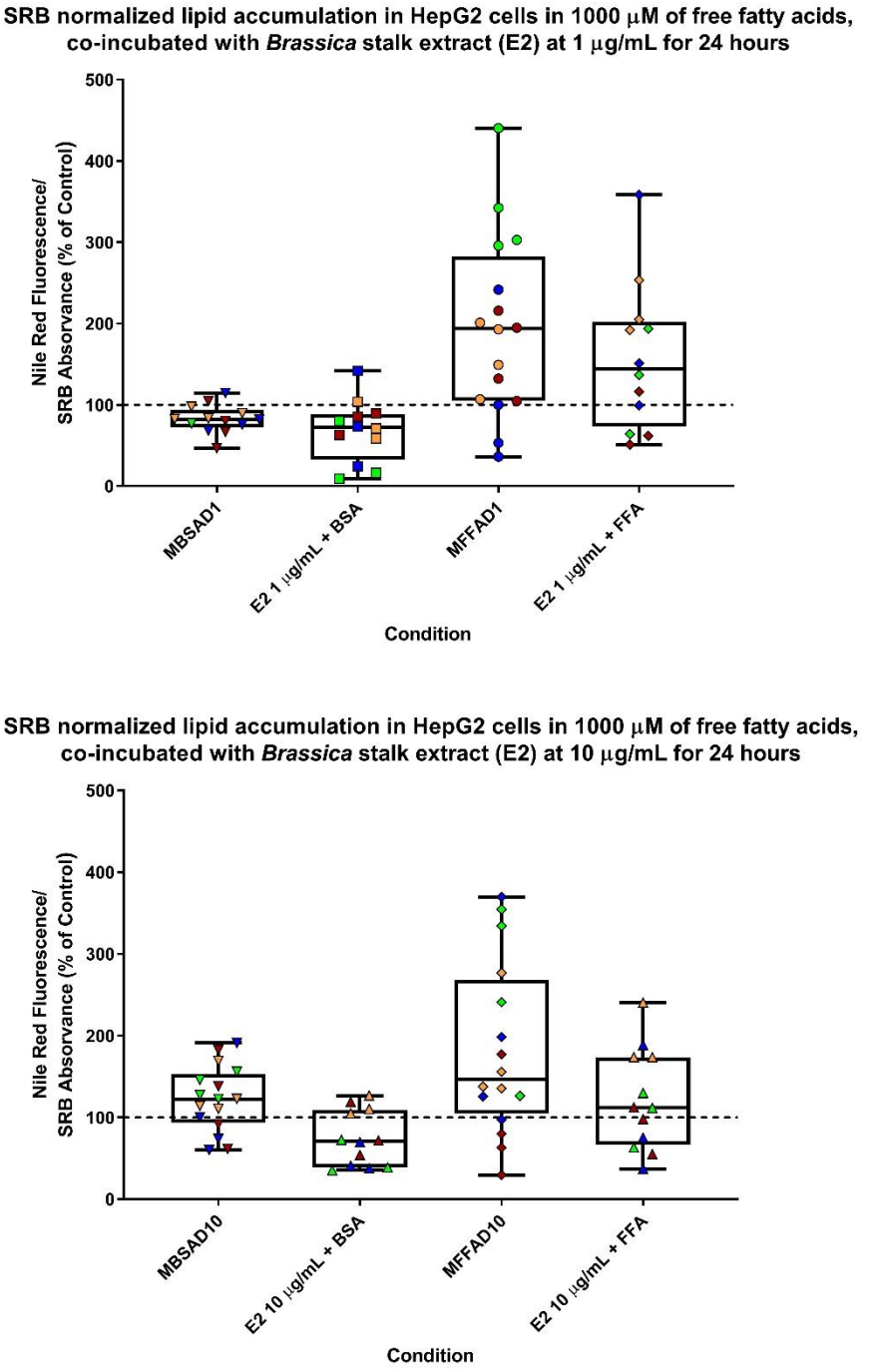


Figure 80 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.

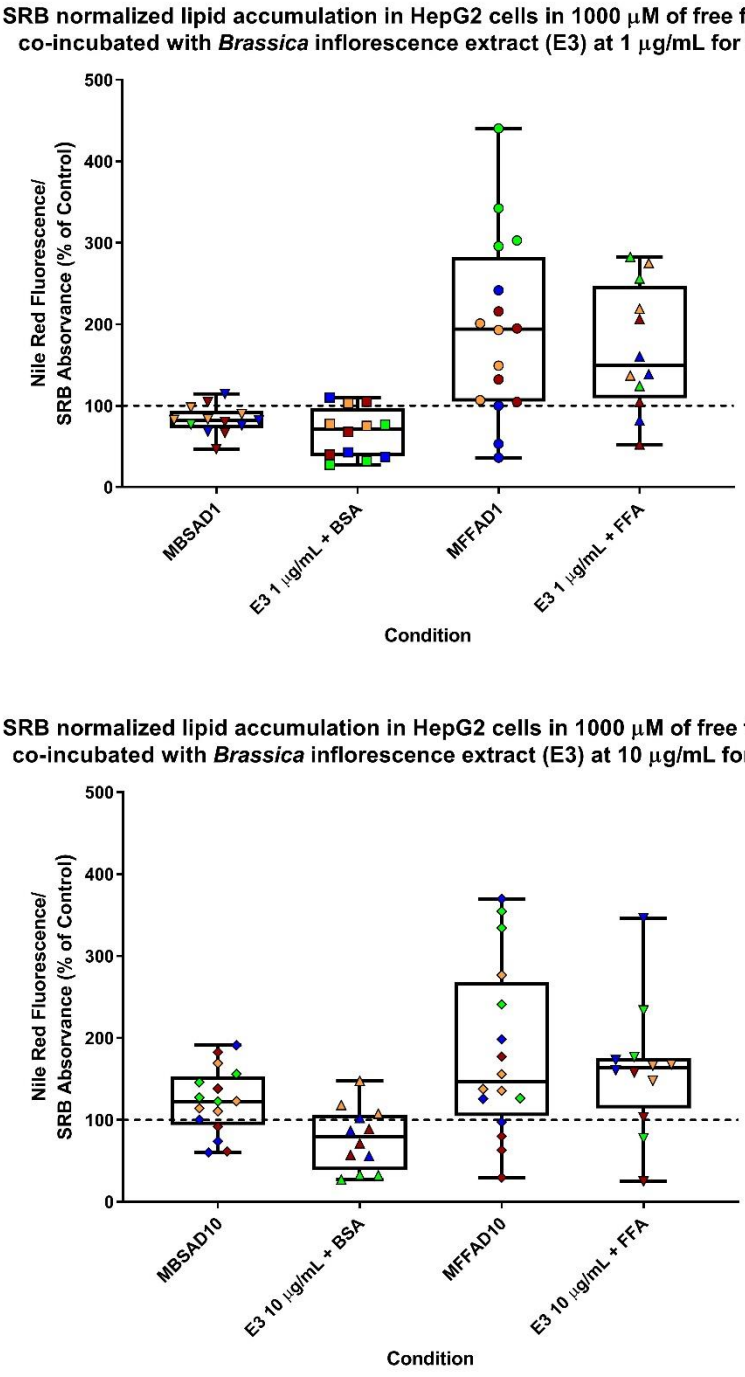


Figure 81 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

4.2.3. Co-incubation of extracts and 2000 µM of free fatty acids for 24 hours

HepG2 cells were co-incubated with the extracts and 1000 µM of FFA for 24 hours. This approach was taken to test the extracts capability of triggering a cell response to deal with a lipotoxic environment.

The leaves extract at 1 and 10 µg/mL statistically significantly reduced cell mass of HepG2 cells compared to control groups, when co-incubated with both BSA and FFA. Leaves extract at 1 µg/mL also caused a statistically significant increase in SRB normalized lipid accumulation.

The stalk and inflorescence extract at 10 µg/mL statistically significantly prevented cell mass loss due to FFA lipotoxicity and caused a statistically significant decreased in SRB normalized lipid accumulation when compared to control groups.

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

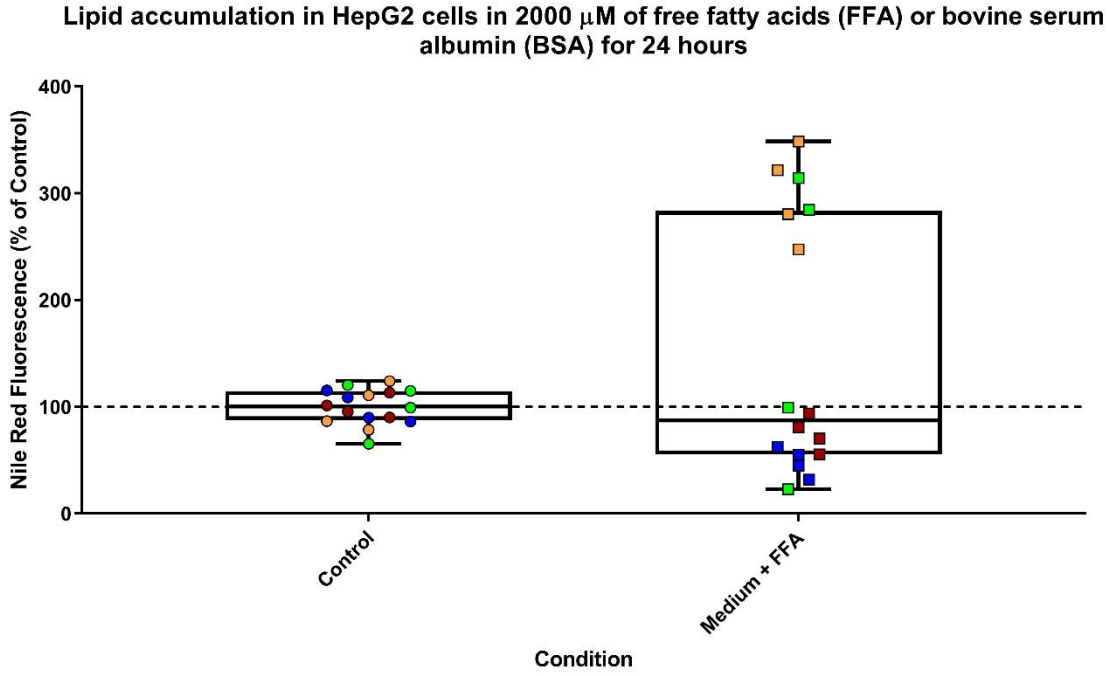


Figure 82 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA) or BSA (4%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **Medium + FFA:** Cell culture medium with 2000 µM of FFA.

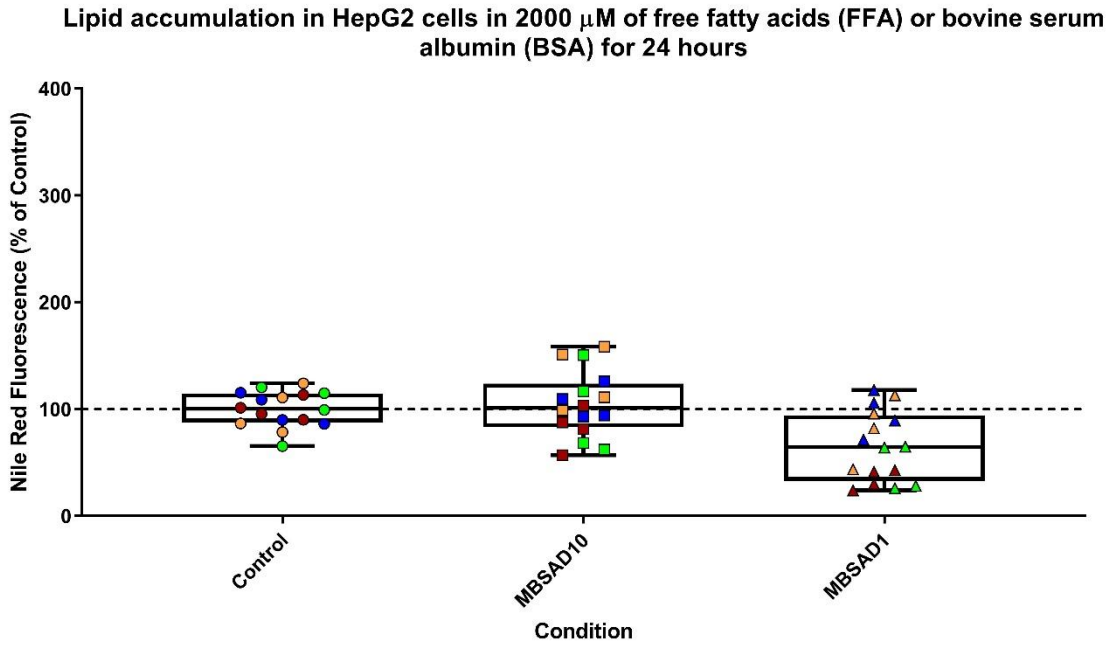


Figure 83 - Lipid accumulation in HepG2 cells in DMSO and BSA (4%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

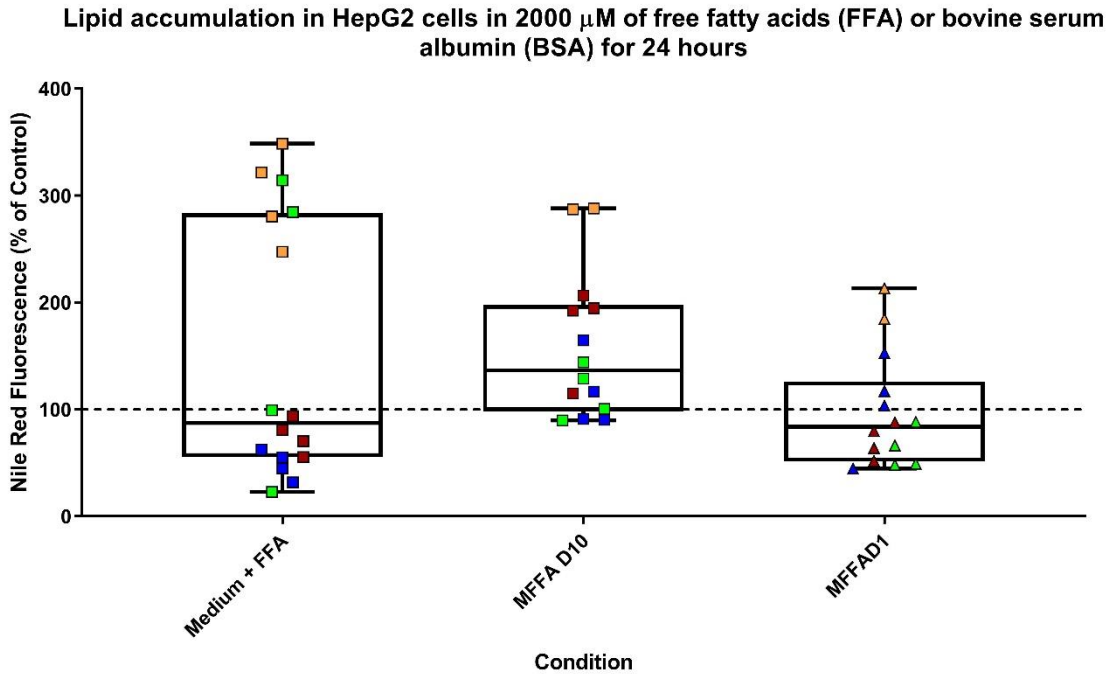


Figure 84 - Lipid accumulation in HepG2 cells in DMSO and 2000 μM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 2000 μM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 μM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 μM of FFA and DMSO (0,002%).

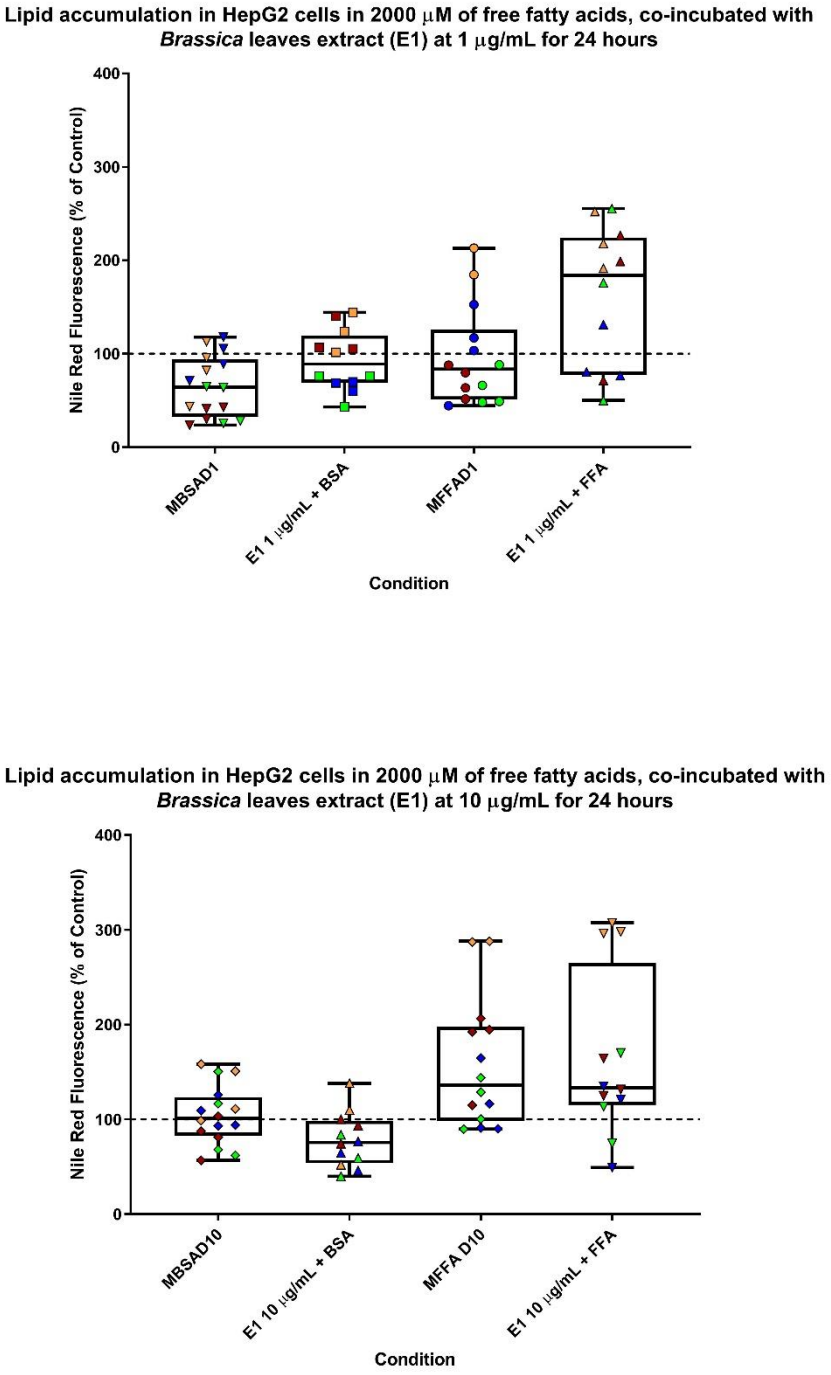
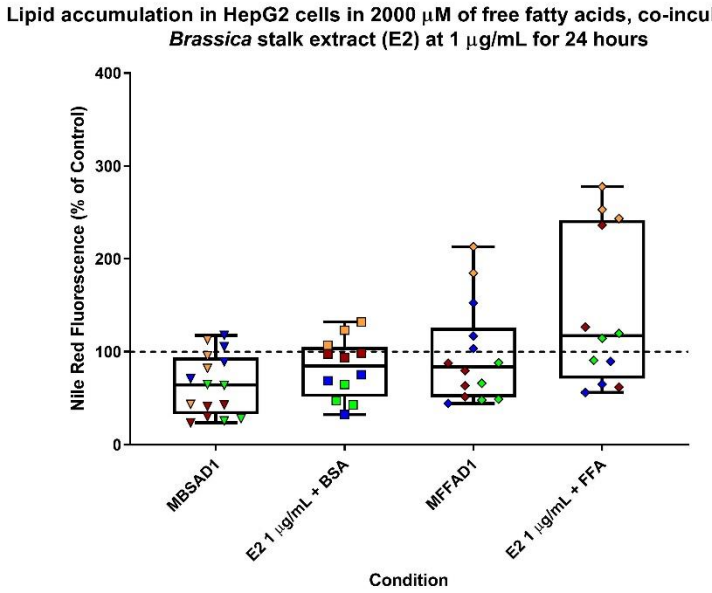


Figure 85 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.



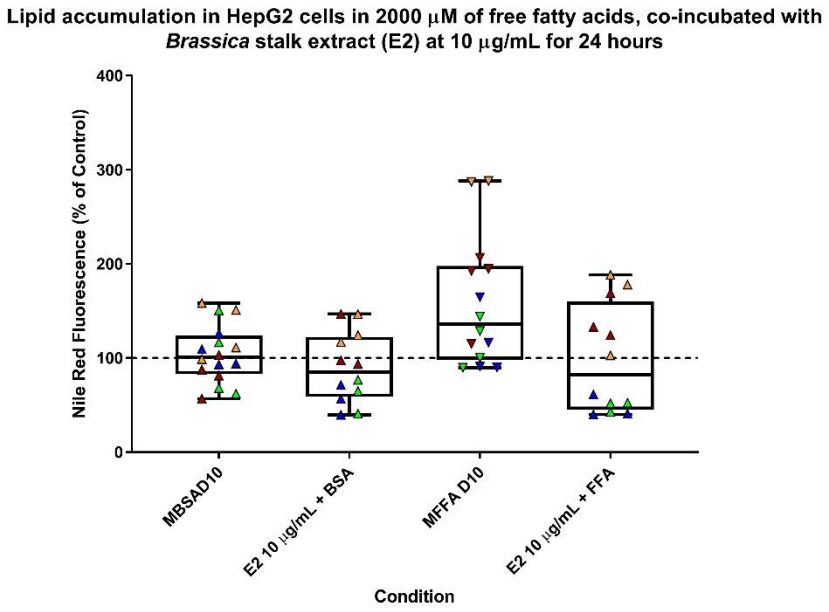


Figure 86 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.

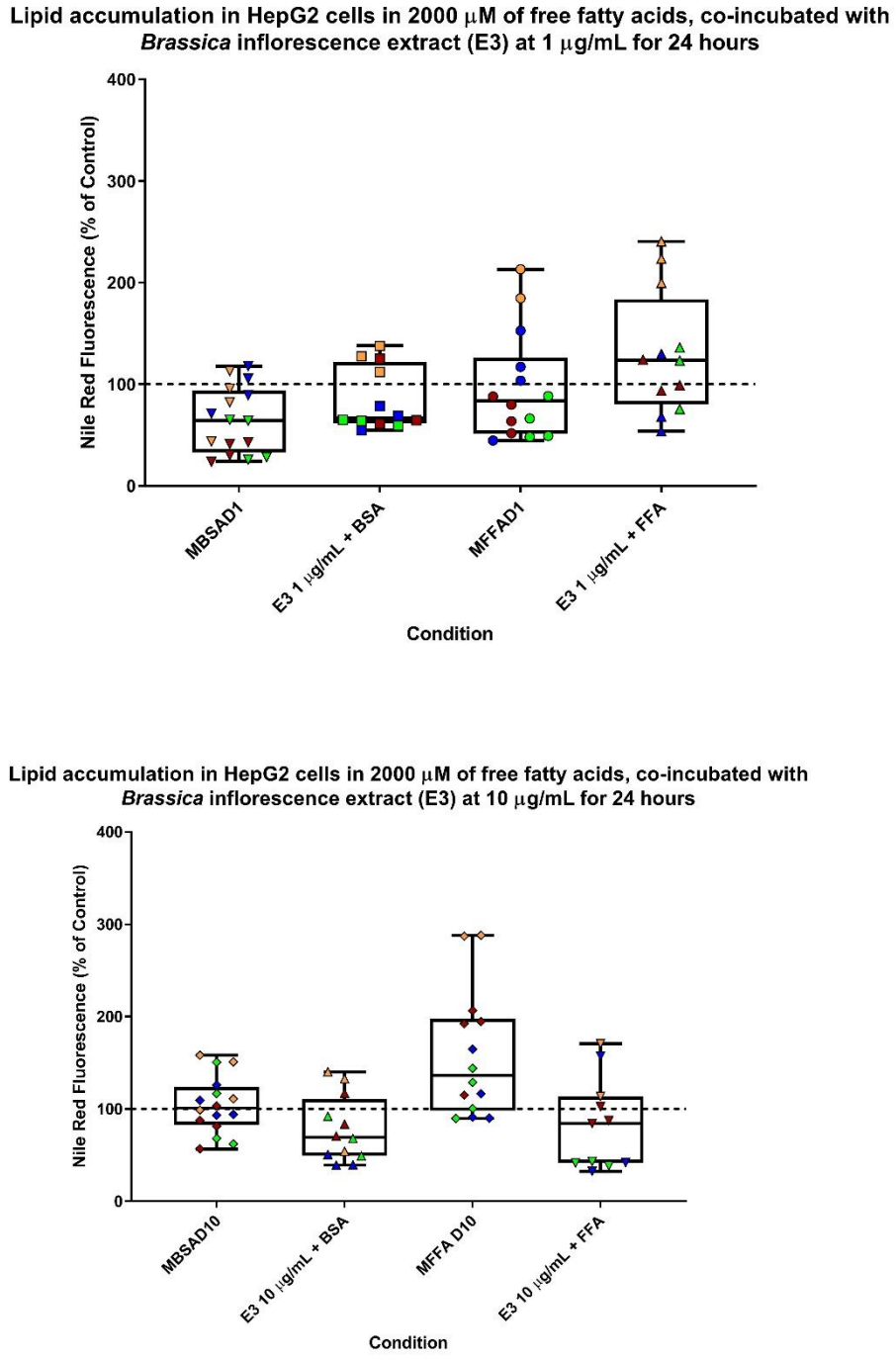


Figure 87 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

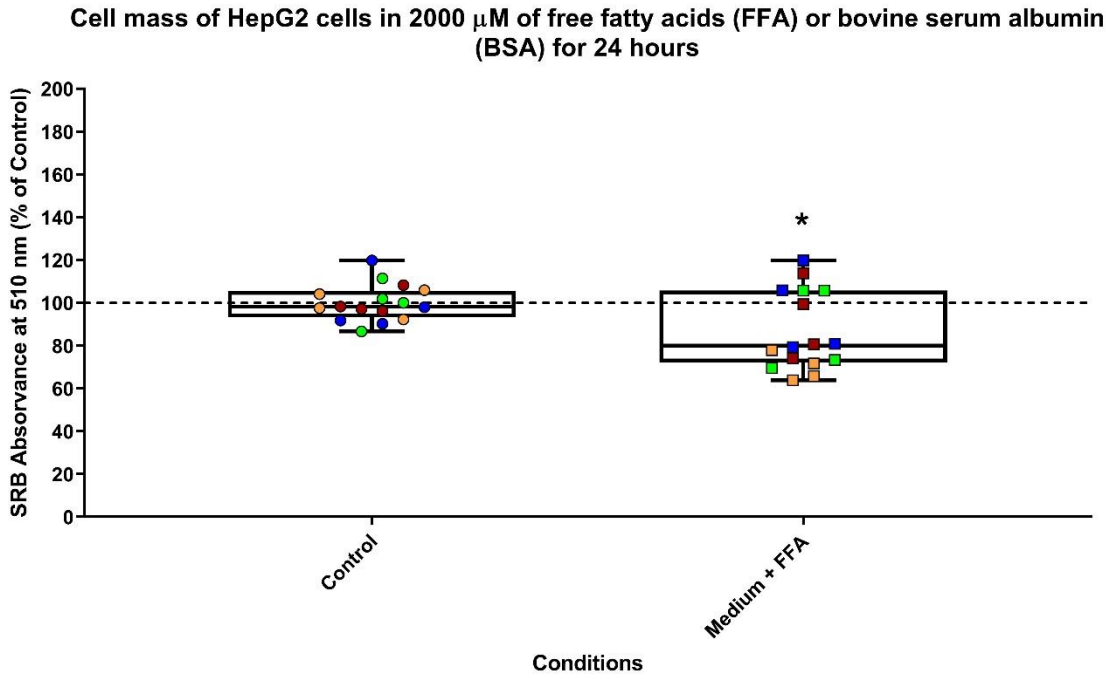


Figure 88 - Cell mass of HepG2 cells in 1000 μM of free fatty acids (FFA) or BSA (4%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0161 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 μM of FFA.

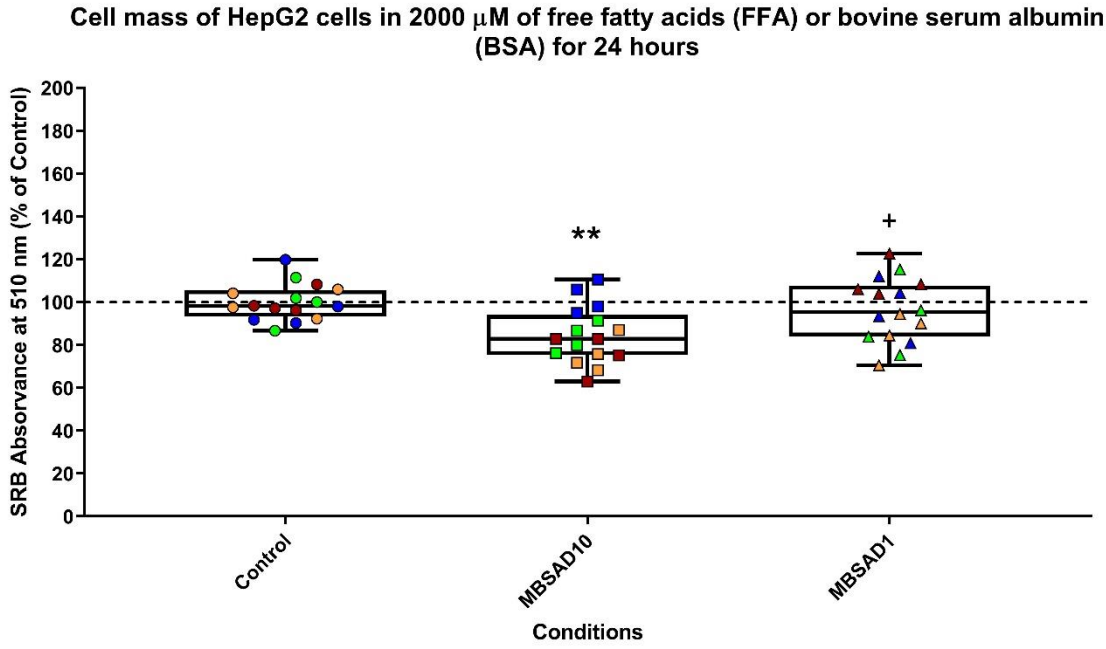


Figure 89 - Cell mass of HepG2 cells in DMSO and BSA (4%) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0028 vs Control; +p=0.0260 vs MBSAD10; **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

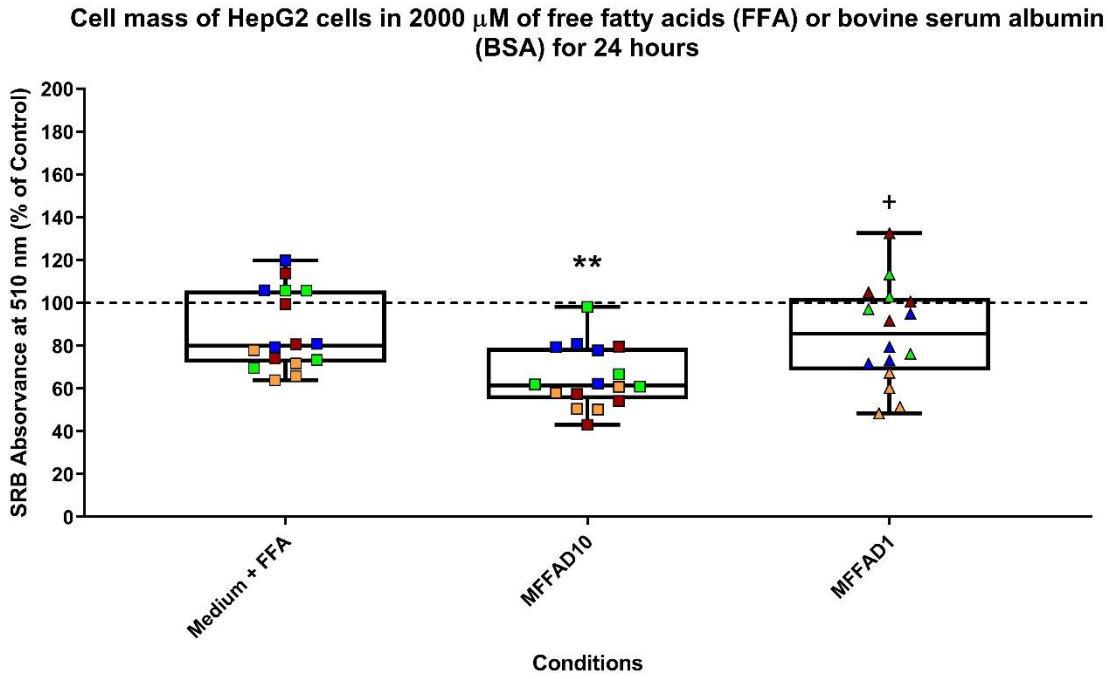


Figure 90 - HepG2 cells in DMSO and 2000 µM of free fatty acids (FFA) for 24 hours. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0069 vs Medium + FFA; +p=0.119 vs MFFAD10. **Medium + FFA:** Cell culture medium with 2000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%).

