Anti-diabetic properties of *Fucus vesiculosus* and pine bark extracts using the adipocyte cell model 3T3-L1

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Sykursýkishamlandi virkni í *Fucus vesiculosus* og furutrjáberki könnuð með notkun 3T3-L1 fitufrumumódels

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Ágrip

Offita er alvarlegt heilsufarsvandamál sem hefur áhrif á líf mörg hundruð milljón einstaklinga í hinum Vestræna heimi. Hún einkennist af miklu magni fitu sem safnast upp í fitufrumum í fituvef, en fituvefurinn gegnir einnig mikilvægu hlutverki í innkirtlavirkni líkamans. Þessi virkni getur orðið fyrir truflunum af völdum þessarar fituuppsöfnunar og þannig stuðlað að þróun ýmissa efnaskiptasjúkdóma líkt og sykursýki. Þar að auki hefur framgangur sykursýki verið tengdur uppsöfnun sindurefna í líkamanum sem einnig er þekkt að geti valdið öðrum hrörnunarsjúkdómum.

Markmið þessa meistaraverkefnis var að kanna áhrif útdrátta úr *Fucus vesiculosus* og furutrjáberki (*Pinus* spp) á fituuppsöfnun í 3T3-L1 fitufrumumódeli, hindrandi áhrif þeirra gegn α-glucosidase ásamt andoxunarvirkni. Þessar plöntur eru rík uppspretta lífvirkra efna, aðallega fjölfenóla og fjölsykra. Þrír þangútdrættir hindruðu fituuppsöfnun í 3T3-L1 frumunum án þess að hafa áhrif á lifun þeirra og sýndi *F. vesiculosus* vatnsútdráttur bestu virknina með 35% hindrun í styrkleika 0,1 mg/mL. *F. Vesiculosus* sýruútdráttur sýndi einnig góða virkni með 19% hindrun í sama styrkleika. Þá höfðu bæði þang- og furutrjábarkar útdrættir sterka andoxunarvirkni svo og hindrunarvirkni gegn α-glucosidase. Furutrjábörkurinn hafði mestu andoxunarvirknina í ORAC með 2869 µmol TE/g og sami útdráttur hafði góða hindrunarvirkni gegn α-glucosidase með 1,3 µg/mL IC_{50} gildi.

Frekari rannsókna er þörf til að bera kennsl á þá ferla sem hindra fituuppsöfnunina. Einnig geta *in vivo* rannsóknir veitt upplýsingar um hvort þessara áhrifa gæti einnig í lifandi verum. Þetta gæti leitt til þróunar og framleiðslu á fæðubótarefni sem hægt væri að nota til að koma í veg fyrir offitu og þá efnaskiptasjúkdóma sem henni tengjast.

Abstract

Obesity is a serious health problem, affecting the lives of several hundred million individuals in the western civilizations. In obese individuals, large amounts of fat are stored in adipose tissue, which also acts as endocrine organ. This function can be affected in obesity, thus contribute to the onset of metabolic disorders like diabetes. Also, the progression of diabetes has been linked to accumulation of free radicals in the body which are as well involved in other degenerative diseases.

The aim of this Master thesis was to investigate the effects of *Fucus vesiculosus* and pine bark (*Pinus* spp) extracts on lipid accumulation in a 3T3-L1 adipocyte model, inhibition against α- glucosidase activity and to determine their antioxidant activity. These plants are rich sources of bioactive compounds, mainly polyphenols and polysaccharides. Three seaweed extracts inhibited lipid accumulation in the 3T3-L1 cells without affecting their viability, whereas water based *F. vesiculosus* extract was the most effective with 35% inhibition in concentration 0.1 mg/mL. An acid based *F. vesiculosus* extract also obtained good anti-adipogenic activity with 19% inhibition in the same concentration. Additionally, both seaweed and pine bark extracts had strong antioxidant activity and α-glucosidase inhibitory activity. The pine bark had the strongest antioxidant activity in the ORAC with 2869 µmol TE/g and the same extract obtained an IC_{50} value of 1.3 µg/mL in the α-glucosidase assay.

Further studies are needed to identify through which pathways the differentiation of the adipocytes is inhibited. Also, *in vivo* studies would provide information if these effects are measurable in whole organisms. This could lead to the development and production of a dietary enrichment product that could be used to alleviate obesity and its secondary metabolic disorders.

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Abbreviations

AAE	Ascorbic acid equivalents
AAPH	2,2 azobis (2-methylpropionamidine) dihydrochloride
ADD1/SREBP-1c	Adipocyte determination and differentiation factor 1
AMPK	AMP-activated protein kinase
ap2	Fatty acid binding protein
ATCC	American Type Culture Collection
AUC	Area under the curve
BAT	Brown adipose tissue
BCS	Bovine calf serum
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPPH	2,2 diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ET	Electron transfer
Ex/em	Excitation/emission
F. vesiculosus	*Fucus vesiculosus*
FBS	Fetal bovine serum
Ferrozine	3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine
FFA	Free fatty acid
GLUT4	Glucose transporter 4
HAT	Hydrogen atom transfer
IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
IL-6	Interleukin-6
kDa	Kilodalton
M	Molar
MC	Metal chelating
mM	Millimolar
MQ	Milli-Q water
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate buffered saline
PGE	Phloroglucinol equivalents

PNP-Gluc	p-nitrophenyl-α-D-glucopyranoside
PPARγ	peroxisome proliferator-activated receptor γ
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RP	Reducing power
RPM	Rounds per minute
RT	Room temperature
SD	Standard deviation
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
TE	Trolox equivalents
TNF-α	Tumor necrosis factor-α human
TPC	Total phenolic content
Trolox	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
U	Units
v/v	Volume/volume
WAT	White adipose tissue
WHO	World Health Organization
WM	Wet milled

1 Introduction

Obesity is a serious health problem, arising from an imbalance in energy homeostasis, which results in an increase in adipose tissue mass which can occur through both the size of individual adipocytes (hypertrophy) and the number of pre-adipocytes differentiating into mature adipocytes (hyperplasia). The differentiation of pre-adipocytes is called adipogenesis and is characterized by fat accumulation. It is regulated by transcription factors and the process is well understood [1-4]. Therefore, adipocytes have become a target for developing drugs and dietary enrichment compounds possessing anti-adipogenic activity in order to prevent and treat obesity and its secondary diseases [2].

Although being a serious health problem by itself, obesity gives rise to various secondary complications, such as cardiovascular diseases and type 2 diabetes mellitus (T2DM) [1, 3]. Commercial drugs for treating obesity and T2DM are available, however they are often linked to negative side effects and therefore studies on novel and healthy compounds for preventing and treating these conditions have been gaining increasing interest [3].

A critical role in the pathogenesis of T2DM, as well as other diseases, is played by oxidative stress, arising from an imbalance in cellular reactive oxygen species (ROS) levels. Essential in maintaining the ROS balance and preventing damage that can be caused by excessive amounts of ROS within the cells are antioxidants [5-8]. Thus, dietary compounds possessing antioxidant activity could provide a promising strategy in preventing cellular damage caused by oxidative stress and thereby its accompanying degenerative diseases [9].

Former studies have reported the potent antioxidant activity of polyphenols [10, 11] and also indicated their anti-adipogenic [3] and anti-diabetic [12] properties. Polyphenols are found in abundancy in brown seaweeds [10] and pine bark [13], thereby indicating their antioxidant and anti-adipogenic activities, as has formerly been reported [6, 10, 14, 15]. However, the anti-adipogenic effects of Icelandic seaweed have not been studied before. *Fucus vesiculosus,* found on Icelandic coasts, is a brown seaweed which are known to contain various bioactive compounds [7, 10] that might serve as interesting candidates for dietary enrichment or drugs for preventing and treating obesity, T2DM and other metabolic diseases.

In this study, the anti-adipogenic effects of seven *F. vesiculosus* extracts, with different extraction methods, as well as two pine bark extracts were examined using a 3T3-L1 cell line model. Their total phenolic content was also measured and their antioxidant properties evaluated using 4 different antioxidant activity assays. Moreover, the inhibitory activity of the extracts against the α-glucosidase enzyme was observed, thereby providing extensive information on their various bioactivity.

2 Review of the literature

2.1 Obesity

In recent years, obesity has been affecting a growing number of individuals and posing a major threat to human health around the world [1, 3, 16]. According to the World Health Organization (WHO) the number of obesity cases have more than doubled since 1980 and in 2014 more than 1.9 billion adults were overweight and thereof over 600 million were obese. Even worse, this condition does not only affect adults since data show that 42 million children under the age of 5 were overweight or obese in 2013 [17].

Obesity is a major risk factor for severe secondary diseases such as cardiovascular diseases, hypertension, hyperglycaemia, dyslipidaemia, insulin resistance and T2DM, thus contributing to high rates of morbidity and premature mortality [1, 3, 18-20]. This is partly connected to dysregulation in the secretion of cytokines, caused by accumulated fat in adipocytes and elevated oxidative stress in obese individuals. Additionally, this oxidative stress can have direct impact on vascular wall cells, thus increasing the risk of hypertension and atherosclerosis cells [5]. Obesity-associated metabolic disorders have also been linked to chronic low-grade inflammatory state [21] but diets containing high amounts of refined carbohydrates, fructose in particular, have been associated with increased inflammatory response in adipose tissue. Fructose is mainly metabolised in the liver and its high amounts results in increased triglyceride accumulation, followed by decreased glucose and lipid metabolism and elevated secretion of pro-inflammatory cytokines by adipocytes. This inflammation can lead to insulin resistance, high blood sugar levels, due to altered lipid and sugar metabolism, and finally T2DM [22].

Obesity occurs when energy intake of the body is higher than its energy output which results in increased adipose tissue mass [1, 3]. It cannot be traced to a single cause, but rather to a combination of contributing factors like age, developmental stage, physical activity and genes [22]. Diet is also closely associated with the development of obesity and represents a contributing factor of a special interest. This interest arises due to dietary factors having an impact on the regulation of adipogenesis as well as adipocyte metabolism, indicating that enriched diet might serve as a promising strategy for preventing and treating obesity [1].

2.2 Adipose tissue

The adipose tissue serves mainly as an energy storage for the body, but has as well important metabolic and endocrine functions. For example, fatty acids are released from the adipose tissue in case of energy deficiency. Two antagonistic processes, lipolysis and lipogenesis, determine the level of stored fat as well as the rate of lipid metabolism [23-25]. Lipogenesis is the process where triglycerides are synthesised and stored in the body for future utilization. It is stimulated by high levels of fat and/or carbohydrates in the diet and is regulated by several hormones, including insulin which enhances this process. Triglyceride synthesis mainly occurs in the adipose tissue but also takes place in the liver, muscles, heart and pancreas [26]. On the contrary, lipolysis is the catalytic process where triglycerides

from adipocytes are hydrolysed into fatty acids and glycerol [1]. It mainly takes place in the adipose tissue and is regulated directly and indirectly by several hormones, such as glucagon, insulin and catecholamines which acts as the most potent stimulator for this process [26].

