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A study of avenanthramides in oats for future applications

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Abstract

Avenanthramides are polyphenols existing exclusively in oats. Studies have shown their antioxidant, anti-inflammatory and anti-proliferatory properties, making avenanthramides interesting therapeutic candidates for treatment of diseases with cardiovascular and dermatological origin. This study has shown a method for enrichment of avenanthramides involving steeping and germination at low pH levels, resulting in comparable amounts to the amounts found physiologically significant in previous studies in mice. Since germination at low pH levels potentially means preservation of other health beneficial compounds such as β-glucan, an application based on this method would not only contain the therapeutic effects from avenanthramides, but also the positive effects originating from β-glucan.

Keywords

Oats, avenanthramides, antioxidants, anti-inflammatory, anti-proliferatory, cardiovascular disease, dermatological disease, germination, β-glucan.

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Populärvetenskaplig sammanfattning

Havre är ett av de vanligaste spannmålen i Sverige och förekommer både som foder åt djur och som matprodukter för människor. Havre innehåller höga nivåer av antioxidanter, vitaminer, mineraler och kostfiber som har visats sig kunna minska skadligt kolesterol i blodet, hjälpa till med insulinreglering och även sänka risken att drabbas av hjärt- och kärlsjukdomar. Avenantramider är ämnen i havre som fungerar som antioxidanter och studier har visat att de även besitter förmågor att bland annat minska celldelning, inflammation och klåda. Dessa egenskaper gör avenantramider till intressanta kandidater för behandling av en mängd olika sjukdomar.

Det är sedan tidigare känt att halterna avenantramider ökar under en groningsprocess, det vill säga där havrekärnor utsätts för behandlingar som får dem att börja gro. Denna studie visar att pH påverkar anrikningen av avenantramider under groningsprocessen och att de högsta avenantramidhalterna fås under groning vid låga pH:n. Dessa halter är jämförbara med halter som i tidigare studier haft fysiologisk effekt i möss, vilket antyder att denna metod för anrikning av avenantramider är fullgod för skapandet av extrakt med terapeutisk potential. Vidare så medför groning vid låga pH:n lägre enzymaktivitet, vilket potentiellt innebär att andra nyttiga ämnen som annars skulle brutits ned bevaras.

Havreextrakt på material som behandlats enligt den föreslagna metoden in denna studie skulle kunna inkluderas i en mängd olika applikationer för behandling av bland annat hjärt- och kärlsjukdomar, hudåkommor, allergier eller ingå i produkter med nutritionellt värde.

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1. Introduction

Oats have been cultivated for more than a thousand years and are used today not only as feeding crop for animals, but also in a variety of different food products for human consumption. The main producers of oats are Finland, Germany and Sweden, with oats landing a third place as the largest crop produced in Sweden. There are many health benefits from consumption of oat based products. The high nutritional content of oats, with high levels of lipids, proteins, dietary fibers such as β-glucan, minerals such as calcium and iron, vitamins like Vitamin E and a wide variety of antioxidants, contribute to reduce plasma cholesterol, improve glucose and insulin regulation and lower the risks for coronary heart disease. The amounts of proteins present in oats are not only higher but also of a higher quality compared to other cereals, containing the essential amino acids lysine, threonine and methionine (Bryngelsson 2002, Meydani 2009 and Skoglund 2008b). Furthermore, oats are considered to be safe for adults suffering from celiac disease, since only trace amounts of gluten protein can be found (Janatuinen *et al.*, cited in Bryngelsson 2002:14).

1.1 The oat grain

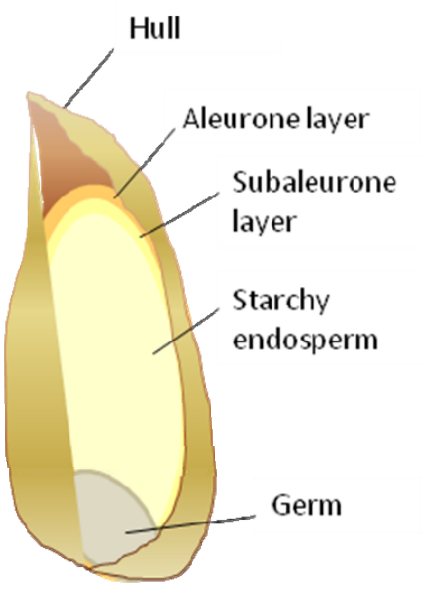


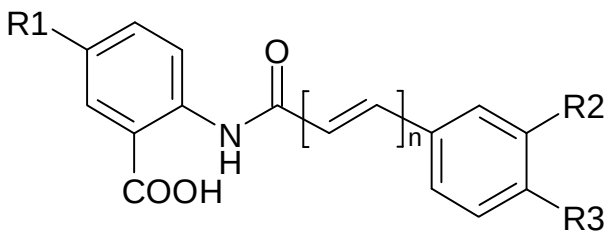
Figure 1. The major structural features of the oat grain.

The oat grain (Figure 1) consists of the oat groat surrounded by the hull which functions as protection from the outer environment. The hull, consisting of mostly cellulose and hemicellulose, becomes dry and brittle, with the metabolic activity decreasing as the grain matures. The groat consists of the starchy endosperm and the germ. The starchy endosperm is a reservoir of nutrients needed during germination. During germination the metabolic activity of the germ rises and nutrients are transported from the starchy endosperm to the embryonic axis. The aleurone and subaleurone layers function as an envelope for the oat groat. The aleurone and subaleurone layers are rich in protein and phenolic compounds and

are a part of the bran, which is a fraction obtained from commercial milling (Bryngelsson 2002 and Skoglund 2008b).

1.2 Avenanthramides

Avenanthramides are low molecular-weight soluble polyphenols that exist exclusively in oats, where they act as phytoalexins produced in response to pathogens (Mayama *et al.* and Miygawa *et al.*, cited in Bryngelsson 2002:27; Skoglund 2008b and Meydani 2009). Formation of avenanthramides is catalysed by the hydroxycinnamoyl-CoA:hydroxyanthranilate N-hydroxycinnamoyl transferase (HHT) by condensation between anthralinic acids and hydroxycinnamoyl-CoA esters (Bryngelsson *et al.* 2003). The majority of the avenanthramides can be found in the oat groat, with the highest concentration in the bran. However since avenanthramides are expressed throughout the oat grains, they are also present to some extent in the oat hull (Skoglund 2008b and Liu *et al.* 2005). The structures of avenanthramides are that of substituted N-cinnamoylanthralinic acids, with one anthralinic acid moiety and one cinnamic acid moiety. The different substitution patterns distinguish the different avenanthramides. The anthralinic moiety can consist of an anthralinic acid (1), 5-hydroxyanthralinic acid (2), 5-hydroxy-4-methoxyanthralinic acid (3), or 4-hydroxyanthralinic acid (4), while the cinnamic moiety can consist of caffeic (c), *p*-coumaric (p), sinapic (s), ferulic (f) or cinnamic acid (a) (Bratt *et al.* 2003 and Skoglund 2008b). Avenanthramides 2p, 2c and 2f are the major forms, with 2c being the most abundant (Guo *et al.* 2008). Figure 2 displays the chemical structure of avenanthramides 2c, 2p, 2f, 2pd and 2fd together with the commercial drug Tranilast. Avenanthramide 2pd and 2fd differs from 2p and 2f in their additional double bond.



	n	R1	R2	R3
2c	1	OH	OH	OH
2p	1	OH	H	OH
2f	1	OH	OCH_3	OH
$2p_d$	2	OH	H	OH
$2f_d$	2	OH	OCH_3	OH
Tranilast	1	H	OCH_3	OCH_3

Figure 2. Chemical structure of the known avenanthramides and Tranilast.

Avenanthramides have been found to exert antioxidant activities both *in vitro* and in vivo, with up to 30 times greater activity than other phenolic compounds found in oats and the highest antioxidant activity has been shown to be exerted by avenanthramide 2c (Bratt 2003 and Meydani 2009). Studies have indicated avenanthramides abilities to inhibit vascular smooth muscle cell proliferation and to suppress expression of adhesion molecules from vascular endothelial cells through inhibition of the cytokine IL-1β (Liu *et al.* 2004; Nie *et al.* 2006a and Guo *et al.* 2008). The avenanthramides have also been shown to have activities that are anti-inflammatory and itch-suppressing, involving the NF-κB-mediated canonical pathway (Kulms and Schwarz 2006 and Sur *et al.* 2008). Furthermore, the structure of avenanthramides is similar to that of the synthetic drug Tranilast, which is used in certain allergy treatments (Sur *et al.* 2008 and Skoglund 2008b). Taken together, this information makes the avenanthramides interesting candidates in the search for new therapies against a variety of human disorders.