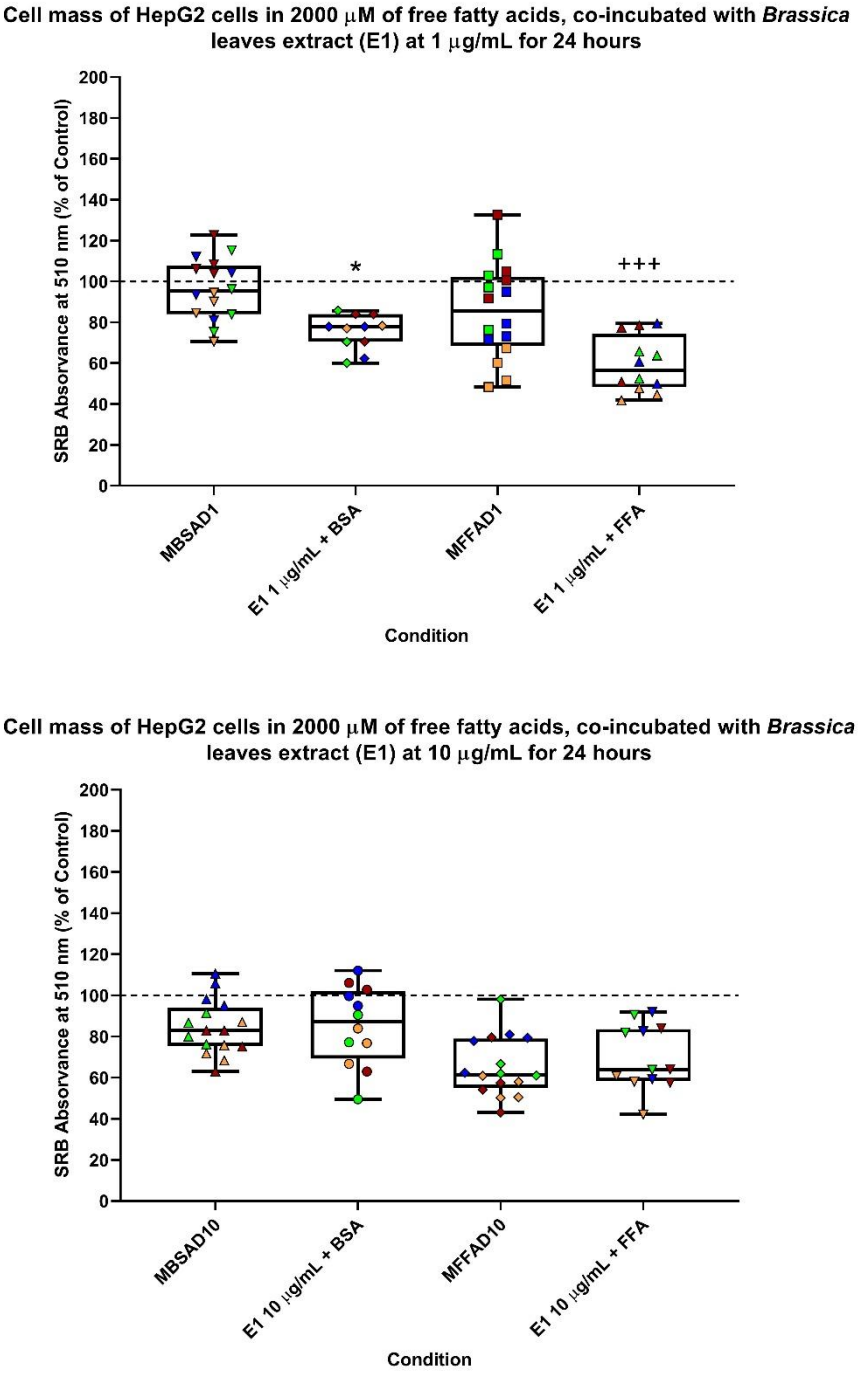


Figure 91 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0116 vs MBSAD1; +++p=0.0009 vs MFFAD1. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.

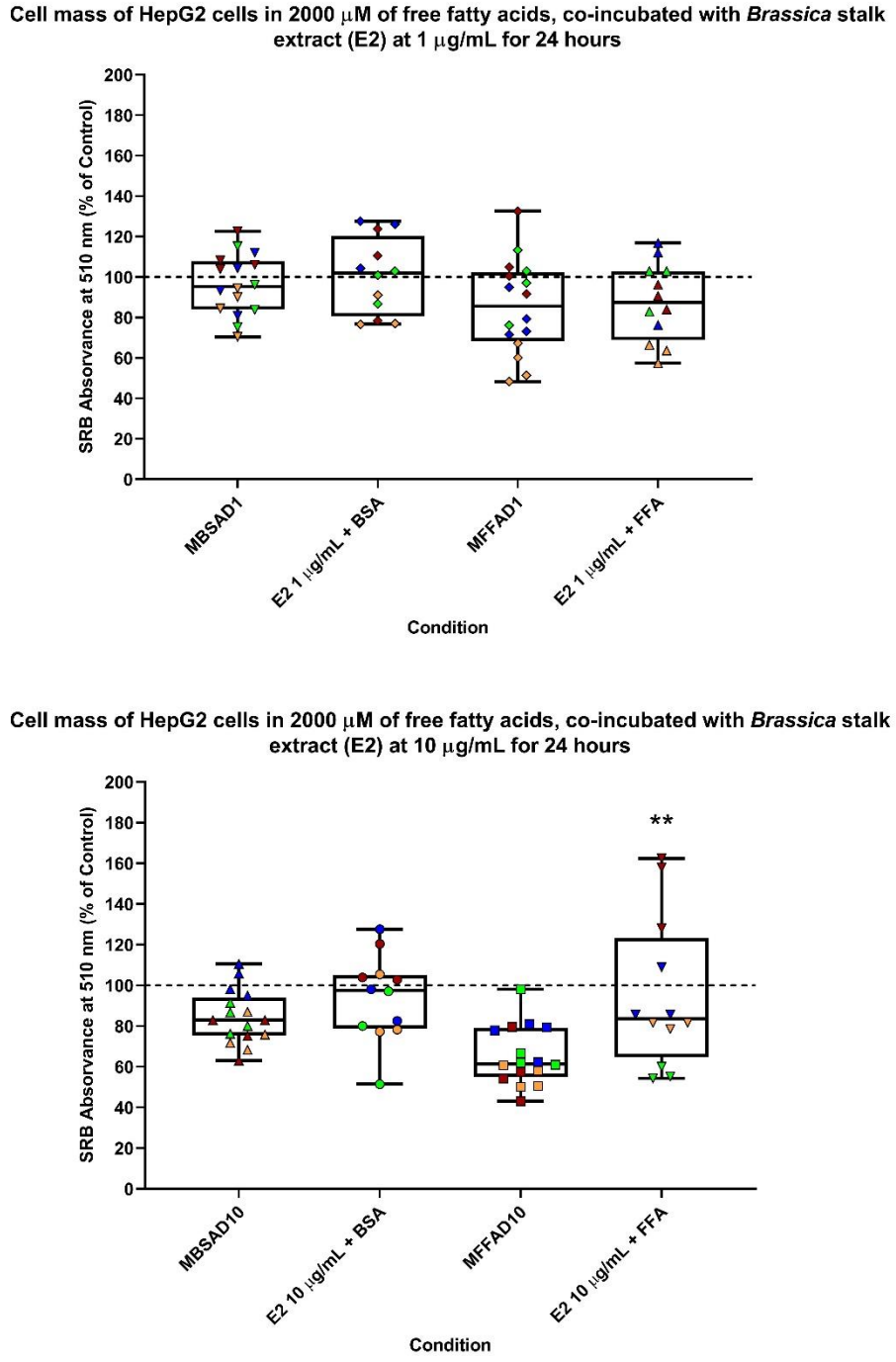
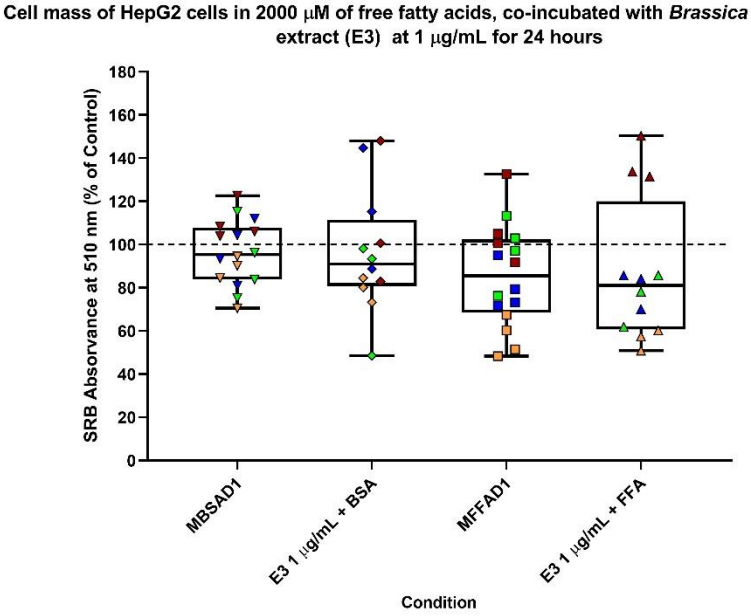


Figure 92 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0048 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.



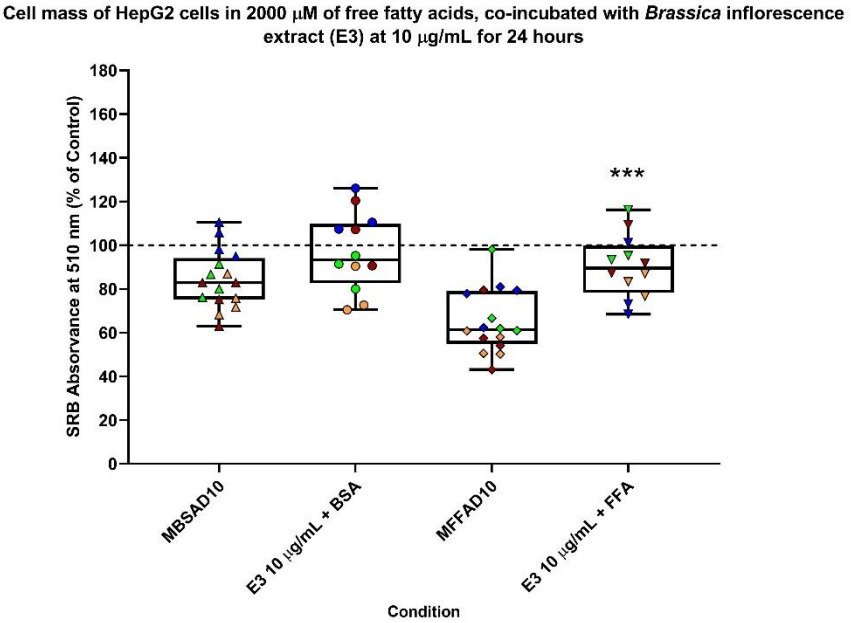


Figure 93 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0003 vs MFFAD10 **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 2000 µM of FFA.

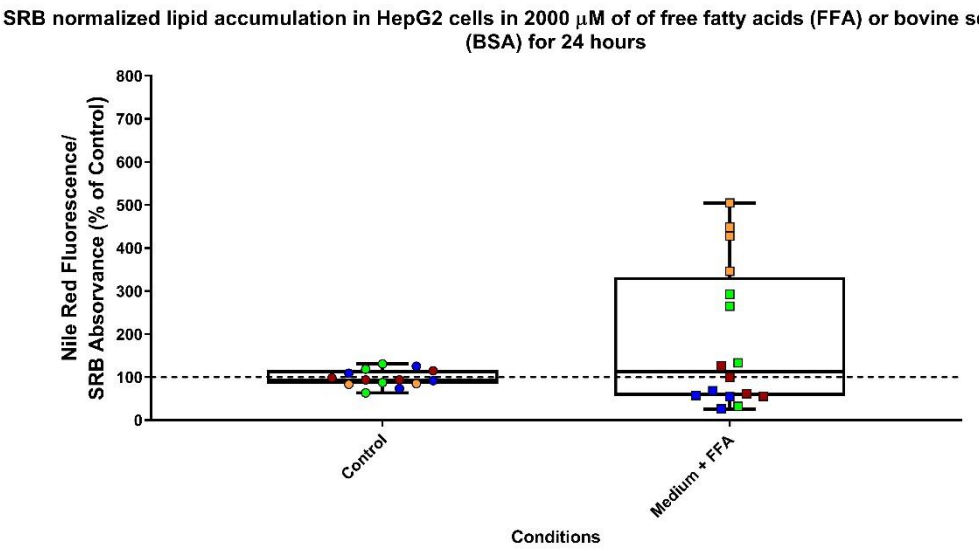


Figure 94 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA) or BSA (4%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **Medium + FFA:** Cell culture medium with 2000 µM of FFA.

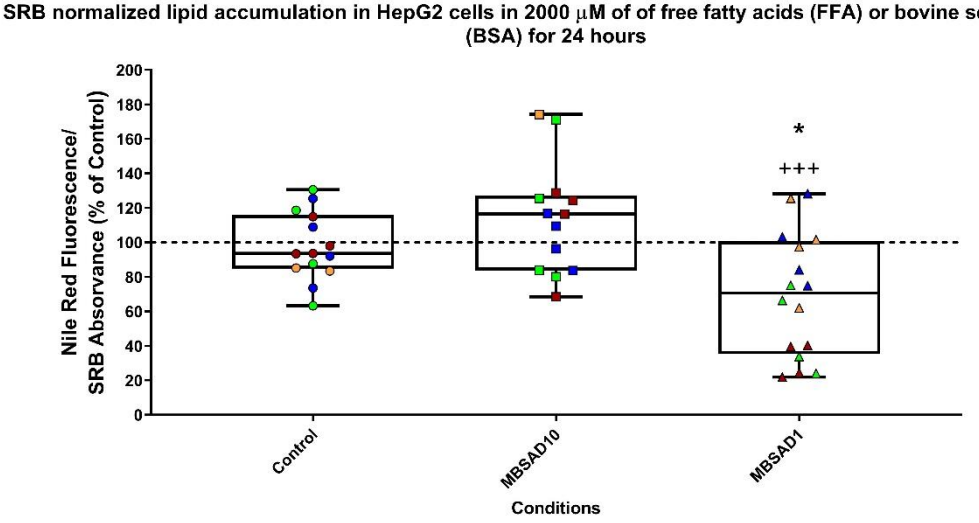


Figure 95 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (4%) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0345 vs Control; +++p=0.0009 vs MBSAD10; **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

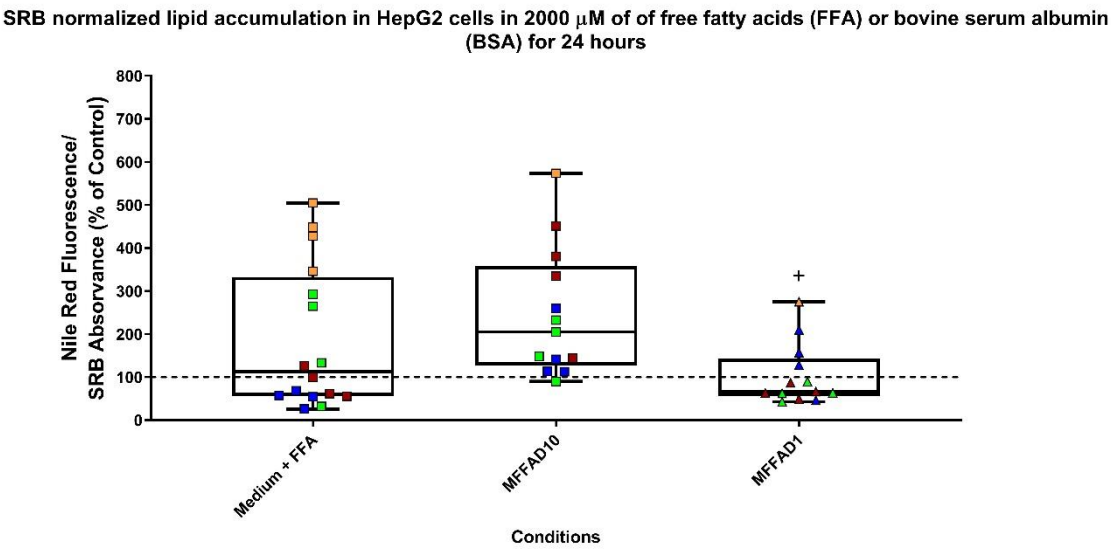


Figure 96 - SRB normalized lipid accumulation in HepG2 cells in DMSO and 2000 µM of free fatty acids (FFA) for 24 hours. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. +p=0.0162 vs MFFAD10; **Medium + FFA:** Cell culture medium with 2000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%).

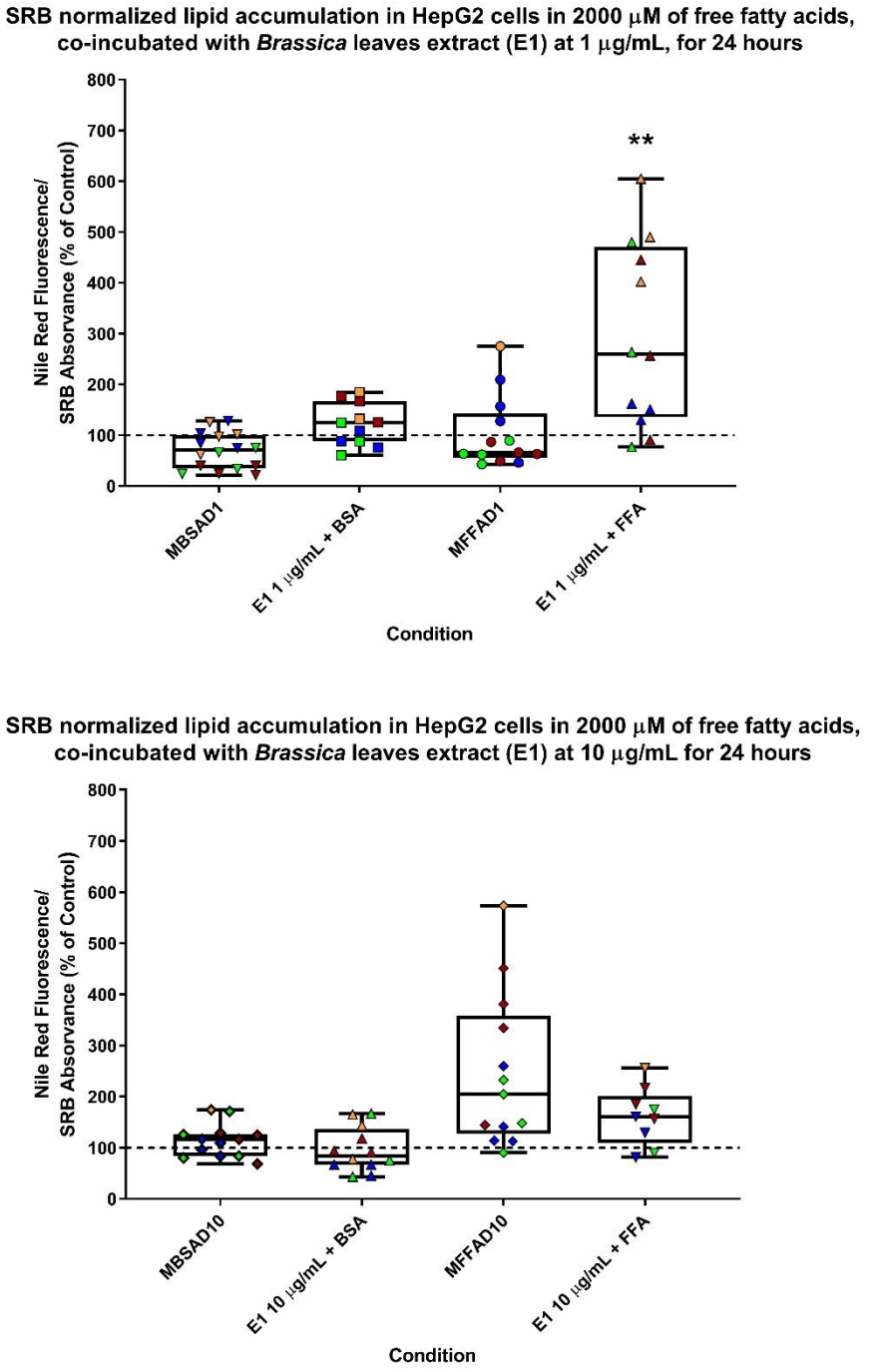
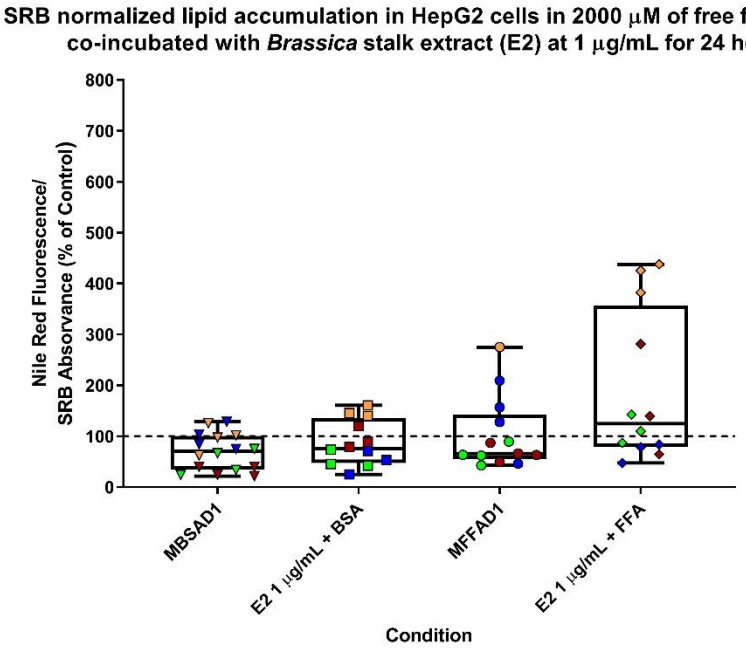


Figure 97 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0078 vs MFFAD1. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.



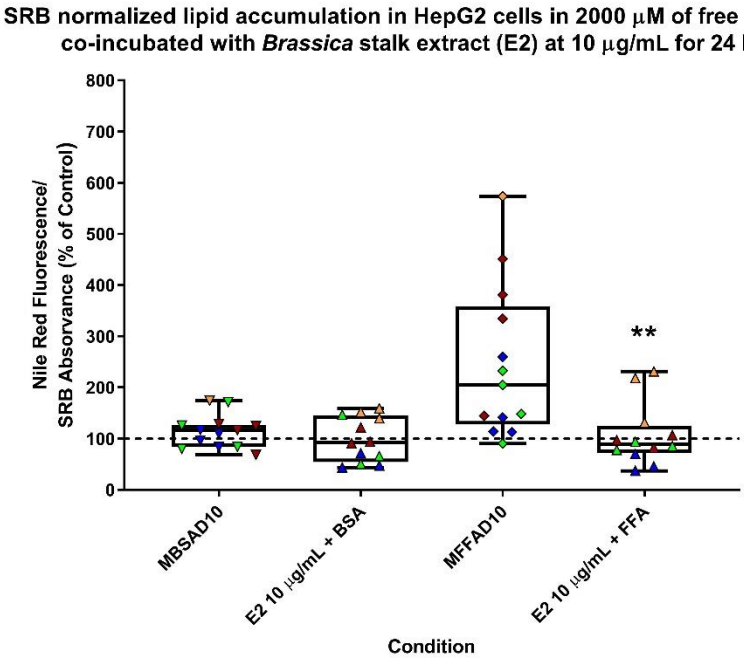


Figure 98 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0059 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.

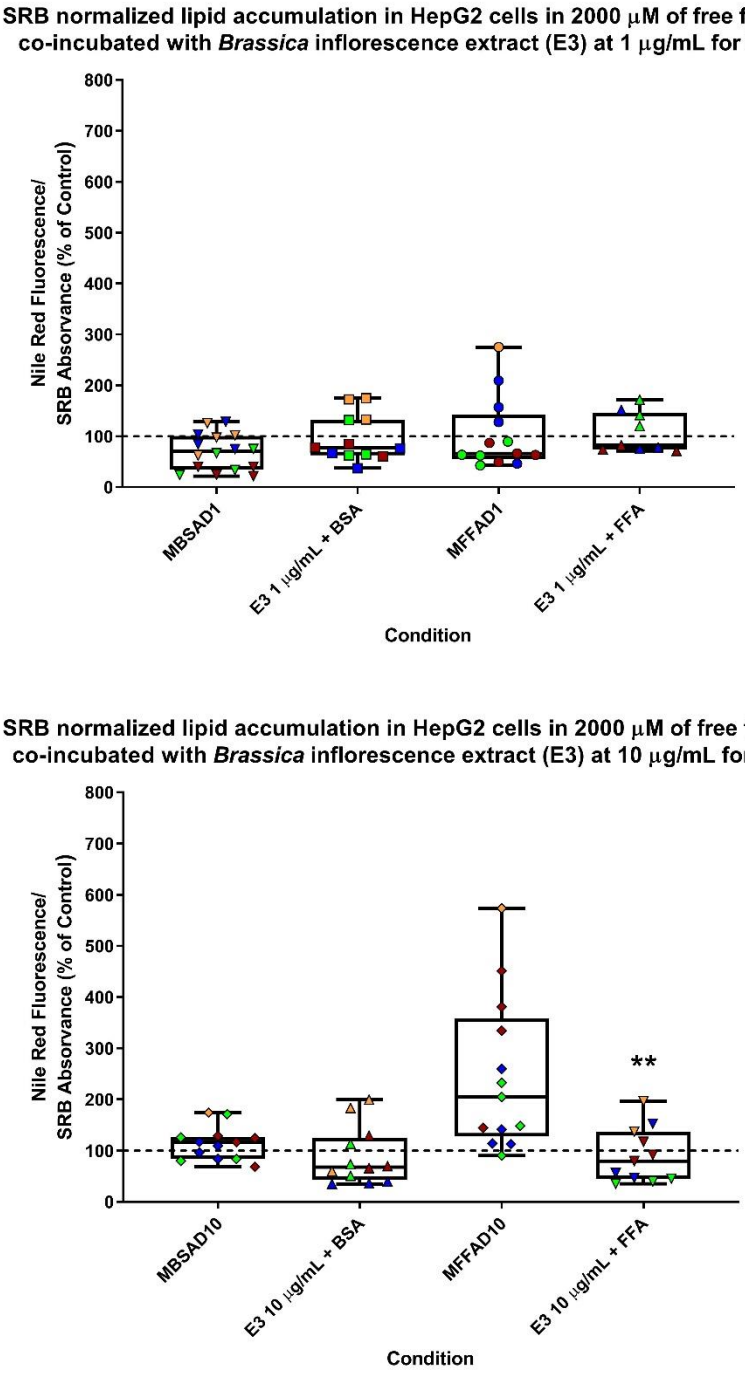


Figure 99 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0050 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 2000 µM of FFA.

4.2.4. Co-incubation of extracts and 250 µM of free fatty acids for 24 hours, after a 24 hour pre-incubation period with the aforementioned extracts

HepG2 cells were pre-incubated with the extracts for 24 hours and then co-incubated with the extracts and 250 µM of FFA for 24 hours. This approach was taken to test the extracts capability of triggering a cell response to deal with a moderate accumulation of lipids. The extra pre-incubation period was added to assess if this response is more immediate or if takes a longer time to fully form.

The leaves extract at 1 µg/mL statistically significantly reduced cell mass of HepG2 cells compared to control groups, when co-incubated with both BSA and FFA and stalk extract at 1 µg/mL statistically significantly reduced cell mass of HepG2 cells compared to the control group, when co-incubated with BSA.

Co-incubation of FFA with the leaves, stalk or inflorescence extracts at 10 µg/mL showed a statistically significant decrease in SRB normalized FFA accumulation on HepG2 cells when compared to their respective control group. The leaves extract at 1 µg/mL statistically significantly increased SRB normalized FFA accumulation on HepG2 cells when compared to the control group.