In humans, adipose tissue can be distinguished in two different types, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is comprised of white adipocytes carrying large lipid droplets and mainly serves as a triglyceride storage but it also plays a key role as an endocrine system regulating various activities like lipid metabolism and insulin sensitivity [27]. It is distributed throughout the body and interestingly, adipose tissue exhibits differences in their metabolic function based on their location there. Actually, increase in visceral WAT negatively affects health and correlates with inflammation and insulin resistance while increase in subcutaneous adipose tissue correlates with improved glucose tolerance [24, 27].

BAT is abundant in newborns and owes its brown colour to the large amount of mitochondria [27]. Although formerly not believed to exist in adult humans, recent studies have demonstrated the presence of active BAT in adults [28]. It plays a role in thermogenesis and produces heat by metabolizing lipids and glucose. It is located in the paravertebral, supraclavicular and periadrenal regions in the body and is able to increase metabolic rate upon activation as well as positively affecting insulin resistance and hyperlipidemia [24, 27].

Additionally, various physiological or hormonal activators can give rise to the induction of beige adipocytes in WAT which share similar functions as the BAT. Therefore this type of adipocytes have gained increasing interest because of their possible impact on lipid metabolism [24].

2.2.1 Adipocytes

Adipocytes represent the major cellular component of the adipose tissue where mature adipocytes carry out both lipolysis and lipogenesis based on the energy status of the body. These cells vary greatly in size, from 20-200 µm in diameter, and are fixed in a connective tissue matrix. The nucleus and cytoplasmic rim are pushed to the edge of the cell to make room for the lipid droplet which represents 90% of the cell volume [3, 29].

The pathogenesis of obesity is characterised by fat accumulation in the body and increased WAT mass [24]. Mature adipocytes do not divide or spread but the adipose tissue mass can increase through two different processes, called hypertrophy and hyperplasia [25]. Hypertrophy is the process where the size of adipocytes increase due to elevated cellular lipid content and is a characteristic of all overweight individuals. Hyperplasia however, describes the process where number of adipocytes increase due to differentiation of pre-adipocytes and is most prominent in seriously obese individuals [1, 2, 30].

Various drugs have been marketed for treating obesity. However, they are often linked with negative side effects and regained weight although having short-term benefits [3]. Indeed, many of them have been withdrawn from the market because of their serious side effects. Orlistat is a currently available anti-obesity drug which inhibits pancreatic and gastric lipase which results in lower absorption of dietary fat. Its side effects include diarrhoea, oily spotting and dyspepsia although serious liver injury has also

been implicated with its use [31]. Therefore, novel bioactive compounds possessing anti-obesity properties without negative side effects have been gaining increasing interest in recent years [3].

WAT can be reduced by suppressing adipocyte differentiation and fat accumulation and several studies have reported various bioactive compounds being able to inhibit adipogenesis and lipogenesis and induce lipolysis and apoptotic death of adipocytes. Additionally, studies have demonstrated suppressive effects of bioactive compounds on WAT and body weight gain as well as hyperglycaemia in animal models [1, 2, 24, 25]. However, too much inhibition of adipocyte differentiation would have as well negative health effects, since their role is not only to store energy but also as an endocrine organ [2]. Among these activities are the maintaining of energy balance and the involvement in regulating physiological function by secreting substances like cytokines, enzymes, growth factors and hormones [1]. Thereby WAT provides a complex network of endocrine, paracrine and autocrine signals influencing the response of various tissues including the hypothalamus, pancreas and liver as well as influencing immune responses, insulin sensitivity and food intake, affecting almost all the organs of the body [23, 25, 29]. An excess of adipose tissue can affect this network and disturb the balance due to the high cellular lipid content which could be followed by insulin resistance and increased risk of T2DM [2].

The signals originating in WAT include adipocytokines although WAT is also responsible for secreting other hormones, growth factors and steroids as well as several regulators of lipoprotein metabolism [29]. The adipocytokines secreted from WAT include leptin, adiponectin, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), resistin, retinol-binding protein-4, dipeptidyl peptidase-4, plasminogen activator inhibitor-1, visfatin, apelin and free fatty acids (FFAs) [23]. Leptin and adiponectin are the main regulators of body weight gain [24]. Leptin reduces appetite and its secretion is elevated as total body fat mass increases. Despite that, leptin resistance in obese individuals prevents its activity [2, 23].

Conversely, adiponectin increases appetite [24] and its secretion is affected by oxidative stress, amongst other factors, and is usually reduced in obese individuals [2, 23]. It is responsible for decreasing lipid accumulation in muscles as well as increasing insulin sensitivity [23] and could be useful in the prevention and treatment of obesity [2].

Inflammation has been linked with obesity as infiltration of macrophages has been described in the adipose tissue in obese animal models. These macrophages produce pro-inflammatory cytokines causing insulin resistance due to their interference with insulin signalling [19, 32]. Among these pro-inflammatory cytokines are TNF-α and IL-6. They are also produced by adipocytes and their secretion is elevated in obese individuals [19]. Both cytokines have been linked to insulin resistance and increased risk of T2DM. This is thought as the link between obesity and T2DM [23].

Adipogenesis is controlled by various transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding proteins (C/EBPα, C/EBPβ and C/EBPδ) and adipocyte determination and differentiation factor 1 (ADD1/SREBP-1c) [4] as shown in figure 1. PPARγ controls the differentiation of adipocytes as well as regulating genes responsible for lipid uptake and glucose metabolism, such as fatty acid transporter, glucose transporter 4 (GLUT4), lipoprotein lipase, acetyl-CoA oxidase and fatty acid binding protein (aP2) [6] whose serum level is connected to the severity of insulin resistance and T2DM [2]. On the contrary, AMP-activated protein kinase (AMPK) serves as an important target for preventing and treating obesity as it inhibits lipogenesis and regulates

energy metabolism [18, 33]. Therefore, the activation of AMPK and the inhibition of PPARγ mRNA expression might be a key event in preventing lipid uptake and adipogenesis and decreasing adipose tissue mass [24, 33].

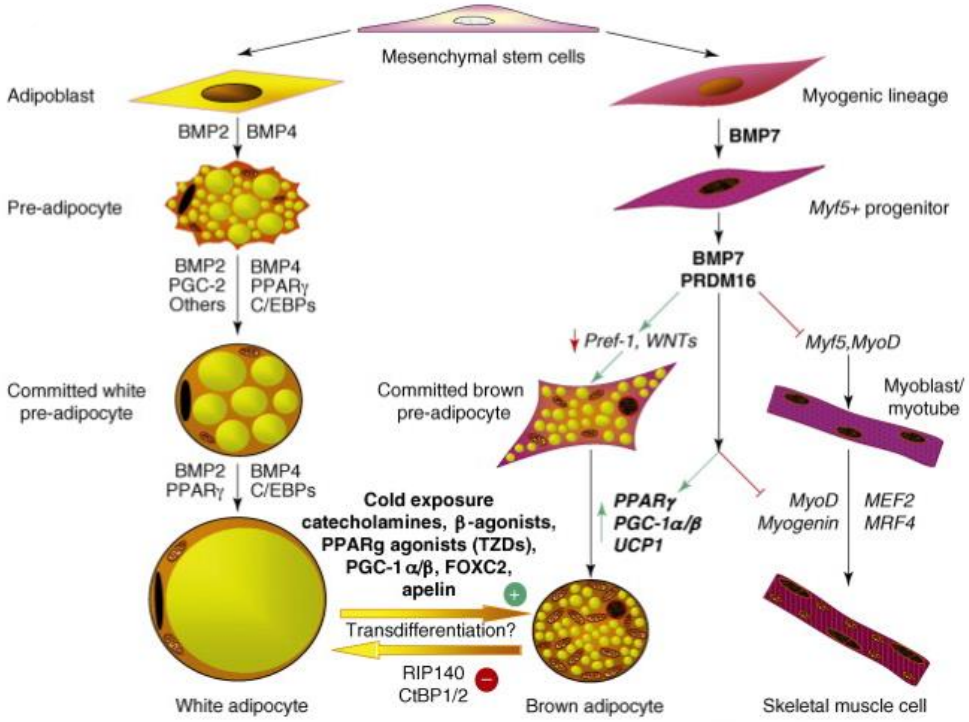


Figure 1. The development of mesenchymal stem cells to white and brown adipocytes and the transcription factors involved in this process. Obtained from [34]

2.2.2 3T3-L1 cell line

3T3-L1 cells (figure 2) are mouse embryo fibroblasts and are widely used as an *in vitro* model for studying adipocyte differentiation [18]. These cells retained the morphological and biochemical properties representative for adipocytes *in vivo* and are able to secrete adipocytokines [1].

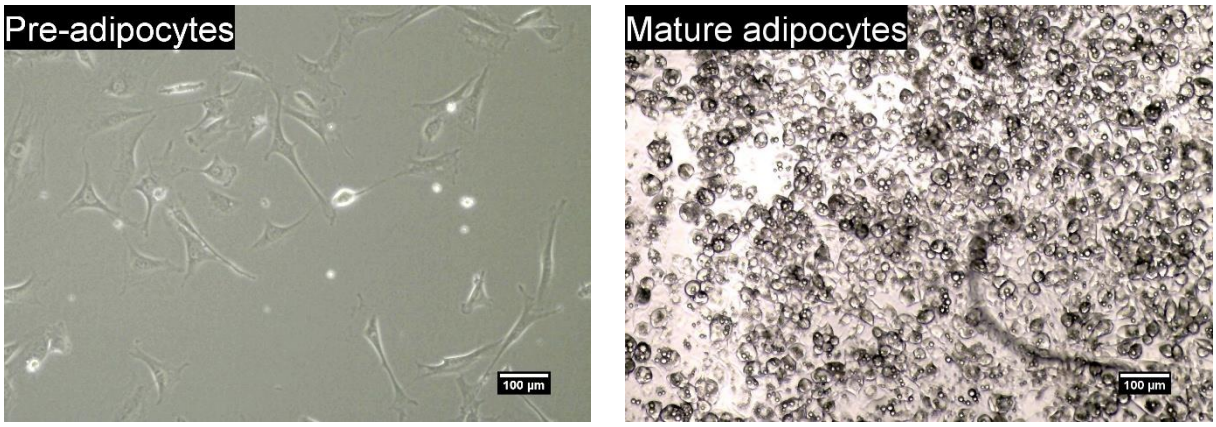


Figure 2. 3T3-L1 pre-adipocytes and mature adipocytes

2.3 Diabetes

Diabetes is usually divided into two categories, type 1 diabetes mellitus (T1DM) and T2DM. T1DM is an autoimmune disorder characterised by complete insulin deficiency due to destruction of pancreatic-β-cells [23]. T2DM, however, is characterised by hyperglycaemia and its main cause is insulin resistance in skeletal muscles, liver and adipose tissue. Insulin resistance refers to the failure of cells to respond normally to insulin. This results in a lower glucose uptake in adipocytes and muscles leading to elevated blood glucose levels [19, 35, 36]. T2DM is a severe metabolic disorder [19, 35] with a growing number of affected individuals as obesity cases continue to increase. In 2014, diabetes was estimated to affect 9% of the world's population aged 18 and over [37]. It poses as a major risk factor for various diseases like atherosclerosis and cancer and has a strong link to obesity as 80% of T2DM patients are obese [23, 36]. Insulin resistance arising from obesity has been linked with inflammation in adipocytes [38] but its development to T2DM has not been fully elucidated [19].