Malting has been shown to enrich the avenanthramides in the oat seeds (Bryngelsson 2002 and Skoglund 2008b). The malting process consists of three steps; steeping, germination and kilning. In the steeping step, the oat kernels are soaked in liquid, enabling the grains to absorb water to a 43-45% increase in moisture content which resumes their metabolic activity. The steeping is followed by a germination process, favored at 16-20°C for about 4-5 days, in which synthesis of enzymes and modifications of the kernel takes place. It is in this step that the major part of avenanthramide enrichment occurs, however other substances such as β-glucan have been shown to decrease due to increasing β-glucanase activity (Skoglund 2008b). It is therefore desirable to modify the germination process to obtain satisfactory avenanthramide enrichment, while preserving other beneficial compounds. Lastly, a kilning process is performed at temperatures ranging from 50-220°C, to dry the grains and stop further metabolic and enzymatic activity and also minimize contamination from microorganisms (Skoglund 2008b and Xu *et al.* 2009). Bryngelsson *et al.* (2003) showed an increase of avenanthramides during steeping and germination of oat grains, together with an increased activity of the avenanthramide synthesizing enzyme HHT. Skoglund *et al.* (2008a) confirmed these findings and proceeded with showing the increase of avenanthramides to be dependent on choice of cultivar and germination conditions.

1.3 Aim of project

The aim of this project was to find a method to enrich avenanthramides in oats through germination, focusing on effects from pH levels, with the main question: Does the pH level in a germination process have any effect on the enrichment of avenanthramides? Additionally, the effect of avenanthramide enrichment on antioxidant activity was determined and previous studies concerning the physiological effects of avenanthramides was investigated. This information may subsequently lead to formulation of future applications with potential nutritional, cardiovascular and dermatological value.

To determine the effect of different pH levels on the enrichment of avenanthramides during a germination process, the oat cultivars Betania, Circle and Matilda was used. These cultivars differ in characteristics such as fat content and dietary fibers. The same experiments were performed on the cultivars. The seed samples was freezed followed by steeping in two different solutions with two different pH levels and subsequently transferred to Petri dishes for the germination process for four days. Samples was taken out and dried after the steeping and after the germination, raw and raw dried samples were also included. The samples were be milled and the avenanthramides extracted and the avenanthramide profile was analyzed by HPLC. Moreover the antioxidant activity was determined through a DPPH-radical scavenging system.

2. Materials and Methods

2.1 Empirics

A study of the available literature regarding the physiological aspects of avenanthramides was performed. The information found was subsequently used to evaluate the avenanthramide amounts needed for physiological effects and to discuss the therapeutic potential of the avenanthramide extracts obtained in this study regarding future applications.

2.2 Oat materials

Hulled seeds of cultivars Betania, Circle and Matilda from a 2009 test cultivation from a non-heated storage, were provided by Lantmännen SW Seed in Svalöv, Sweden. The fat content of the grains from the 2009 harvest was for Betania 5.9%, while Circle and Matilda contained 4.7 and 10.2%, respectively. The β-glucan content was 5.9, 5.2 and 4.7% for Betania, Circle and Matilda, respectively.

2.3 Chemicals

Acetonitrile, ethanol, methanol and Na_2HPO_4 were supplied by Merck (Darmstadt, Germany) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) by Sigma Chemicals (St. Louis, USA). Commercial sodium hypochloride (Klorin®), manufactured by Colgate Palmolive and citric acid was purchased from a grocery store. All solvents were of analytical grade and were used without further purification.

2.4 Raw seeds

Raw seeds from each cultivar, in duplicates, were dried at 105°C for 24h and used, together with raw seeds, as reference.

2.5 Freezing

Seeds of the respective cultivar were put in plastic cups and transferred to a -20°C freezer over night to remove dormancy.

2.6 Steeping

Citrate-phosphate solution in tap water (0.1 M, pH4.1) was prepared and autoclaved for 30 min together with tap water with pH 6. The seeds were surface sterilized using a 10% chlorine solution for 10 min, followed by rinsing in deionized water. The seeds from the respective cultivars were split in two groups of 200 seeds and steeped in the respective solutions (100 ml solution/100 seeds) at 20°C for 24h. Following the steeping, the 200 seeds from each cultivar not designated for germination were dried at 105°C for 24h.

2.7 Germination

The 200 seeds from each cultivar designated for germination were transferred to 95% EtOH-sterilized Petri dishes in replicates (100 seeds/dish*2) with filter papers in the bottom and in the lid. The filter papers were moist with the respective pH solution and the Petri dishes were transferred to a constant room at 20°C and incubated for four days. After the germination, the germination frequency of the respective cultivar was calculated as the ratio between the number of germinated seeds through the total seed amount. The germination criteria were visible roots or shoots of at least 1 mm. Subsequently, the roots and shoots were separated from the seeds and weighed separately before the seeds were dried at 105°C.

2.8 Extraction of avenanthramides

The dried seeds, as well as raw untreated seeds were milled in an ultra-centrifugal type ZM 1 Retsch mill to a particle size of <0.5 mm and weighed to 1 g into 30 ml screw-capped tubes with duplicates. Avenanthramides from the 1 g samples were extracted three times at 50°C for 20 minutes using 8 ml 80% aqueous EtOH with pH 2, followed by centrifugation at 2500 rpm for 10 minutes. The supernatants were combined and the ethanol evaporated in a BÜCHI Multivapor P-12 at 50°C under vacuum. The residuals were resuspended in 2 ml MeOH, transferred into Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes. The extracts were subsequently transferred into HPLC vials and analysed in duplicates by HPLC. Raw seed flours (0.1 g) were weighed in and transferred to the 105°C oven over night for dry matter calculations. The flours from the grains already exposed to a drying process were

considered adequately dried and dry matter calculations were therefore not performed on these samples.

2.9 High performance liquid chromatography (HPLC) analysis

The avenanthramides were analyzed using an Agilent HPLC system with reversed-phase Hypersil ODS column (5 µm, 5.0x125 mm), with solvent A (0.01 M formic acid, pH 2.9, 5% acetonitrile) and solvent B (acetonitrile) as mobile phases. A linear gradient from 0 to 40% of solvent B in A for 40 minutes was used with a flow rate of 1 ml min^{-1}. The injection volume was 10 µl and the UV-absorbing substances were detected at 340 nm. The peaks were manually integrated and identification and quantification of the known avenanthramides were performed using retention time, UV-spectra and calibration curves of external references.

2.10 Antioxidant activity assay, DPPH-radical scavenging system

The respective extracted samples (100 µl) and 2.9 ml of DPPH (1,1-diphenyl-2-picrylhydrazyl) methanolic solution (76.1 mm) were mixed in a cuvette, placed in a spectrophotometer and the absorbance at 517 nm was recorded during 10 min. For the pH 6 germinated samples, a two times' dilution in MeOH prior to the mixing with DPPH solution was necessary to avoid absorbance decrease below the detection limits. To determine the start absorbance of each sample, the linear equation of the spontaneous decrease in DPPH absorbance by time was calculated ($y = -0{,}0005x + 1{,}0193$, $R^2 = 0{,}5741$). This start value was subsequently used to calculate the antioxidant activity given in absorbance units calculated as: $(A_{t=0min} - A_{t=10min})*100$, where A represents the absorbance at 517 nm.

2.11 Statistical analysis

Results from the HPLC and DPPH-radical scavenging system analyses were statistically evaluated by analysis of variance (ANOVA General Linear Model) and Pearson correlation using the MiniTab 15 Statistical Software. The level of significance was set to $p<0.05$ (with $p<0.05$ represented as *, $p<0.01$ as ** and $p<0.001$ as ***).

3. Empirics

3.1 Antioxidant and antigenotoxic effects of avenanthramides

In normal cell metabolism, low concentrations of radicals or reactive oxygen species, ROS, and reactive nitrogen species, RNS, are produced. At homeostatic conditions, these substances take part in various cellular transduction pathways and defenses against pathogens. In high amounts however, these radical substances can afflict cell death and tissue damage by degradation of proteins, lipids, DNA and RNA, leading to diseases such as cancer or atherosclerosis. Antioxidants are substances able to inhibit the initiation and propagation of oxidizing cascades, thus hindering the damaging oxidation caused by ROS (Skoglund 2008b and Xu *et al.* 2009). Avenanthramides have been found to exert antioxidant activities both *in vitro* and *in vivo*, with up to 30 times greater activity than other phenolic compounds found in oats and avenanthramide 2c has been shown to exhibit the highest antioxidant activity of the avenanthramides *in vitro* (Meydani 2009).

The antioxidant abilities of phenolic compounds were shown in a study performed *in vitro* by Chen *et al.* (2004), where the ability of an oat extract to protect human low-density lipoprotein (LDL) was examined. Oat phenolics with concentrations ranging from 0.52 to 1.95 µmol/L attenuated the calcium induced oxidation of LDL in a dose dependent manner. This attenuation was extended when adding 5 µmol/L vitamin C, showing a synergistic interaction between the phenolic compounds and the vitamin C. This finding indicates that avenanthramides may display synergistically interactions to other antioxidative compounds, decreasing the oxidative stress in biological systems.