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

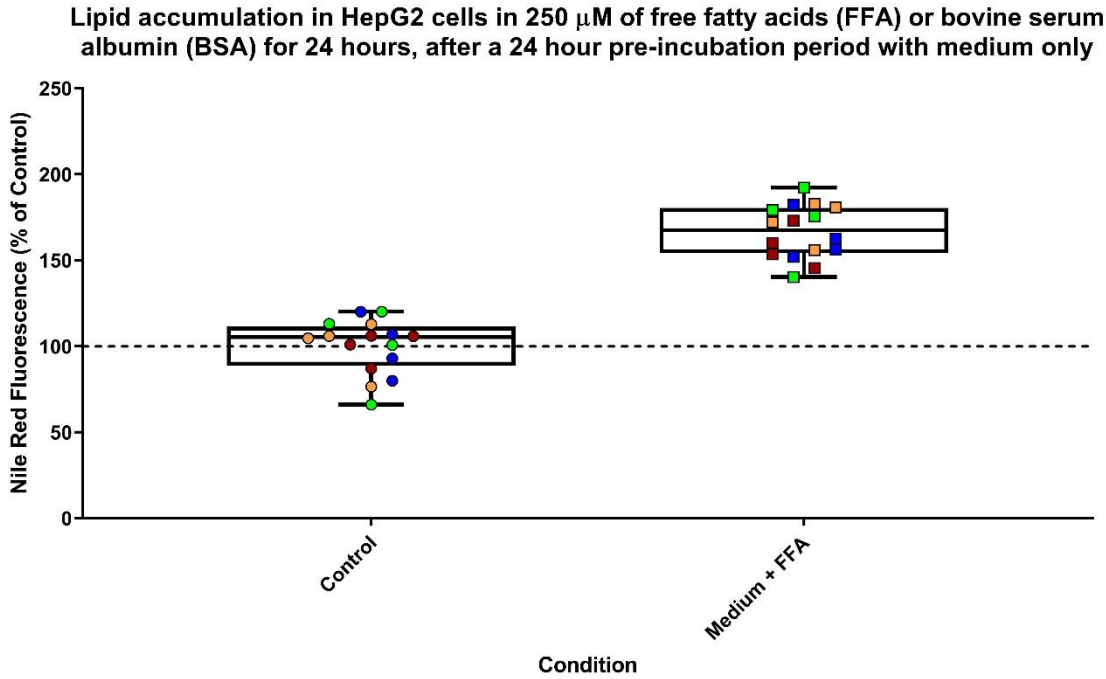


Figure 100 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA) or BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

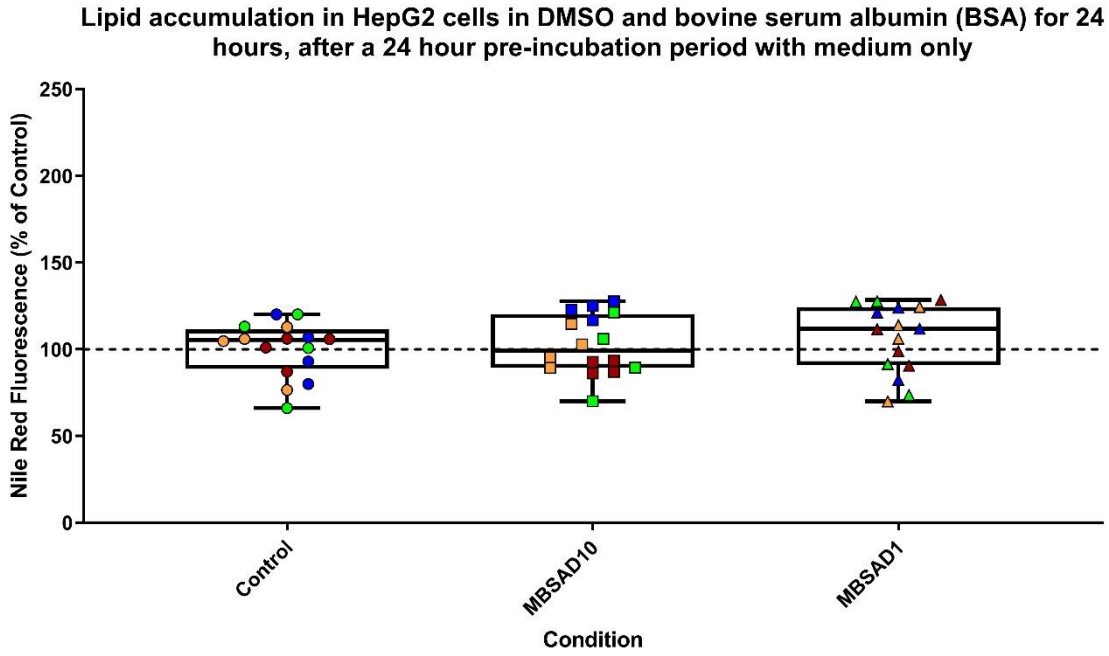


Figure 101 - Lipid accumulation in HepG2 cells in DMSO and BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

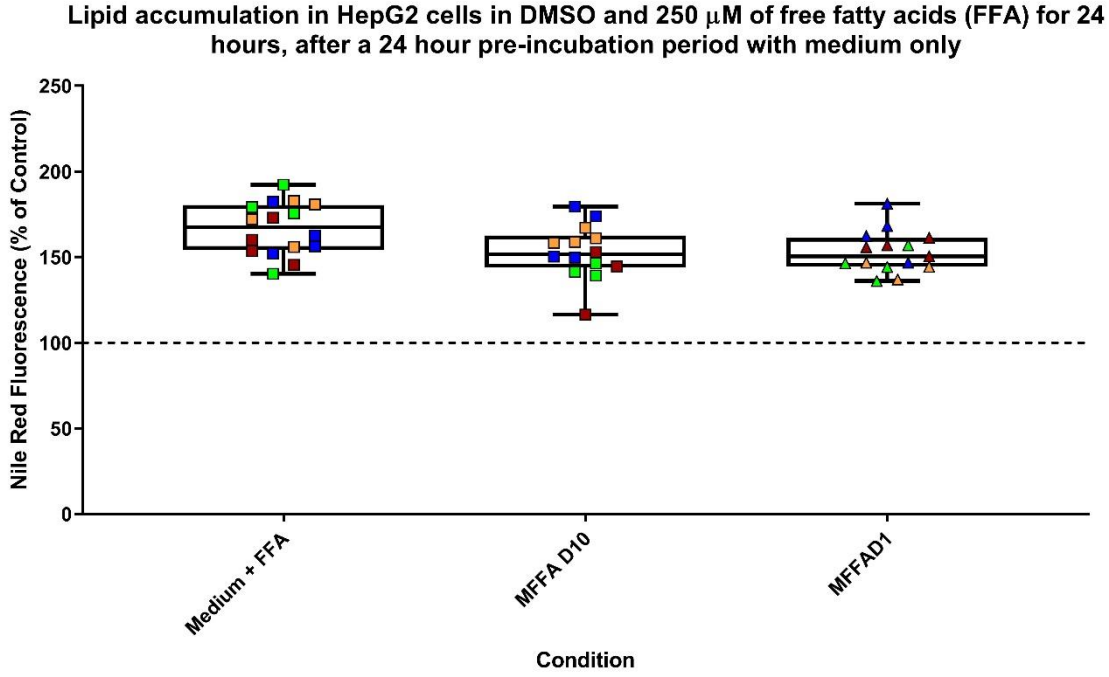
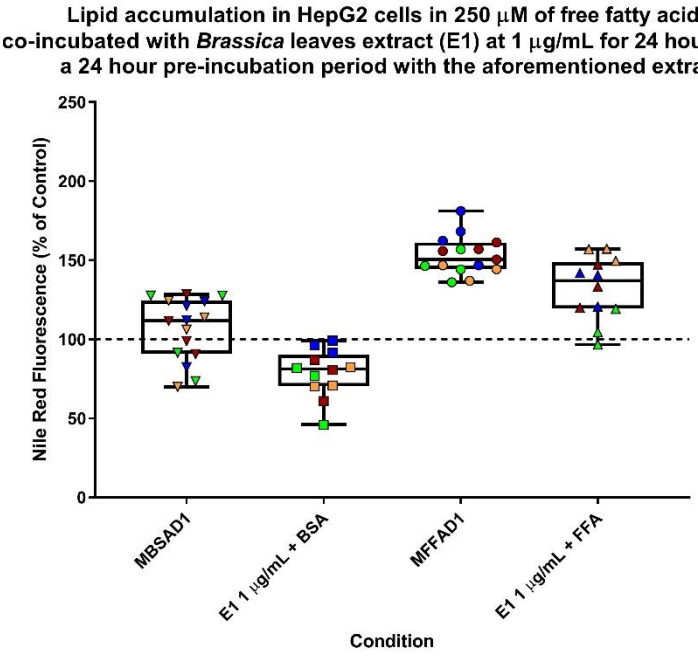


Figure 102 - Lipid accumulation in HepG2 cells in DMSO and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 250 µM of FFA; **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).



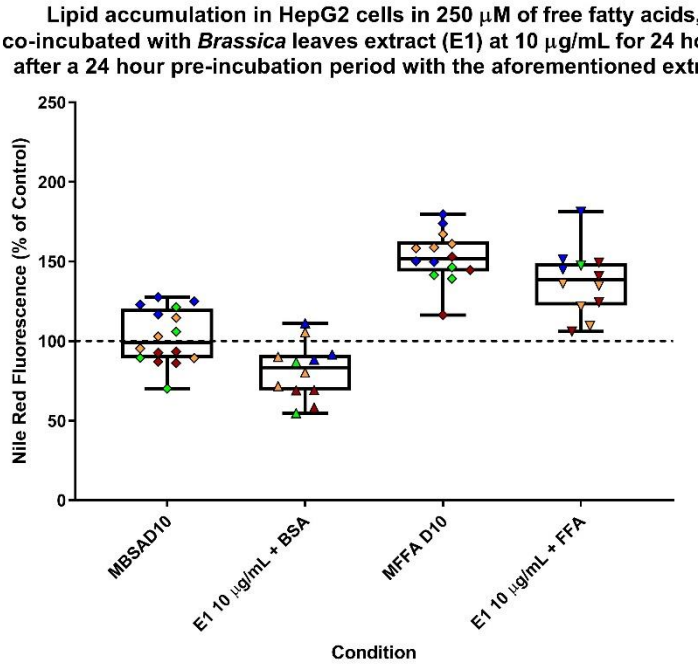
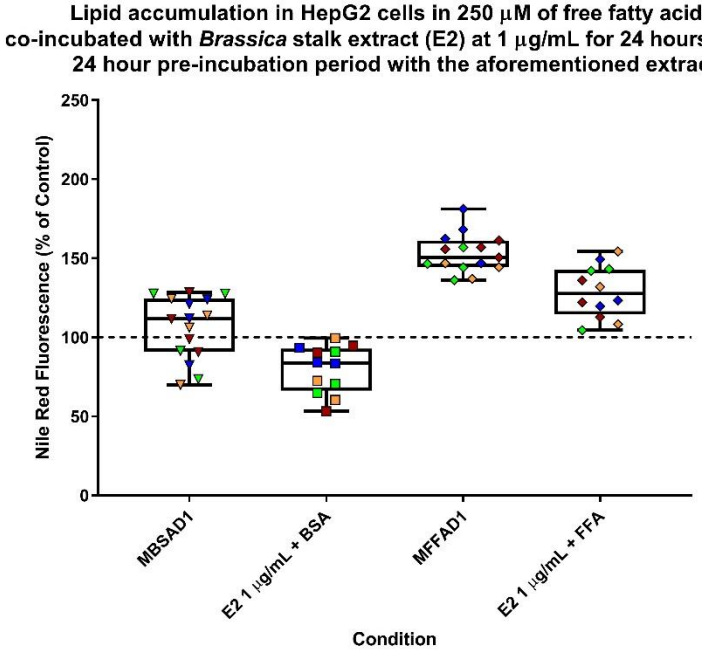


Figure 103 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.



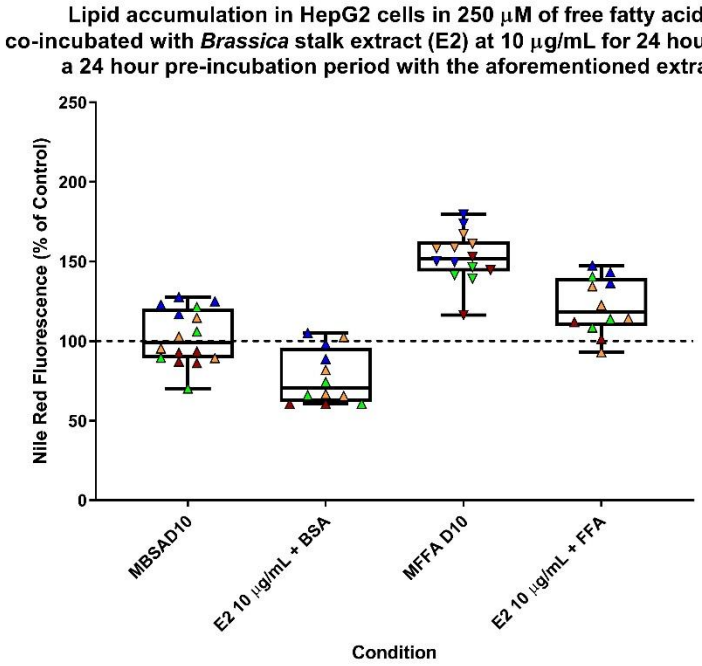


Figure 104 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

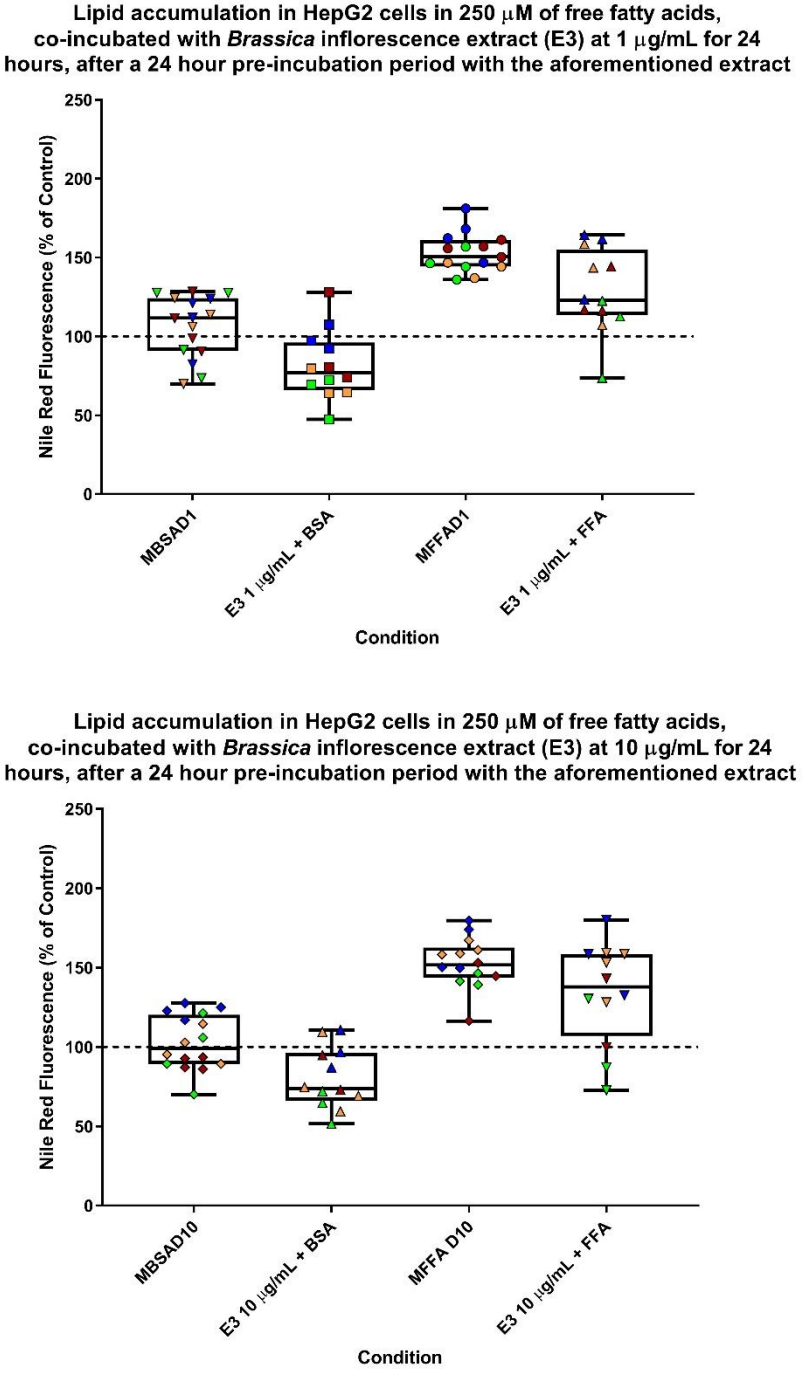


Figure 105 - Lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

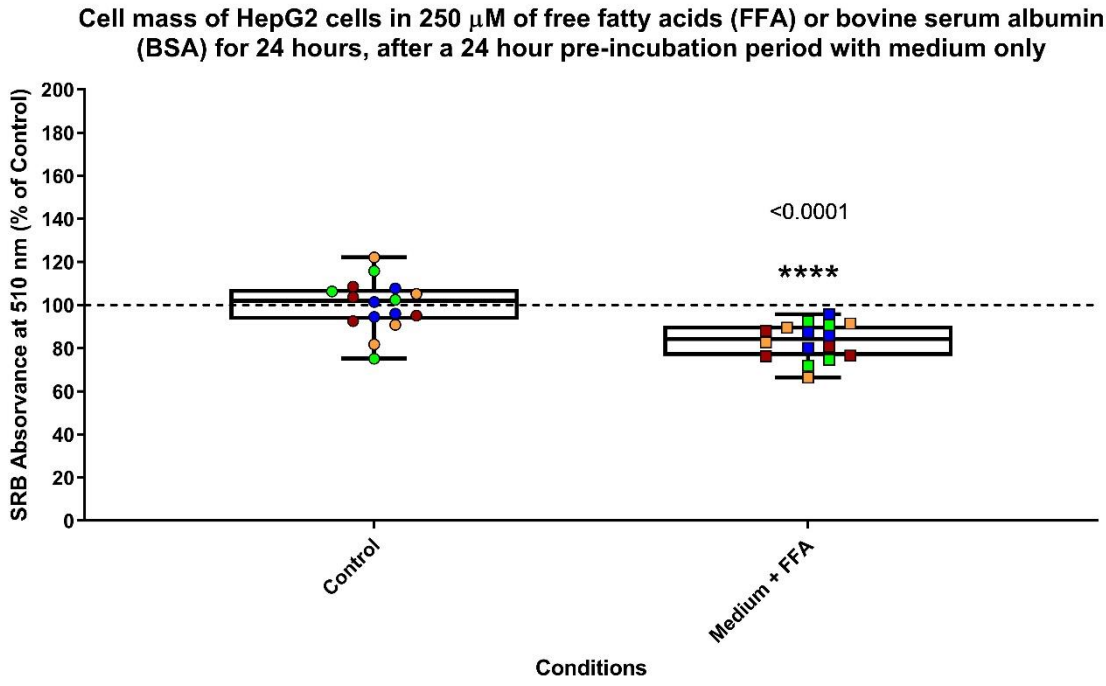


Figure 106 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA) or BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

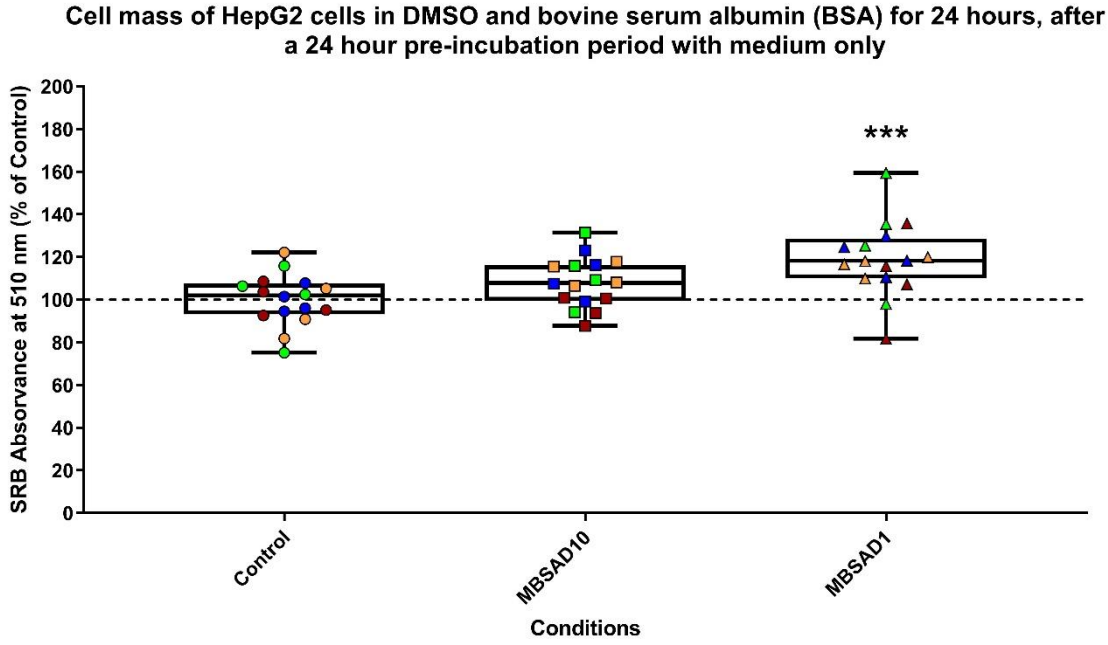


Figure 107 - Cell mass of HepG2 cells in DMSO and BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0009 vs Control. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

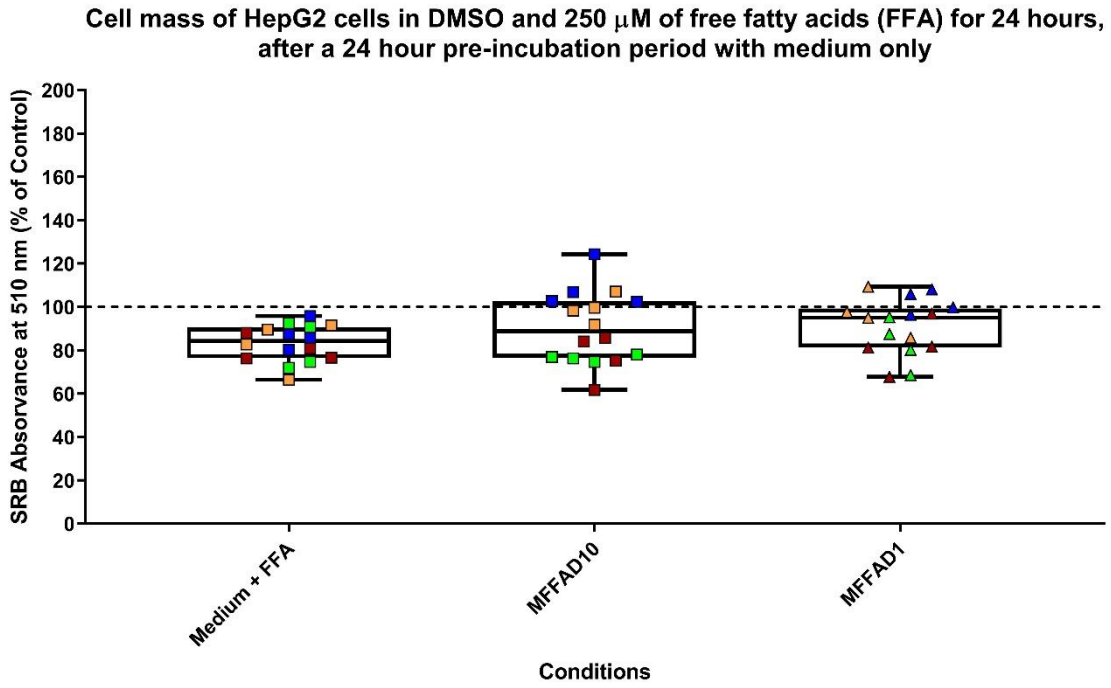
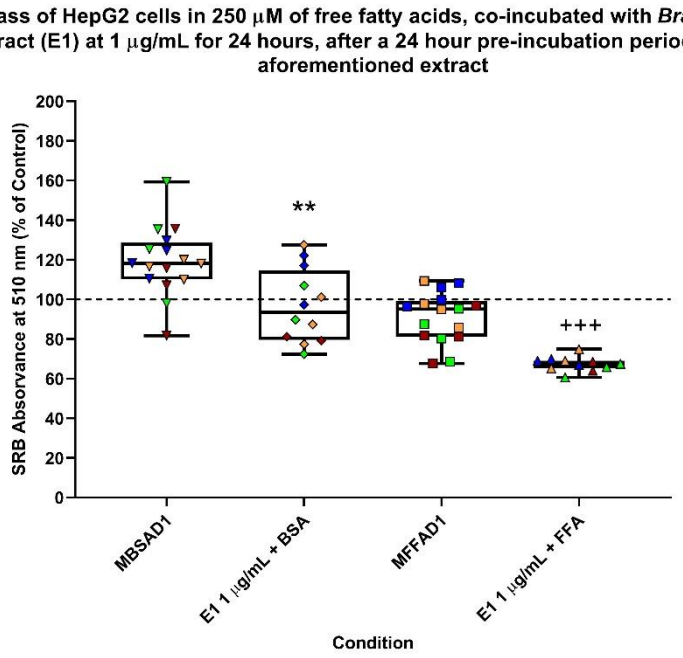


Figure 108 - Cell mass of HepG2 cells in DMSO and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 250 µM of FFA; **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).



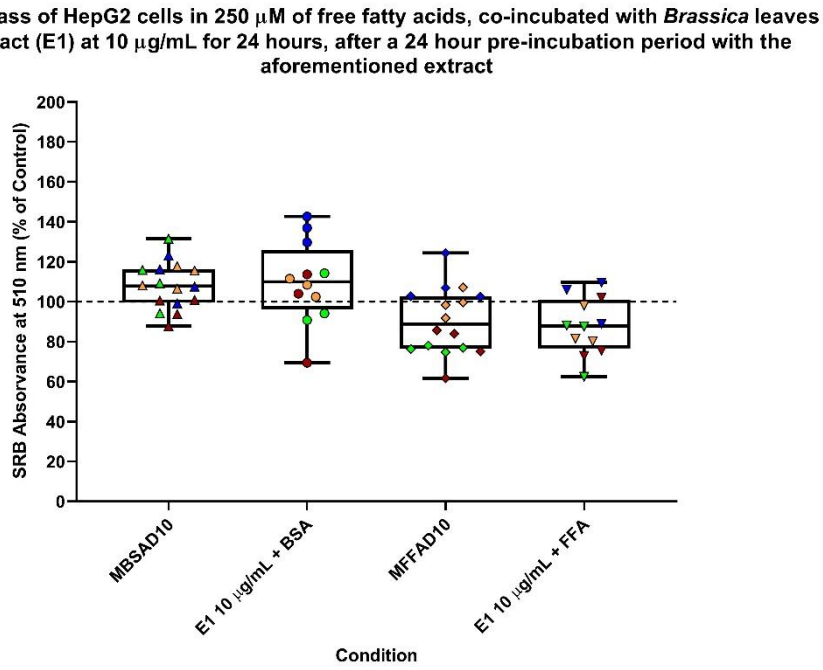


Figure 109 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0010 vsMBSAD1; +++p=0.0007 vs MFFAD1. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

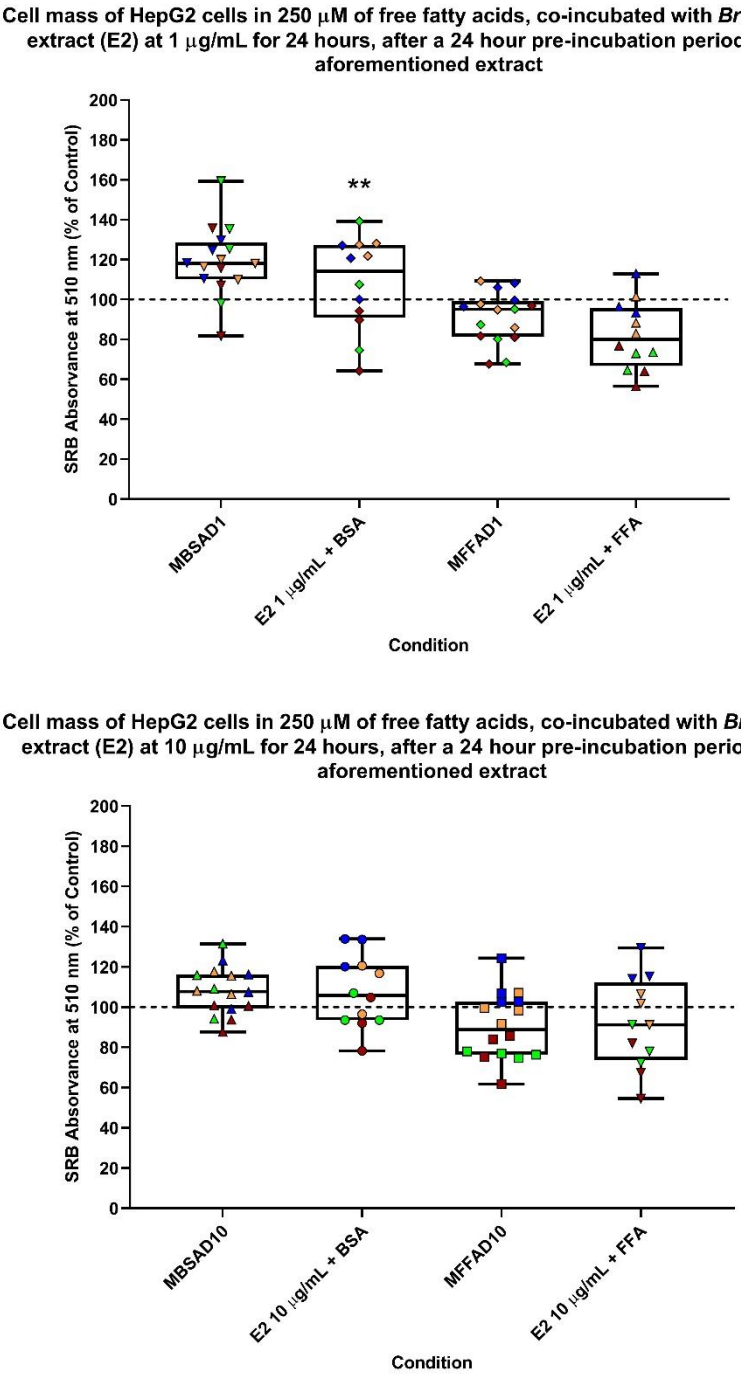


Figure 110 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0092 vs MBSAD1. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

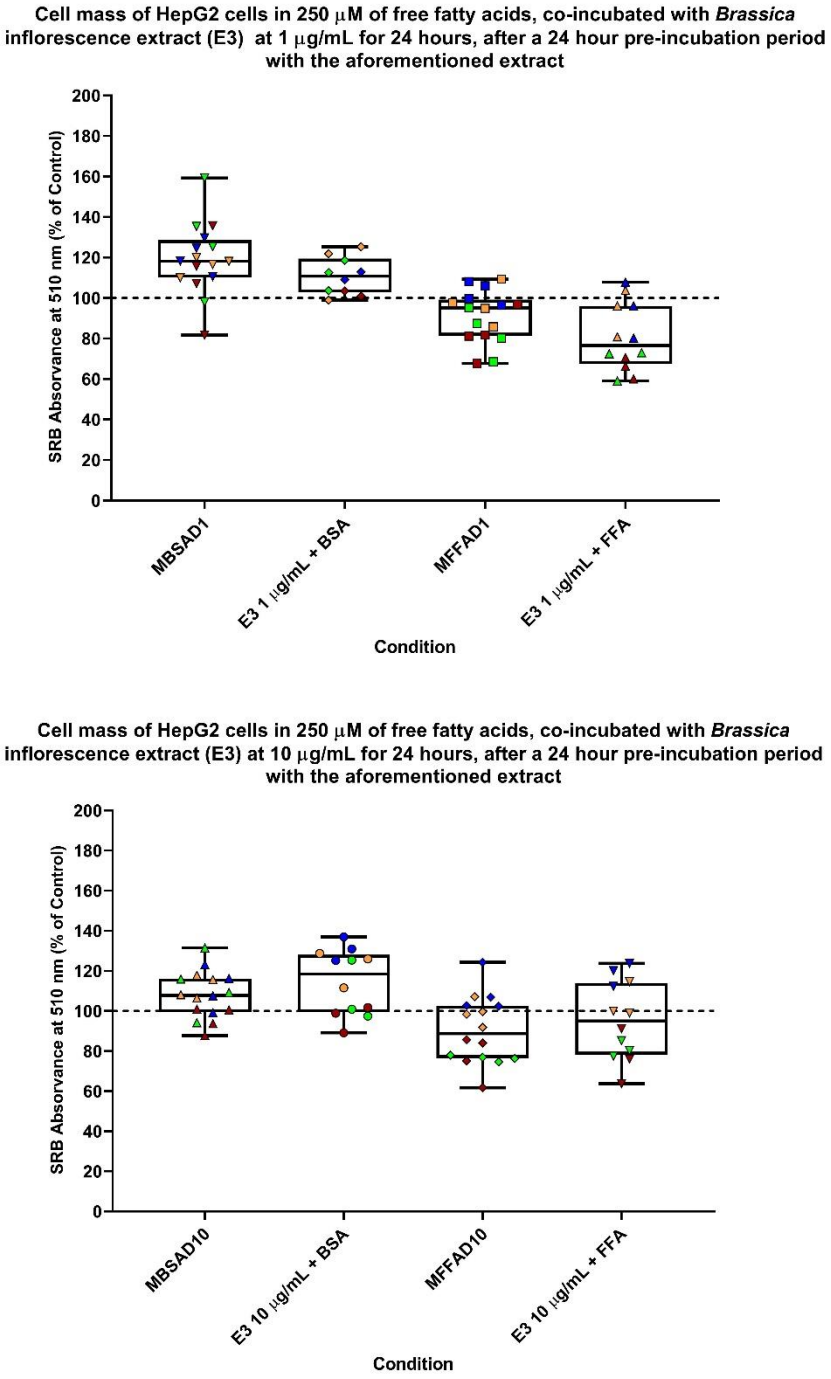


Figure 111 - Cell mass of HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

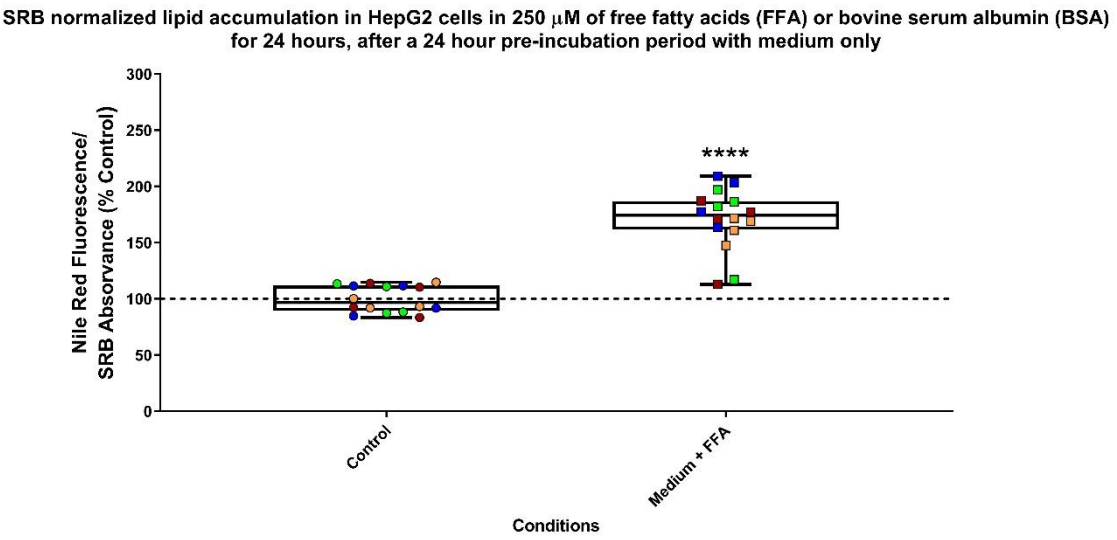


Figure 112 - SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA) or BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

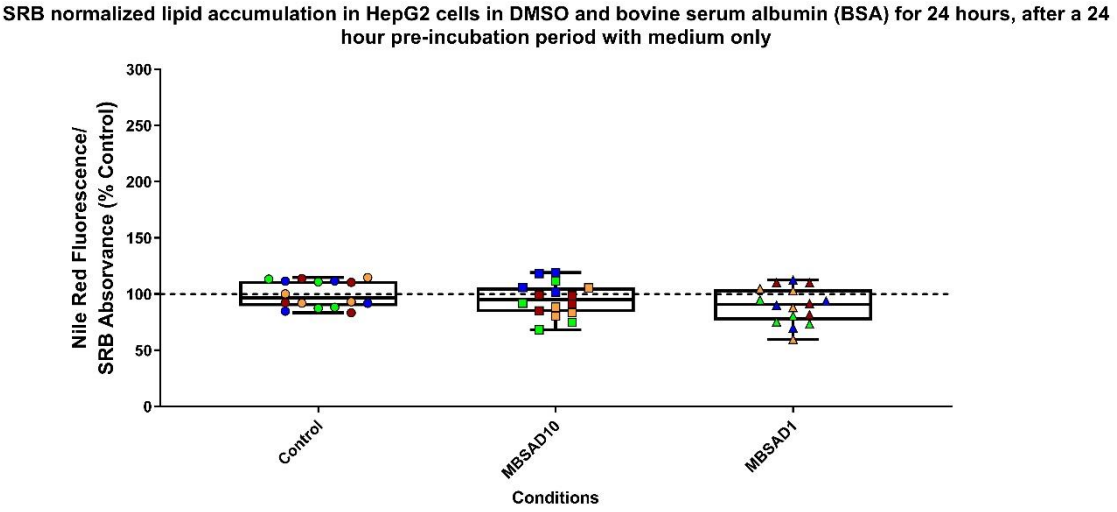


Figure 113 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (0,5%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (0.5%); **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%).