Diabetes is linked to the amount and structure of carbohydrates in the diet. Starch digestion is catalysed by α-amylase in the small intestine, the reaction releasing both linear maltose and branched isomaltose oligosaccharides. These are further hydrolysed to absorbable glucose in the presence of α-glucosidase. Thus, high levels of refined carbohydrates in the diet increases the risk of postprandial hyperglycaemia [35, 39]. The commercial drugs Acarbose and Voglibose are non-hydrolyzable sugar analogs which are used to inhibit the digestion of dietary carbohydrates in order to treat T2DM [35, 40, 41].

These drugs inhibit α-amylase and α-glucosidase, so starch digestion is pushed into the colon where bacteria are able to ferment it and cause negative gastrointestinal side effects like abdominal pain, diarrhoea, renal tumours and liver disorders [35, 40].

A better approach might therefore be to use compounds exhibiting strong inhibition against α-glucosidase and weak inhibition against α-amylase to prevent the bacterial starch fermentation but simultaneously prevent postprandial hyperglycaemia [40]. Therefore, effort have been made recently to find novel compounds that are able to inhibit these digestion enzymes, thereby providing treatment for T2DM without the severe side effects accompanying the existing commercial drugs [35].

2.4 Reactive oxygen species (ROS)

ROS are highly reactive, oxygen containing molecules and include both radicals and non-radical compounds, such as superoxide, hydroxyl and peroxyl radicals, singlet oxygen and hydrogen peroxide [7, 8]. These molecules are produces by cells and represent byproducts from their aerobic respiration [42]. They are produced continuously and then detoxified in the aerobic metabolism [43]. Additionally ROS are generated under various stress conditions like inflammation, nutrient distress, ultraviolet light and exposure to oxidants [42, 44]. Although they play a role in gene expression and regulating signal transduction, their overproduction by both endogenous and exogenous factors leads to oxidative stress and cellular dysfunction, demonstrating an important balance between ROS production and antioxidant defences [7].

ROS cause cell damage by reacting with nucleic acids, proteins, lipids and other cellular molecules, giving rise to various degenerative diseases including immune system decline, cardiovascular diseases and cancer [7, 8]. Oxidative stress also plays a key role in the pathogenesis of T2DM, as it decreases glucose uptake in adipose tissue and muscles and reduces insulin secretion by pancreatic β-cells [5, 6].

The human body possesses the ability to maintain the balance in ROS levels using a complex system of antioxidant defences. These includes cellular enzymes, like superoxide dismutase and glutathione peroxidases. Additionally, antioxidants ingested with the food are also able to scavenge or quench ROS, thereby preventing oxidative stress and its degenerative effects [7, 8].

2.4.1 Antioxidant activity

Antioxidants delay or inhibit oxidation and can be divided into two categories, primary or secondary antioxidants. Primary antioxidants inhibit radical initiation or propagation while secondary antioxidants delay the rate of oxidation. The antioxidant activity of compounds can be evaluated using antioxidant activity assays that can be divided into two types, based on their reaction. Hydrogen atom transfer (HAT)-based assays determine the ability of a sample to quench free radicals while electron transfer (ET)-based assays measure the ability of a sample to react with a fluorescent or coloured probe. Antioxidants are of great interest for various reasons, as they are both able to prevent rancidity in foods as well as protect the human body against damage due to oxidative stress [8]. When measuring antioxidant activity, a single test is considered inadequate as it is unable to identify different reaction mechanisms [10]. Therefore, various assays are usually selected, as in this study where four different antioxidant activity assays, both testing primary and secondary antioxidant capacities and both reaction types, were carried out to evaluate the antioxidant properties of the extracts.

1. The Oxygen Radical Absorbance Capacity (ORAC) assay is an HAT-based assay [8] used to measure the ability of samples to scavenge peroxyl free radicals that are generated via thermal degradation of AAPH. Samples with antioxidant potentials donate hydrogen atoms to the radicals which otherwise combine with the fluorescent molecule that leads to its loss of fluorescence. Fluorescence curves are monitored over time which allows the calculation of radical scavenging activity, compared to a Trolox standard curve, based on the AUC [45, 46].
2. Reducing power is an ET-based assay [8] used for measuring the ability of samples to prevent oxidation by reducing potential oxidizers. When potassium ferricyanide is reduced to potassium ferrocyanide, it forms a Perl's Prussian blue complex with ferric chloride. This complex has a strong blue colour, but in the absence of antioxidants the test solution remains yellow. This allows determination of the reducing power of samples that can be calculated based on an L-ascorbic acid standard curve [47].
3. Iron is an essential factor in various biological processes where it for instance serves as an enzyme cofactor. However, it also catalyses the oxidation of biomolecules and therefore gives rise to cell damage. The metal chelating activity assay measures the ability of samples to chelate divalent iron that otherwise would form a stable magenta complex with ferrozine [48, 49].
4. DPPH radical scavenging activity assay is an ET-based assay which measures the ability of samples to scavenge the DPPH radical [8]. When the radical receives a hydrogen atom its

colour turns from deep purple to yellow and the difference in absorbance can be used to calculate the radical scavenging activity of the samples compared to a blank with no scavenging activity [8, 50].

2.5 Polyphenols

Phenols are molecules that are produces by plant secondary-metabolism and contain a hydroxyl group (-OH) bonded to an aromatic hydrocarbon group [51, 52]. The number of phenol rings and the structural components that bind them together is what categorizes polyphenols into diferuloylmethanes, stilbenes, flavonoids, phenolic acids and tannins (figure 3) [53, 54]. Normally, phenolic compounds are less soluble in water than polar organic solvents and recommended extractants are aqueous mixtures of methanol, ethanol and acetone [10].

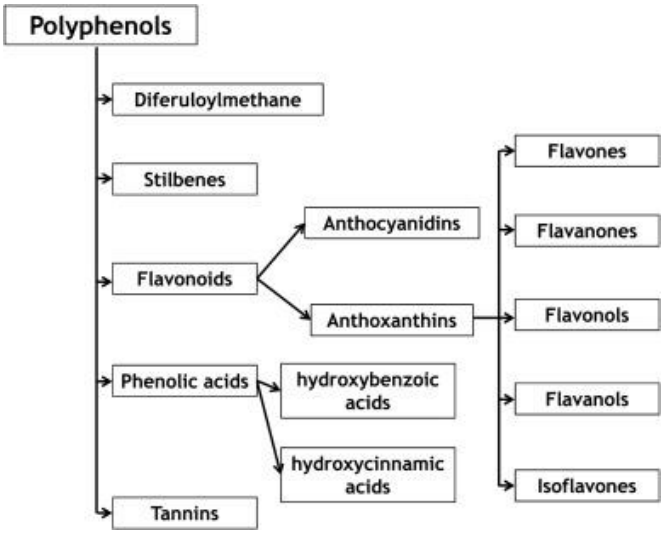


Figure 3. Classification of polyphenols. Obtained from [54]

Polyphenols can be found in terrestrial plants as derivations of gallic and ellagic acid and in algae as derivations of polymerised phloroglucinol units. Brown seaweeds are much richer in polyphenols than both red and green seaweeds and represent an abundant source of the heterogeneous phenol group phlorotannins [51] which defend the algae against herbivores, UV-light and microorganisms. Additionally, studies have indicated their role in reproduction of the algae as well as in the construction of their cell wall [52, 55].

Concentration of phlorotannins varies greatly between different seaweed species from <1% up to 14% of their dry weight [51, 55]. It also differs within the thallus of an individual and between geographical locations [55]. Submerged seaweeds generally contain lower concentrations of polyphenols as it is protected against various environmental stresses [51].

Polyphenols possess various bioactive properties, including antioxidant, antibiotic and antidiabetic activities. The bioavailability of polyphenols has been debated, but it is determined by the fraction that reaches the systemic circulation after their ingestion [51, 53]. Studies have observed elevated

antioxidant capacity of plasma after the ingestion of polyphenols which could facilitate their various applications, such as in functional foods or cosmetics [51].

2.6 Seaweed

Seaweeds represent a heterogeneous category of plants that habitat both intertidal and subtidal areas in the sea. Unlike terrestrial plants, they don't have roots, leaves or vascular system and therefore their nutrient uptake is carried out trough osmosis. Through the years, seaweeds have been used as a food source due to their low lipid content and richness in polysaccharides, minerals, polyunsaturated fatty acids and vitamins. Besides these, they are known to generate secondary metabolites with wide variety of bioactivities which they produce for their protection [56, 57]. Their tissue composition and bioactive components are influenced by multiple external factors, including light, nutrient availability, salinity, temperature and season [58] but can also vary within the thallus of individuals due to their structural complexity [59]. This variability can also be connected to differences in geographical location because of genetic distinction into locally adapted strains or phenotypically adapted individuals exposed to various environmental factors [58].

When seaweeds are exposed to extreme environmental conditions, such as strong light, high concentration of oxygen and temperature fluctuations, as well as pollution and stress, they elevate their production of secondary compounds [56, 57, 60]. Among the bioactivity exhibited by these compounds is antioxidant activity. Seaweeds are known for being a rich source of antioxidants with the ability to scavenge free radicals, chelate metal ions and prevent chain initiation, among other. These include polysaccharides, carotenoids and polyphenols, which are known to be powerful ROS scavengers due to their number of hydroxyl groups [7, 18].

Seaweeds are generally categorised as green, red and brown seaweeds, mainly based on their pigmentation [61]. Brown seaweeds, which owe their colour to the xanthophyll pigment fucoxanthin, are known to possess various bioactive compounds including fucoidan and phlorotannins [7, 18, 56]. Fucoidans are polysaccharides containing sulphated L-fucose and <10% of other monosaccharides. They are found in abundance in brown seaweeds where they serve as cell wall reinforcing molecules as well as providing protection against dehydration during low tide. They can represent up to 20% of the dry weight of seaweeds, with *F. vesiculosus* being the richest source of these polysaccharides which exhibit various bioactive properties [51]. *F. vesiculosus* (figure 4) has been widely studied for its bioactivity, including antioxidant [10] and anti-cancer [62] activity. It habitats intertidal and upper subtidal zones of the Barents and White seas as well as the Northern Atlantic where the intensity of water movement varies as well as the salinity [63]. Besides fucoidan, *Fucus* spp are considered a rich source of polyphenols with phlorotannin representing 12% of their dry weight [51].

Figure 4. *Fucus vesiculosus*. Obtained from [64]

Bioactive substances from seaweeds have been extracted using various methods. Using water as an extractant, water soluble polysaccharides, proteins and organic acids can be extracted [10]. Besides water, acid solution serves as an effective extractant for fucoidans. However, using solvent can limit the bioactivity of fucoidans due to degradation [51]. Seaweeds have been gaining increasing interest in recent years as a source of bioactive compounds which has been contributed by their advantages such as abundancy, fast growth rate and low collection cost [65].