O'Moore *et al.* (cited in Meydani 2009:732) investigated the antioxidant effect of avenanthramide-enriched extracts of oats in laboratory rats. When supplementing the diet with avenanthramide-enriched extracts of oats at 200 mg/kg diet, the exercise-induced production of ROS were attenuated, the supplement of 200 mg/kg diet corresponding to about 40 mg avenanthramides/kg body weight in rats.

The antioxidant and antigenotoxic activity of synthetic avenanthramides was investigated by Lee-Manion *et al.* (2009). When examining the antioxidant activity using the DPPH assay, evaluating the free radical scavenging capacity of the test compounds, the most reactive

avenanthramide was found to be 2c followed by 2p and 2f. The result coincided with the results from the Ferric Reducing Antioxidant Potential (FRAP) assay, measuring the combined reducing power of electron-donating antioxidants. The antigenotoxic activity was determined using the Comet assay, which is a single-cell gel electrophoresis assessing the ability of the test compounds to protect human colon adenocarcinoma cells, (HT-29 cells) stressed with hydrogen peroxide, from DNA damage. The results proved the DNA-protective ability of avenanthramides, with the most protective compound being avenanthramide 2c, decreasing DNA damage by 50% at a concentration of only 0.9µmol/L. The antigenotoxic effect of the three major avenanthramides decreased in a similar fashion as the antioxidant activity, however no significant relationship between the two could be found, indicating that no direct relationship exist between these two parameters.

3.2 Avenanthramides and atherosclerosis

According to the World Health Organization (2010), cardiovascular diseases, CVDs, are by far the number one cause of death globally. 17.1 million people died from CVDs in 2004, and it is estimated that in 2030 almost 24.6 million people will die from CVDs, one of the main causes being coronary heart diseases, CHDs, which are diseases affecting the blood vessels supplying blood to the heart. High intake of oats has been shown in both epidemiological and clinical studies to reduce the risk of CHD, with the effects on the blood vessels attributing to the avenanthramides. Atherosclerosis is a CHD where inflammatory processes interacts with the vascular endothelium, causing adhesion of leukocytes to the endothelium leading to plaque formation and thus thickening of the arterial walls (Liu *et al.* 2005; Nie *et al.* 2006a; Nie *et al.* 2006b and Guo *et al.* 2008).

Interleukin-8 (IL-8), interleukin-6 (IL-6) and monocyte-chemoattractant protein-1 (MCP-1) are cytokines produced by a variety of cells, e. g. endothelial cells, keratinocytes, smooth muscle cells and macrophages and act on a variety of different targets leading to a spectrum of different effects. IL-8, while thought to be a chemokine with lymphocyte attracting abilities, is also a potent angiogenic factor influencing growth of blood vessels in atherosclerotic lesions. MCP-1 also expresses chemokine properties, attracting monocytes and macrophages to early atherosclerotic lesions. IL-6 increases the adherence of lymphocytes to endothelial cells, the expression of intracellular adhesion molecule-1 (ICAM-

1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells and is, together with IL-8, responsible for activating the phenotype transformation of vascular smooth muscle cells, causing proliferation and migration to the intimal layer of the endothelium. This in turn leads to accumulation of lipids and plaque formation causing the disease (Nie *et al.* 2006a).

Activation of Nuclear Factor-κB (NF-κB) is associated with expression of these pro-inflammatory cytokines, rendering NF-κB as a candidate for regulatory control. There are five structurally related members that make up the NF-κB family of transcription factors, all existing in the cytoplasm of the cell as homo- or heterodimers. These include p50, RelA (p65), p52, RelB and Rel (c-Rel) and the precursors p105 and p100 for p50 and p52 respectively, with the most abundant form being the RelA/p50 heterodimer. There are two pathways for the activation of NF-κB, the canonical pathway and the non-canonical pathway. The non-canonical pathway is involved in the adaptive immune response and will not be described here. The canonical pathway, however, is activated by a large group of different stimuli, all triggering the innate immune response. These stimuli involve pro-inflammatory cytokines, virulence factors, Pathogen-Associated Molecular Patterns (PAMPs) and stress factors like UV-radiation or oxidative stress, activating receptors such as Toll Like Receptors (TLR), tumor necrosis-factor receptors (TNFR) or IL-1 receptors. When activated, the dimer translocates from the cytoplasm to the nucleus where the N-terminal Rel Homology Domain (RHD) binds to the DNA leading to transcription of the targeted gene. In the cytoplasm the NF-κB dimer is in its resting form, interacting with a NF-κB inhibitor (IκB). There are several members in the family of IκBs, the most important being IκBα, IκBβ and IκBε. These proteins bind the RHD and hinder the translocation to the nucleus by disrupting of the Nuclear Localization Signal (NLS). For activation of the NF-κB dimer, disposal IκB is necessary, which is done by phosphorylation of the IκB proteins by the activated IκB kinase complex (IKK complex). This complex consists of three subunits, IKK1 and IKK2 which are catalytical and NEMO which is regulatory. Activation of one or more receptors activates the IKK complex leading to the phosphorylation. This is subsequently followed by polyubiquitylation, marking the IκB for proteasomal degradation, rendering the NF-κB dimer activated and free to translocate and activate transcription of pro-inflammatory proteins such as IL-6, IL-8 and

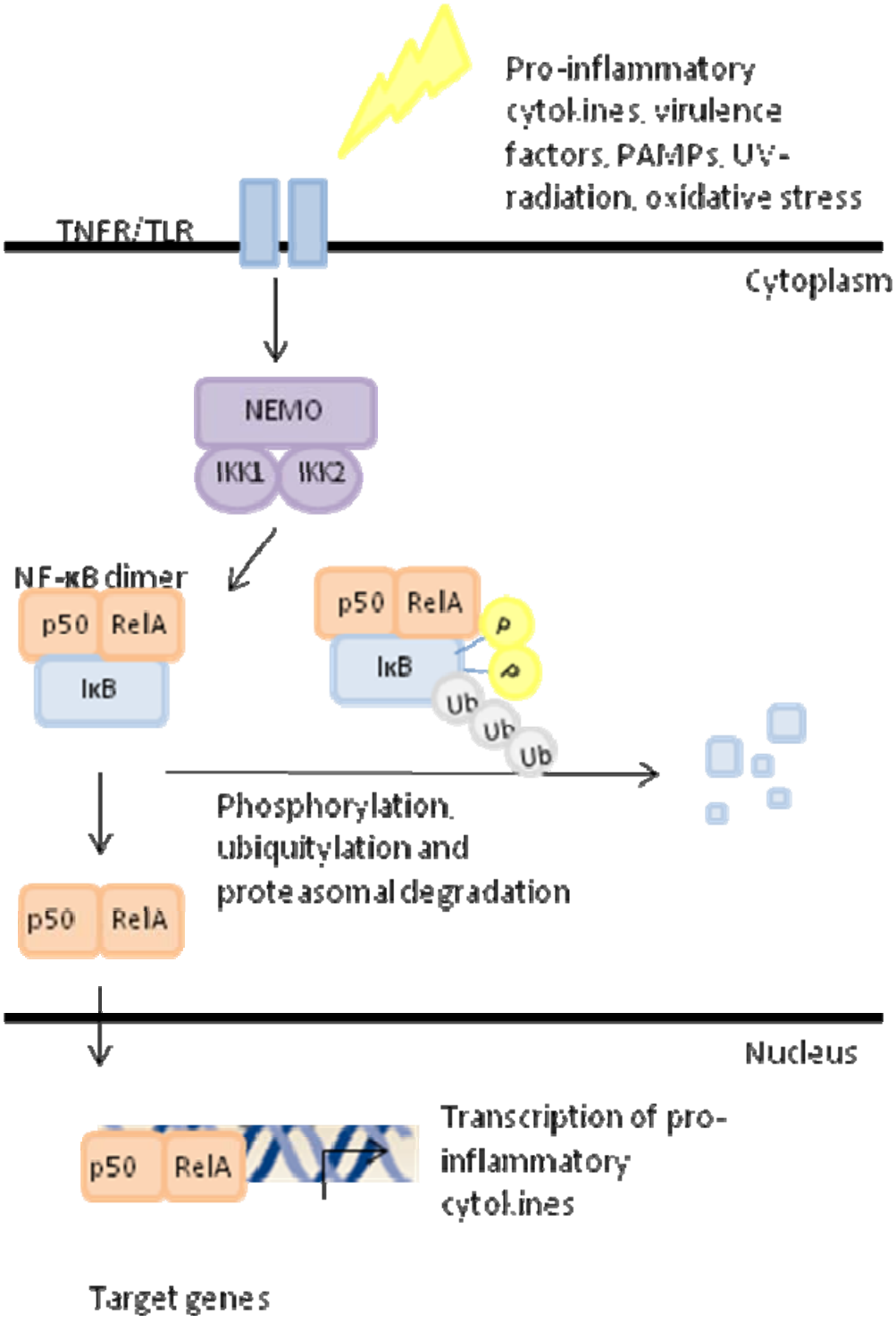


Figure 3. Mechanism of the NF-κB canonical pathway. In resting cells, inactive NF-κB dimers, associated with IκB inhitors, are sequestered in the cytoplasm. Activation of receptors leads to activation of the IKK complex, which subsequently phosphorylates the IκB protein triggering the polyubiquitylation that marks the IκB for proteasomal degradation. The activated NF-κB dimer translocates to the nucleus, where it binds to the promoters of the target genes and activates transcription of mainly pro-inflammatory cytokines.