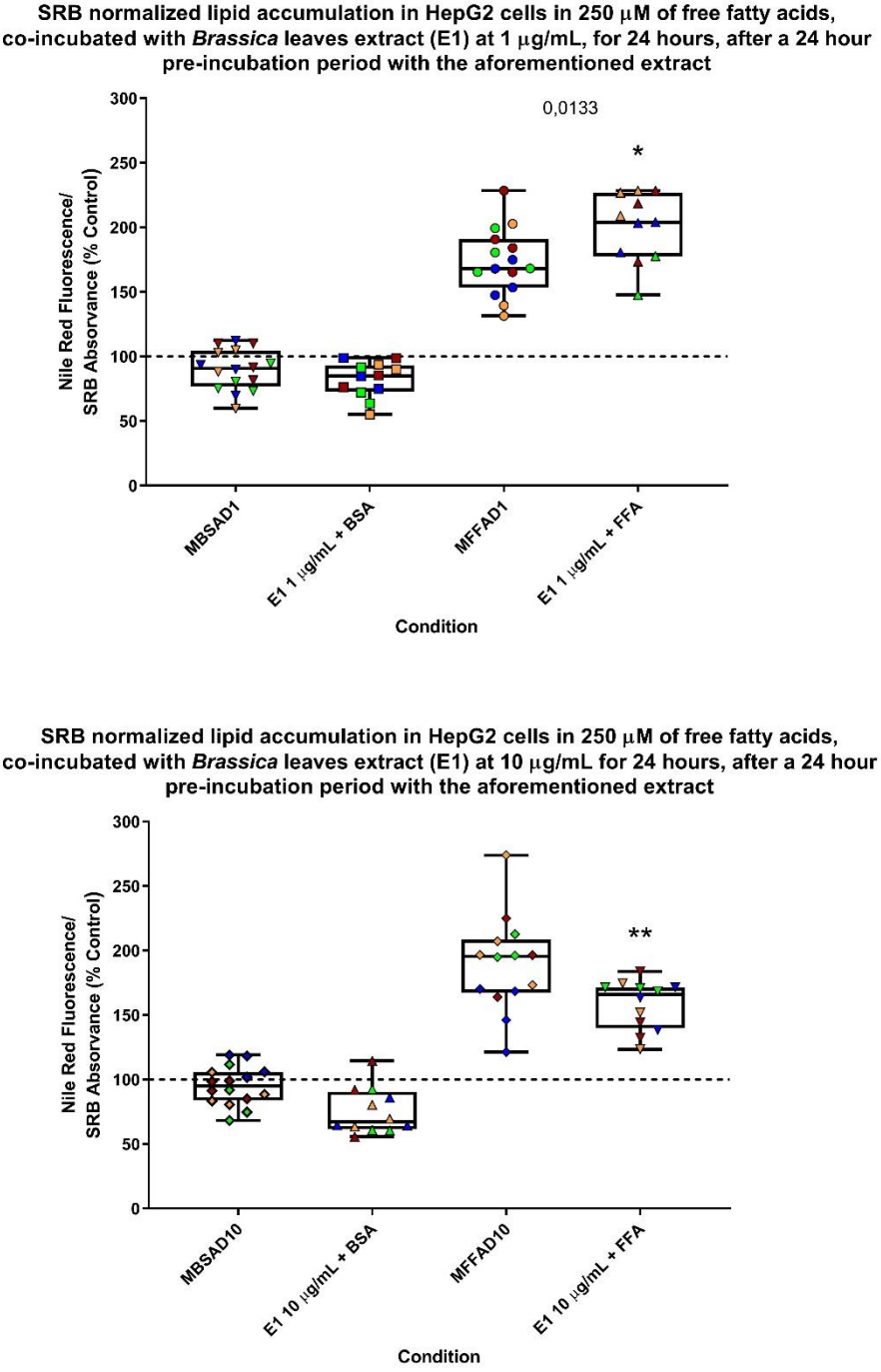


Figure 114 - SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0133 vs MFFAD1; **p=0.0090 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

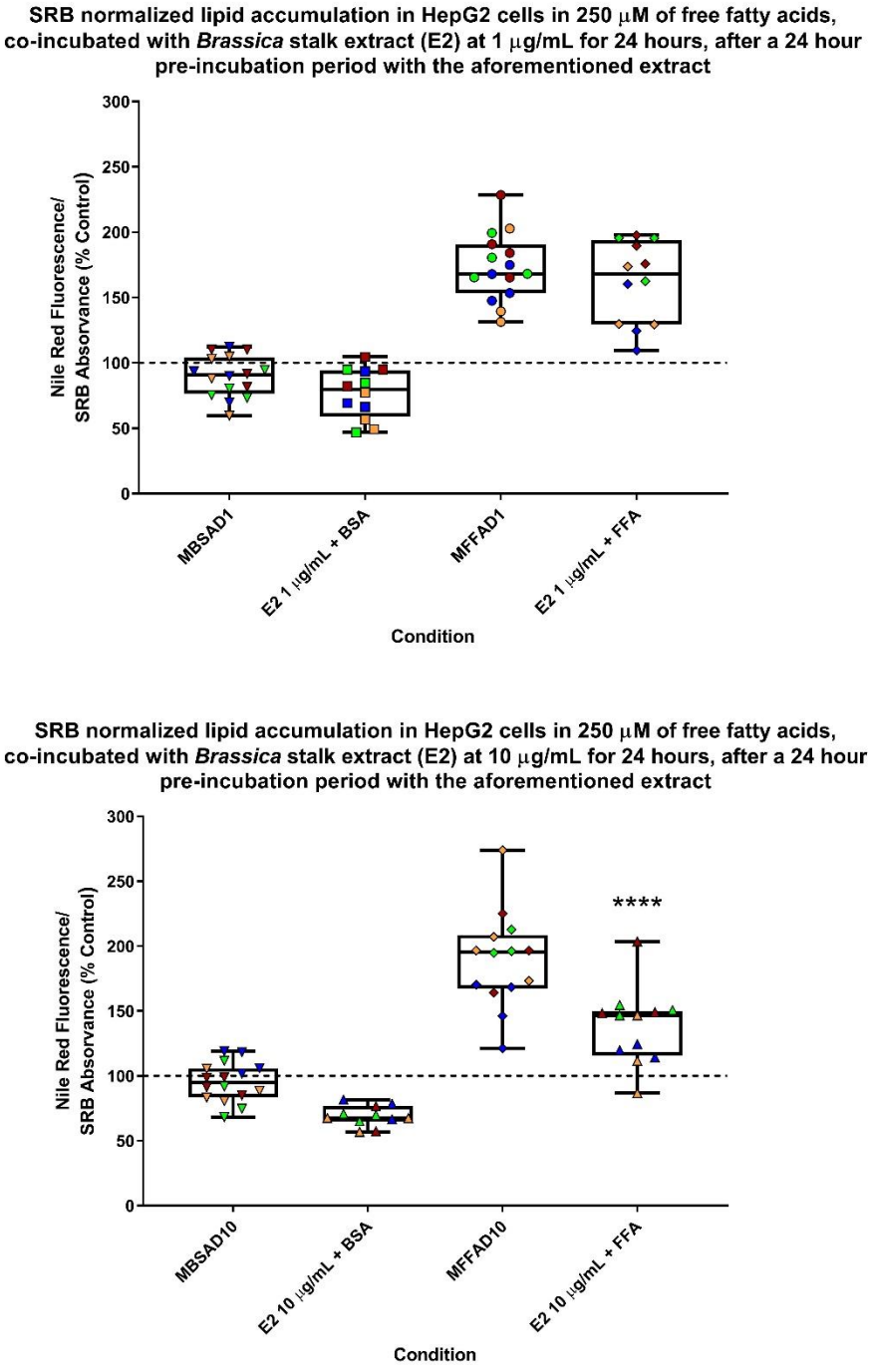
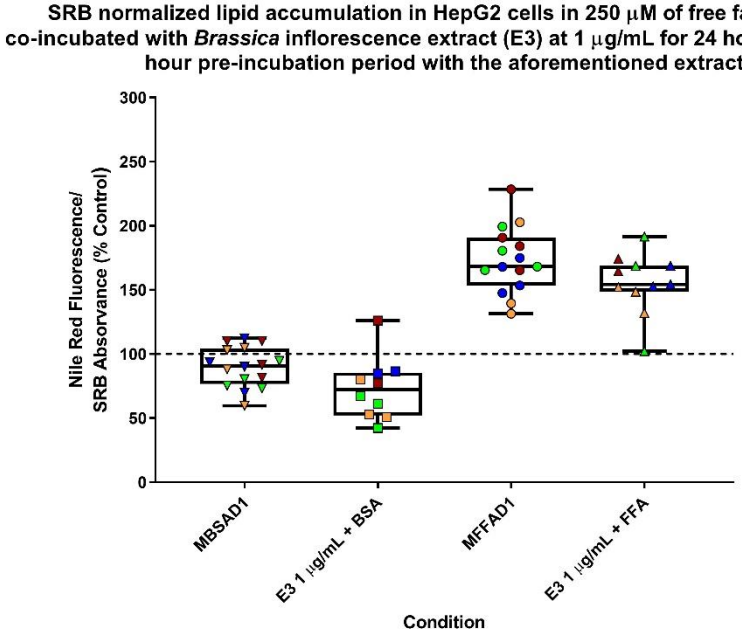


Figure 115 - SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.



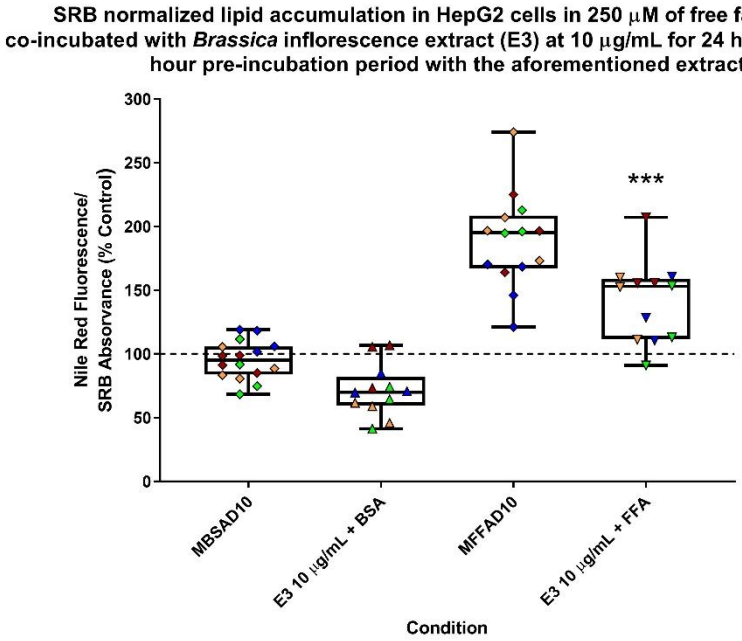


Figure 116 - SRB normalized lipid accumulation in HepG2 cells in 250 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of five independent experiments of either two or four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and either three or four replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0003 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (0,5%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD1:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 250 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **MFFAD10:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.5. Co-incubation of extracts and 1000 µM of free fatty acids for 24 hours, after a 24 hour pre-incubation period with the aforementioned extracts

HepG2 cells were pre-incubated with the extracts for 24 hours and then co-incubated with the extracts and 1000 µM of FFA for 24 hours. This approach was taken to test the extracts capability of triggering a cell response to deal with an elevated accumulation of lipids. The extra pre-incubation period was added to assess if this response is more immediate or if takes a longer time to fully form.

The leaves and stalk extract at 1 µg/mL statistically significantly reduced cell mass of HepG2 cells compared to control groups, when co-incubated with FFA.

The inflorescence extract at 10 µg/mL statistically significantly prevented cell mass loss due to FFA lipotoxicity.

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

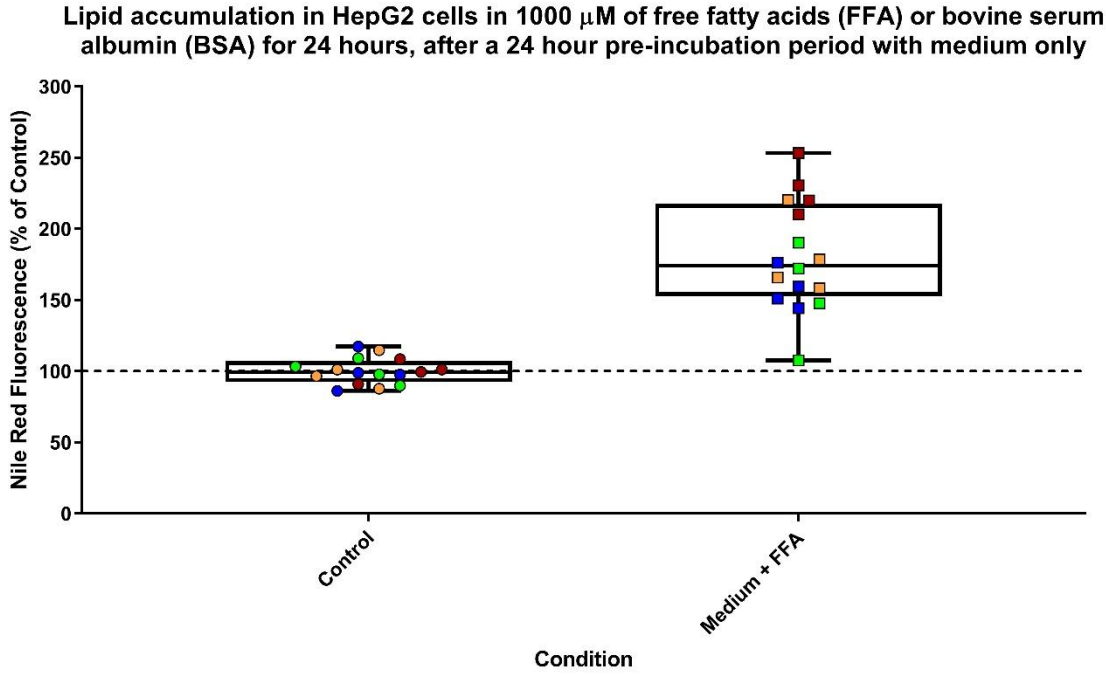


Figure 117 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **Medium + FFA:** Cell culture medium with 1000 µM of FFA.

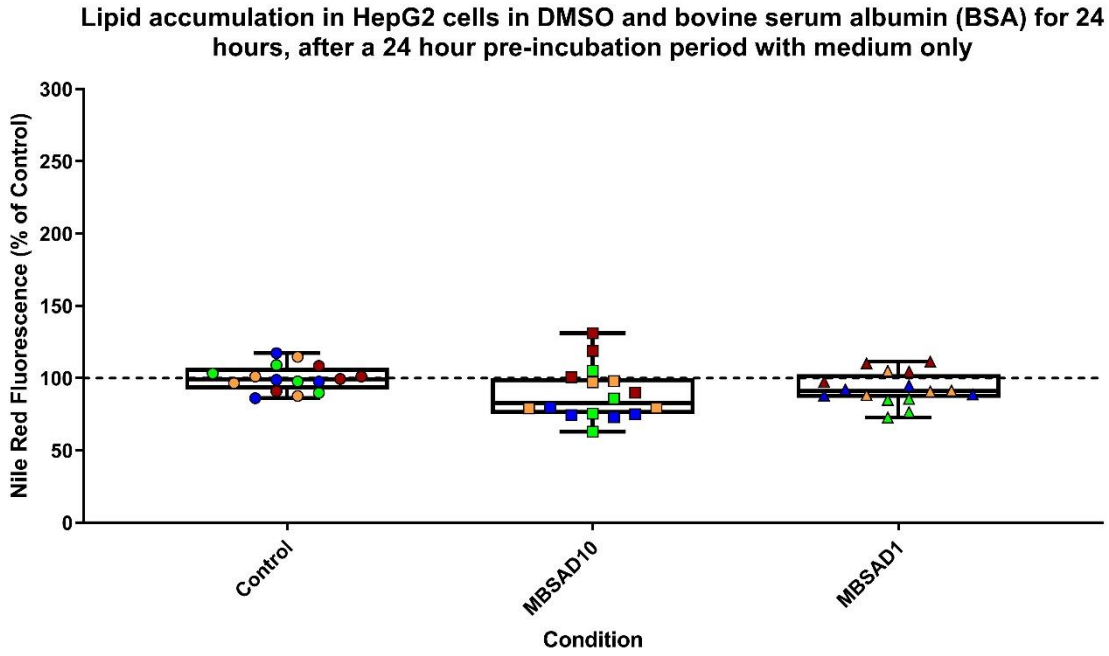


Figure 118 - Lipid accumulation in HepG2 cells in DMSO and BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

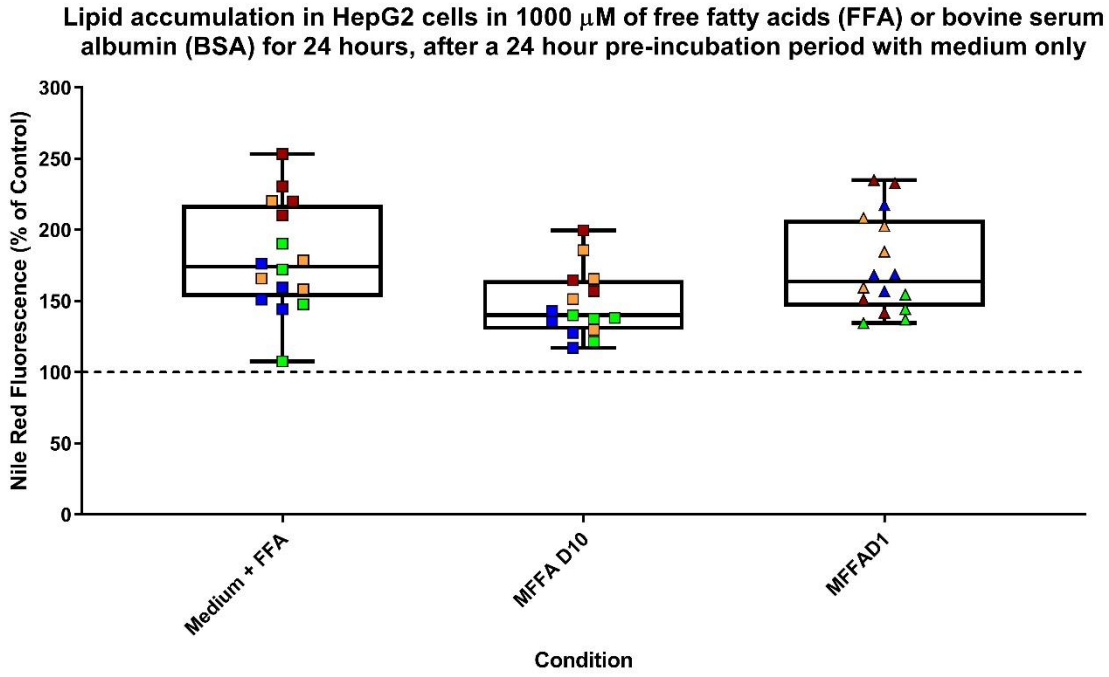


Figure 119 - Lipid accumulation in HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

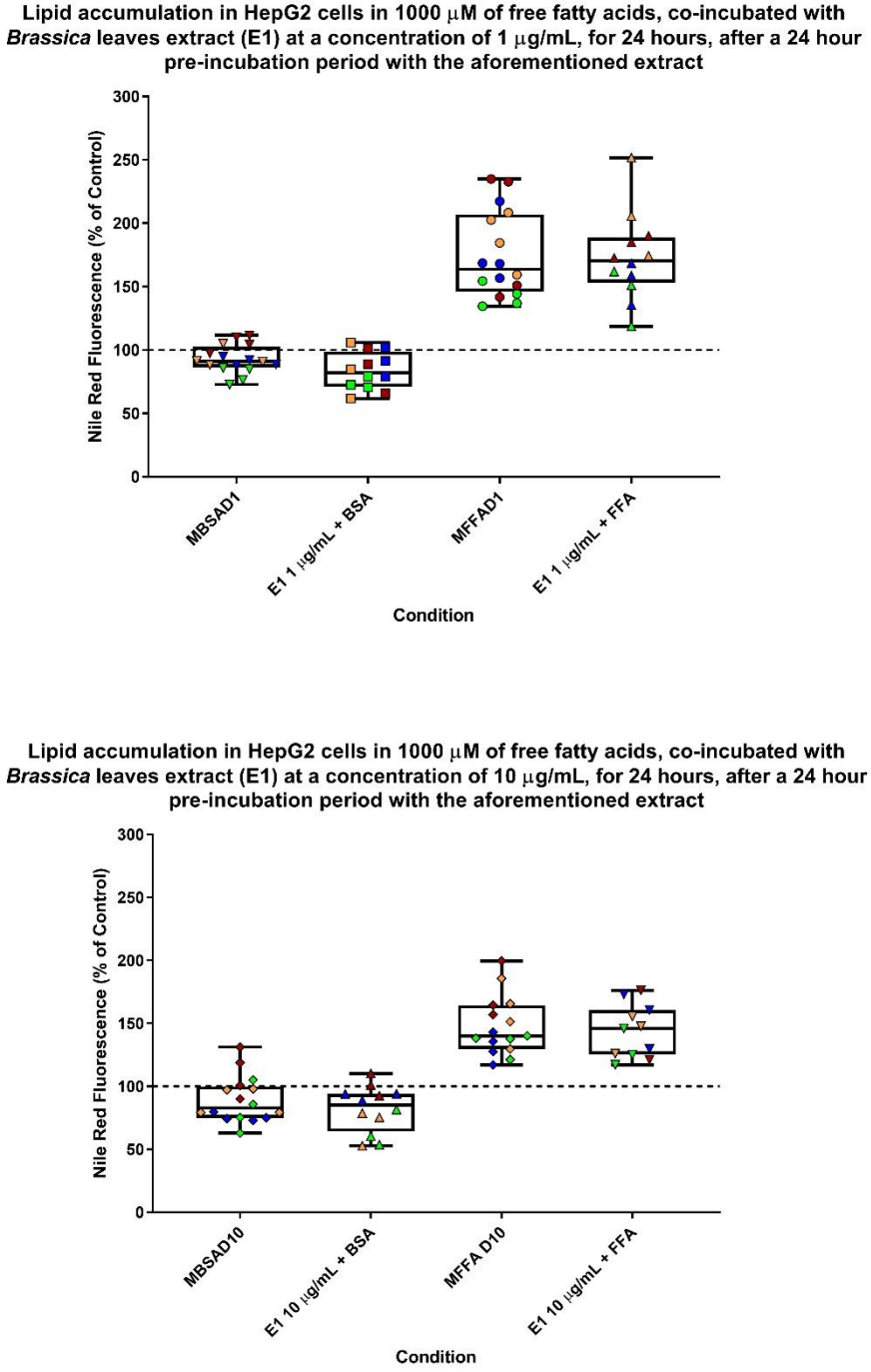
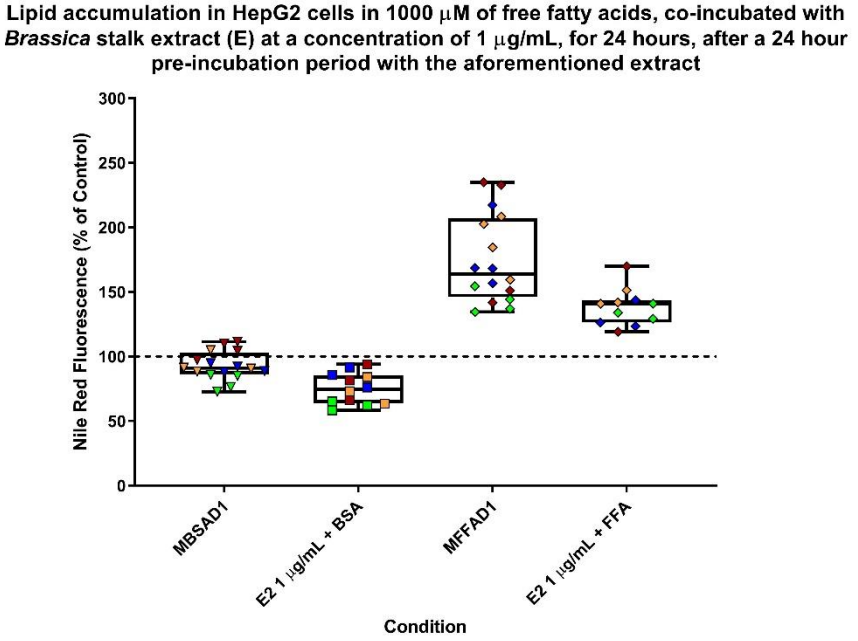


Figure 120 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.



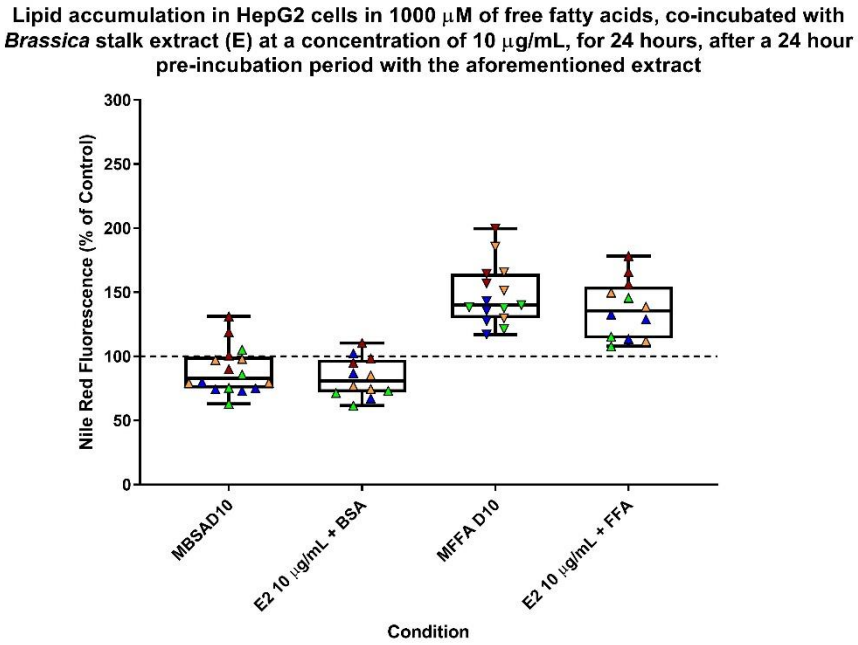
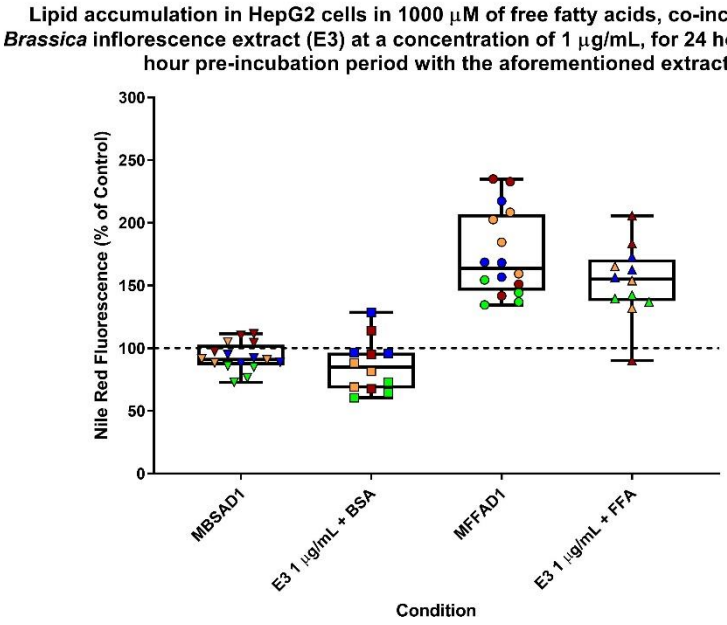


Figure 121 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.