2.7 Pines

Pines (*Pinus* spp) are evergreen tree species characterized by bearing their leaves in clusters of 2-5 needles. They are industrially important as they are utilized for lumber, wood pulp, turpentine and edible seeds. Additionally, the inner part of their bark is used as a food source in some countries, including Finland and Sweden [66].

During wood industrial processing, great amounts of organic waste are generated, including pine bark. This waste is generally combusted or wasted although it could be used for adding value to this product and gaining economic benefit due to the several bioactive compounds it contains. These compounds include phenols which are known for their antioxidant activity as well as being able to reduce the risk of several diseases, including cardiovascular diseases and cancer [67-69]. Indeed, pine bark has been used since ancient times as a drug [70].

Various bioactive compounds are generated within pines as their defence mechanism against numerous external threats, including herbivores, microbial pathogens and invertebrate pests. The amount of these compounds varies significantly between species, from 1% to 33% of their dry weight. Their content also varies within species and within individuals, as higher amounts are found in the bark, heartwood, roots, branch bases and wound tissues [71]. Environmental stress causes elevated production of secondary metabolites in plants. Atmospheric pollution, herbivores, high temperatures and UV-radiation are all factors that have been shown to increase phenol production in pines [72].

Numerous methods are available in order to extract bioactive compounds from pine bark and some have even been standardized. Pycnogenol is a standardized extract of the French maritime pine (*Pinus pinaster*, figure 5) obtained by the extraction of fresh pine bark using water and ethanol [66]. It is a complex mixture of compounds, including monomeric phenols and condensed flavonoids [70, 73]. The pine species it is derived from is mainly cultivated for its lumber and habitats several Mediterranean countries and some North African countries as well [67]. Pycnogenol has been reported for its antioxidant activity, as well as providing positive effects on several diseases, and studies have shown that the whole extract exhibits greater bioactivity than its individual components. Due to its bioactivity, pycnogenol has been used as a dietary enrichment product in European countries [73].

Figure 5. *Pinus pinaster* and its bark. Obtained from [74]

3 Materials and Methods

3.1 Reagents and equipment

Mouse embryo pre-adipocytes (3T3-L1) were obtained from ATCC, UK. Dulbecco's modified eagle medium (DMEM), Fetal bovine serum (FBS), Bovine calf serum (BCS) and Penicillin/streptomycin were obtained from Gibco by LifeTechnologies. PrestoBlue reagent was obtained from Invitrogen and AdipoRed reagent from Lonza. All other chemicals were obtained from Sigma-Aldrich.

The clear 96-well Nunc microplates were obtained from ThermoScientific, the opaque 96-well microplates from Corning and the cell culture microplates from Falcon. All absorbance and fluorescent measurements were carried out in a PolarStar OPTIMA microplate reader from BMG Labtech.

3.2 Preparation of plant extracts

Pine bark extracts A and B were obtained commercially and the *F. vesiculosus* and fucoidan extracts were obtained from Matís ohf. The extracts were prepared for the assays by weighing and dissolving them in concentration 10 mg/mL where Milli-Q water (MQ) was used as a solvent for all assays, except for the DPPH radical scavenging assay where 70% ethanol was used as a solvent. The extracts were then put on a shaker at 800 RPM for 1 hour and finally centrifuged at 4500 RPM for 5 minutes. For the cell based assays, an additional filtration step through 0.45 µm filter was carried out after the centrifugation to sterilize the extracts.

3.3 Chemical and cell based assays

All extracts except for the fucoidan extract were tested *in vitro* for their total phenolic content (TPC) as well as their antioxidant capacity using four different antioxidant activity assays. All extracts were tested for their anti-diabetic properties using an enzymatic assay and an adipocyte cell line model.

3.4 TPC

TPC of the extracts was analysed with the method developed by Singleton et al. [75] with some modifications to adapt it to microplate format. Briefly, 20 µL of sample were mixed with 100 µL of 0.2N solution of Folin-Ciocalteu (diluted in MQ) in quadruplicate in a clear 96-well microplate which was subsequently incubated at room temperature (RT) for 5 minutes in the dark. Then 80 µL of 7.5% solution of Na_2CO_3 (dissolved in MQ) were added to the microplate which was then agitated at 400 RPM on a shaker for 30 minutes at RT. Phloroglucinol (dissolved in methanol and diluted in MQ) was used as a standard and MQ was used as a blank. The absorbance was read at 720 nm and results were expressed as grams of phloroglucinol equivalents (PGE) per 100 grams of extract.

3.5 ORAC

The oxygen radical absorbance capacity of the extracts was analysed with the ORAC assay according to [45] and [46] with slight modification. Briefly, 60 µL of 10 nM fluorescein solution (dissolved and diluted in 10 mM phosphate buffer (10 mM $Na_2HPO_4 \cdot 2H_2O$ and 10 mM $NaH_2PO_4 \cdot H_2O$ in ratio 5:2 (v/v), pH 7.4)) were added to opaque 96-well microplate along with 10 µL of sample, blank (MQ) and standard (Trolox) in triplicate. The microplate was incubated in the microplate reader at 37°C for 10 minutes before 30 µL of 120 mM solution of 2,2 azobis (2-methylpropionamidine) dihydrochloride (AAPH, dissolved in 10 mM phosphate buffer) were injected to each well, using integrated injector. The reaction was conducted at 37°C and the fluorescence signal (ex/em 485/520 nm) was recorded every minute for 100 minutes in the microplate reader. Results were calculated based on the area under the fluorescence curve (AUC) and expressed as micromoles of Trolox equivalents (TE) per gram of extract.

3.6 Reducing Power

The reducing power of the extracts was analysed according to [47] with slight modification. Briefly, 300 µL of 0.2 M phosphate buffer (0.26M $NaH_2PO_4 \cdot H_2O$ and 0.16M $Na_2HPO_4 \cdot 2H_2O$ in ratio 1:1 (v/v), pH 6.6) and 300 µL of 1% $K_3Fe(CN)_6$ (dissolved in MQ) were mixed in an Eppendorf vial along with 60 µL of sample, standard (L-ascorbic acid) and blank (MQ) in triplicate. The vials were incubated at 50°C for 30 minutes. Then, 300 µL of 10% trichloroacetic acid solution (TCA, dissolved in MQ) was added to each vial and 200 µL of the supernatant were added to a clear 96-well microplate in quadruplicate. 40 µL of 0.1% $FeCl_3$ solution (dissolved in MQ) were added to the microplate and subsequently the absorbance was read at 700 nm. Results were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of extract.

3.7 Metal Chelating

The metal chelating activity of the extracts was analysed according to [48] with slight modification. Briefly, 100 µL of sample, positive control (EDTA) or blank (MQ) were added in triplicate to a clear 96-well microplate and mixed with 50 µL of 0.2 mM solution of $FeCl_2$ (dissolved and diluted in MQ). Then 100 µL of 0.5 mM solution of 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine, dissolved and diluted in MQ) or MQ (for control blank and control samples) were added to the microplate which was then agitated at 400 RPM on a shaker for 30 minutes in the dark. The absorbance was read at 520 nm and results were expressed as the IC_{50} in milligrams per millilitre.

3.8 DPPH Radical Scavenging activity

The DPPH radical scavenging activity of the extracts was analysed according to [50] with slight modification. Briefly, 150 µL of sample (dissolved and diluted in 70% ethanol), positive control (L-ascorbic acid) or blank (70% ethanol) were added to a clear 96-well microplate in triplicate. Then 50 µL of 0.2 mM solution of 2,2 diphenyl-1-picrylhydrazyl (DPPH) dissolved at 2 mM in methanol and diluted

at 1:10 (v/v) in 70% ethanol) were added to the microplate which was then agitated at 400 RPM on a shaker for 30 minutes in the dark. The absorbance was read at 520 nm and results were expressed as the IC_{50} in micrograms per millilitre.

3.9 α-glucosidase inhibition

The capacity of the extracts to inhibit the α-glucosidase enzyme was analysed according to [39] with slight modification. Briefly, 80 µL of samples in triplicate were mixed with 20 µL of 1 U/mL α-glucosidase solution (dissolved in 0.1 M phosphate buffer (3.09 g $NaH_2PO_4 \cdot H_2O$ and 4.56 g $Na_2HPO_4 \cdot 2H_2O$ dissolved in 500 mL MQ, pH 6.9) in a clear 96-well microplate and incubated at 37°C for 3 minutes in the microplate reader. Acarbose in concentration range 0.25-10 mg/mL was used as a positive control and 0.1 M phosphate buffer was used as a blank. Then, 100 µL of 4 mM solution of p-nitrophenyl-α-D-glucopyranoside (PNP-Gluc, dissolved in 0.1 M phosphate buffer) were injected to each well, using integrated injector. The reaction was conducted at 37°C and the absorbance at 405 nm was monitored every minute for 40 minutes and results expressed as the IC_{50} in µg/mL.

3.10 3T3-L1 cell culture and differentiation

3T3-L1 cells were grown in DMEM containing 10% BCS and 50 U/mL penicillin and 50 µg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For assessing the anti-diabetic properties of the extracts, the cells were differentiated by chemical induction according to [76]. Briefly, cells were seeded in DMEM supplemented with 10% BCS on opaque, clear bottom, 96-well microplate (for viability and AdipoRed staining assays) or clear 24-well microplate (for Oil Red O assay) at density of 1.03×10^4 cells/cm^2. After incubation for 48 hours the cell culture medium was replaced with 100 µL of fresh medium and the cells were incubated for additional 48 hours. The differentiation of the cells was induced 4 days after seeding (day 0) by replacing the medium with identical volume of differentiation medium (DMEM containing 10% FBS, 1.0 µM dexamethasone solution (dissolved in 70% ethanol), 0.5 mM methylisobutylxanthine solution (IBMX, dissolved in dimethyl sulfoxide (DMSO) and 1.0 µg/mL insulin solution (dissolved at 2 mg/mL in 0.01 M HCl). This medium was replaced by identical volume of adipocyte maintenance medium (DMEM containing 10% FBS and 1.0 µg/mL insulin solution) on days 3 and 5 of differentiation. Samples were diluted in medium and added to the cells on day 0, and replaced with the medium on days 3 and 5. The Oil Red O and AdipoRed staining assays were carried out on day 7 of differentiation.

3.11 Viability assay

The cytotoxic effect of the extracts on the pre-adipocyte 3T3-L1 cells was evaluated according to [77]. Briefly, cells were incubated for 24 hours after seeding and then the medium was replaced by 80µL of DMEM containing 10% BCS and dilutions of the samples. Untreated cells were only exposed to DMEM containing 10% BCS. When the cells had been exposed to the samples for 48 hours, 20 µL of PrestoBlue

(diluted 1:1 (v/v) with DMEM) viability reagent was added to the plate with subsequent incubation at 37°C for 2 hours. The fluorescence signal (ex/em 560/610 nm) was measured and the cell viability was calculated as % viability of untreated cells.