MCP-1 (Kulms and Schwarz 2006 and Pasparakis 2009). The mechanism for the canonical pathway can be seen in Figure 3.

Liu *et al.* (2004) investigated the antiatherogenic effects of avenanthramides in human aortic endothelial cells (HAECs) focusing on the adherence to monocytes, the expression of adhesion molecules and the production of cytokines and chemokines. To induce an inflammatory state in the HAECs similar to that in vivo, the cells were treated with IL-1β prior to the different experiments, a pro-inflammatory cytokine normally produced by infiltrating inflammatory cells. The cell adhesion was investigated using a U937 cell adhesion assay, where the ability of an avenanthramide-enriched mixture (AEM) to inhibit the adherence of activated HAECs to U937 monocytic cells was determined. Pretreatment of activated HAECs with 4, 20 and 40 ng/ml AEM reduced the adherence to U937 with 20, 40 and 45% respectively, indicating the avenanthramides ability to reduce cell adherence in a concentration-dependent manner. Studying the effect of avenanthramides on the expression of adhesion molecules showed an effective concentration-dependent inhibition of IL-1β-stimulated expressions of ICAM-1, VCAM-1 and E-selectin, all of which mediates the migration of leukocytes to the endothelium leading to plaque formation. An AEM with concentration 40 μg/ml rendered the most effective inhibition of expression of all three adherence molecules. Furthermore, the AEM displayed a significant ability to reduce the activated HAEC production of cytokines IL-8, IL-6 and MCP-1 in a concentration dependent manner. AEM of 20 and 40 μg/ml reduced the production with 30 and 50% respectively for all the three cytokines. Since activation of the transcription factor NF-κB is necessary for the induction of the adhesion molecules and cytokines, the authors suggested the NF-κB canonical pathway as a potential mediator of the avenanthramide inhibition.

Consumption of oat products has been shown to improve the function of the endothelial walls of the blood vessels. Nie *et al.* (2006a) investigated the effect of avenanthramides on human smooth muscle cell (SMC) proliferation, a contributing factor in the development of atherosclerosis enhanced by the cytokine IL-8. Moreover, the effect of avenanthramides on nitric oxide (NO) biosynthesis by endothelial cells and VSMCs was examined since several studies have showed an impaired NO synthesis present in the pathogenesis of this disease. NO is known to be a powerful vasodilator, additionally it prevents platelet aggregation,

VSMC proliferation, adhesion of leukocytes and expression of atherogenesis associated genes, making it an important antiatherosclerotic compound. The effect of the synthetically prepared avenanthramide 2c on SMC proliferation was determined using a [^{3}H] thymidine incorporation assay, with Fetal Bovine Serum (FBS)-induced SMC proliferation. Treatment with synthetic 2c inhibited the FBS-stimulated DNA synthesis in the SMC, thus reducing the proliferation. Concentrations of 40, 80 and 120 µM of 2c reduced the proliferation by 41, 62 and 73% respectively after 96h of treatment. To determine the NO production, the 5.5 diaminofluorescein (DAF-2) fluorescence assay was used, where fluorescence of the supernatants from activated and DAF-2 treated SMC and HAEC cultures were measured at 485 and 538nm. Treatment with 2c increased NO production significantly in both VSMCs and HAECs. A three-fold increase was seen in SMCs at a concentration of 120 µM, while the HAECs produced a nine-fold increase in NO at a concentration of 80 µM. To further investigate the mechanism by which avenanthramide 2c affects the NO production, the effect of 2c on endothelial NO Synthase (eNOS) mRNA levels was studied by real-time PCR. At a concentration of 80 µM, 2c rendered a 2.2-fold increase in mRNA levels in VSMCs, while in the HAECs, the same concentration gave a 2.4-fold increase in mRNA levels. Both the inhibiting effect on proliferation and the increased NO production are contributing factors to the prevention and progression of CHD. The inhibition of the proliferation is furthermore considered to be of great importance in the prevention of restenosis, which means narrowing of the arterial walls following angioplasty.

Nie *et al.* (2006b) continued their research by investigating the ability of avenanthramide 2c to block the cell cycle progression in VSMCs in order to determine the molecular mechanism by which avenanthramides exert their inhibiting properties. The VSMC cycle is regulated by retinoblastoma protein (pRB) phosphorylation which is, in turn, regulated by the signal molecules p53, p21cip1, p27kip1 and cyclin D1. The effect of the avenanthramides on cell cycle distribution was determined by flow cytometry, using the primary rat embryonic aorta cell line A10, a common model for human VSMCs. Treatment with avenanthramide 2c decreased the number of cells in S phase, where the DNA replication occurs, and increased the number of cells in G0/G1 phase, where no proliferation takes place. Since hyperphosphorylation of pRb is necessary for the transition of the cells from the G1 phase to the S phase, the effect of 2c on the phosphorylation of pRb was measured by Western blot

analysis. The pRb phosphorylation significantly decreased after treatment with avenanthramide 2c, causing the cells to remain in the G1 phase. The complex responsible for the pRb phosphorylation in the G1 phase is the cyclin D/CDK complex. When examining the effect of avenanthramide 2c on cyclin D1 expression, 2c, with concentrations ranging from 0-120μM, was found to attenuate cyclin D1 expression in a dose dependent manner, indicating that 2c acts on pRb phosphorylation through inhibition of cyclin D1. Furthermore, 2c was found to increase levels of the tumor suppressor p53, implicated in cell growth control of VSMCs, by increasing the half life of the protein. The main target of p53 in cell cycle inhibition is p21cip1, while upregulation of p21cip1 is associated with prevention of pRb phosphorylation. Treatment of A10 cells with 2c increased the level of expression of p21cip1 in a dose dependent manner. However, 2c had no effect on p27kip1 expression, another gene regulated by p53.

Guo *et al.* (2008) further investigated the ability of avenanthramides to suppress IL-1β-stimulated secretion of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1 by HAECs with interest in the NF-κB canonical pathway. The inhibitory effect of an avenanthramide enriched mixture (AEM) on the activation of NF-κB was studied by measuring the DNA binding activity of NF-κB p50. The AEM was found to decrease the IL-1β induced activity of the NF-κB p50 in a concentration dependent manner in Human Aortic Endothelial Cells (HAECs). The same results were obtained using a synthetically prepared avenanthramide 2c. A methyl ester of avenanthramide 2c (CH3-Avn-c) was investigated in respect to IL-1β induced production of proinflammatory cytokines and their mRNA expression in HAECs. At concentrations of 10, 40 and 100 μM, secretion of IL-6 was reduced by 39, 55 and 60%, IL-8 by 21, 41 and 90% and MCP-1 by 24, 42 and 58% respectively, coinciding with a reduction in mRNA levels of the respective cytokine. Treatment of IL-1β stimulated HAECs with CH3-Avn-c lead to a significant, concentration dependent reduction in activation and translocation of NF-κB p50 and NF-κB p65, as measured by ELISA-based TransAM NF-κB p50 and p65 kit and luciferase assay. It was furthermore found that the avenanthramide inhibition on NF-κB activity was executed in an indirect fashion. Therefore the effect of CH3-Avn-c on the phosphorylation of the IκB kinase subunits IKK1/IKK2 and the NF-κB inhibitor IκB in Human Umbilical Vein Endothelial Cells (HUVECs,) along with the IL-1β induced degradation of IκB, were examined by Western blotting. CH3-Avn-c stabilized the NF-κB inhibitor IκB and

prevented phosphorylation of IKK1/IKK2. Additionally CH3-Avn-c suppressed the proteasome activity, which lead to a concentration dependent accumulation of ubiquitin conjugates. These results point to the potential of avenanthramides and their synthetic derivatives as drugs against atherosclerosis and CHDs.

3.3 Avenanthramides and colloidal oat extracts in dermatology

The abilities of poultices of oats and colloidal oatmeal to relieve different skin conditions have been known for quite some time. Remedies of oats withholding avenanthramides have indicated positive effects in dermatological conditions such as sunburn, eczema atopic dermatitis and allergic or contact dermatitis (Sur *et al.* 2008). These conditions often involve malfunctioning epidermal barrier, pruritus (itch), inflammation of the skin and increased levels of pro-inflammatory substances like IL-8, tumor necrosis factor-α (TNF-α) and arachidonic acid (Aries *et al.* 2005). Itch can come from damaged or over stimulated neurons (neuropathic and neurogenic itch, respectively) or be due to skin disorders such as irritation, inflammation or other damages (cutaneous itch). Itch and other disorders are often accompanied by loss of barrier function in the skin, leading to penetration of different irritants exacerbating the conditions. It is therefore important that treatments of these conditions also express hydrating abilities able to restore the barrier function of the outer layers of the skin (Sur *et al.* 2008 and Pacifico *et al.* 2005).