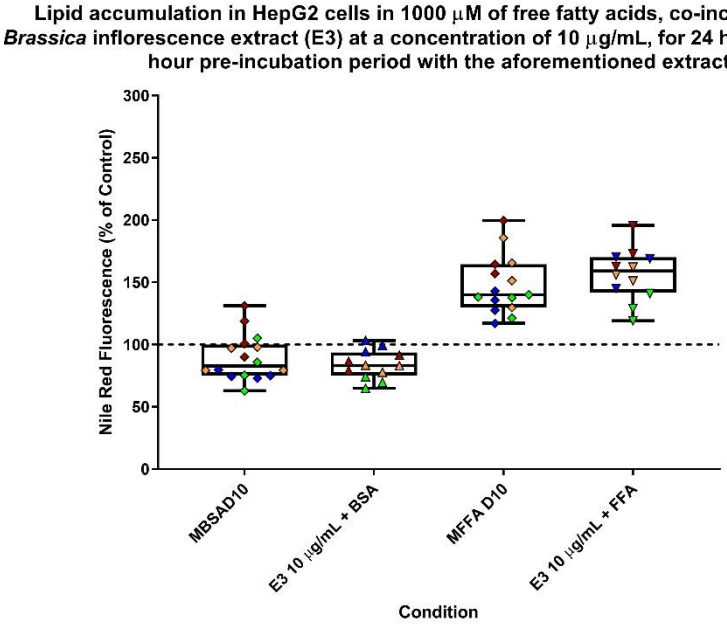


Figure 122 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

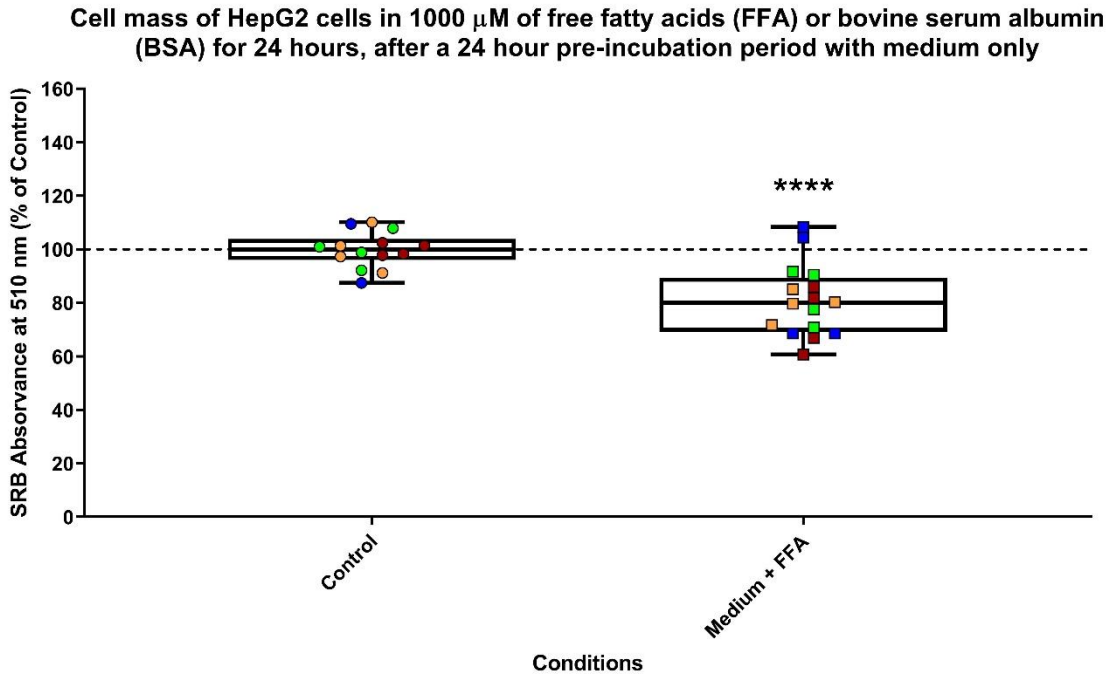


Figure 123 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

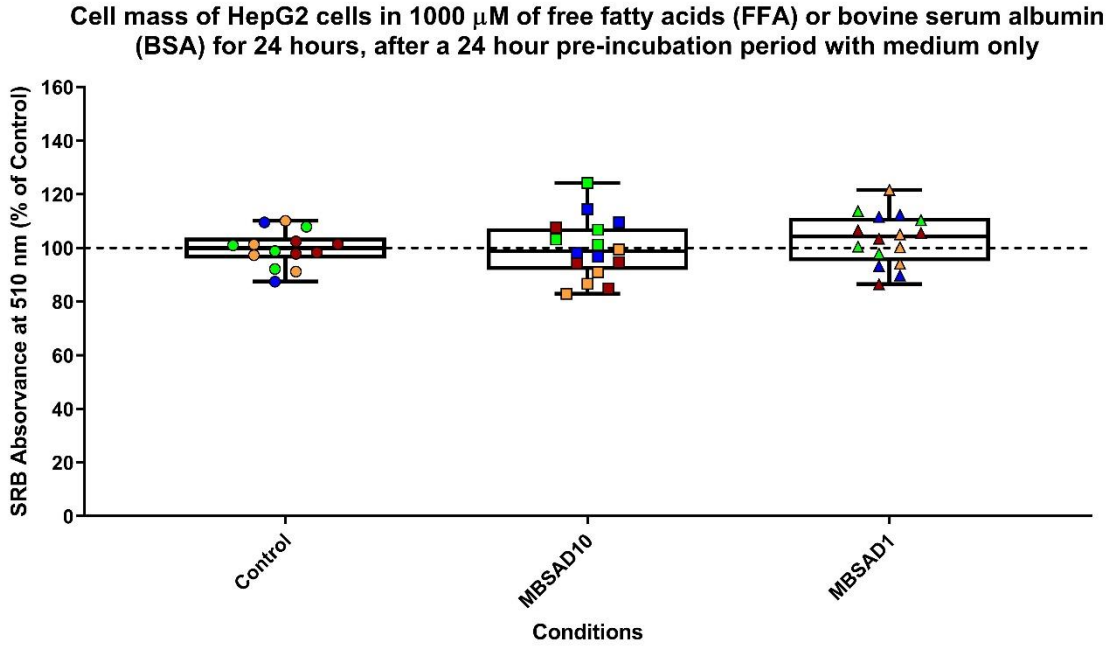


Figure 124 - Cell mass of HepG2 cells in DMSO and BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

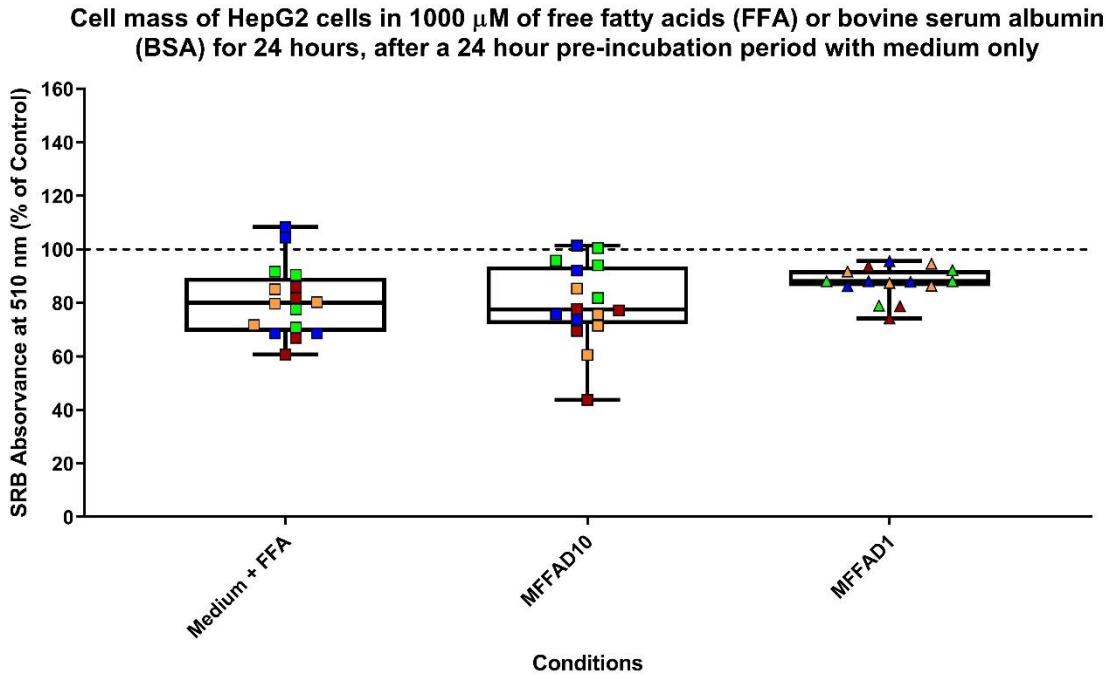


Figure 125 - Cell mass of HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture media with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

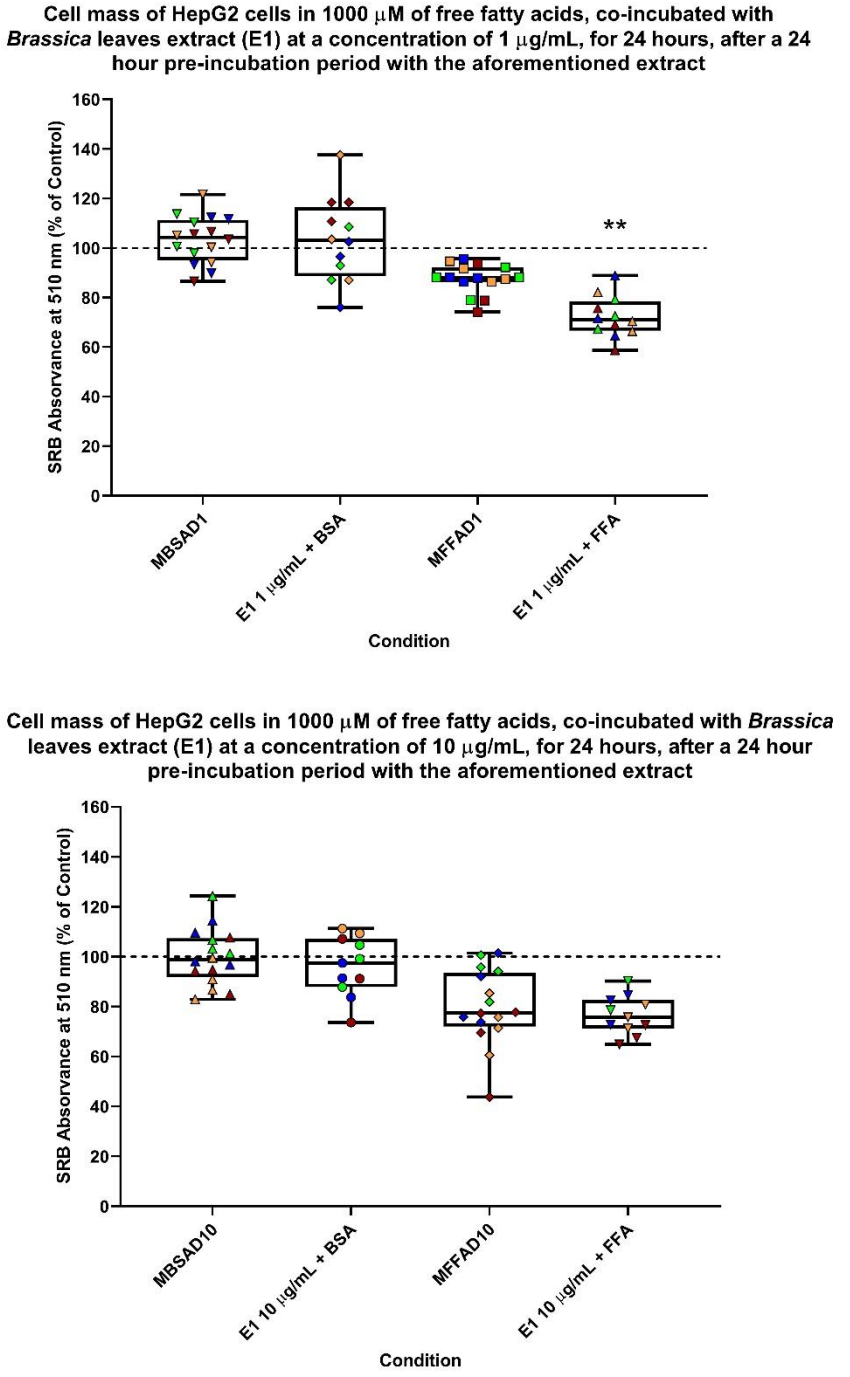


Figure 126 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0028 vs MFFAD1. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.

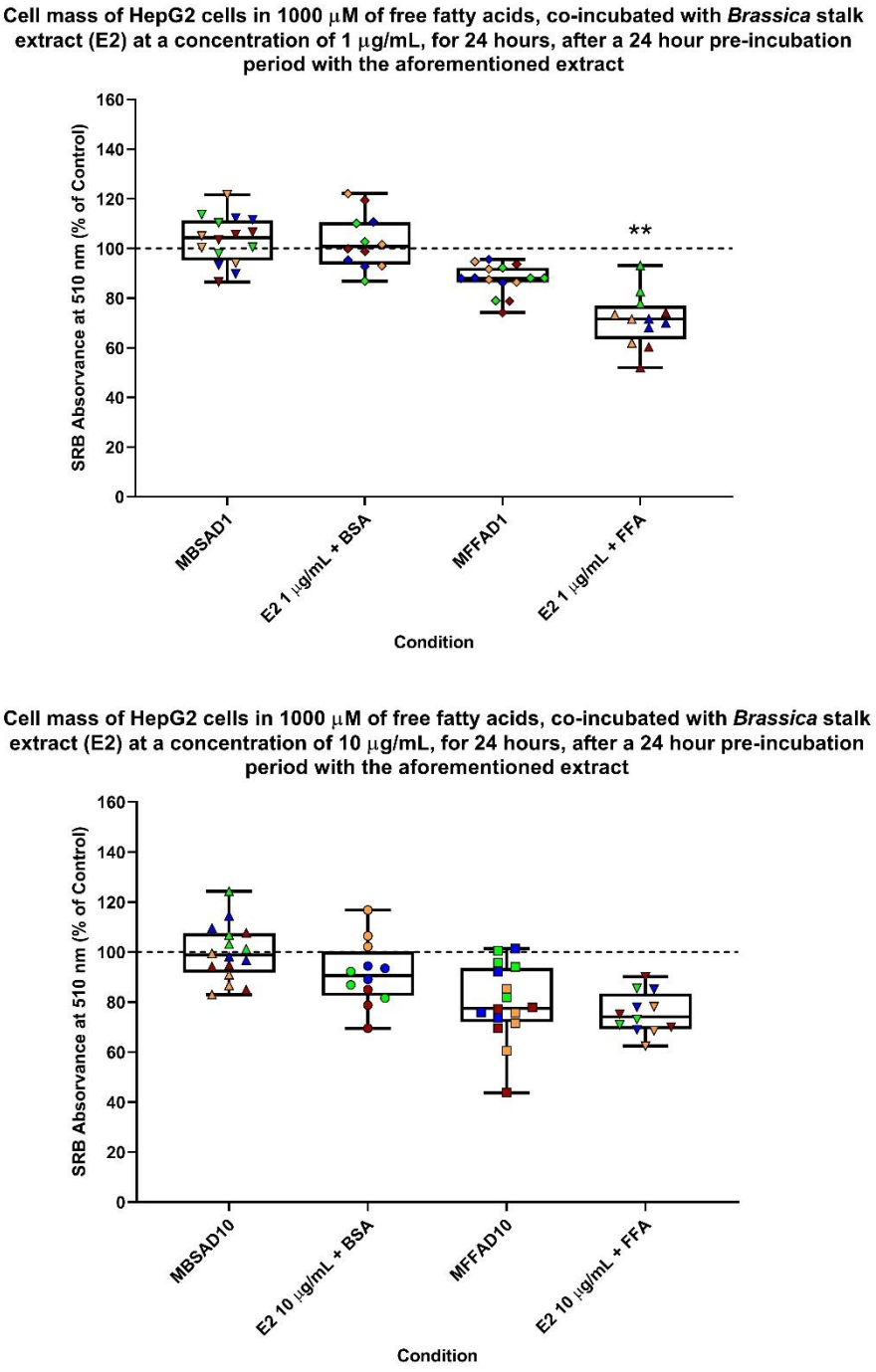
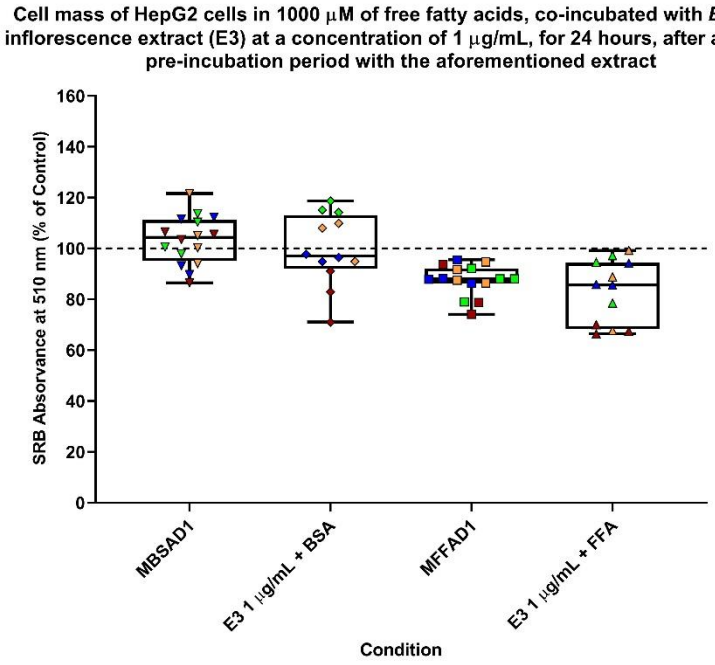


Figure 127 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0003 vs MFFAD1. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.



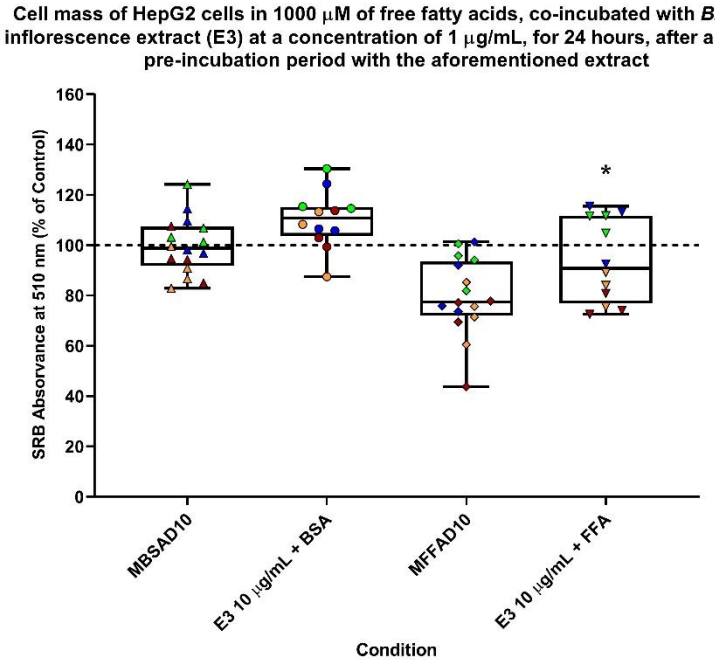


Figure 128 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0476 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

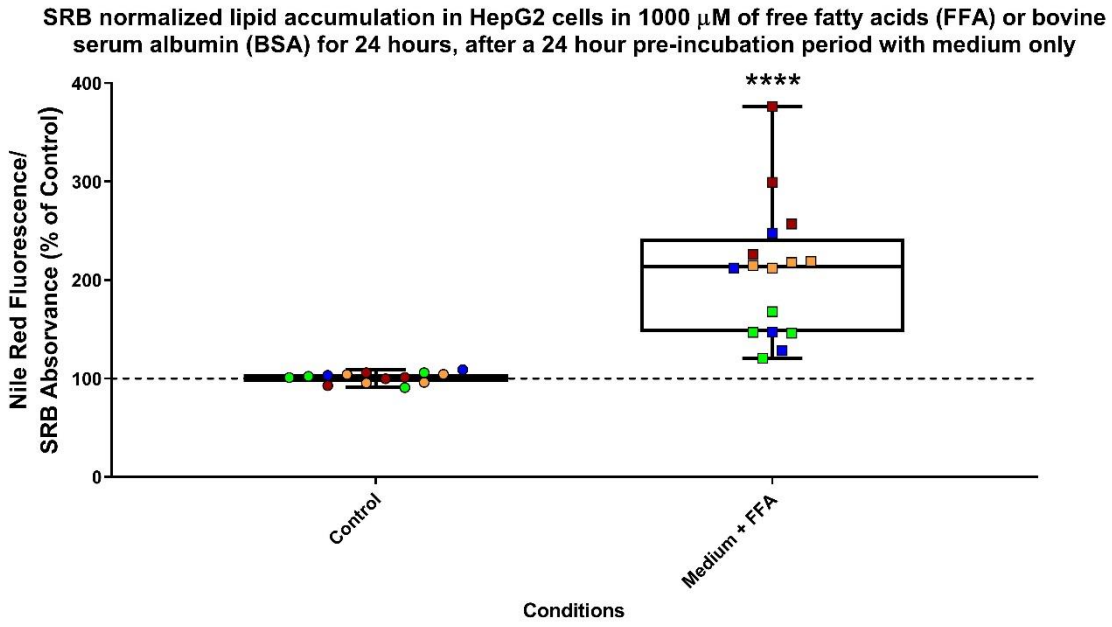


Figure 129 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (2%); **Medium + FFA:** Cell culture medium with 1000 µM of FFA.

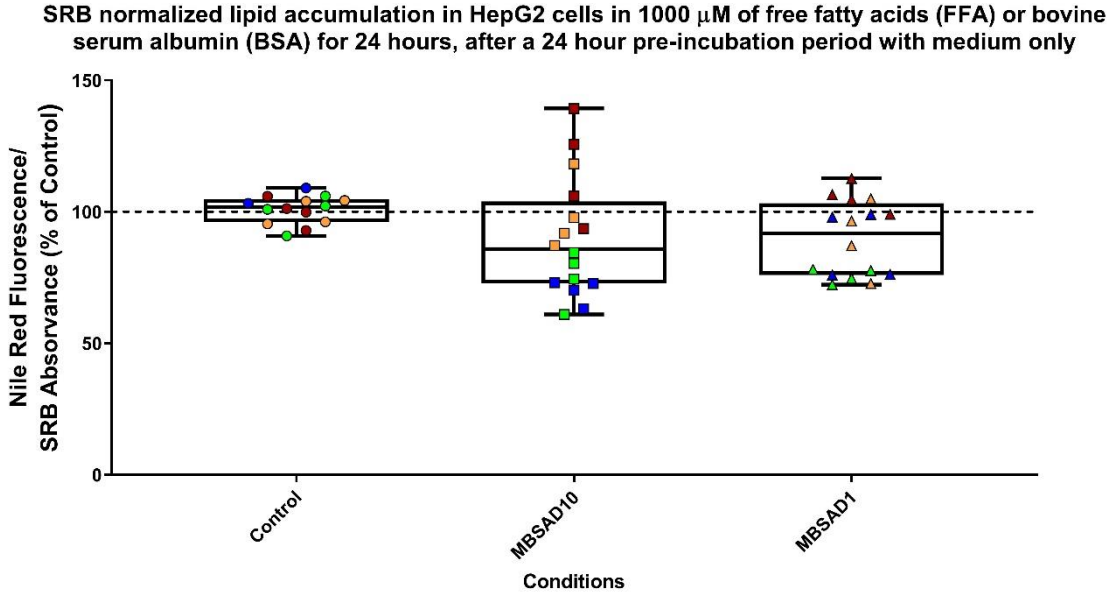


Figure 130 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (2%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (2%); **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%).

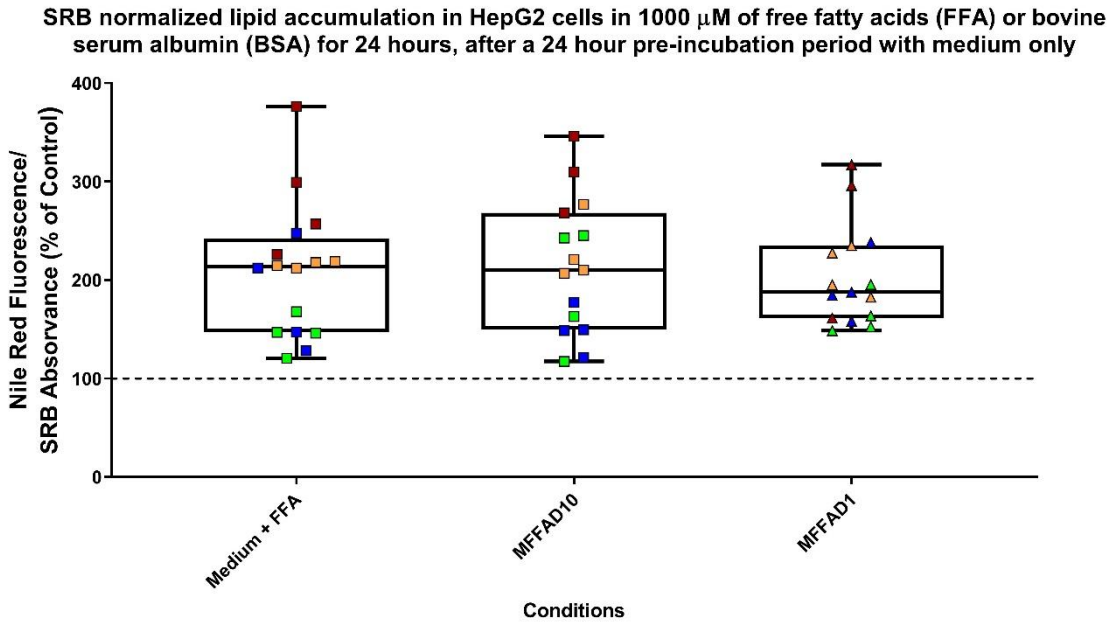


Figure 131 - SRB normalized lipid accumulation in HepG2 cells in DMSO and 1000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 1000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%).

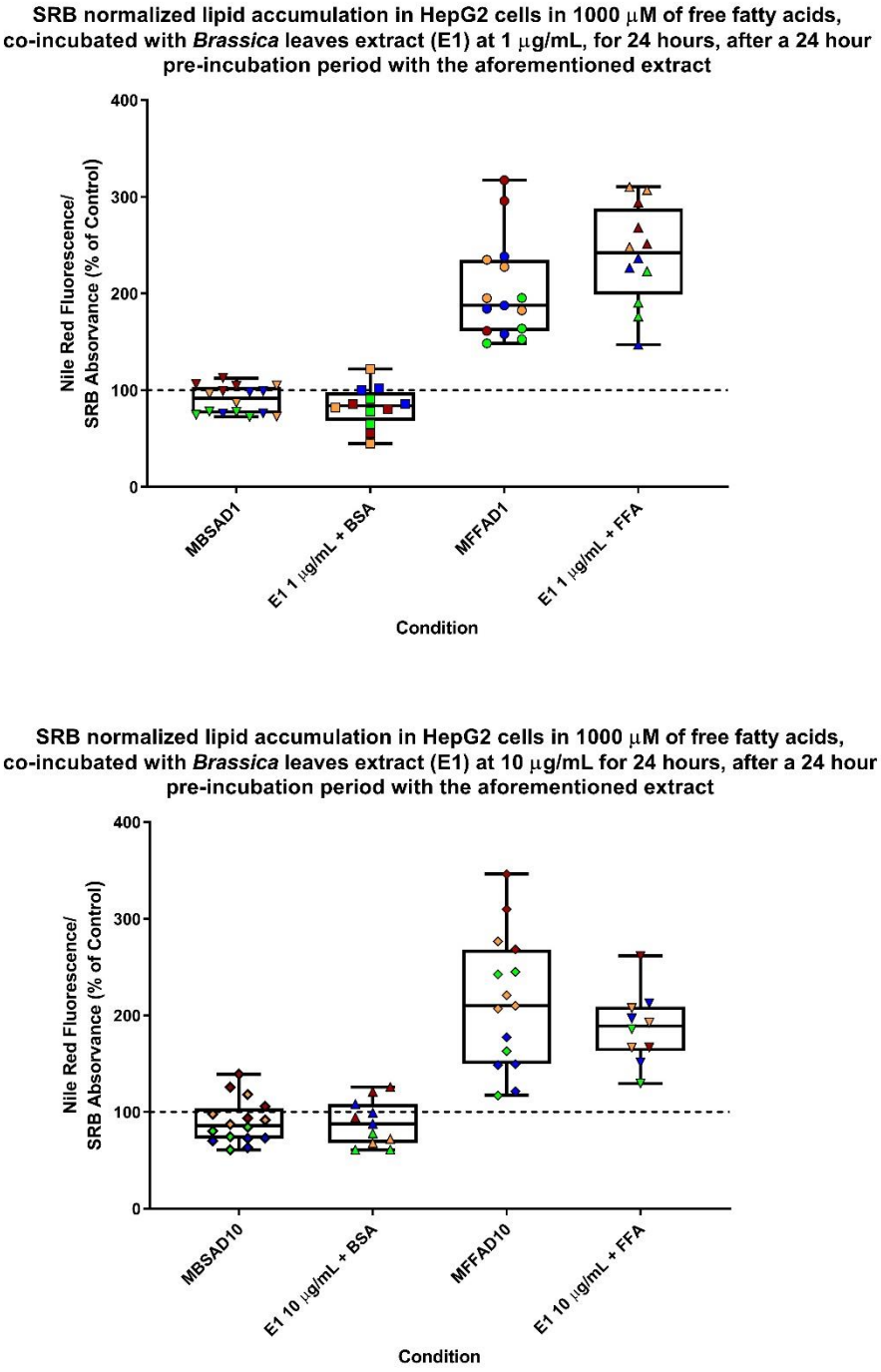


Figure 132 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 1000 µM of FFA.