3.12 Proliferation assay

The effect of the extracts on the proliferation of pre-adipocyte 3T3-L1 cells was evaluated according to [78] using a CyQuant® Cell Proliferation Assay Kit. Cell number standard curve was generated by harvesting 3T3-L1 cells by trypsinization as previously described and preparing a concentrated cell suspension of 10^5 cells in medium. The cells were centrifuged at 2000 RPM for 3 minutes before the supernatant was discarded and the cell pellet was put in -80°C freezer overnight. The next day the cell pellet was thawed at RT and resuspended in 200 µL of the CyQuant® GR dye/cell-lysis buffer (prepared as described by [78]). A serial dilution of the cells was prepared in the buffer on opaque, clear bottom, 96-well microplate with cell numbers ranging from 250-50000 cells per well, including a blank containing only 200 µL of the buffer. The cells were incubated at RT for 5 minutes in the dark before the fluorescence signal (ex/em 485/520 nm) was measured and a standard curve was generated.

The proliferation of the cells was analysed after the viability measurements as previously described. Cells were washed once with 100 µL of PBS and then put in -80°C freezer overnight. The next day the cells were thawed at RT and 200 µL of the proliferation reagent was added to each well of the plates. The microplates were incubated for 5 minutes at RT in the dark before the fluorescence signal was measured as previously described. The effect of the extracts on the proliferation was analysed by calculating the number of cells in each well of the microplate by linear regression using the generated standard curve. Number of treated cells were presented as the % number of untreated cells.

3.13 Oil Red O staining

The extent of cell differentiation was evaluated by Oil Red O lipid staining according to [6]. Briefly, cells were fixed in 10% formaldehyde solution for 1 hour at RT, washed once with MQ and let dry. Cells were stained with 0.5% Oil Red O solution (dissolved in 6:4 (v/v) isopropanol:MQ solution, filtered through 0.45 µm filter) for 30 minutes at RT, washed four times with MQ and let dry. The amount of lipid accumulation was evaluated visually and images obtained by EPSON Perfection V750 PRO.

3.14 AdipoRed fluorescence staining

Adipocyte differentiation was also determined by AdipoRed staining according to [79]. Briefly, microplates were removed from the incubator (37°C) and allowed to cool to RT. The cells were washed with 200 µL of PBS prior to adding 200 µL of PBS to each well. Microplates were placed in the microplate reader where 5 µL of the AdipoRed reagent were injected to each well of the plate by an integrated injector. The microplates were incubated in the dark for 10 minutes at RT before the fluorescence signal

(ex/em 485/585 nm) was measured and results were expressed as the % lipid accumulation inhibition of untreated cells.

4 Results

4.1 TPC

Pine bark extract A had the highest phenol content of this study with 68 g PGE/100 g extract (table 1), it was three times higher than pine bark extract B (22 g PGE/100 g extract). Of the *F. vesiculosus* extracts the water extract had the highest phenol content (24 g PGE/100 g extract). The lowest phenol content was found in the HCl <100 kDa (6 g PGE/100 g extract) and the byproduct *F. vesiculosus* extracts (7 g PGE/100 g extract).

4.2 Antioxidant properties

The highest ORAC value was obtained by pine bark extract A with 2869 µmol TE/g extract (table 1). Of the *F. vesiculosus* extracts, the byproduct extract showed no activity and the HCl <100 kDa extract showed moderate activity around 600 µmol TE/g extract. All the other seaweed extracts obtained ORAC values in the range of 825-1211 µmol TE/g extract. A strong relation is observed between the phenol content of the extracts and their ORAC values with correlation factor of 0.93.

The highest reducing power activity was obtained by pine bark extract A with 309 mg AAE/g extract (table 1). Of the *F. vesiculosus* extracts, the lowest activity was exhibited by the HCl <100 kDa extract and the byproduct extract which both exhibited activity lower than 100 mg AAE/g extract. Other seaweed extracts exhibited activity in the range of 110 – 167 mg AAE/g extract.

All extracts exhibited less metal chelating activity than the positive control of EDTA. The highest metal chelating activity was obtained by the HCl <100 kDa *F. vesiculosus* extract with IC_{50} of 561 µg/mL (table 1). Low metal chelating activity was exhibited by the byproduct extract with IC_{50} value of 5454 µg/mL. Both pine bark extracts had no metal chelating activity and there was no correlation between TPC and chelating ability.

All extracts exhibited less DPPH radical scavenging activity than the positive control of ascorbic acid with IC_{50} value of 5 µg/mL. The highest DPPH radical scavenging activity was obtained by pine bark extract A with IC_{50} of 17 µg/mL (table 1). The lowest DPPH radical scavenging activity was obtained by the *F. vesiculosus* byproduct extract and the HCl <100 kDa extract with IC_{50} values of 179 and 134 µg/mL respectively. The lowest IC_{50} value of the *F. vesiculosus* extracts was obtained by the water extract with IC_{50} value of 57 µg/mL. TPC and DPPH radical scavenging activity had a correlation factor of -0.74. Results from antioxidant assays are summarized in table 1.

Table 1. TPC and antioxidant properties of the extracts

	TPC[a] (g PGE/100 g)	ORAC[b] (µmol TE/g)	RP[c] (mg AAE/g)	MC[d] (IC_{50} µg/mL)	DPPH[e] (IC_{50} µg/mL)
Pine bark ext A	68 ± 1	2869 ± 22	309 ± 15	No activity	17 ± 3
Pine bark ext B	22 ± 1	1714 ± 69	92 ± 6	No activity	46 ± 4
Fv water ext	24 ± 1	1840 ± 51	167 ± 16	1499 ± 63	57 ± 8
Fv HCl >100 kDa	17 ± 0,3	1211 ± 32	126 ± 8	762 ± 89	82 ± 9
Fv HCl <100 kDa	6 ± 0,3	583 ± 12	39 ± 3	561 ± 39	134 ± 19
Fv byproduct, WM HCl >100 kDa	7 ± 0,3	No activity	61 ± 3	5454 ± 508	179 ± 9
Fv WM HCl >100 kDa	15 ± 0,3	825 ± 48	110 ± 3	1161 ± 108	70 ± 7
Fv WM H_2SO_4 >10 kDa	19 ± 0,3	902 ± 61	129 ± 4	940 ± 74	68 ± 2
Positive control[1]	-	-	-	2 ± 0.2	5 ± 0.5

[a]TPC, Total phenolic content

[b]ORAC, Oxygen radical absorbance capacity

[c]RP, Reducing power

[d]MC, Metal chelating

[e]DPPH, DPPH radical scavenging activity

[1]The positive controls of MC and DPPH were EDTA and ascorbic acid, respectively

All values are presented as the means of triplicates within 3 separate measurements ± SD

4.3 α-glucosidase inhibition

All extracts tested had lower IC_{50} values than Acarbose (1313 µg/mL) with the highest inhibitory activity shown by the HCl >100 kDa *F. vesiculosus* extract (IC_{50} of 0.7 µg/mL). This extract showed almost 1900 times higher activity than Acarbose. Pine bark extract A showed similar activity as both the *F. vesiculosus* water extract and the H_2SO_4 >10 kDa extract (IC_{50} of 1.3, 1.3 and 1.6 µg/mL, respectively). The fucoidan extract had the lowest inhibitory activity (IC_{50} 128 µg/mL).

Table 2. Inhibitory activity of the extracts on α-glucosidase

	α-glucosidase[a] (IC_{50} µg/mL)
Pine bark ext A	1.3 ± 0.2
Pine bark ext B	14 ± 2
Fucoidan	128 ± 36
Fv water ext	1.6 ± 0.4
Fv HCl >100 kDa	0.7 ± 0.1
Fv HCl <100 kDa	30 ± 7
Fv byproduct, WM HCl >100 kDa	2.8 ± 0.2
Fv WM HCl >100 kDa	2.6 ± 0.3
Fv WM H_2SO_4 >10 kDa	1.3 ± 0.1
Acarbose (positive control)	1313 ± 135

[a]α-glucosidase. The inhibitory effect on the α-glucosidase enzyme is expressed as the IC_{50} in µg/mL

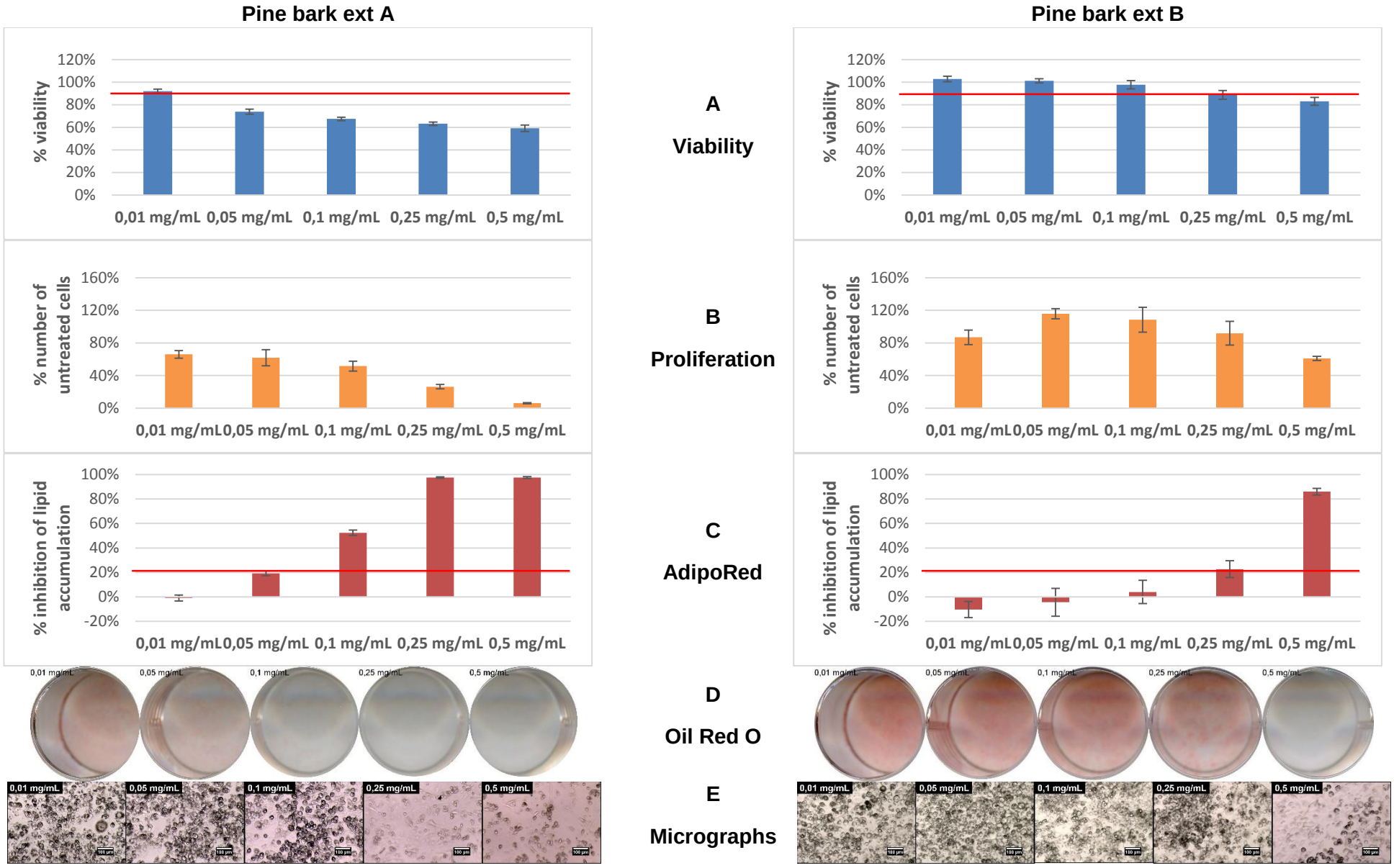
Values from the α-glucosidase assay were presented as the means of triplicates within 3 separate measurements ± SD

4.4 Inhibitory effect on lipid accumulation in 3T3-L1 cells

All extracts showed inhibitory activity on the lipid accumulation in the cells in a dose dependant manner, except for the fucoidan extract which had no influence on lipid accumulation (figure 6C and D). Results on the anti-adipogenic effects of the extract were considered in relation to the viability and proliferation results (figure 6A and B). Extracts which were able to lower viability below 90% were considered as cytotoxic and their anti-adipogenic results therefore excluded.