Apart from being expressed in SMCs, endothelial cells and macrophages, IL-8 is expressed in keratinocytes upon stimulation with IL-1α, IL-1β, interferone-γ (IFN-γ) or TNF-α. It has strong chemotactic effects on neutrophils and lymphocytes, increases intercellular calcium concentrations and induces granule exocytosis. TNF-α is a pro-inflammatory cytokine, inducing expression of cutaneous and endothelial adhesion molecules which contributes to the development of inflammation. Normally, TNF-α is stored in epidermal mast cells, however following stimulation it can be produced by keratinocytes. Arachidonic acid (AA) is a long-chain polyunsaturated fatty acid situated in the phospholipids of cell membranes. It is released by enzymatic activity of the cytosolic phospholipase A_2 ($cPLA_2$) which upon activation translocates from the cytosol to the membrane of the cells, where it acts upon the membrane phospholipids. $cPLA_2$ is highly dependent on calcium levels and for its activation, a rise in free intracellular calcium and phosphorylation by serine/threonine protein kinase

(MAPK) is required. Following release, the AA is subsequently metabolized by cyclooxygenases and 5-lipoxygenase into prostaglandins, prostacyclins and thromboxanes or leukotrienes and hydroxyeicosatetraenioc acids (HETEs), respectively. These metabolites are actively synthesized in normal skin epidermis where they modulate a variety of different physiological functions, however increased levels of the metabolites and increased activity of PLA_2 are associated with inflammatory skin disorder (Aries *et al.* 2006 and Welss *et al.* 2005).

Sur *et al.* (2008) performed a study of the mechanism behind the anti-inflammatory effects of avenanthramides in human keratinocytes. Treatment of keratinocytes with TNF-α lead to a degradation of IκBα, a decrease that was significantly reduced in the presence of 1 ppb avenanthramides. Results from a luciferase activity assay, measuring the NF-κB activity in TNF-α stimulated cells, in the absence and presence of avenanthramides, confirmed these findings, as 1 ppb avenanthramides inhibited NF-κB activity by 1.7-fold. Since NF-κB is a major regulatory transcription factor for a variety of different compounds, the effect of avenanthramides on the production of the pro-inflammatory cytokine IL-8 from keratinocytes was subsequently studied. Treatment of TNF-α stimulated keratinocytes with avenanthramides significantly reduced the IL-8 production by 1.4-fold, demonstrating that the anti-inflammatory effects of the avenanthramides are exerted via the NF-κB pathway. The authors progressed with investigating the ability of the avenanthramides to suppress contact hypersensitivity, neurogenic inflammation and itch responses in a mouse model. Contact hypersensitivity was induced by challenging the mice in the left ear with oxazolone, a powerful allergen, inducing edema. Topical treatment with avenanthramides post oxazolone challenge significantly inhibited contact hypersensitivity and decreased the edema of the ear in a dose dependent manner. Doses of 2 and 3 ppm avenanthramides gave a 42.89 and 67.2% decrease, respectively. As a model for neurogenic dermatitis, resiniferatoxin-induced ear edema was used. Resiniferatoxin (RTX) activates small diameter sensory neurons which results in neurogenic inflammation. As in the case of the contact hypersensitivity, 2 and 3 ppm of avenanthramides significantly reduced edema with 31.58 and 46.09%. Histamine is a powerful inducer of itch released by mast cells in response to pathogens. Compound 48/80 induces itch sensations due to histamine release. In the itch model followed by Sur *et al.* (2008), mice were treated with compound 48/80, and the scratching was documented. Animals treated with 3 ppm avenanthramides showed

significantly less scratching, with a reduction by 40.7% that was comparable to anti-itch responses due to hydrocortisone treatment. This indicates that avenanthramides modulate nerve responses and possess potential therapeutic abilities against pruritic skin diseases. Additionally, by modulating responses from sensory neurons leading to a reduction of itch, and subsequently scratching, the avenanthramides may reduce the risk of the secondary inflammation that follows with the disrupted barrier function of the skin common in dermatological disorders like atopic dermatitis and eczema.

The effects of the oatmeal extract Avena Rhealba® (AR) on arachidonic acid (AA) metabolism and $cPLA_2$ expression in human keratinocyte cell line HaCaT was examined by Aries *et al.* (2005). Avena Rhealba® is a colloidal oatmeal extract composed of a white species of the oat *Avena sativa*, selected for its cultivation properties and chemical composition. AR is produced by a patented process where dehulled seeds are milled and the resulting flour subjected to turboselection in a dry process leading to the colloidal extract. 0.1% AR showed a significant ability to inhibit A23187-induced arachidonic acid-mobilization as well as arachidonic acid metabolites from both the cyclooxygenase and the 5-lipoxygenase pathways with a reduction by 27, 30 and 29%, respectively. Investigation of $cPLA_2$ protein expression in A23187-stimulated and AR treated cells showed a highly significant reduction by 85% with the lowest dose of AR of 0.01% and a 100% reduction with AR doses of 0.05 and 0.1%. The authors continued with using TNF-α as inducer of $cPLA_2$ expression to study the effects of AR in a more pathological and physiological environment. The results coincided with previous findings, AR reduced TNF-α-induced $cPLA_2$ expression by 67, 77 and 95% with the doses 0.01, 0.05 and 0.1%, respectively and it was furthermore found to reduce $cPLA_2$ mRNA expression in a dose dependent manner.

The anti-inflammatory effects of Avena Rhealba® have previously been investigated by Vié *et al.* (2002) in a clinical trial using the sodium lauryl sulfate (SLS) irritation model. Two colloidal oatmeal extracts, AR and *Avena sativa* in petrolatum ointment vehicle, were topically applied to test areas on the volar surface of the upper arm of the test subjects under occlusion for 2h followed by application of 1% SLS solution for 24h. The inflammation of the subjected skin was determined using a chromameter, evaluating the redness, and a laser-Doppler PF3, measuring the cutaneous blood flow in perfusion units. At a concentration of

20%, both AR and *Avena sativa* significantly reduced the SLS-induced redness of the skin, however no significant difference could be observed between the two. It is nevertheless evident that oatmeal extracts display properties rendering them able to prevent alterations of the cutaneous barrier function and vasculature associated with skin irritation.

Pacifico *et al.* (2005) performed a clinical study on patients with pruritus and cutaneous xerosis, evaluating the efficacy and tolerability of Aveeno Skin Relief Moisturizing Lotion, containing colloidal oatmeal from *Avena sativa* and menthol. Patients suffering from these conditions were treated with the lotion once a day for three weeks and the efficacy and tolerability was evaluated as changes in pH, skin hydration and clinical appearance. After the three week treatment, a significant improvement of the cutaneous lesions and the pruritus was observed in 96% of the patients, with complete regression in 88.9%. The cutaneous hydration increased by 36.9 and 46.7% after one and three weeks, respectively, with a simultaneous decrease in cutaneous pH levels by -4% after one week and -6.3% at the end of the trial. Although no information regarding avenanthramides were included in the studies of AR and *Avena sativa*, these results together with previous findings indicate that the health benefits and effects from these colloidal extracts are correlated to the avenanthramides.

3.4 Bioavailability of avenanthramides

The bioavailability of avenanthramides in humans was investigated by Chen *et al.* (2007) in a clinical trial, where six subjects consumed 360 ml milk alone or containing 0.5 or 1 g of an avenanthramide enriched mixture (AEM) with concentrations of 154, 109 and 111 µmol/g for avenanthramides 2p, 2f and 2c, respectively. Samples of plasma were collected over a 10h period and the maximum plasma concentrations of the three major avenanthramides was determined as 112.9, 14.2 and 41.4 nmol/L for 2p, 2f and 2c, respectively after consumption of 0.5 g AEM. While concentrations after consumption of the 1 g AEM was 375.6, 96 and 89 nmol/L for 2p, 2f and 2c, respectively. When comparing the bioavailabilities a significant difference was observed between 2p and 2f, with 2p displaying a 4-fold greater bioavailability than that of 2f following a 0.5 g intake of AEM. The highest concentrations of plasma avenanthramides were obtained 1.75 -2.3h after intake and the levels were dimidiated after 1.75-3h. Furthermore, intake of 1 g AEM lead to an increase of a reduced

form of glutathione, indicating that the levels of avenanthramides obtained after intake were physiologically sufficient for the avenanthramides to exert their antioxidant capacities.

In a previous study on bioavailability of oat phenolics made in hamsters, by Chen *et al.* (2004), plasma concentrations peaked at 40 min with levels abolished after 120 min. Taken together with the results in the human study, absorption and metabolism of avenanthramides seem species dependent. Furthermore, the bioavailability of 2p and 2f in humans was 18- and 5-fold greater, respectively, compared to the bioavailability in the hamster trial. However, more studies are needed to prove that consumption of oat products lead to plasma concentrations that have significant physiological effects. Furthermore it is not clear whether prolonged intake of oat products can accumulate avenanthramides in higher concentrations in other tissues than in the blood.