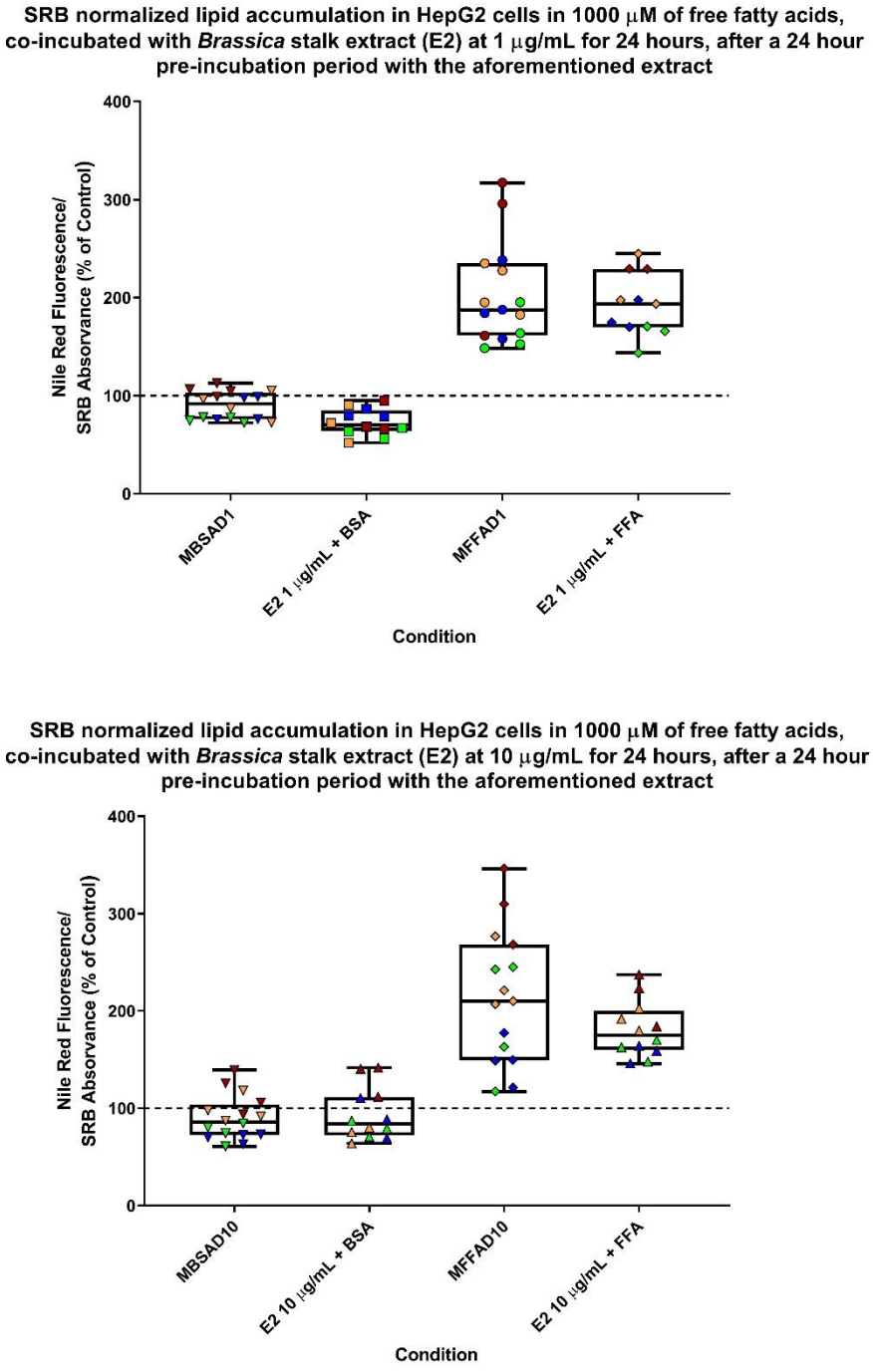
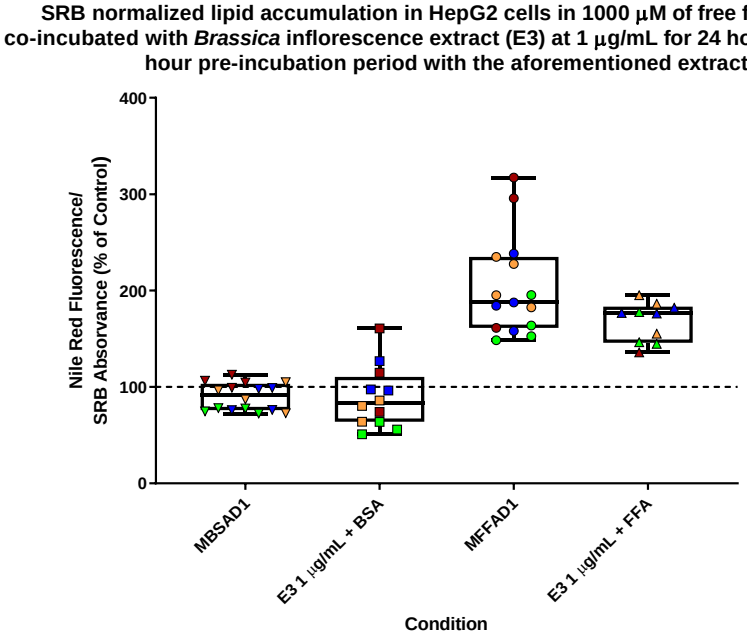


Figure 133 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 1000 µM of FFA.



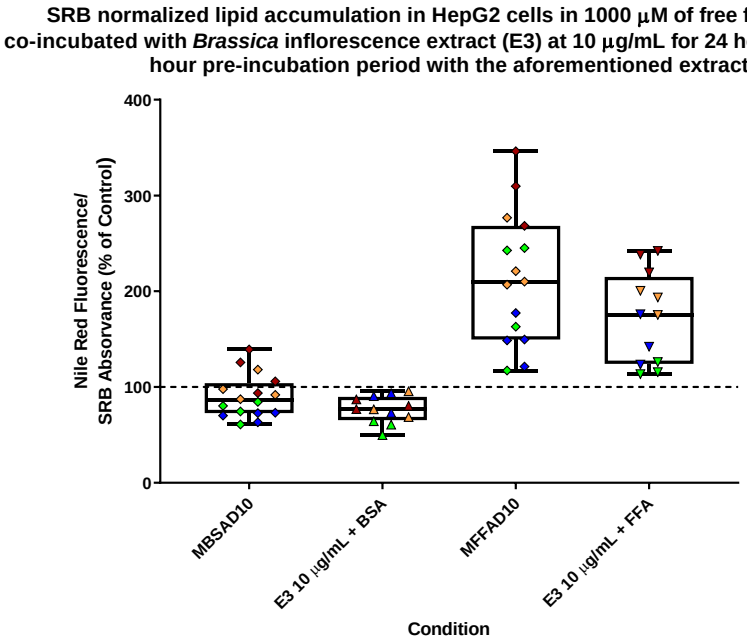


Figure 134 - SRB normalized lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

4.2.6. Co-incubation of extracts and 2000 µM of free fatty acids for 24 hours, after a 24 hour pre-incubation period with the aforementioned extracts

HepG2 cells were pre-incubated with the extracts for 24 hours and then co-incubated with the extracts and 2000 µM of FFA for 24 hours. This approach was taken to test the extracts' capability of triggering a cell response to deal with a lipotoxic environment. The extra pre-incubation period was added to assess if this response is immediate or if takes longer to occurs.

The leaves extract at 1 µg/mL statistically significantly reduced cell mass of HepG2 cells compared to the control group, when co-incubated with BSA.

Co-incubation of FFA with the leaves, stalk or inflorescence extracts at 10 µg/mL showed a statistically significant decrease in SRB normalized FFA accumulation on HepG2 cells when compared to their respective control group. The stalk extract at 1 µg/mL also statistically significantly decreased SRB normalized FFA accumulation on HepG2 cells when compared to the control group.

Non-normalized lipid accumulation results have no statistical results, not because they may not exist, but because they lose meaning to the statistical analysis of normalized lipid accumulation results.

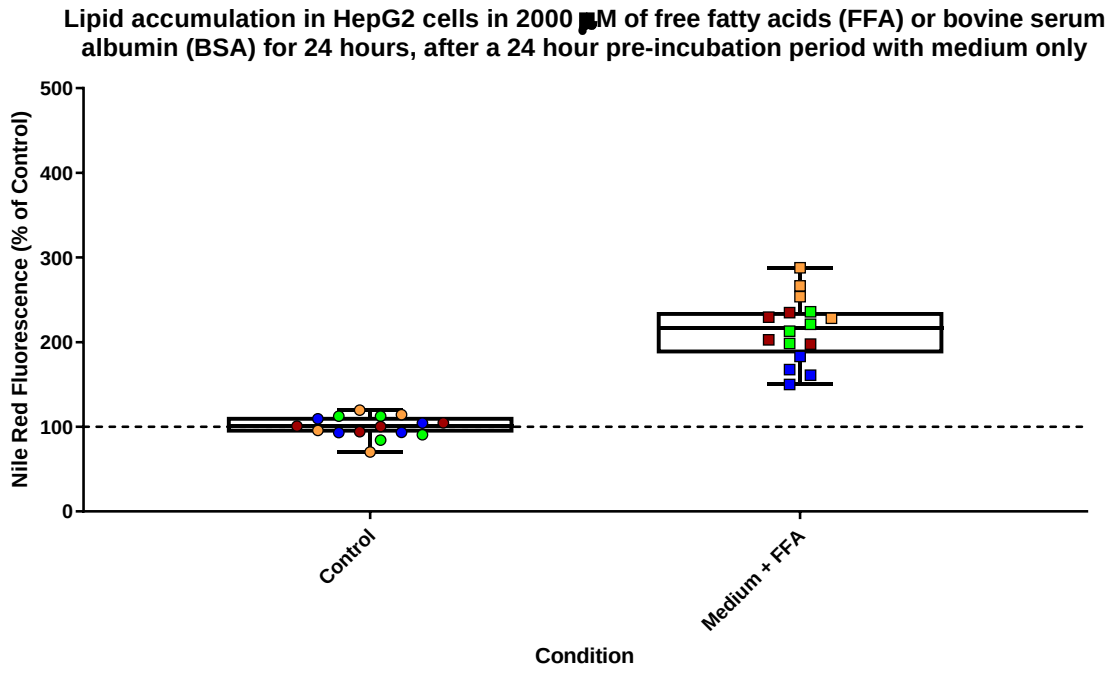


Figure 135 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA) or BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **Medium + FFA:** Cell culture medium with 2000 µM of FFA.

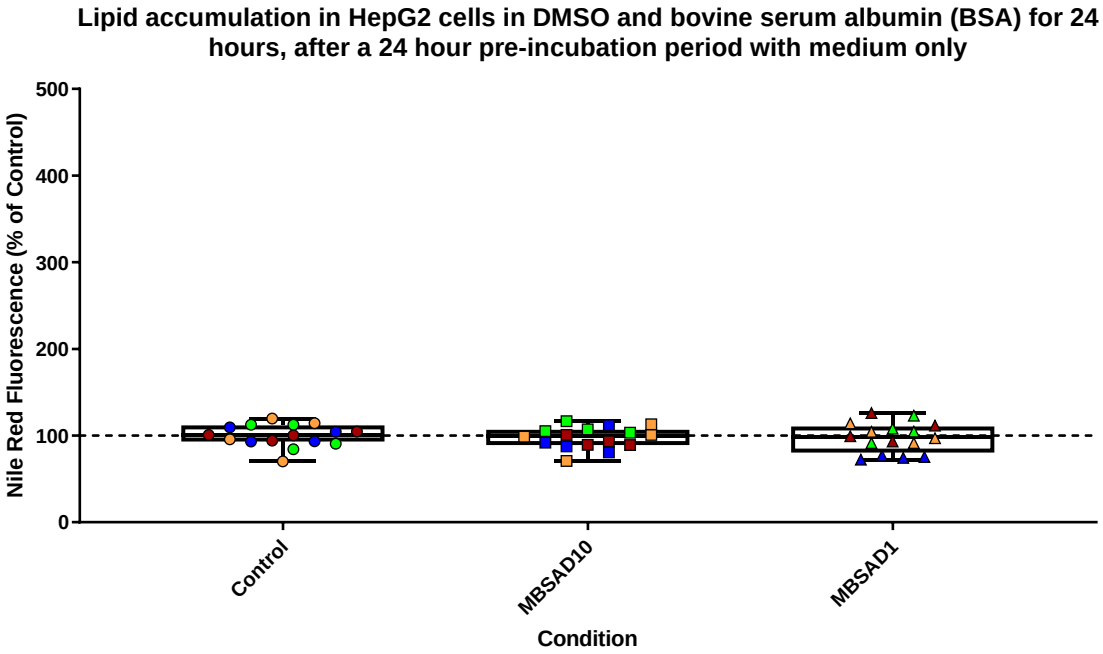


Figure 136 - Lipid accumulation in HepG2 cells in DMSO and BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

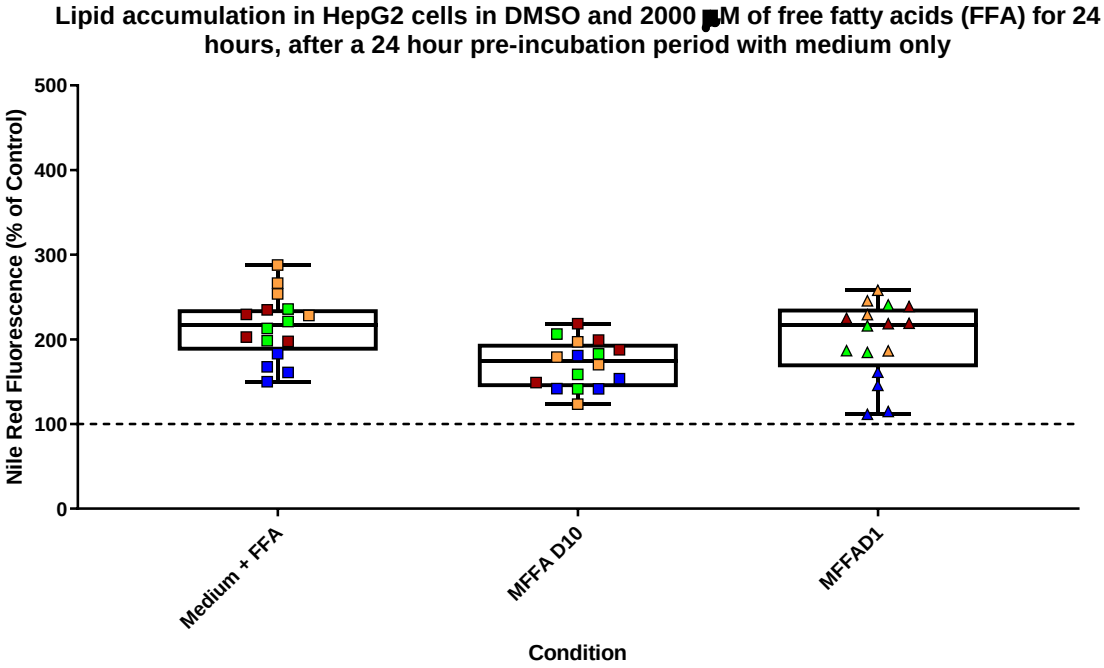


Figure 137 - Lipid accumulation in HepG2 cells in DMSO and 2000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Medium + FFA:** Cell culture medium with 2000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%).

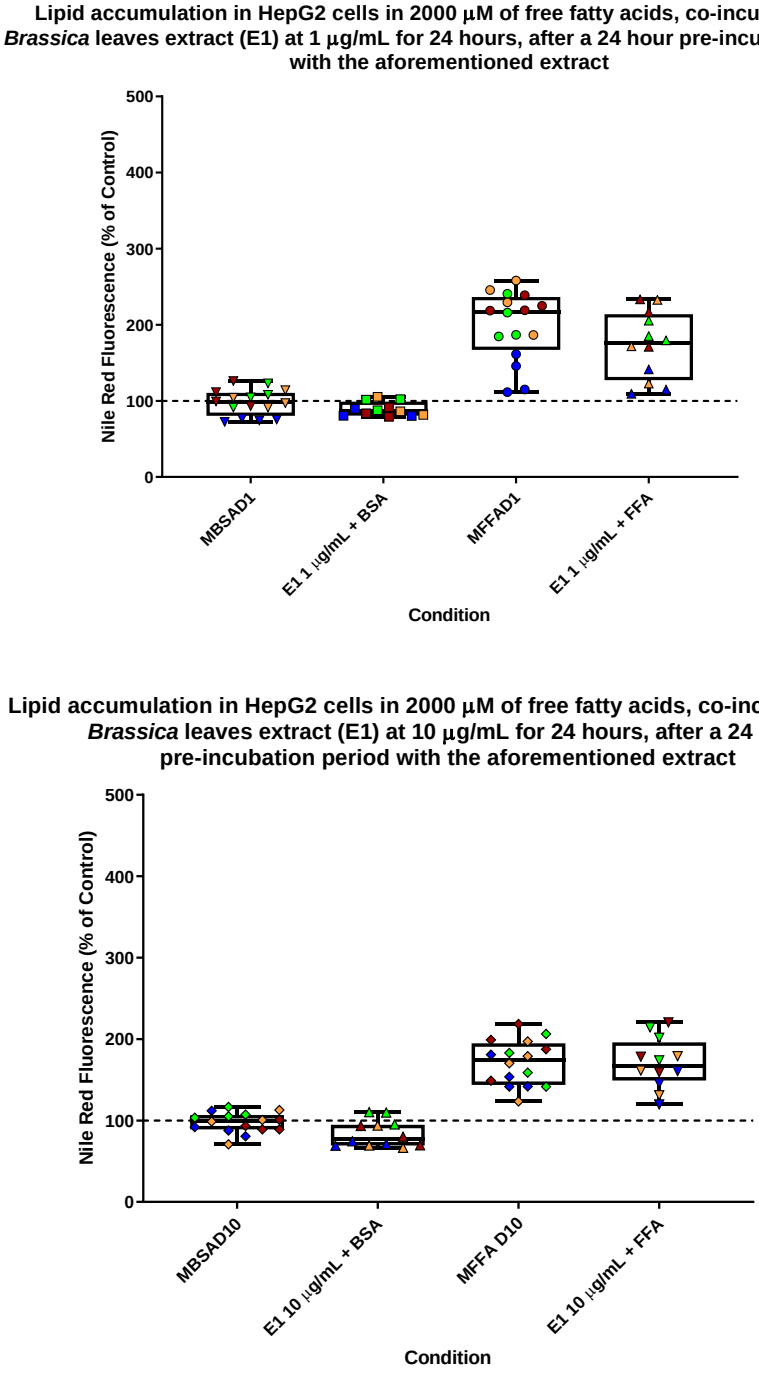
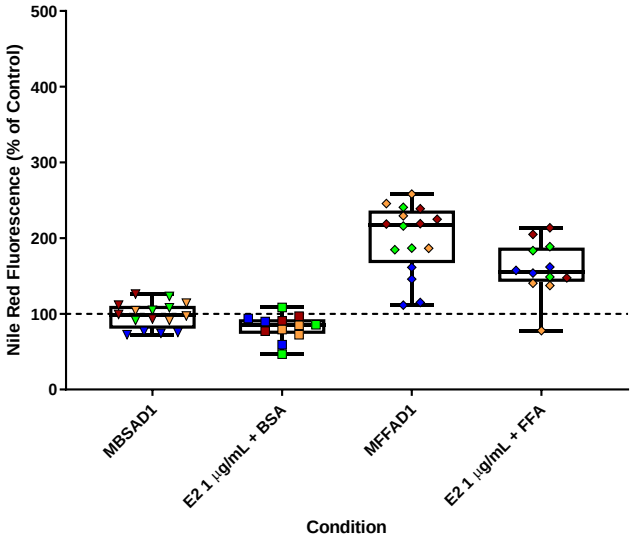


Figure 138 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.

Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 1 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract



Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 10 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract

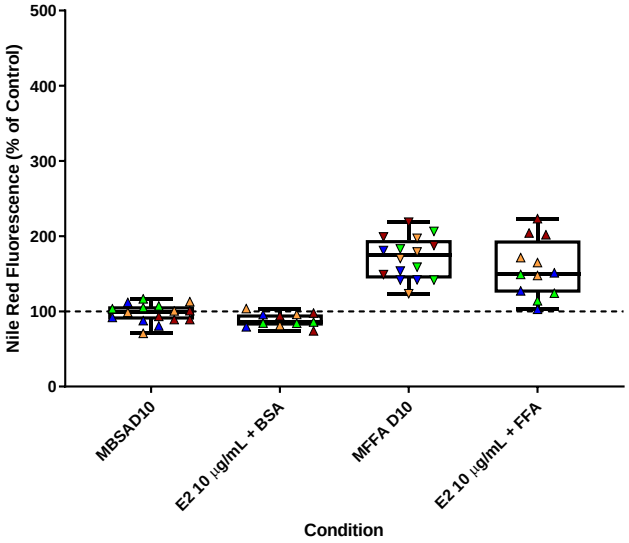
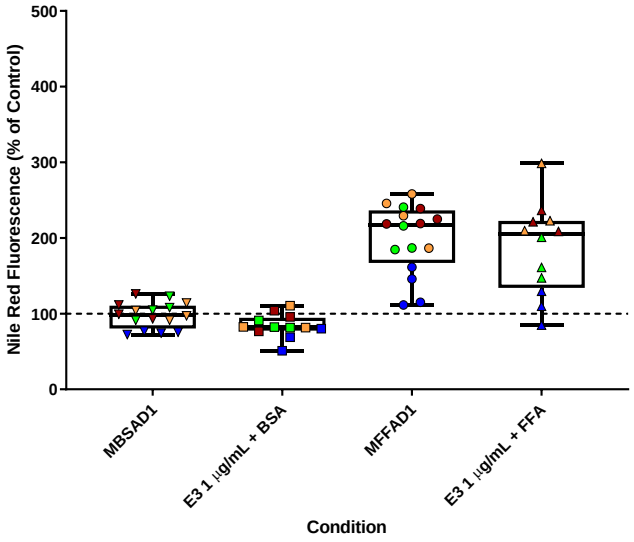


Figure 139 - Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.

Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E3) at 1 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract



Lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E3) at 10 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract

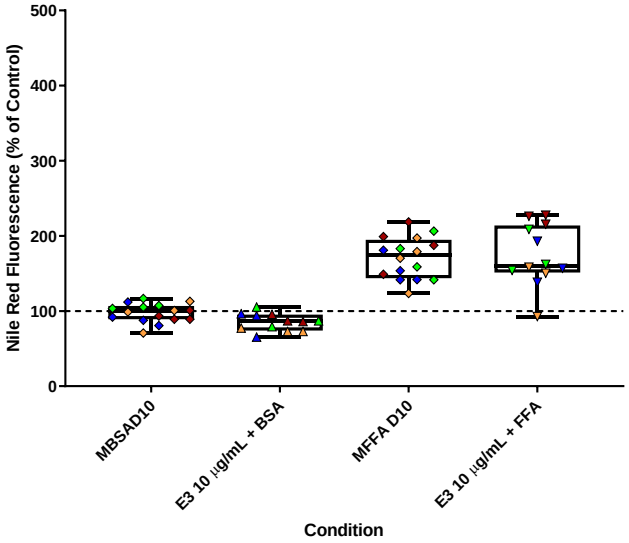


Figure 140 - Lipid accumulation in HepG2 cells in 1000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (2%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (2%); **MFFAD1:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 1000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (2%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (2%); **MFFAD10:** Cells with culture medium treated with 1000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 1000 µM of FFA.

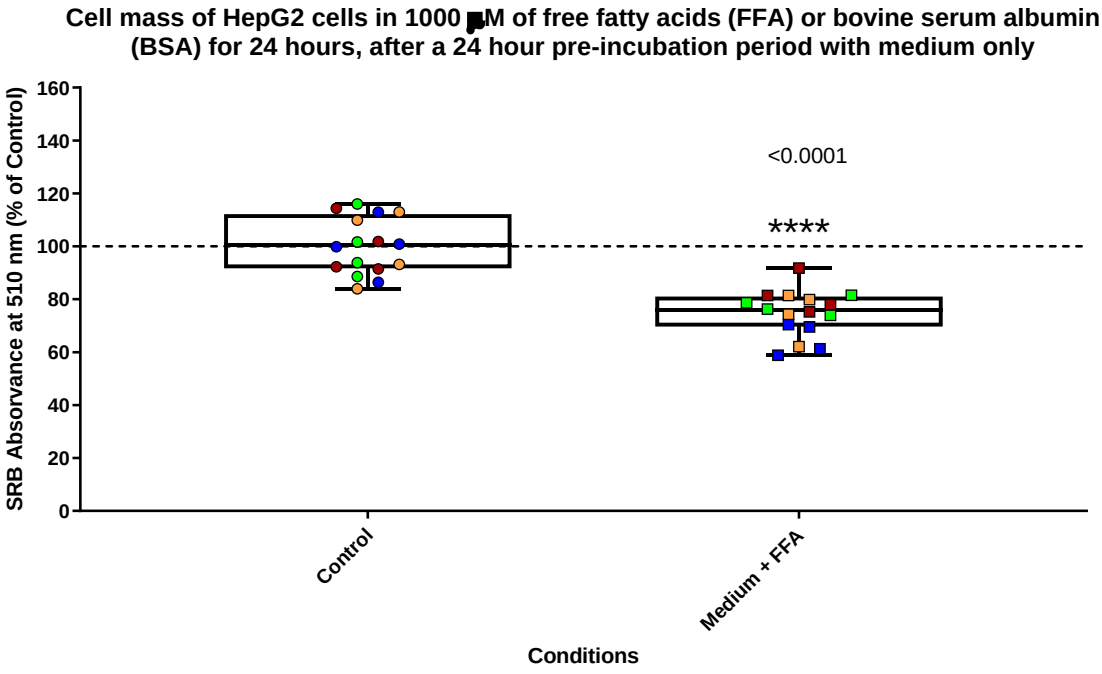


Figure 141 - Cell mass of HepG2 cells in 1000 µM of free fatty acids (FFA) or BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (0.5%); **Medium + FFA:** Cell culture medium with 250 µM of FFA.

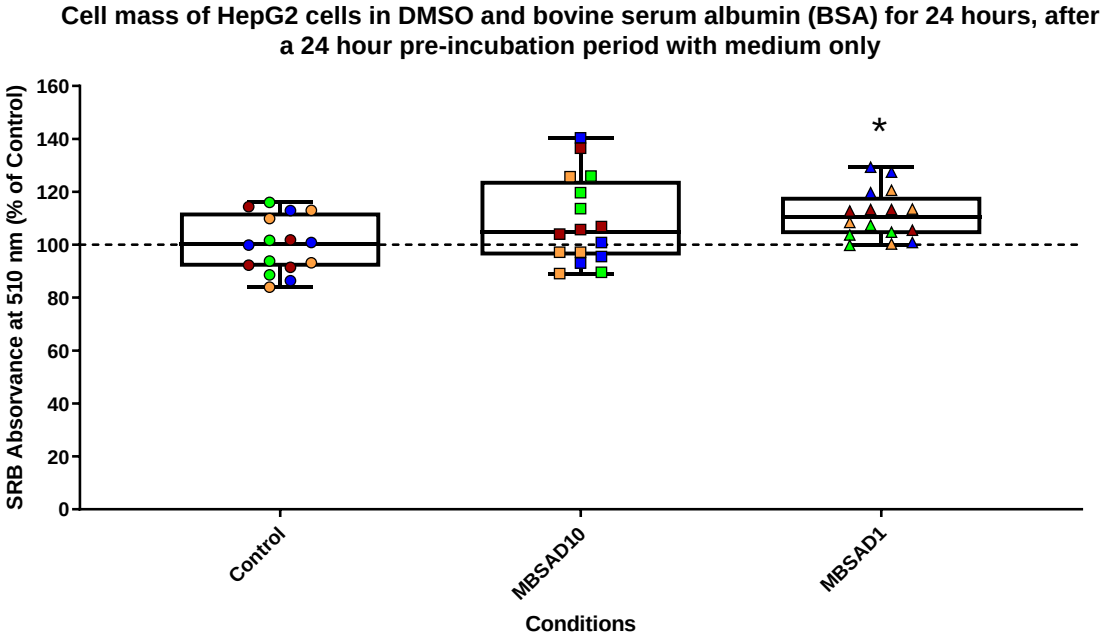


Figure 142 - Cell mass of HepG2 cells in DMSO and BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0367 vs Control; **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

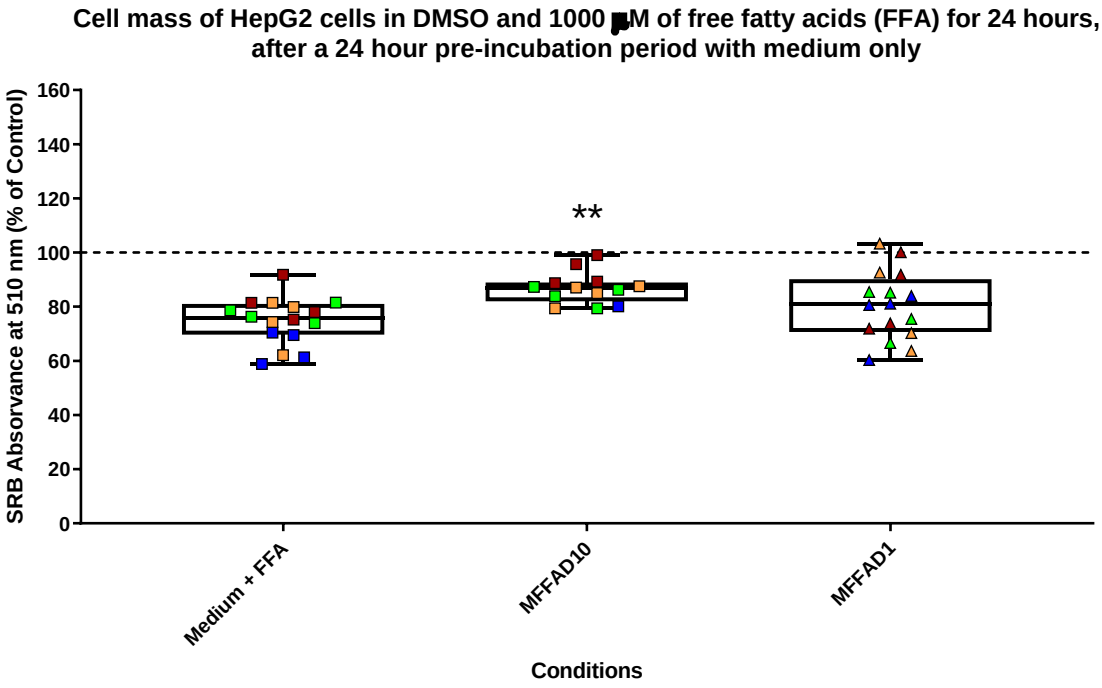
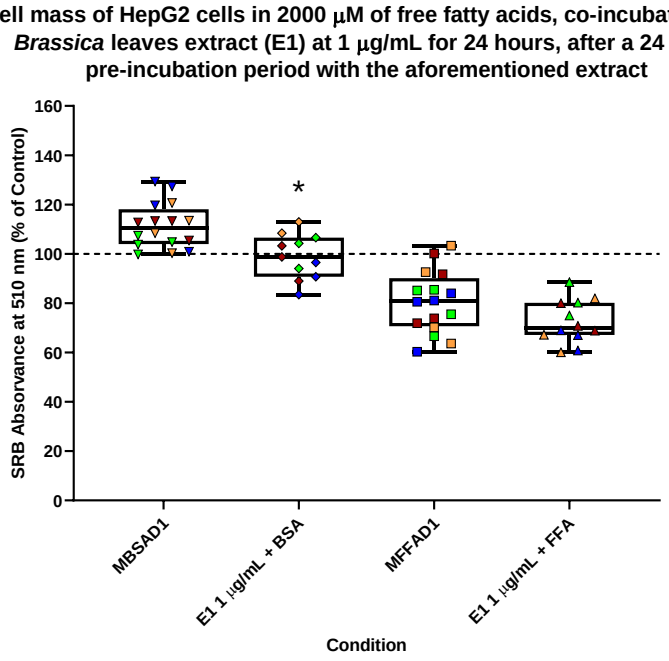


Figure 143 - HepG2 cells in DMSO and 2000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0045 vs Medium + FFA; +p=0.119 vs MFFAD10. **Medium + FFA:** Cell culture medium with 2000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%).



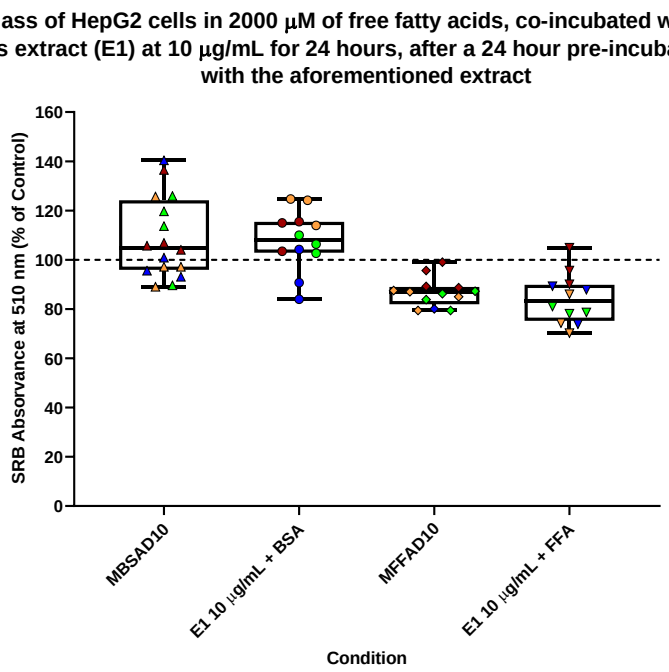
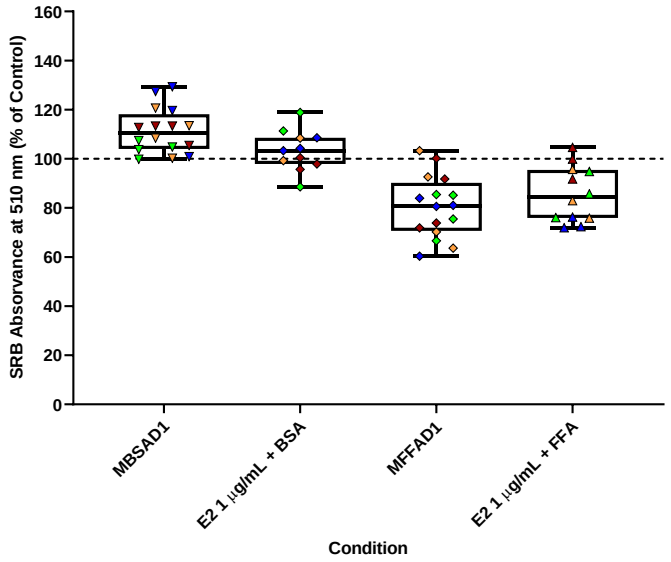


Figure 144 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0164 vs MBSAD1. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.