No visual inhibition of lipid accumulation according to the Oil Red O staining, and less than 20% inhibitory activity according to the AdipoRed staining assay, was considered as no activity. Microscopic images (figure 6E) obtained on day 7 of differentiation were also used to evaluate the inhibitory activity of the extracts on the lipid accumulation, indicating similar results as obtained in the AdipoRed and Oil Red O staining assays. Undifferentiated cells (figure 7) form a confluent layer and have no visible intracellular lipid droplets. Untreated cells also form a confluent layer but have much rounder shape and many intracellular lipid droplets, as pointed out in figure 2. The suppression of differentiation was visible on the microscopic images as reduction of intracellular lipid droplets.

A total of three *F. vesiculosus* extracts exhibited inhibitory activity on lipid accumulation in concentrations that did not affect cell viability, the water extract (35%), HCl >100 kDa extract (55%) and the byproduct extract (30%). The concentrations needed to obtain these effects were 0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL for the water extract, HCl >100 kDa extract and the byproduct extract, respectively. Neither pine bark extract exhibited inhibitory activity on the lipid accumulation that could not be explained by their cytotoxic effects.


Pine bark ext A
Pine bark ext B
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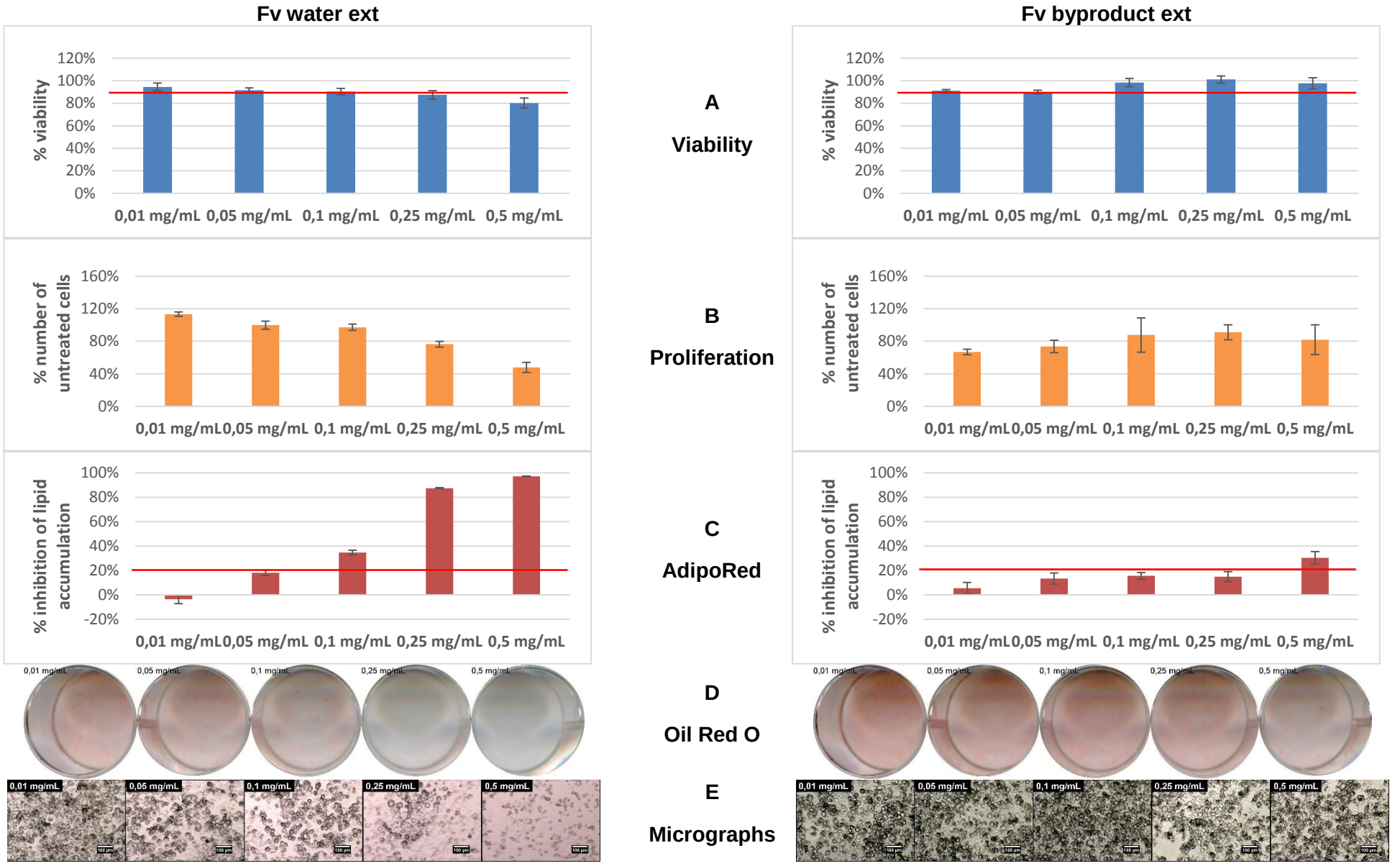
Micrographs


Fv water ext
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% viability
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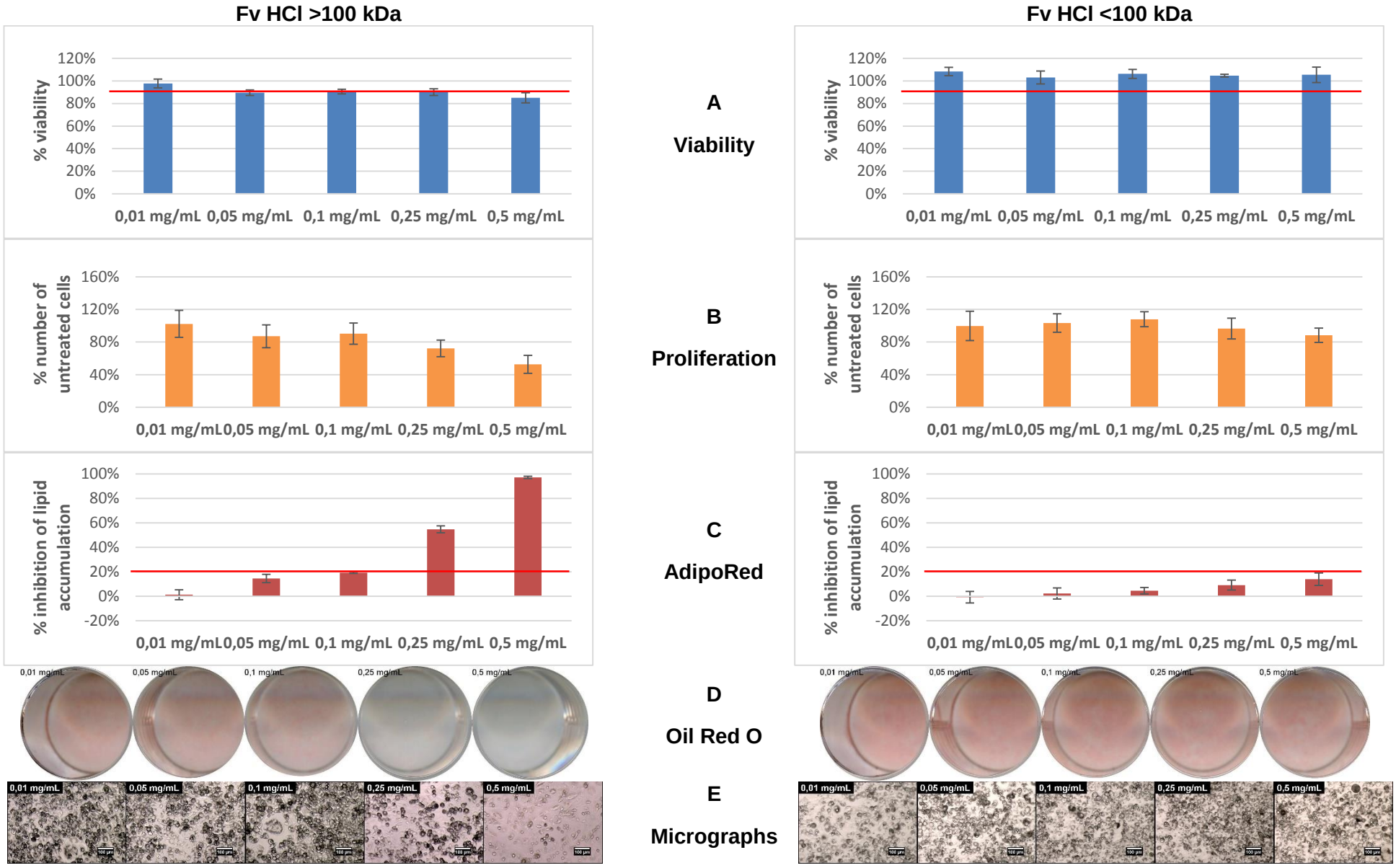
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B
Proliferation
% number of untreated cells
160%
120%
80%
40%
0%
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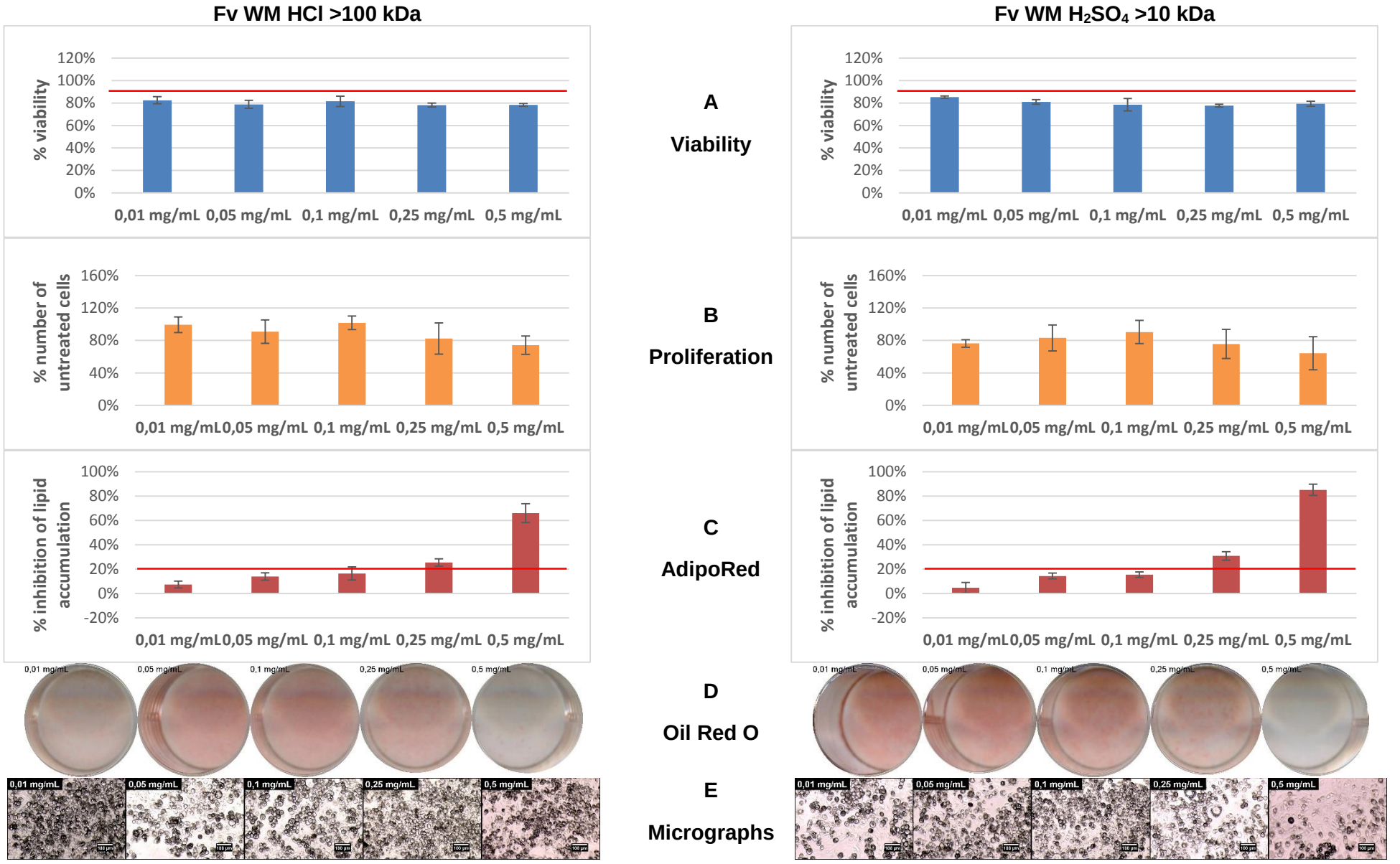
E
Micrographs