3.5 Tranilast

The structure of avenanthramides is similar to that of the compound Tranilast, which has been used as drug against allergy for the treatment of bronchial asthma, atopic dermatitis and allergic conjunctivitis, with effects due to inhibition of degranulation of mast cells. Additionally, Tranilast inhibits proliferation of fibroblasts and functioning smooth muscle cells *in vitro* showing potential antiatherogenic properties. Taken together with its anti-histamine, anti-inflammatory and anticancer properties and ability to inhibit angiogenesis, Tranilast displays a number of potential therapeutic effects and the structural similarity between Tranilast and avenanthramides suggests that the compounds also share similar biological effects (Isaji *et al.* 1997; Isaji *et al.* 1998; Sur *et al.* 2008 and Lee-Manion *et al.* 2009).

Spiecker *et al.* (2002) investigated the effects of Tranilast on the transcription factor NF-κB and the induction of pro-inflammatory cytokines and adhesion factors. 100µg/ml Tranilast significantly reduced the TNF-α-stimulated expression of VCAM-1, ICAM-1 and E-selectin by 38, 32 and 32% in Human Umbilical Vein Endothelial Cells (HUVECs), respectively. Investigating the TNF-α-induced IL-6 secretion it was found that 100µg/ml Tranilast caused a reduction by 67%. Since these compounds are dependent on NF-κB-induction, and activation of this transcription factor is in turn regulated by phosphorylation and degradation of IκB

proteins, the authors progressed with examining the effect of Tranilast on TNF-α-induced IκB degradation. Results showed no effect of Tranilast on protein degradation independent of concentration. This coincided with the findings that Tranilast was unable to inhibit translocation of the NF-κB subunit RelA to from the cytoplasm to the nucleus, leading to the conclusion that the inhibitory effect of Tranilast on transcriptional activity of NF-κB occurs without affecting the DNA binding ability of NF- κB. Therefore, the transcriptional co-activator cAMP response element-binding protein binding protein (CREBBP, CBP) was examined. In HUVECs treated with 50µg/ml Tranilast, association between CBP and NF-κB was inhibited, furthermore, treatment with Tranilast resulted in a reduced expression of CBP, which might explain the inhibitory effect.

The *in vitro* antioxidant activity and antigenotoxic effects of Tranilast were investigated in a study done by Lee-Manion *et al.* (2009). Tranilast displayed no antioxidant activity in neither the DPPH or the FRAP assay, however a significant decrease in DNA damage was observed in stressed HT29 cells treated with Tranilast in the Comet assay, indicating the antigenotoxic properties of Tranilast. These findings indicate that although in the absence of antioxidant activity, an antigenotoxic, protective effect can be found.

4. Results from Experiments

4.1 Germination frequency

The different cultivars germination frequency is shown in Table 1, based on a mean value of two replicates. All of the cultivars showed the best germination in pH 6. Matilda germinated in pH 6 displayed the highest frequency expressed as percentage, with 87% compared to 85% and 80% for Circle and Betania, respectively. For the germination in pH 4.1, Circle displayed the highest percentage of 36%.

Table 1. The germination frequency of seeds from the three cultivars Betania, Circle and Matilda, steeped and germinated at pH 4.1 or pH 6. The value, represented as percentage, rely on mean value of two replicates.

Cultivar	Germination frequency (%)
Betania pH 4.1	17
Betania pH 6	80
Circle pH 4.1	36
Circle pH 6	85
Matilda pH 4.1	35
Matilda pH 6	87

4.2 Influence of drying on avenanthramide amounts

When comparing the raw and the 105°C dried seeds independent of cultivar, a significant decrease in avenanthramide amounts could be seen (Table 2a-b). The three major avenanthramides 2c, 2p and 2f decreased with 45, 40 and 52% respectively due to drying. The peaks that were not significant also showed this trend.

Table 2a. Avenanthramide amounts in raw grains and raw grains dried at 105°C, independent of cultivar. The values rely on mean value of 6 analyses.

AVA	Raw (nmol/g) Mean ± SD	Raw dried (nmol/g) Mean ± SD	P*
2c[1]	394 ± 54	216 ± 98	**
2p	365 ± 81	221 ± 86	*
2f	417 ± 148	202 ± 83	*

*Significance set to * p<0.05, ** p<0.01, *** p<0.001
1. See figure 4.

Table 2b. Avenanthramide amounts in raw grains and raw grains dried at 105°C, independent of cultivar. The values rely on mean value of 6 analyses.			
AVA	**Raw (Area/g) Mean ± SD**	**Raw dried (Area/g) Mean ± SD**	**P***
2pd[1]	362 ± 128	249 ± 236	ns
2fd	170 ± 34	95 ±46	**
Peak 2	70 ± 22	32 ±11	**
Peak 3	49 ±23	23 ±12	*
Peak 4	38 ±15	21 ±11	ns
Peak 7	81 ±21	47 ±16	**
Peak 8	139 ±47	66 ±22	**
Peak 9	40 ±18	30 ±20	ns
*Significance set to * p<0.05, ** p<0.01, *** p<0.001 1. See figure 4.			

4.3 Influence of pH and germination on avenanthramide amounts

Figure 4, visualises a typical chromatogram of extracts of the cultivar Matilda steeped and germinated in pH 4.1 solution. The peaks marked 2c, 2p, 2f, 2pd and 2fd represents known avenanthramides and the peaks marked with numbers represents compounds that are believed to be avenanthramides, but have yet to be identified.

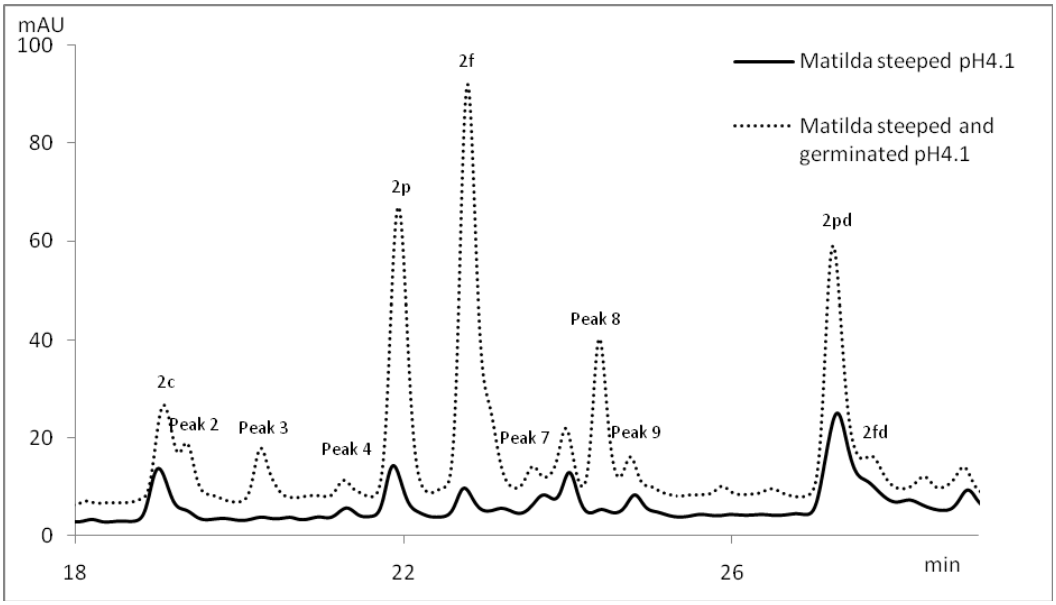


Figure 4. Typical chromatogram of ethanol extracts of the cultivar Matilda steeped in pH4.1 and steeped and germinated in pH4.1.

Tables 3a and 3b represents the avenanthramide amounts when comparing the different treatments independent of pH and cultivars. A slight increase in avenanthramide amounts can be seen during steeping compared to the raw dried samples, however this increase is not significant in the majority of the avenanthramides. A highly significant increase in amounts is visible when comparing raw dried grains and steeped and germinated grains, leading to the assumption that it is during the germination process that the highest enrichment of avenanthramides occur. This was true for all the avenanthramides except peak 7, where no significant difference could be seen. These trends were also visible when taking the different cultivars into account (Table 4a-b), although a slightly significant decrease of peak 7 during germination was seen in Circle. The effects of steeping and germination on the three major avenanthramides, independent of cultivar and pH, are additionally visualized in Figure 5. The major avenanthramides 2c, 2p and 2f have increased in amounts by 219, 197 and 358%, respectively, between the untreated and the germinated samples.

Table 3a. Avenanthramide amounts in raw grains, steeped grains and steeped and germinated grains, all dried at 105°C, independent of cultivar and pH. Mean values rely on 6, 12 and 24 analyses for raw, steeped and steeped and germinated samples respectively.