Cell mass of HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 1 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract



Cell mass of HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 10 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract

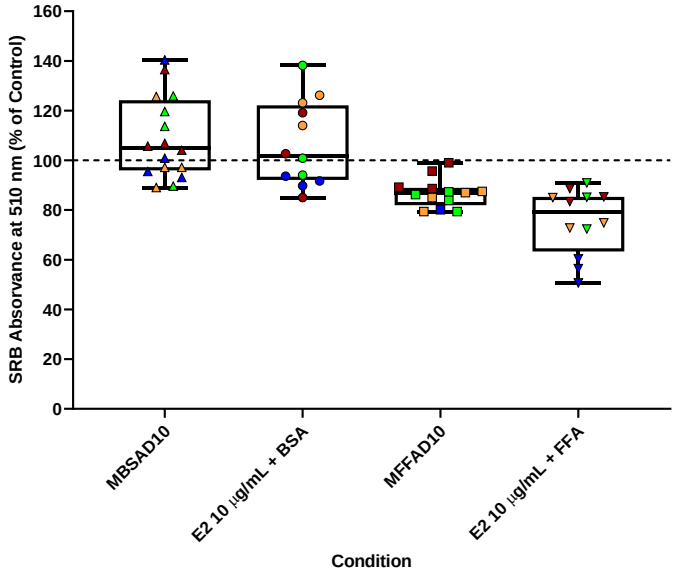
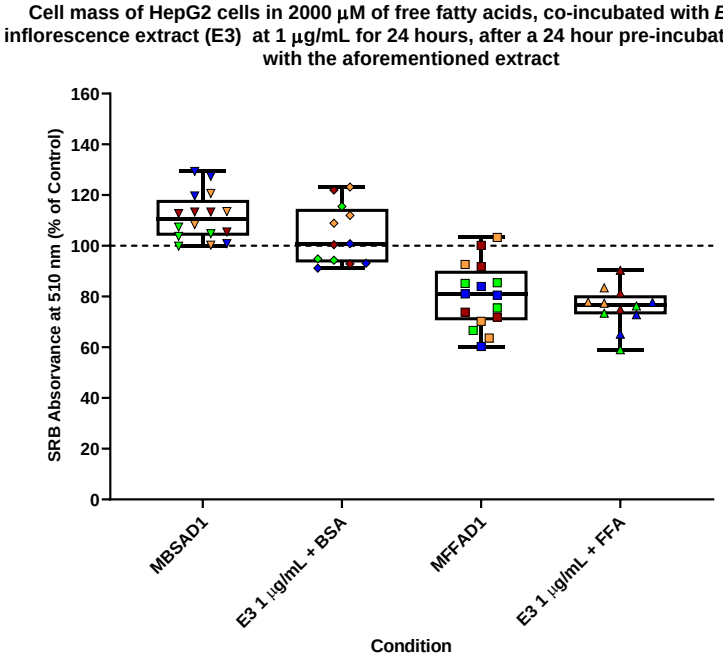


Figure 145 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0048 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.



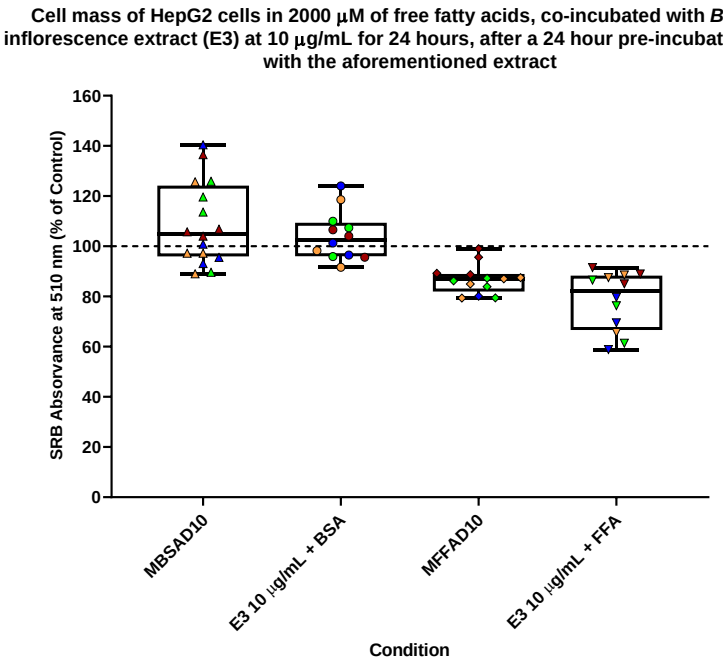


Figure 146 - Cell mass of HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 2000 µM of FFA.

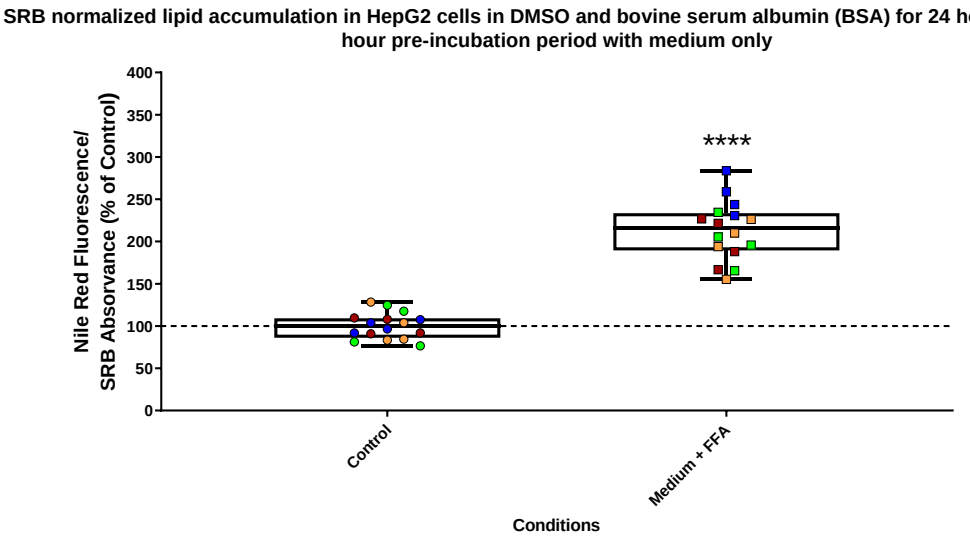


Figure 147 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA) or BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p<0.0001 vs Control. **Control:** Cell culture medium with BSA (4%); **Medium + FFA:** Cell culture medium with 2000 µM of FFA.

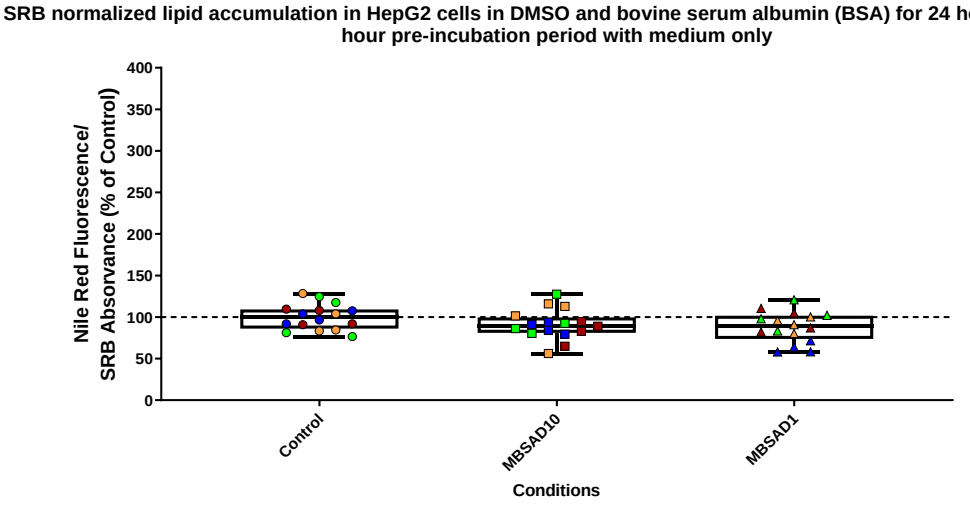


Figure 148 - SRB normalized lipid accumulation in HepG2 cells in DMSO and BSA (4%) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **Control:** Cell culture medium with BSA (4%); **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%).

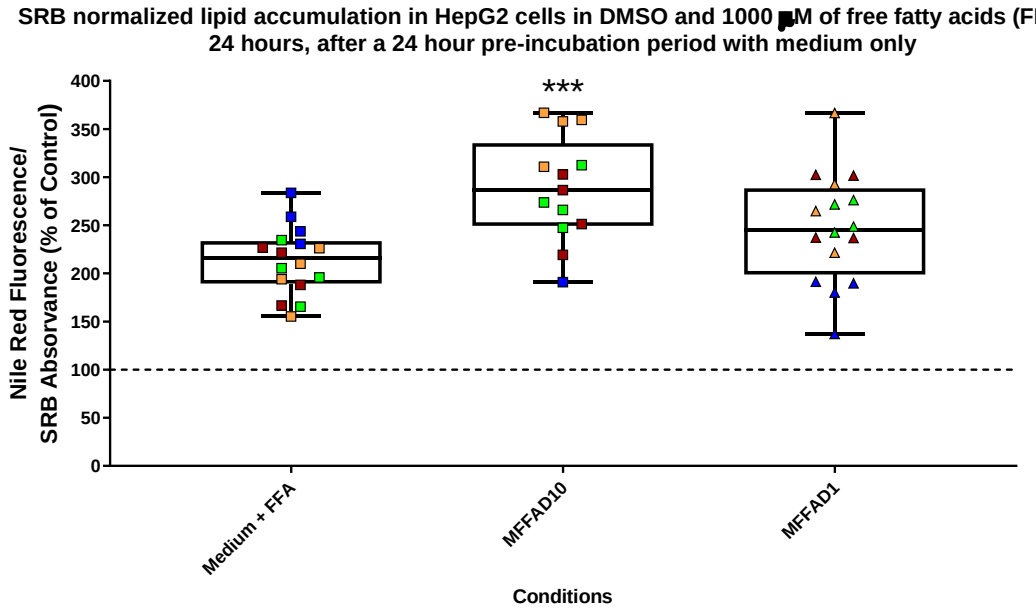
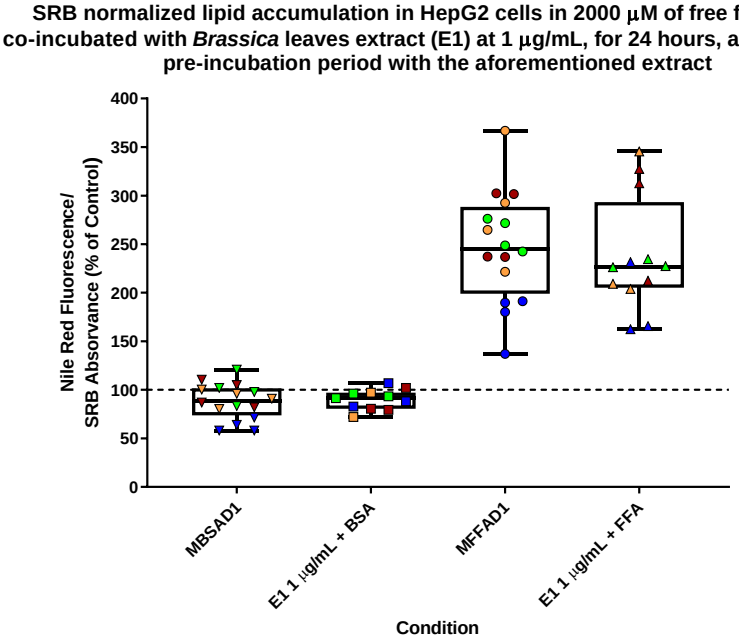


Figure 149 - SRB normalized lipid accumulation in HepG2 cells in DMSO and 2000 µM of free fatty acids (FFA) for 24 hours, after a 24 hour pre-incubation period with medium only. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0005 vs Medium + FFA; **Medium + FFA:** Cell culture medium with 2000 µM of FFA; **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%).



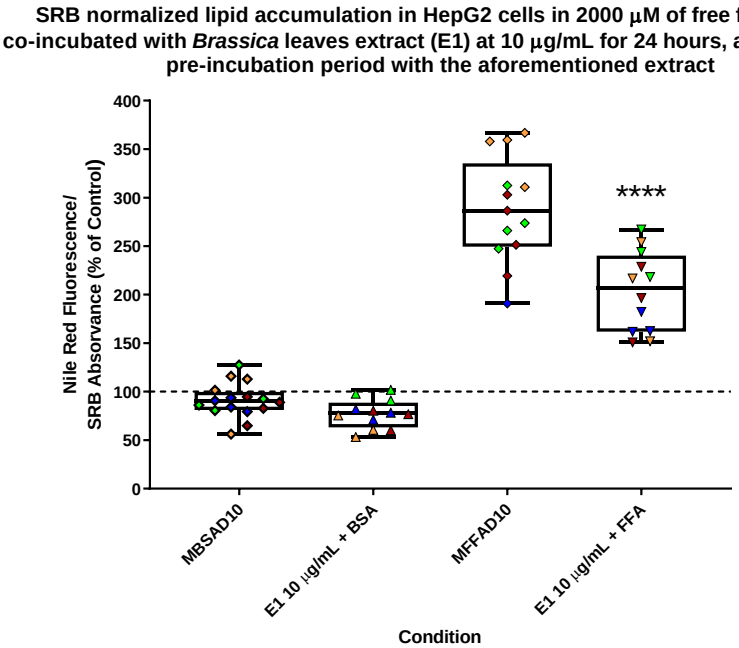
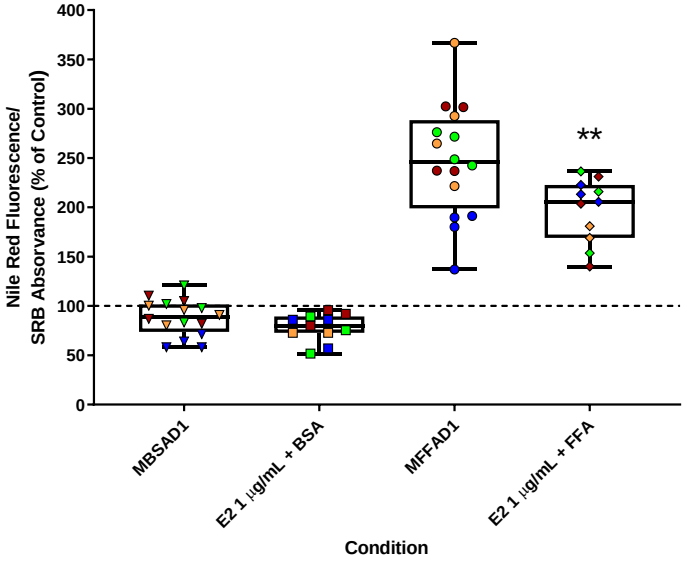


Figure 150 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* leaves extract (E1), at 1 or 10 µg/mL, at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ****p=0.0001 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E1 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of leaves extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E1 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of leaves extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E1 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E1 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of leaves extract and 2000 µM of FFA.

SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 1 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract



SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids, co-incubated with *Brassica* stalk extract (E2) at 10 µg/mL for 24 hours, after a 24 hour pre-incubation period with the aforementioned extract

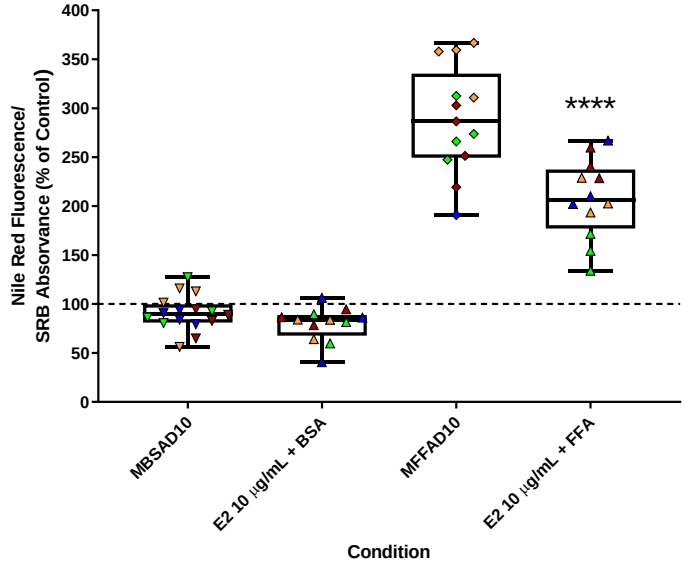
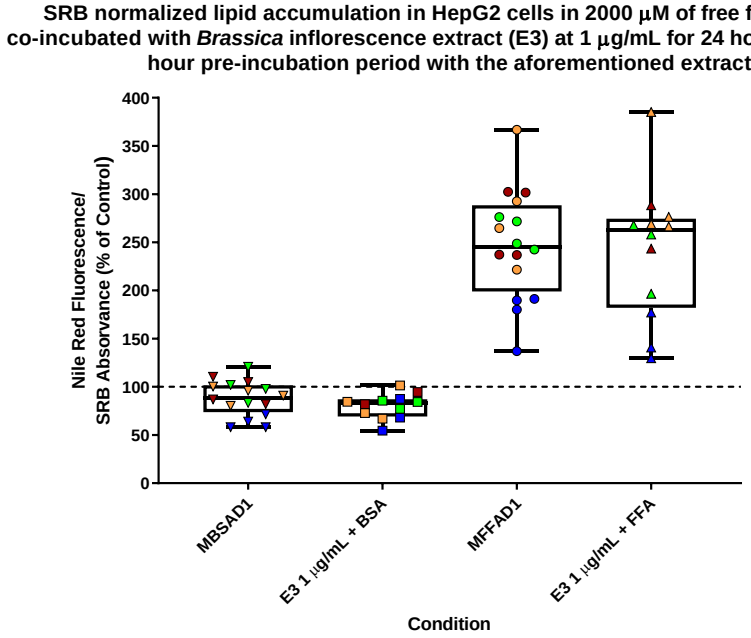


Figure 151 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* stalk extract (E2), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **p=0.0048 vs MFFAD1; ****p=0.0001 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E2 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of stalk extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E2 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of stalk extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E2 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E2 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of stalk extract and 2000 µM of FFA.



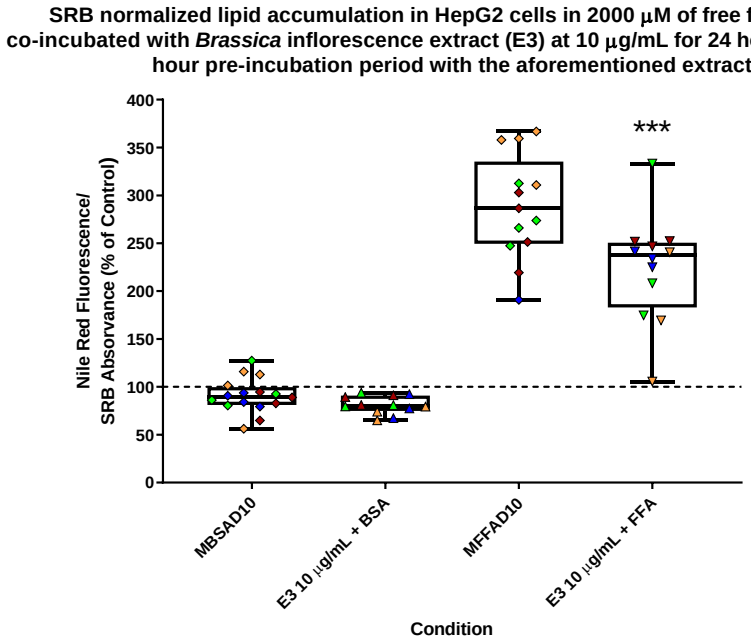


Figure 152 - SRB normalized lipid accumulation in HepG2 cells in 2000 µM of free fatty acids (FFA), co-incubated with *Brassica* inflorescence extract (E3), at 1 or 10 µg/mL, after a 24 hour pre-incubation period with the aforementioned extracts. Lipid accumulation was measured using the Nile red colorimetric assay and cell mass was measured using the SRB assay. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of four replicates for MBSAD1, MBSAD10, MFFAD1 and MFFAD10 and three replicates for every other condition tested. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0009 vs MFFAD10. **MBSAD1:** Cells with culture medium, BSA (4%) and DMSO (0,002%); **E3 1 µg/mL + BSA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and BSA (4%); **MFFAD1:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,002%); **E3 1 µg/mL + FFA:** Cells with culture medium treated with 1 µg/mL of inflorescence extract and 2000 µM of FFA; **MBSAD10:** Cells with culture medium, BSA (4%) and DMSO (0,02%); **E3 10 µg/mL + BSA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (4%); **MFFAD10:** Cells with culture medium treated with 2000 µM of FFA and DMSO (0,02%); **E3 10 µg/mL + FFA:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 2000 µM of FFA.

4.2.7. Oxygen consumption rate (OCR) in HepG2 cells, after exposure to 250 µM of FFA: The potential of BBP to prevent mitochondrial damage – co-incubation approach

In an attempt to have a better understanding of the cellular and molecular mechanisms behind the results previously obtained with 250 µM of FFA in the co-incubation approach, oxygen consumption rate (OCR) of several parameters of mitochondrial were measured under those same conditions. The effects of FFA, if any, were then observed and extracts capacity to prevent those effects were assessed. Only extracts at 10 µg/mL were tested, as this is the concentration where the extracts performed better in the previous assays.

There was a statistically significant decrease in oxygen consumption rate in basal respiration, maximal respiration, and spare respiration capacity of HepG2 cells incubated with 250 µM of FFA when compared to cells incubated with BSA.

Inflorescence extract at 10 µg/mL caused a statistically significant reduction of oxygen consumption rate in spare respiratory capacity in cells co-incubated with 250 µM of FFA when compared to cells co-incubated with 250 µM FFA and DMSO.

No further statistically significant differences were observed between the control groups with 250 µM of FFA plus DMSO and the corresponding groups with 250 µM of FFA plus the extracts, among the various parameters of mitochondrial respiration.

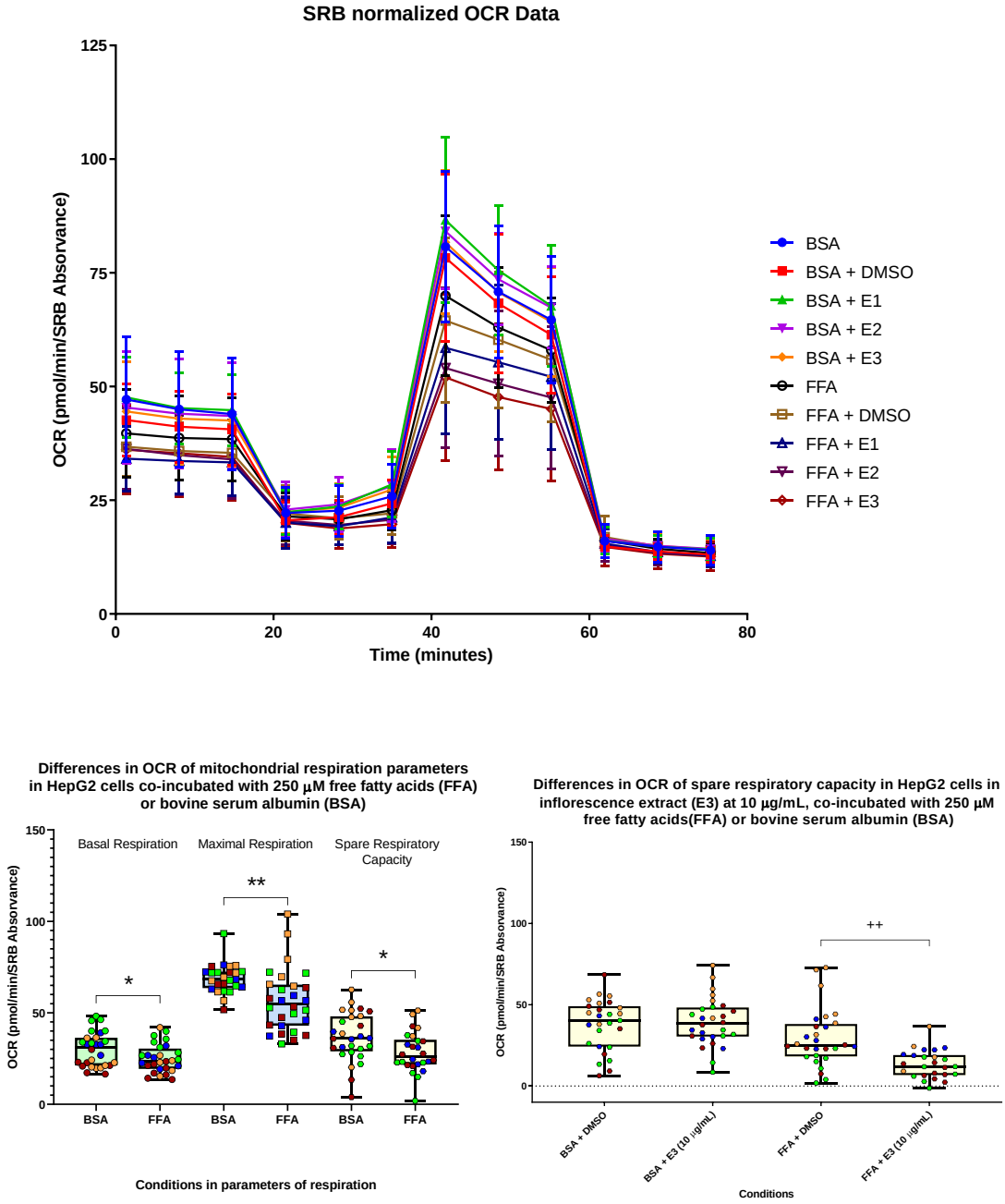


Figure 153 - Seahorse XF Cell Mito Stress profile of HepG2 cells co-incubated with extracts and 250 µM of FFA for 24 hours. Significant differences found are highlighted as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of four independent experiments of eight replicates, where every color represents an independent experiment and every same colored symbol represent a biological replicate. *p<0.04; **p=0.0022; ++p=0.0048. **BSA:** Cell culture medium with BSA (0.5%); **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **BSA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **BSA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **FFA:** Cell culture medium with 250 µM of FFA; **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **FFA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA; **FFA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA; **FFA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.8. Oxygen consumption rate (OCR) in HepG2 cells, after exposure to 250 µM of FFA: The potential of BBP to prevent mitochondrial damage – pre-incubation approach

In an attempt to have a better understanding of the cellular and molecular mechanisms behind the results previously obtained with 250 µM of FFA in the pre-incubation approach, oxygen consumption rate (OCR) of several parameters of mitochondrial were measured under those same conditions. The effects of FFA, if any, were then observed and extracts capacity to prevent those effects were assessed. Only extracts at 10 µg/mL were tested, as this is the concentration where the extracts performed better in the previous assays.

No statistically significant differences were observed in the oxygen consumption rate between the control groups with 250 µM of FFA plus DMSO and the corresponding groups with 250 µM of FFA plus the extracts, nor between the control groups with FFA or BSA only among the various parameters of mitochondrial respiration.

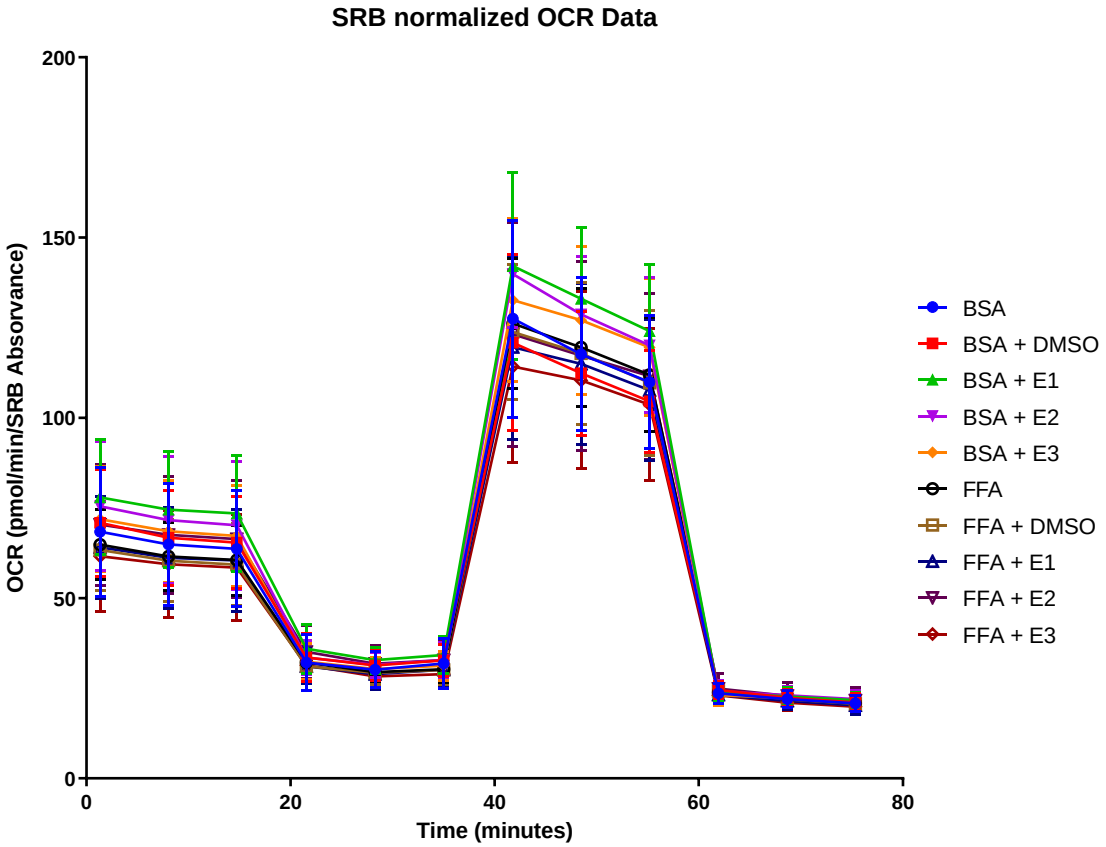


Figure 154 - Seahorse XF Cell Mito Stress profile of HepG2 cells, pre-incubated with extracts for 24 hours, and co-incubated with extracts and 250 µM of FFA for another 24 hours. **BSA:** Cell culture medium with BSA (0.5%); **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **BSA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **BSA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **FFA:** Cell culture medium with 250 µM of FFA; **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,02%); **FFA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA; **FFA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA; **FFA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.9. Intracellular ATP content in HepG2 cells after exposure to 250 µM of FFA for 24h, in presence and absence of BBP – Extracts and FFA Co-incubation approach

These assays were made to complement the results of the metabolic flux analysis, specifically the OCR associated with ATP production. It also done to get a general view of how the extracts affect intracellular ATP content over-all. The following results are for HepG2 co-incubation with extracts and 250 µM of FFA for 24 hours. Only extracts at 10 µg/mL were tested, as this is the concentration where the extracts performed better in the previous assays.

Although no statistically significant differences were observed in the ATP content between the control groups with 250 µM of FFA or bovine serum albumin, with or without DMSO, DMSO appears to have reduced ATP content in control conditions when compared to controls without DMSO.

Inflorescence extract at 10 µg/mL increases ATP content in HepG2 cells when co-incubated with 250 µM of FFA or bovine serum albumin, when compared to control groups without the extract.