Fv WM HCl >100 kDa
Fv WM H_2SO_4 >10 kDa
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Viability
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0,1 mg/mL
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Proliferation
% number of untreated cells
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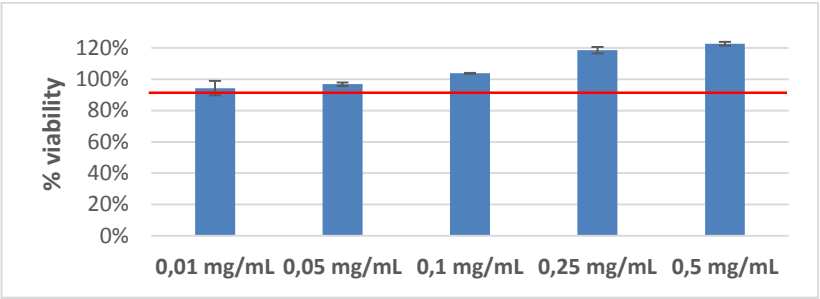
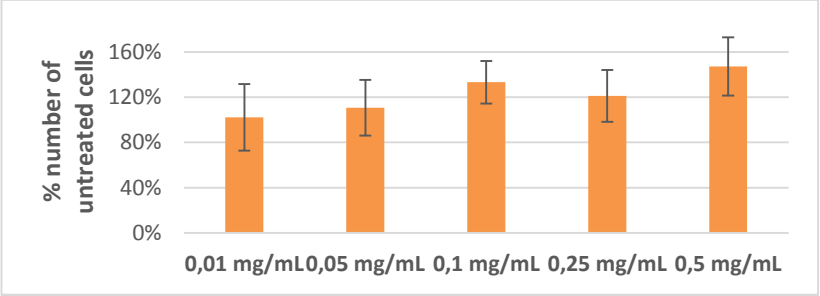
E
Micrographs

Fucoidan



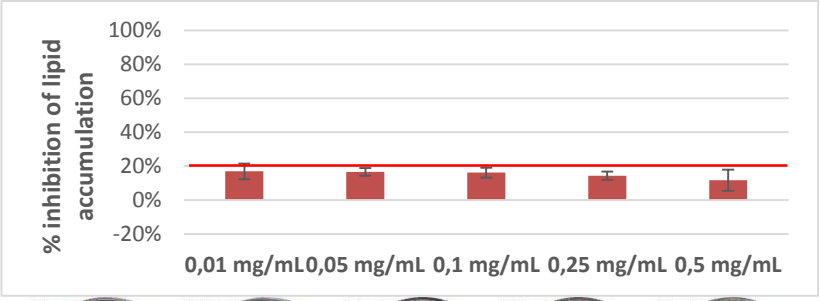
A

Viability



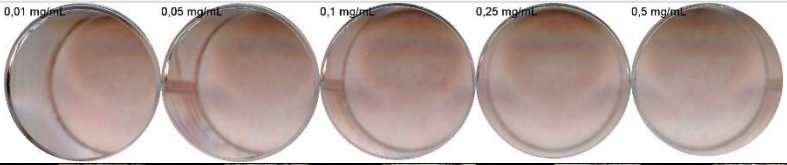
B

Proliferation



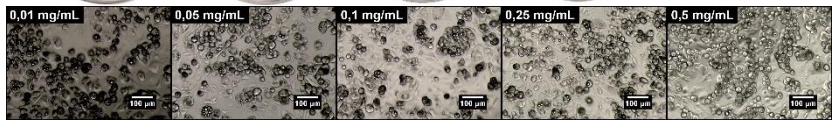
C

AdipoRed



D

Oil Red O



E

Micrographs

Figure 6. Effects of extracts on anti-adipogenic 3T3-L1 cell line model. (A) Viability of 3T3-L1 pre-adipocytes was determined using PrestoBlue reagent after 48 hour exposure to the extracts. Results were expressed as the % viability of treated cells compared to untreated cells. Extracts lowering the viability below 90% were considered cytotoxic. (B) Proliferation of 3T3-L1 pre-adipocytes was determined using CyQuant assay kit after 48 hour exposure to the extracts. Results were expressed as the number of treated cells as the % of untreated cells. (C) Inhibition of lipid accumulation was determined by AdipoRed fluorescence staining and results were expressed as the inhibition of lipid accumulation by the extracts in treated cells compared to untreated cells. (D) Visual inhibition of lipid accumulation determined by Oil Red O staining of the cells on day 7 of differentiation. Images were obtained by EPSON Perfection V750 PRO. From left: Cells treated with extracts in concentrations 0.01, 0.05, 0.1, 0.25 and 0.5 mg/mL, respectively. (E) Micrographs (taken with EVOS XL Core Imaging system, Life Technologies, 100x magnification in phase contrast) were obtained of cells on day 7 of differentiation. From left: Cells treated with extracts in concentrations 0.01, 0.05, 0.1, 0.25 and 0.5 mg/mL, respectively. All values were presented as the means of triplicates within 3 separate measurements ± SD

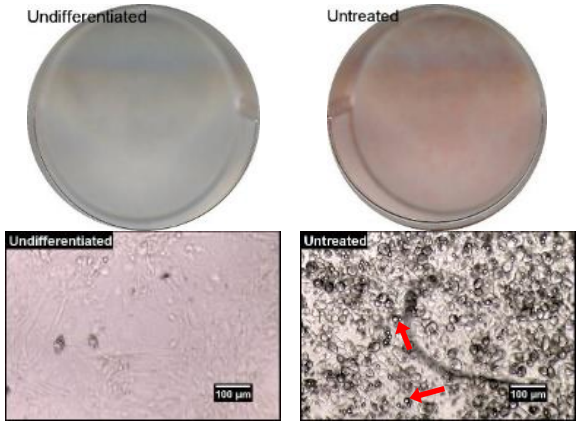


Figure 7. Undifferentiated and untreated 3T3-L1 cells. (A) Oil Red O staining of cells on day 7 of differentiation. From left: undifferentiated cells and untreated cells, respectively. (B) Micrographs (taken with EVOS XL Core Imaging system, Life Technologies, 100x magnification in phase contrast) were obtained of cells on day 7 of differentiation. From left: undifferentiated and untreated cells, respectively. Lipid droplets are pointed out in the micrograph of the untreated cells.

5 Discussion

In this study, the antioxidant and anti-adipogenic properties of two pine bark and seven *F. vesiculosus* extracts, with different extraction protocols were examined. Results demonstrate high antioxidant activity of the extracts as well as potent anti-adipogenic activity of some of the *F. vesiculosus* extracts. Antioxidants are essential in preventing damage in cells caused by oxidative stress [7]. As the ferrous ions are very powerful pro-oxidants, metal chelating capacity is considered an important mechanism of antioxidant activity [10]. Oxidative stress plays a critical role in the pathogenesis of various diseases, including T2DM [5-8], while the differentiation of adipocytes are considered a key factor in the development of obesity and its secondary complications [3]. This suggests that components from these extracts could serve as active ROS scavengers and metal chelators as well as inhibitors of adipocyte differentiation with the possibilities to be used as dietary factors to prevent the development and pathogenesis of obesity, T2DM and other serious diseases.

As the seaweed extracts used in this study are produced using different extraction protocols, their active substances vary. Fucoidan is a complex sulphated polysaccharide with a large average size of 100 kDa [80]. Therefore, this substance would be excluded in the extract filtered for a size under 100 kDa but most probably be present in the other extracts. Polyphenols are an abundant group of substances which vary significantly in size [81] and phlorotannins have been reported to be the only phenolic group detected in brown seaweeds [10]. They normally occur in the size range of 10-100 kDa, but *F. vesiculosus* has been reported to possess large amount of low molecular weight phlorotannins of less than 1.2 kDa [82]. This indicates that phlorotannins are present in all the seaweed extracts, although the larger ones are excluded from the extract filtered for a size under 100 kDa. As the byproduct extract is extracted from seaweed residues from previous extraction process it can be estimated that it contains lower amounts of bioactive substances, as most of them have already been extracted.

Antioxidant activity of Icelandic seaweed has previously been described but its anti-adipogenic effects are being reported for the first time in this study. The various extraction methods of *F. vesiculosus* also provide a different approach on similar studies which mainly focus on one specific extract throughout their study. Other studies have carried out antioxidant assays in parallel with anti-adipogenic measurements, although little effort has been made to examine it alongside enzymatic diabetic assays. Therefore, this study provides novel aspects on the anti-adipogenic effects of seaweed through its origin, extraction methods and its connection to an enzymatic anti-diabetic assay.