AVA	Raw dried (nmol/g) Mean ± SD	Steeped (nmol/g) Mean ± SD	P	Steeped and germinated (nmol/g) Mean ± SD	P*
2c	216 ± 98	275 ± 107	ns	688 ± 171	***
2p	221 ± 86	240 ± 120	ns	656 ± 242	***
2f	202 ± 83	288 ± 187	ns	924 ± 452	**

*Significances refer to values within a row, set to * p<0.05, ** p<0.01, *** p<0.001

Table 3b. Avenanthramide amounts in raw grains, steeped grains and steeped and germinated grains, all dried at 105°C, independent of cultivar and pH. Mean values rely on 6, 12 and 24 analyses for raw, steeped and steeped and germinated samples respectively.

AVA	Raw dried (Area/g) Mean ± SD	Steeped (Area/g) Mean ± SD	P	Steeped and germinated (Area/g) Mean ± SD	P*
2pd	249 ± 236	258 ± 113	*	652 ± 158	***
2fd	95 ±46	141 ± 53	ns	325 ± 159	**
Peak 2	32 ±11	40 ± 21	ns	146 ±62	***
Peak 3	23 ±12	28 ± 20	ns	134 ±69	***
Peak 4	21 ±11	34 ±16	*	137 ± 69	***
Peak 7	47 ±16	65 ± 19	ns	54 ± 24	ns
Peak 8	66 ±22	77 ±58	***	489 ±132	***
Peak 9	30 ±20	37 ± 19	ns	72 ± 23	**

*Significances refer to values within a row, set to * p<0.05, ** p<0.01, *** p<0.001

Table 4a. Avenanthramide amounts in steeped grains and steeped and germinated grains from the cultivars Betania, Circle and Matilda independent of pH. Mean values rely on 4 and 8 analyses for the steeped and the steeped and germinated samples respectively.

AVA	Betania Steeped (nmol/g) Mean ± SD	Betania Steeped and germinated (nmol/g) Mean ± SD	Betania P	Circle Steeped (nmol/g) Mean ± SD	Circle Steeped and germinated (nmol/g) Mean ± SD	Circle P	Matilda Steeped (nmol/g) Mean ± SD	Matilda Steeped and germinated (nmol/g) Mean ± SD	Matilda P*
2c	312 ± 148	791 ± 123	***	289 ± 75	733 ± 144	***	225 ± 95	539 ± 145	**
2p	308 ± 171	621 ± 135	**	265 ± 60	677 ± 156	**	148 ± 42	670 ± 384	*
2f	334 ± 214	719 ± 229	*	424 ± 101	1003 ± 288	**	105 ± 13	1049 ± 680	*

*Significances refer to values within a row, set to * p<0.05, ** p<0.01, *** p<0.001

Table 4b. Avenanthramide amounts in steeped grains and steeped and germinated grains from the cultivars Betania, Circle and Matilda independent of pH. Mean values rely on 4 and 8 analyses for the steeped and the steeped and germinated samples respectively.

AVA	Betania Steeped (Area/g) Mean ± SD	Betania Steeped and germinated (Area/g) Mean ± SD	Betania P	Circle Steeped (Area/g) Mean ± SD	Circle Steeped and germinated (Area/g) Mean ± SD	Circle P	Matilda Steeped (Area/g) Mean ± SD	Matilda Steeped and germinated (Area/g) Mean ± SD	Matilda P*
2pd	168 ± 107	590 ± 185	**	235 ±71	602 ±125	***	372 ± 42	763 ±104	***
2fd	124 ± 68	322 ±76	**	182 ± 47	500 ±92	***	117 ± 15	153± 24	*
Peak 2	53 ± 34	176 ±62	**	39 ± 9	161 ± 48	**	28 ± 4	100 ± 53	*
Peak 3	34 ± 24	119 ± 53	*	42 ± 9	192 ± 61	**	7 ±1	92 ± 53	*
Peak 4	49 ± 18	206 ±54	***	24 ± 5	143 ± 27	***	29 ±6	63 ±10	***
Peak 7	71 ± 29	52 ± 20	ns	60 ±15	39 ± 12	*	64 ± 11	71 ± 26	ns
Peak 8	106 ± 70	551 ± 113	***	105 ± 27	544 ±106	***	20 ±3	372 ± 98	***
Peak 9	39 ± 9	63 ± 16	*	16 ± 5	64 ± 21	**	57 ±9	90 ± 24	*

*Significances refer to values within a row, set to * p<0.05, ** p<0.01, *** p<0.001

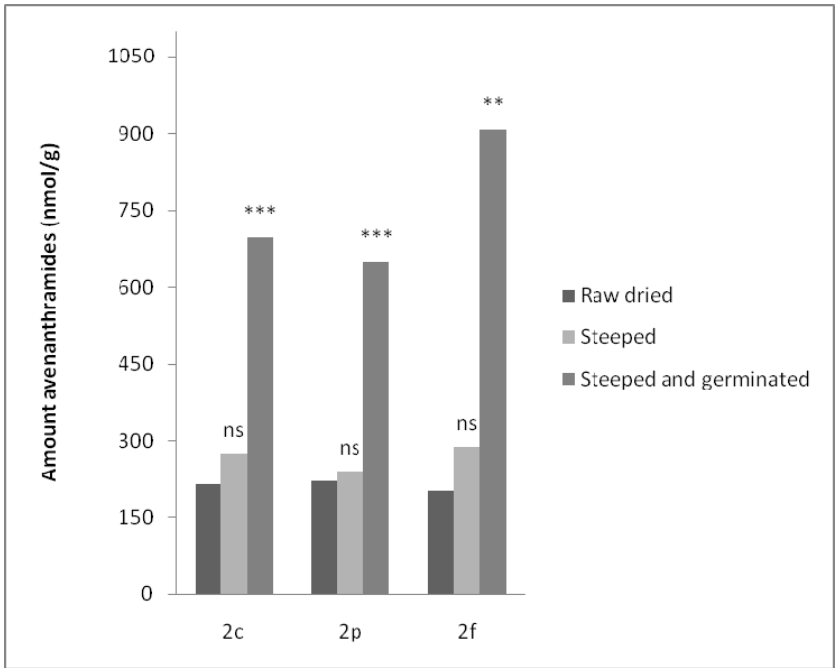


Figure 5. Amounts of the major avenanthramides 2c, 2p and 2f in ethanol extracts from untreated dried grains, steeped and steeped and germinated grains independendent of both cultivar and pH level. Significances are between the untreated dried and the steeped and steeped and germinated samples, respectively.

Table 5a. Amounts of avenanthramides in steeped and germinated grains in pH 4.1 or pH 6, independent of cultivar. Mean values rely on 12 analyses.

AVA	Steeped and germinated in pH 4.1 (nmol/g) Mean ± SD	Steeped and germinated in pH 6 (nmol/g) Mean ± SD	P*
2c	728 ± 151	647 ± 187	ns
2p	827 ± 194	485 ± 145	***
2f	1255 ± 393	593 ± 182	***

*Significance set to * p<0.05, ** p<0.01, *** p<0.001

When considering the effect of pH on avenanthramide enrichment, it was evident that germination in pH 4.1 renders the highest amounts of the avenanthramides 2p, 2f, peak 2, peak 3, peak 7 and peak 9 (Table 5a-b) with 71, 112, 77, 49, 70and 38% higher amounts respectively, compared to the pH 6 amounts. Avenanthramide 2c, 2pd and peak 8, although not significant, displayed the same trend. The result for the major avenanthramides was due mainly to the influence of Matilda and Betania, since no significance could be seen in Circle (data not shown). Avenanthramides 2fd and peak 4 displayed a higher enrichment in the pH 6 germination, however not significant.

Table 5b. Amounts of avenanthramides in steeped and germinated grains in pH 4.1 or pH 6, independent of cultivar. Mean values rely on 12 analyses.

AVA	Steeped and germinated in pH4.1 (Area/g) Mean ± SD	Steeped and germinated in pH6 (Area/g) Mean ± SD	P*
2pd	653 ± 167	650 ± 156	ns
2fd	312 ± 153	338 ± 171	ns
Peak 2	186 ± 51	105 ± 43	***
Peak 3	178 ± 57	91 ± 49	**
Peak 4	134 ± 66	141 ± 74	ns
Peak 7	68 ± 24	40 ± 11	**
Peak 8	538 ± 127	440 ± 123	ns
Peak 9	84 ± 26	61 ± 14	*

*Significance set to * p<0.05, ** p<0.01, *** p<0.001

The increase in avenanthramide amounts between steeping and germination of the cultivar Matilda in pH 4.1 solution is visualized in a chromatogram, with the highest avenanthramide enrichment observed in peaks 2p, 2f, 8 and 2pd (Figure 4). From Figure 6 it was clear that the cultivar responding the best to the treatment was Matilda, with the highest total amount of the major avenanthramides after germination.