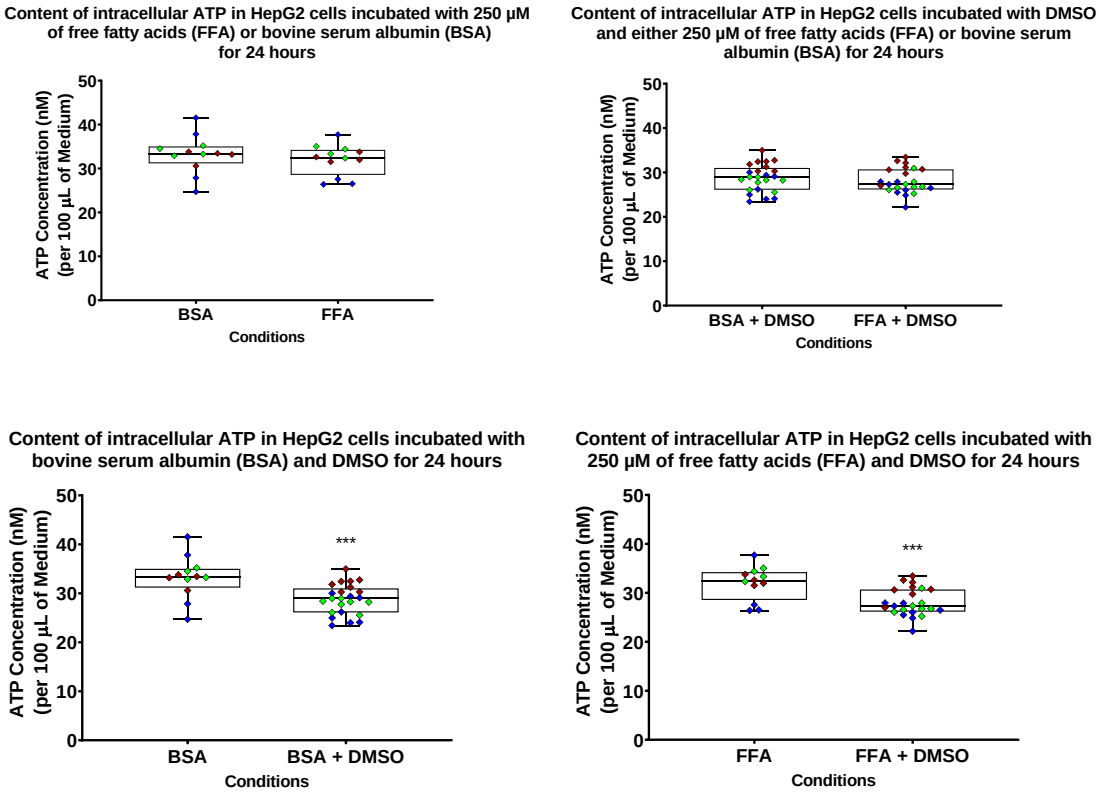


Figure 155 – Intracellular ATP content in HepG2 cells, across control conditions. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either four or eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. ***p=0.0010 vs BSA and FFA. **BSA:** Cell culture medium with BSA (0.5%); **FFA:** Cell culture medium with 250 µM of FFA; **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).

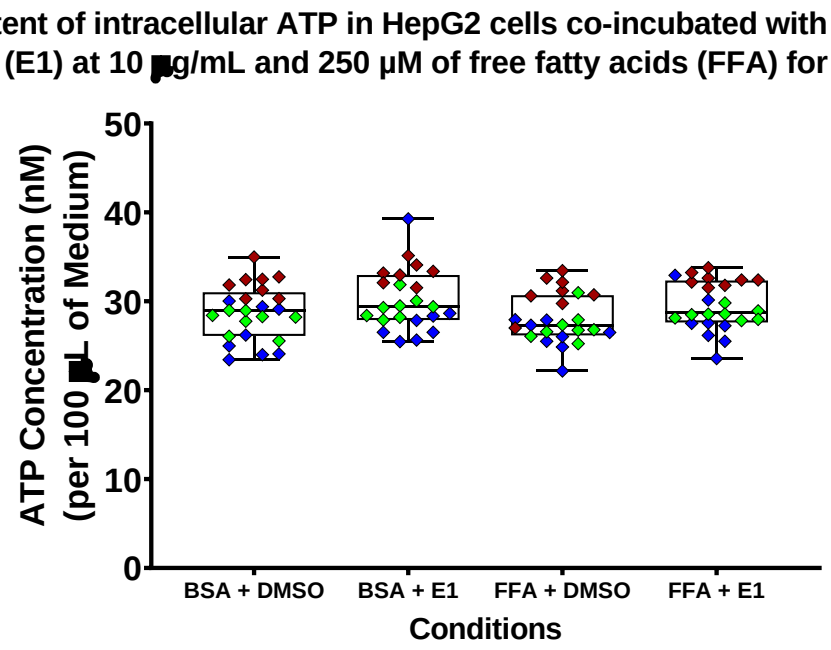


Figure 156 - Intracellular ATP content in HepG2 cells co-incubated with leaves extract (E1) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA.

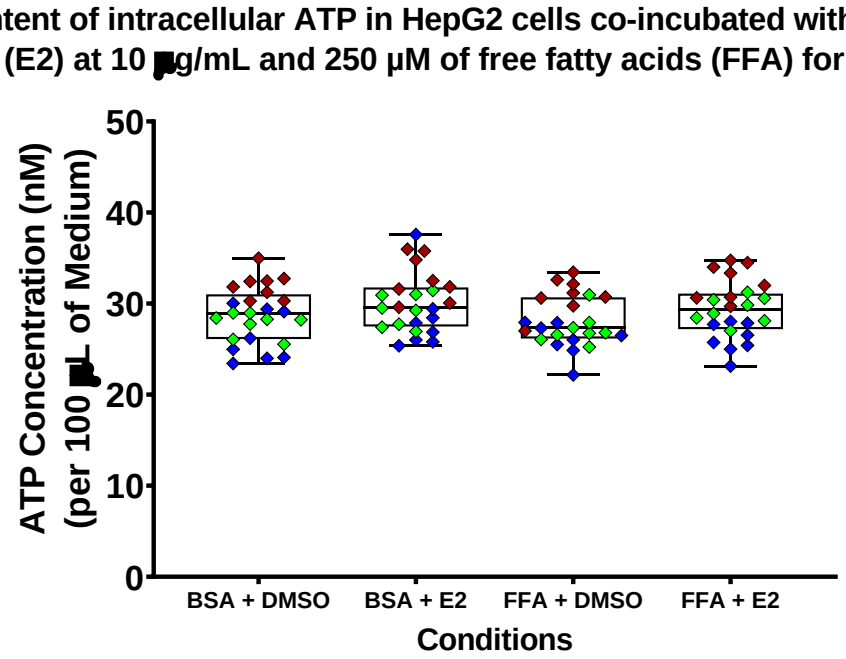


Figure 157 - Intracellular ATP content in HepG2 cells co-incubated with stalk extract (E2) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

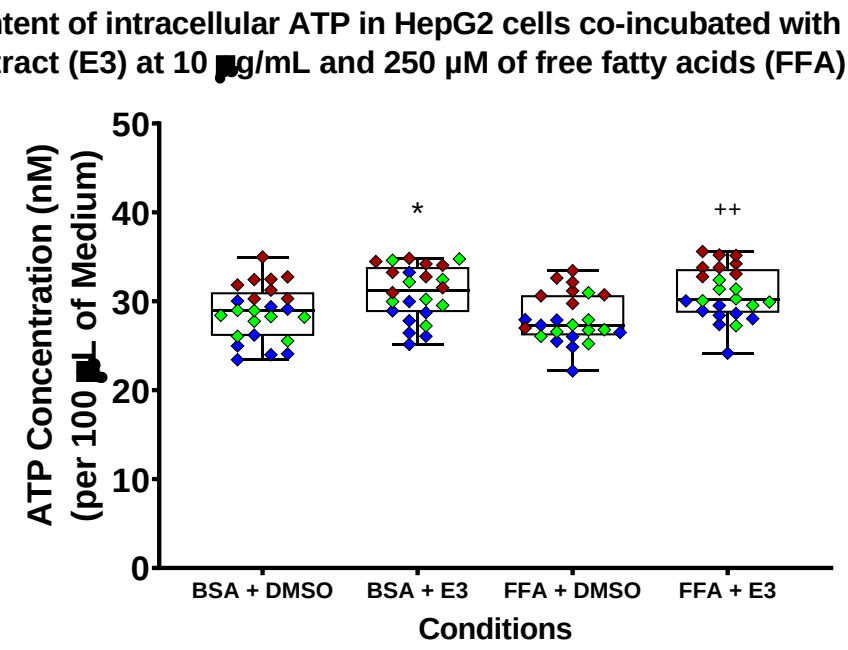


Figure 158 - Intracellular ATP content in HepG2 cells co-incubated with inflorescence extract (E3) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0495 vs BSA + DMSO; ++p= 0.0073 vs FFA + DMSO. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.10. Intracellular ATP content in HepG2 cells after exposure to 250 µM of FFA for 24h, in presence and absence of BBP – pre-incubation approach

These assays were made to complement the results of the metabolic flux analysis, specifically the OCR associated with ATP production. It also is done to get a general view of how the extracts affect intracellular ATP content over-all. The following results are for HepG2 pre-incubation with extracts for 24 hours, followed by co-incubation with extracts and 250 µM of FFA for another 24 hours. Only extracts at 10 µg/mL were tested, as this is the concentration where the extracts performed better in the previous assays.

No statistically significant differences were observed in ATP content between the control groups with 250 µM of FFA plus DMSO and the corresponding groups with 250 µM of FFA plus the extracts, nor between the control groups with FFA or BSA only.

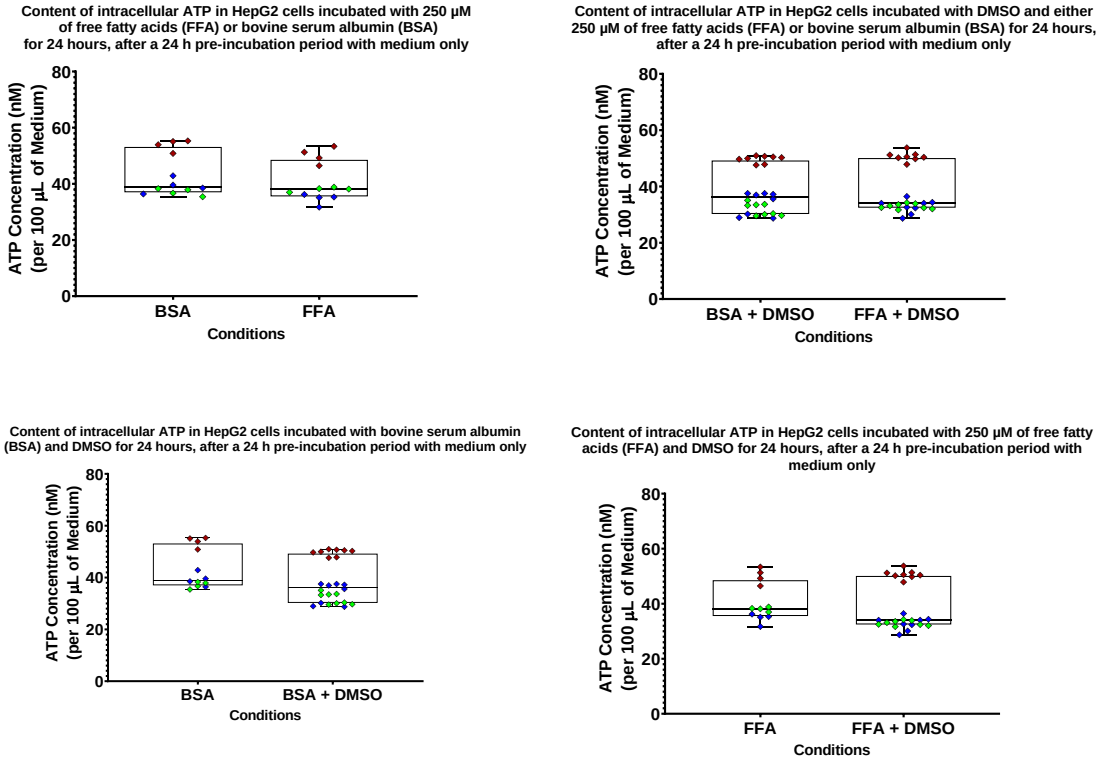


Figure 159 - Intracellular ATP content in HepG2 cells, across control conditions. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of either four or eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **BSA:** Cell culture medium with BSA (0.5%); **FFA:** Cell culture medium with 250 µM of FFA; **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%).

Content of intracellular ATP in HepG2 cells co-incubated with leaves extract (E1) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 h pre-incubation period with the aforementioned extract

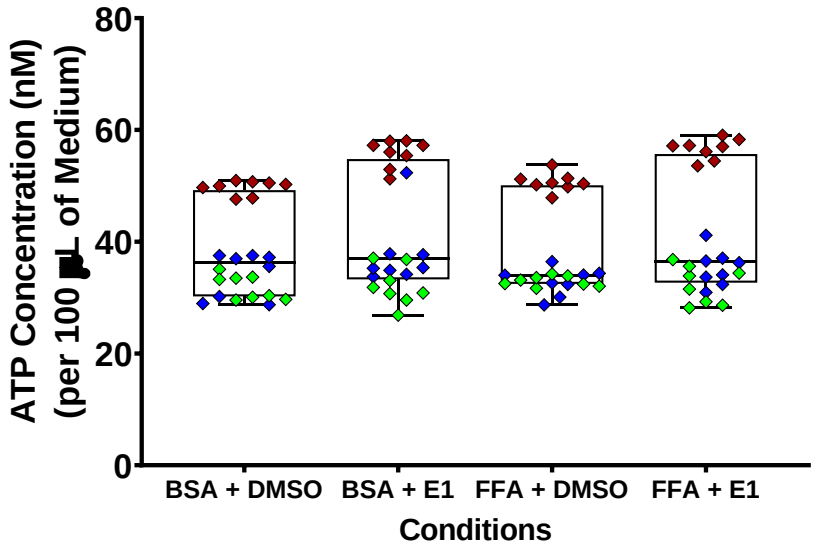


Figure 160 - Intracellular ATP content in HepG2 cells co-incubated with leaves extract (E1) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 h pre-incubation period with the aforementioned extract. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E1:** Cells with culture medium treated with 10 µg/mL of leaves extract and 250 µM of FFA

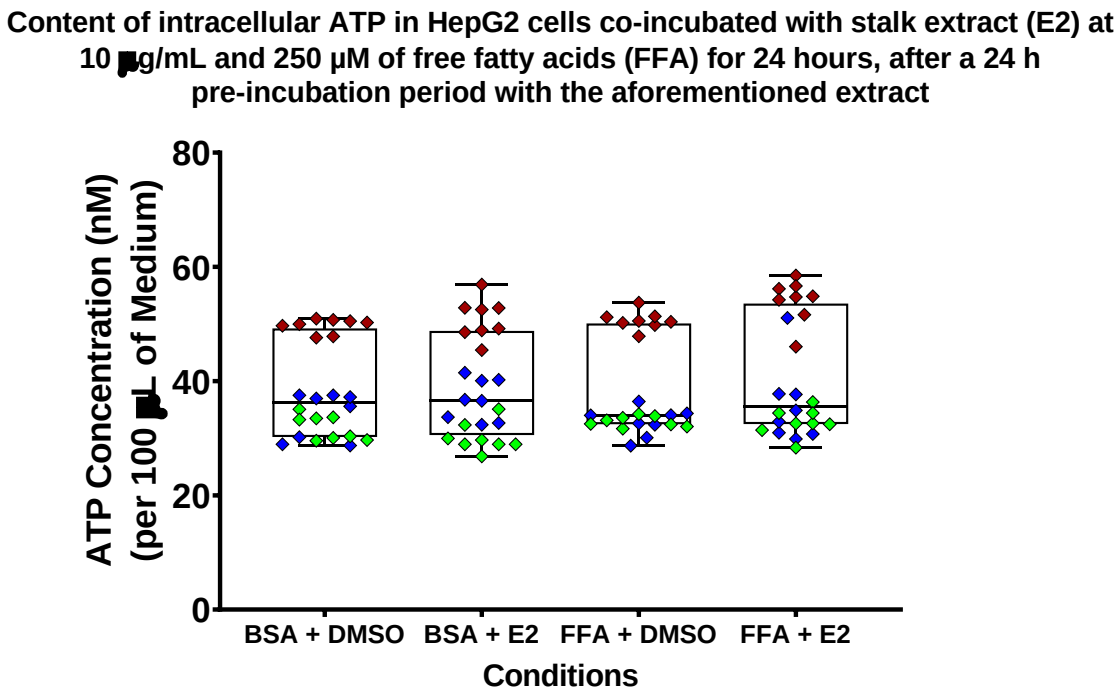


Figure 161 - Intracellular ATP content in HepG2 cells co-incubated with stalk extract (E2) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 h pre-incubation period with the aforementioned extract. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E2:** Cells with culture medium treated with 10 µg/mL of stalk extract and 250 µM of FFA.

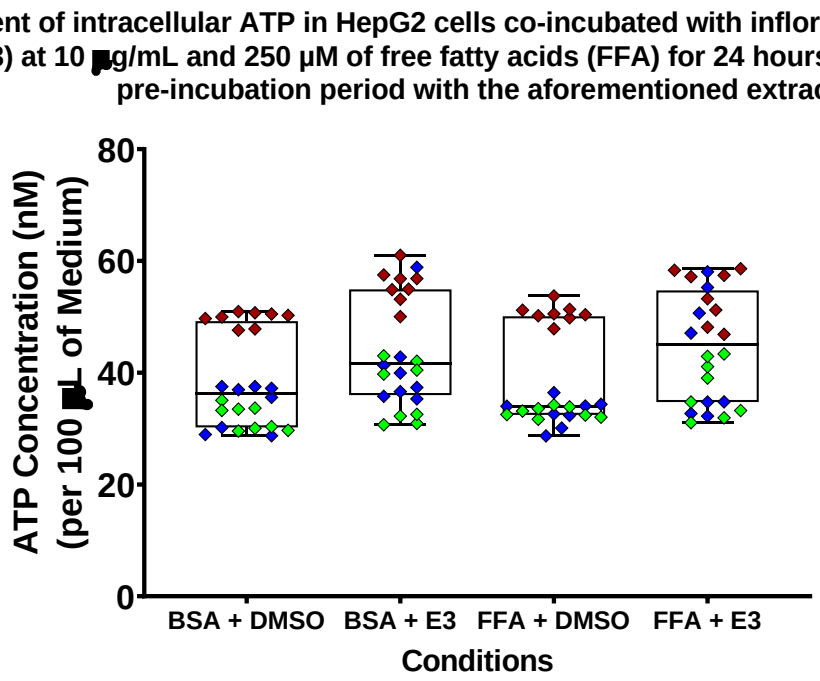


Figure 162 - Intracellular ATP content in HepG2 cells co-incubated with inflorescence extract (E3) at 10 µg/mL and 250 µM of free fatty acids (FFA) for 24 hours, after a 24 h pre-incubation period with the aforementioned extract. Data are expressed as box plots, bordered at the 25th and 75th percentiles of the y variable, with a median line at the 50th percentile, showing all points of three independent experiments of eight replicates. Every color represents an independent experiment and every same colored symbol represent a biological replicate. *p=0.0495 vs BSA + DMSO; ++p= 0.0073 vs FFA + DMSO. **BSA + DMSO:** Cells with culture medium, BSA (0,5%) and DMSO (0,02%); **BSA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and BSA (0,5%); **FFA + DMSO:** Cells with culture medium treated with 250 µM of FFA and DMSO (0,002%); **FFA + E3:** Cells with culture medium treated with 10 µg/mL of inflorescence extract and 250 µM of FFA.

4.2.11. Summary results of the potential prevention capacity of *Brassica* by-products in a model of hepatic accumulation of lipids and protection against a model of hepatic lipotoxicity damage, induced by free fatty acids

Table 5 - Summary results of cell mass assays in HepG2 cells, with extracts and FFA. **E1.1:** Cell culture medium with1 µg/mL of leaves extract; **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E2.1:** Cell culture medium with1 µg/mL of stalk extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E3.1:** Cell culture medium with1 µg/mL of inflorescence extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **250 µM:** 250 µM of free fatty acids; **1000 µM:** 1000 µM of free fatty acids; **2000 µM:** 2000 µM of free fatty acids **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Cell Mass						
	24h Co-Incubation			24 h Pre-Incubation + 24 h Co-Incubation		
	250 µM	1000 µM	2000 µM	250 µM	1000 µM	2000 µM
E1.1	ns	ns	<	<	<	ns
E1.10	ns	ns	<	ns	ns	ns
E2.1	ns	ns	ns	ns	<	ns
E2.10	ns	ns	>	ns	ns	ns
E3.1	ns	ns	ns	ns	ns	ns
E3.10	ns	ns	>	ns	>	ns

Table 6 - Summary results of SRB normalized lipid accumulation assays in HepG2 cells, with extracts and FFA. **E1.1:** Cell culture medium with1 µg/mL of leaves extract; **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E2.1:** Cell culture medium with1 µg/mL of stalk extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E3.1:** Cell culture medium with1 µg/mL of inflorescence extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **250 µM:** 250 µM of free fatty acids; **1000 µM:** 1000 µM of free fatty acids; **2000 µM:** 2000 µM of free fatty acids **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

SRB Normalized Lipid Accumulation						
	24h Co-Incubation			24 h Pre-Incubation + 24 h Co-Incubation		
	250 µM	1000 µM	2000 µM	250 µM	1000 µM	2000 µM
E1.1	ns	ns	>	>	ns	ns
E1.10	ns	ns	ns	<	ns	<
E2.1	ns	ns	ns	ns	ns	<
E2.10	<	ns	ns	<	ns	<
E3.1	ns	ns	ns	ns	ns	ns
E3.10	<	ns	ns	<	ns	<

Table 7 - Summary results of metabolic flux analysis (I) in HepG2 cells, with extracts and FFA. **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **BR:** Basal respiration; **MR:** Maximal respiration; **PL:** Proton Leak; **ATP:** OCR associated with ATP production; **SRC:** Spare respiratory capacity; **NM-OCR:** Non-mitochondrial oxygen consumption rate; **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Metabolic Flux Analysis (I)							
		24h Co-Incubation					
		BR	MR	PL	ATP	SRC	NM-OCR
E1.10	+ 250 µM FFA	ns	ns	ns	ns	ns	ns
E2.10		ns	ns	ns	ns	ns	ns
E3.10		ns	ns	ns	ns	<	ns
E1.10	+ BSA	ns	ns	ns	ns	ns	ns
E2.10		ns	ns	ns	ns	ns	ns
E3.10		ns	ns	ns	ns	ns	ns

Table 8 - Summary results of metabolic flux analysis (II) in HepG2 cells, with extracts and FFA. **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **BR:** Basal respiration; **MR:** Maximal respiration; **PL:** Proton Leak; **ATP:** OCR associated with ATP production; **SRC:** Spare respiratory capacity; **NM-OCR:** Non-mitochondrial oxygen consumption rate; **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Metabolic Flux Analysis (II)							
		24 h Pre-Incubation + 24 h Co-Incubation					
		BR	MR	PL	ATP	SRC	NM-OCR
E1.10	+ 250 µM FFA	ns	ns	ns	ns	ns	ns
E2.10		ns	ns	ns	ns	ns	ns
E3.10		ns	ns	ns	ns	ns	ns
E1.10	+ BSA	ns	ns	ns	ns	ns	ns
E2.10		ns	ns	ns	ns	ns	ns
E3.10		ns	ns	ns	ns	ns	ns

Table 9 - Summary results of metabolic flux analysis (II) in HepG2 cells, with extracts and FFA. **E1.10:** Cell culture medium with 10 µg/mL of leaves extract; **E2.10:** Cell culture medium with 10 µg/mL of stalk extract; **E3.10:** Cell culture medium with 10 µg/mL of inflorescence extract; **ns:** Non-significant differences; **< :** decrease in measured parameter; **> :** increase in measured parameter.

Intracellular ATP Content					
		24h Co-Incubation		**24 h Pre-Incubation + 24 h Co-Incubation**	
		vs 250 µM FFA + DMSO	vs BSA + DMSO	vs 250 µM FFA + DMSO	vs BSA + DMSO
E1.10	+ 250 µM FFA	ns	-	ns	-
E2.10		ns	-	ns	-
E3.10		>	-	ns	-
E1.10	+ BSA	-	ns	-	ns
E2.10		-	ns	-	ns
E3.10		-	>	-	ns

5. Discussion

In the field of agricultural industry, a lot of organic waste is generated. This is because only a small part of the harvested plant is deemed edible for humans and goes on to be sold. The remaining parts of the plant are disposed of. In many cases these methods of disposal are not environmentally friendly. Furthermore, it represents a huge economic waste. *Brassica oleracea* is the perfect example of this, and one of the main reasons it was chosen as the focus of this project. To prevent these negative side effects of broccoli harvest, the discovery of biomedical value for future applications was proposed. Based on existing literature, the antioxidant properties of different kinds of *Brassica* waste were put to the test.

In the experiments performed in presence tBHP no protection capacity was observed from the extracts against tBHP toxicity. However, a semblance of protection was observed in stalk extract at 1 and 10 µg/mL and inflorescence extract at 10 µg/mL, when compared to a control group without the extracts' vehicle, DMSO. This observation might point to a slight protective property of DMSO against tBHP toxicity. In fact, Sanmartín-Suárez et al. reported that DMSO reduces lipid peroxidation and protein carbonyl formation induced by ferrous chloride/hydrogen peroxide in rat brain homogenates, suggesting antioxidant properties of this solvent [132]. Still, the concentration of tBHP used might have been too high for the cell density used, as this was not the lowest toxic concentration tested. However, we choose a concentration of 100µM of tBHP to make the results more robust. Since the concentration chosen does not mimic the actual oxidative stress that cells go through in obesity related morbidities, the model could be adjusted, by using a lower yet still toxic concentration of tBHP. One final deciding factor might be the fact that, as mentioned in section 2.7, HepG2 cells lack cytochrome P450 enzymes expression, which are part of one of the pathways tBHP is metabolized [78,105]. Without cytochrome P450 enzymes, tBHP will only be detoxified by way of glutathione peroxidase. One possibility for the antioxidant activity in BPP is glucosinolates.

Glucosinolates trigger a cascade of events that lead to increased expression of antioxidant enzymes such as glutathione peroxidase. Since out of the two possible pathways to metabolize tBHP, only one is available, perhaps the excessive tBHP in HepG2 cells may suppress an eventual overexpression in antioxidant enzymes, negating any possibly beneficial effects from the extracts. In conclusion, these findings do not show any protective capabilities from the extracts against tBHP, but they appear to suggest their existence.

When it comes to the FFA assays, the highlights go to stalk (E2) and inflorescence (E3) extracts at 10 µg/mL. 10 µg/mL of E2 has prevented cell mass loss due to lipotoxicity in co-incubation with 2000 µM of FFA and reduced lipid accumulation in co-incubation with 250 µM and in pre plus co-incubation with 250 and 2000 µM of FFA. On the other hand, 10 µg/mL of E3 prevented cell mass loss due to lipotoxicity in co-incubation with 2000 µM of FFA and in pre plus co-incubation with 1000 and 2000 µM of FFA. Furthermore, it prevented lipid accumulation in co-incubation with 250 µM and in pre plus co-incubation with 250 and 2000 µM of FFA. Finally, E3 increased the content of intracellular ATP, when co-incubated with FFA with BSA only. Suggesting that, independelty the conditions, E3 increases intracellular ATP levels. There was however a drawback: at 10 µg/mL, E3 caused a decreased in the spare respiratory capacity parameter of mitochondrial respiration in HepG2 cells co-incubated with 250 µM of FFA.

Stalks and leaves are the most studied and characterized of the 3 extracts since they are the ones with less commercial value and therefore the ones that need value. Under fall season conditions, the conditions under which the plants that originated the extracts were harvested, *Brassica oleracea* L. has been shown to possess a greater quantity of glucosinolates and myrosinase activity in the stalk than in the leaves [133]. In the fall (and also winter) temperatures and light exposure periods are lower and there is easier access to water. These two factors however are independent from each other as no correlation between the two has been found [134], meaning that a higher concentration of glucosinolates does not necessarily cause a higher activity of myrosinase, for example. Following the hypothesis that sulforaphane or other isothiocyanate is responsible for the effects observed, increased glucosinolate content and higher myrosinase activity may explain the better results obtained with E2 than with leaves extract (E1). Although out of the two, E2 had the best results, E1 should not be ruled out, since it had some success in preventing lipid accumulation. However, the negative effects of at 1 µg/mL should be cause for caution and further analysis if a definitive biomedical value is to be attributed. In the available literature, E3 has been shown to have superior glucosinolate content than E1 but lower than E2 [135]. Since E3 was arguably the best performing extract, save for the reduction in OCR in spare respiratory capacity, this was a bit surprising. This means that although it could be argued that glucosinolate content is a factor in BBP tested properties (the extract with the lowest concentration performed the poorest), it is not the only one. Myrosinase has been shown to be less active in *Brassica* plants, as the plant matures [136]. Since the inflorescence is in the less mature stage of all three extracts, myrosinase activity might make up for the low glucosinolate content. However, both glucosinolate content and myrosinase activity are highly dependent on culture conditions (light, type of agriculture, available water, temperature) and so all these explanations are merely a hypothesis to explain the obtained results. Glucosinolate content and myrosinase activity should be determined in the extracts used in the experiment,

however, technical difficulties prevented that from happening, leaving only existing literature to rely on for the formulation of this hypothesis.

The results of the co-incubation with extracts and 2000 µM of FFA should be taken with a grain of salt since the “quality control” for the experiment yielded no significant differences in the normalized accumulation of lipids between the control and FFA groups. This might be due to lipotoxicity and a sort of natural selection: only the cells that accumulate fewer lipids survive, while the others die. In this case the normalized accumulation of lipids would be much lower than expected for such concentration of FFA.

In the metabolic flux analysis of the co-incubation of extracts with 250 µM of FFA, the FFA decreased OCR in three parameters: basal respiration, maximal respiration, and spare respiratory capacity. None of the extracts was able to prevent the decline in OCR in those parameters. The metabolic flux analysis yielded no significant differences across all the parameters and all conditions in pre and co-incubation with the extracts and 250 µM of FFA. One hypothesis is that 250 µM of FFA could be a low concentration to elicit alterations in mitochondrial respiration after HepG2 cells have had 48 hours to grow, or in other words, there are too many cells for the available FFA.

In intracellular ATP content quantification, only E3 at 10 µg/mL increased in ATP content by when co-incubated with FFA or BSA. No differences were found between control groups (BSA vs FFA or BSA + DMSO vs FFA + DMSO) or among other conditions. This increase might be associated with antioxidative action. With the increased lipid accumulation in HepG2 cells, β-oxidation would be occurring, as mentioned in section 2.5. With increased protection against OS generated by the β-oxidation, the cells could reap the full energetic benefits of this process: acetyl-CoA to feed into the citric acid cycle, producing more energy without the mitochondrial dysfunction that might arise from increased OS. This might also be due to a metabolic shift to a glycolytic pathway.

6. Conclusions

While the road to take full advantage of BBP is still a long one, these results show intrinsic biomedical value in biological materials being thrown away. Even before moving to animal studies, several other studies can be performed in order to better understand, at the molecular level, the results presented here and how to best take advantage of BBP properties. A repetition of the pro-oxidant studies at a lower tBHP concentration and of the metabolic flux analysis with either a lower cell density for 250 µM or 2000 µM. These should round up the already obtained results nicely. New assays such as ROS measurements and assessment of expression and activity of antioxidant enzymes (catalase and glutathione peroxidase for example) would provide new insight into the action mechanisms of BBP.

Prospective applications for the BBP include food supplements, as a way to prevent the development of NAFDL and complementary therapy for mitigation of NAFLD for patients who already have it.

7. Acknowledgments

No conflicts of interest to declare.

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A very special thanks for my girlfriend Catarina Reis Lopes, for all the support and for helping me with the stress of the last day of my master thesis. I know it could not have been easy.

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