5.1 TPC and antioxidant properties

Results suggest that the phenols in the extracts were the active components in all antioxidant assays, except for the metal chelating assay. As previous studies using extract with phenolic compounds have shown there is a positive correlation between TPC and antioxidant activity [10, 83-85]. However, there are opposing opinions regarding the correlation between TPC and metal chelating activity. While some studies have demonstrated phenolic compounds being active chelators [86, 87], others have stated that there is no correlation between TPC and chelating activity [10, 88, 89]. In accordance with the results

from this study, a former study reported EDTA being a potent metal chelator with much higher activity than phenolic rich extracts [10]. Since the highest metal chelating activity was exhibited by the *F. vesiculosus* extract using HCl as extractant and filtered for a size under 100 kDa, further studies need to concentrate on this extract to identify the active chelating component.

The antioxidant capacity of the extracts differed significantly between the two types of pine bark and also between extraction methods of the seaweed. The large difference between the bioactivity of the two pine bark extracts can be explained by their source as they were extracted from different pine species, which are known to be different in their active components [15]. The highest phenol content and the highest antioxidant activity in all assays, except the metal chelating assay, was exhibited by pine bark extract A. This is in accordance with former studies which also have reported high antioxidant activity in pine bark extracts [15, 90, 91] which can be traced to their composition of antioxidants, such as flavonoids, phenolic acids and cinnamic acids [92]. The difference in results from the metal chelating activity assay and the other antioxidant assays used in this study was expected, since the metal chelating activity demonstrates the ability of a compound to prevent oxidation by chelating the potential oxidant [87], while the other antioxidant assays measure the ability of a compound to quench free radicals [8].

When comparing the different *F. vesiculosus* extracts, the water extract had the highest phenol content and the greatest antioxidant activity in all assays but low chelating capacity. Phenol content varied with extraction method. The type of acid did not influence phenol content, nor did the additional wet milling step prior to the extraction. However, filter size and the original source of seaweed had the largest effect on antioxidant activity since the HCl<100 kDa and the byproduct extracts exhibited the lowest phenol content. These results are not unexpected since the larger phenols have been excluded from the <100 kDa extract and most of the phenols have already been extracted from the byproduct extract. This is in accordance with studies reporting different phenol content in seaweed subjected to different extraction methods [93, 94]. Several studies have reported good antioxidant activity in brown seaweeds, which are known to be rich in polyphenols as well as other secondary components, such as carotenoids and polysaccharides [7, 10, 95].

As oxidative stress causes cell damage and is involved in the pathogenesis of various diseases, it can be concluded that components from these extracts could serve as active ROS scavengers and metal chelators which could be utilized to prevent complications arising from oxidative stress, such as T2DM mellitus and other metabolic diseases.

5.2 α-glucosidase inhibition

Results from this study suggest that all *F. vesiculosus* extracts are more potent α-glucosidase inhibitors than Acarbose which is used as a commercial drug in the treatment of diabetes [39]. Most dietary carbohydrates are composed of poly- and oligosaccharides. After ingestion of linear and branched isomaltose oligosaccharides, α-glucosidase hydrolyses these and releases the absorbable monosaccharides glucose and fructose. A high proportion of refined carbohydrates in a meal can lead to a very rapid hydrolysis and cause postprandial hyperglycaemia. Therefore, an effective dietary anti-diabetic strategy is to inhibit α-glucosidase activity, thereby balancing the postprandial blood glucose

levels [35, 39]. By this process, commercial drugs such as Acarbose and voglibose are currently used for treating T2DM but their undesirable side effects, such as flatulence, diarrhea, abdominal pain and liver disorders, have driven the search for natural substitutes exhibiting α-glucosidase inhibitory activity [35, 40, 41]. Although former studies demonstrate that polyphenolic compounds are active α-glucosidase inhibitors [12, 96], the inhibitory activity of the extracts tested in this study did not completely correlate with their phenol content. Former studies have reported the inhibitory activity of polysaccharides [96, 97] and glycoprotein [98] from seaweed on α-glucosidase activity. This suggests that the inhibition could be more complicated and due to other components besides polyphenols in the extracts, providing synergistic effects on the inhibitory activity.

Different extraction methods of *F. vesiculosus* extracts did not have an effect on the inhibitory activity of the extracts, however a lower activity was obtained by the HCl <100 kDa sample, which indicates that the smaller molecules present in this extract are not as efficient α-glucosidase inhibitors as the large molecules present in the other extracts. A very potent inhibitory activity was observed by pine bark extract A, and as in other assays there is a large difference between the activities of the two pine bark extracts.

Studies have formerly reported α-glucosidase inhibitory activity from both pine bark extracts [99, 100] and seaweed [12, 96, 98, 101, 102]. In this study, pine bark extracts A and B exhibited around 1000 and 100 times higher inhibitory activity than Acarbose, respectively. Another study on pine bark extract reported an IC_{50} value of 5 µg/mL and an Acarbose IC_{50} value of 1000 µg/mL [99] which demonstrates a 200 fold more potent activity of the pine bark extract than Acarbose. The same study reported stronger inhibitory activity using fractions containing oligomers compared to polyphenolic monomers [99]. This suggests that the different results could be traced to different extraction methods, as they are known to extract different bioactive substances [103], or to difference in pine species since active components in pine bark extracts vary between species [15].

In this study, the *F. vesiculosus* water extract and the fucoidan extract exhibited 800 times and 8 times more potent inhibitory effects on α-glucosidase than Acarbose, respectively. Other studies have reported 470 times more potent activity of a *F. vesiculosus* water extract than Acarbose [12] and a 20 times higher activity of a fucoidan extract from *F. vesiculosus* than Acarbose [96]. This difference could be traced to the seaweed being harvested at different geographical locations, as reported to have a great impact on their bioactive constituents [104]. The low inhibitory activity of the fucoidan extract indicates that the fucoidan alone might not be an effective inhibitor of the enzyme itself but could contribute to the inhibition alongside other components

In summary, all extracts that were tested in this study exhibited more potent inhibitory activity on the α-glucosidase enzyme than Acarbose and could therefore represent interesting candidates for the prevention and treatment of diabetes.

5.3 Effect of extracts on adipogenesis in a 3T3-L1 cell line model

Results show that *F. vesiculosus* extracts lowered lipid accumulation in the cells without affecting the viability. The water extract exhibited the most potent activity requiring the lowest concentration to reach

the threshold value of 20% inhibition of lipid accumulation. Since dietary compounds do not reach adipose tissue at high doses [2] this concentration can be considered physiologically relevant of those needed for other extracts to reach the threshold value. These results suggest the *F. vesiculosus* water extract being an effective inhibitor of lipid accumulation and adipocyte differentiation which play a key role in the development of obesity, giving a rise to severe secondary diseases such as T2DM [3, 4, 38].

Seaweeds are known to possess a great variety of bioactive substances and former studies have also reported their anti-adipogenic activity [6, 105, 106]. A study on adipogenesis using 5 different species of brown seaweed demonstrated that three polyphenol compounds of phlorotannins exhibited anti-adipogenic activity [18]. Among them, dieckol exhibited the most potent inhibitory activity as well as it down regulated the expression of key transcription factors for adipogenesis. Another study reported suppressive effects of fucoxanthin, a carotenoid found in seaweeds, on 3T3-L1 pre-adipocyte differentiation [25]. Additionally, fucoxanthin was converted to fucoxanthinol in the cells which also reduced lipid accumulation in the cells and suppressed the activity of glycerol-3-phosphate dehydrogenase, which is an indicator of differentiation.

In this study, results demonstrated that no inhibitory activity was obtained by the fucoidan extract on lipid accumulation in the 3T3-L1 cells. Therefore it can be excluded as an active anti-adipogenic component in the extracts. These results are in disagreement with other studies reporting anti-adipogenic effects of fucoidan [16, 106-108]. A study on this matter reported the inhibitory activity of lipid accumulation in 3T3-L1 cells by fucoidan being 50% in concentration 0.1 mg/mL [16]. Another study reported 32.8% inhibitory activity on lipid accumulation in 3T3-L1 cells by fucoidan in concentration 0.1 mg/mL [107]. However, the fucoidan used in these studies is stated to be extracted from brown seaweed but the species is not specified. Therefore this difference in results might be traced to the fucoidan being extracted from another species of brown seaweed than *F. vesiculosus*, as a study has reported a great variation in the bioactivity of fucoidan extracted from different seaweed species [96].

It was not possible to study the effect of pine bark extract on adipocyte differentiation as the high cytotoxicity interferes with the fat content measurements although a former study has reported anti-adipogenic effects of pine bark extract [14]. As in the other bioactivity assays, a large difference in anti-adipogenic activity is observed between extraction methods. It has been shown that the inhibitory activity of extracts on adipogenesis in 3T3-L1 cells can be traced to their phenolic and flavonoid contents [1, 3, 18]. However, because of the differences in cytotoxicity it is not possible to draw similar conclusions in this study. Further studies would be required on the different components present in the extracts to declare what is the active anti-adipogenic ingredient.

A complete inhibition of adipocyte differentiation is not a desirable result as the adipose tissue does not only serve as an energy storage, but also plays a critical role in secreting adipocytokines that regulate physiological functions like immune response, food intake and insulin sensitivity [4, 25]. The function of adipocytes, along with their secretion of adipocytokines, can be interrupted by high cellular lipid content which may lead to insulin resistance and elevate the risk of T2DM [2]. Several cellular and molecular studies have reported that changes in the adipose tissue can be triggered by dietary factors [1, 16] and a blueberry peel extract rich in polyphenols has been reported to decrease weight gain and

prevent fat accumulation in a high-fat-diet induced obese rats [3]. Therefore an enriched diet with anti-adipogenic compounds might represent a promising strategy for preventing obesity and diabetes.

The most effective inhibition of adipogenesis in the 3T3-L1 cell model was observed by the *F. vesiculosus* water extract which exhibited good inhibitory activity on the lipid accumulation in the most physiologically relevant concentration without being cytotoxic. These results could give rise to further identification of the active components in this extract which could later result in the development of a dietary enrichment compound exhibiting anti-obesity and anti-diabetic properties.

Conclusion

Results from this study suggest extracts from *F. vesiculosus* being effective inhibitors of lipid accumulation in a 3T3-L1 cell model. Additionally, both the *F. vesiculosus* and pine bark extracts exhibited potent antioxidant activity and α-glucosidase inhibitory activity. These results could give rise to further identification of the active components in these extracts which could lead to development of a dietary enrichment compound exhibiting antioxidant, anti-obesity and anti-diabetic properties.

6 Future perspectives

Further studies are proposed on different components in the extracts to indicate what active ingredient is responsible for high metal chelating activity, α-glucosidase inhibitory activity and anti-adipogenic activity. Conducting further studies on the anti-adipogenic effects of the extracts showing good activity in this study would also be interesting. Using western blotting and qPCR, the effects of the extracts on the expression of adipogenic and diabetes transcription factors could be carried out. Additionally, using fluorescence staining, the ROS status in the 3T3-L1 cells during differentiation could be evaluated to see if the extracts possess similar antioxidant activities within living cells as in the chemical based assays. Later, animal studies could be carried out to see if extracts possess the same activity in living organisms which could give rise to the development of a dietary compound possessing anti-diabetic activities.

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