4.4 Antioxidant activity of avenanthramides

The differences in antioxidant activity between the different treatments and cultivars are visible in Figure 7 and Tables 6a-d. In Figure 7, the antioxidant activity is presented in absorbance units, the higher the units, the higher the activity. The negative percentage values (Table 6a-d.) represent lower antioxidant activity and for example raw dried vs steeped represent the activity of the steeped samples compared to the raw dried. When comparing the raw and the raw dried seed extracts independent of cultivar and pH, a significant higher activity could be observed in the dried seeds. After the steeping process the antioxidant activity was significantly lower compared to the activity in the extracts from the raw dried seeds, while the germinated samples had significantly higher activity compared both to the raw dried and the steeped seeds, independent of cultivar and pH. When investigating the effect of pH on the antioxidant activity a distinctly higher activity was visible in the pH 6 germinated seed extracts compared to pH 4.1, a result that coincided in all three of the different cultivars. In the steeping process, however, no significant difference could be seen between pH 4.1 and pH 6.

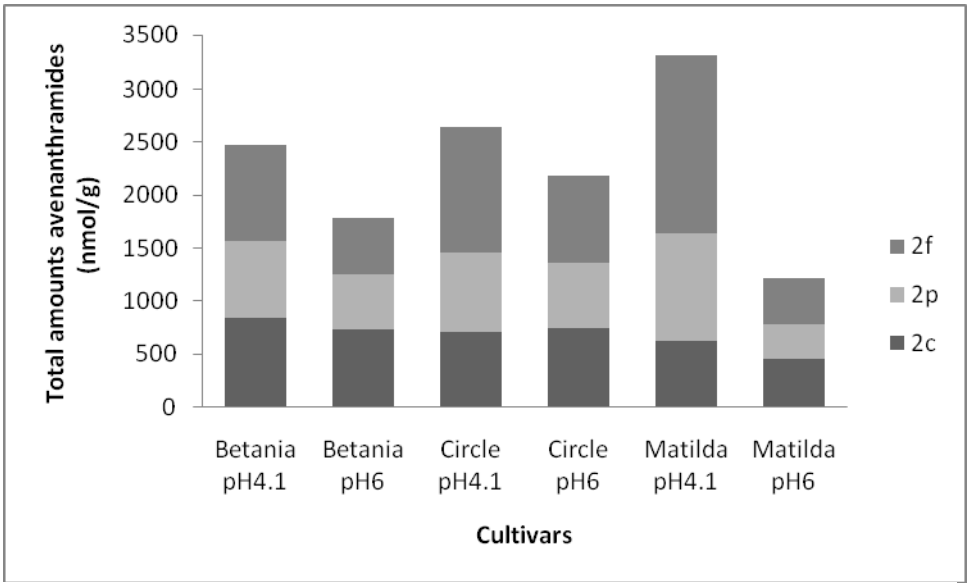


Figure 6. Total amounts of the major avenanthramides 2c, 2p and 2f in ethanol extracts of grains from the cultivars Betania, Circle and Matilda steeped and germinated at pH4.1 or pH6.

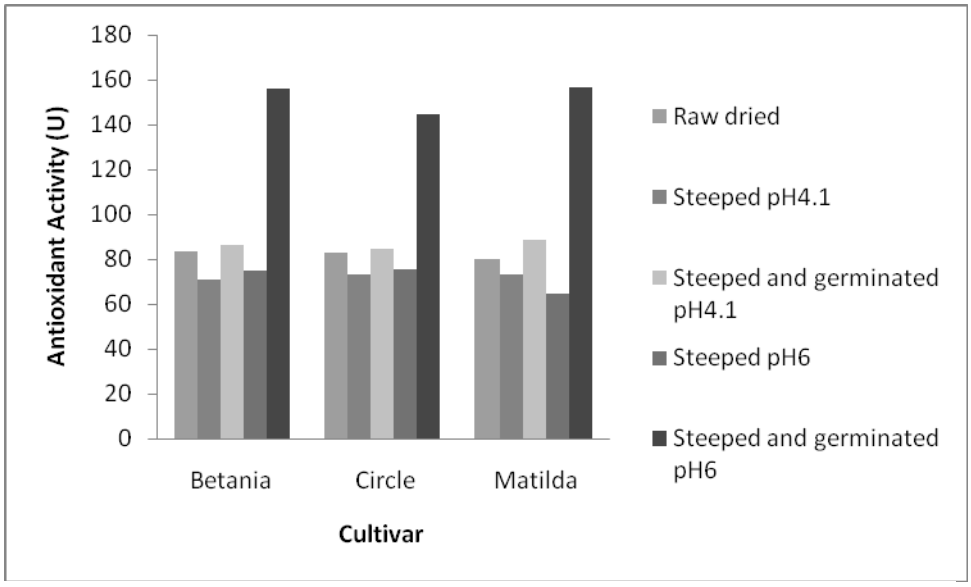


Figure 7. Antioxidant activity in ethanol extracts of grains from the cultivars Betania, Circle and Matilda steeped and germinated at pH4.1 or pH6.

Table 6a. The difference in Antioxidant activity as measured by DPPH, represented as percentage

Treatments analysed independent of cultivar and pH	Difference in activity (%)	P*
Raw dried vs. Steeped	-12	***
Raw dried vs. Steeped and germinated	33	*
Steeped vs. Steeped and germinated	41	***

*Significance set to * p<0.05, ** p<0.01, *** p<0.001

Table 6b. The difference in Antioxidant activity as measured by DPPH, represented as percentage

Treatments analysed independent of cultivar	Difference in activity (%)	P*
Steeped in pH 4.1 vs. pH 6	-0.01	ns
Steeped and germinated in pH 4.1 vs. pH 6	44	***
Steeped vs. Steeped and germinated in pH 4.1	18	***
Steeped vs. Steeped and germinated in pH 6	54	***

*Significance set to * p<0.05, ** p<0.01, *** p<0.001

Table 6c. The difference in Antioxidant activity as measured by DPPH, represented as percentage		
Treatments of the different cultivars analysed independent of pH	**Difference in activity (%)**	**P***
Betania - Steeped vs. Steeped and germinated	41	*
Circle - Steeped vs. Steeped and germinated	37	*
Matilda - Steeped vs. Steeped and germinated	45	*
*Significance set to * p<0.05, ** p<0.01, *** p<0.001		

Table 6d. The difference in Antioxidant activity as measured by DPPH, represented as percentage		
Treatments of the different cultivars	**Difference in activity (%)**	**P***
Betania - Steeped and germinated in pH 4.1 vs. pH 6	45	***
Circle - Steeped and germinated in pH 4.1 vs. pH 6	42	***
Matilda - Steeped and germinated in pH 4.1 vs. pH 6	44	***
*Significance set to * p<0.05, ** p<0.01, *** p<0.001		

4.5 Statistical analysis

Table 7 represents a Pearson correlation analysis between avenanthramide content and the germination frequency. Significance could be seen in all peaks except for avenanthramide 2c and 2fd. Only avenanthramide 2pd displayed a significant positive correlation to germination frequency, while a significant negative correlation was visible for avenanthramides 2p and 2f.

Table 7. Correlation between the amounts of the known avenanthramides 2c, 2p, 2f, 2pd and 2fd and germination frequency		
Avenanthramides	**Pearson correlation**	**Significance**
2c	-0.39	ns
2p	-0.73	**
2f	-0.69	*
2pd	0.74	**
2fd	0.25	ns
*Significances set as * p<0.05, ** p<0.01, *** p<0.001		

The correlation between the amounts of the known avenanthramides and antioxidant activity is shown in Table 8. Negative correlation was visible only for avenanthramide 2fd, while the remaining avenanthramides correlated to antioxidant activity in a positive manner, however no significance could be found for 2p and 2f. Furthermore, the germination frequency displayed a highly significant correlation to antioxidant activity ($p<0.001$), data not shown.

Table 8. Correlation between the amounts of the known avenanthramides 2c, 2p, 2f, 2pd and 2fd and antioxidant activity as measured by DPPH

Avenanthramides	Pearson correlation	P*
2c	0.45	**
2p	0.17	ns
2f	0.09	ns
2pd	0.88	***
2fd	-0.6	***

*Significances set as * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5. Discussion

5.1 Germination frequency

An interesting observation is that the germination of the seeds was more pronounced in the pH 6 germinated samples, with long visible shoots and roots, whereas the germination in the pH 4.1 samples had just begun with only a hint of roots visible, while it was the pH 4.1 germination that rendered the highest avenanthramide amounts. The negative correlation between the avenanthramide amounts and germination frequency coincided with the indications of higher frequency in the pH 6 germination samples, which showed lower avenanthramide enrichment. It is therefore possible that high amounts of certain avenanthramides affect the ability of the oat seed to germinate in a negative manner (Amarowicz *et al.* 2001 and Kong *et al.* 2008).

5.2 Influence of drying on avenanthramide amounts

When comparing raw and untreated dried seeds it was evident that the avenanthramide amounts decreases when drying at 105°C. This decrease obstructs direct comparison between the raw and the steeped and germinated samples. Therefore, comparisons have been done only between the samples that have undergone the drying process.

There are several explanations as to what may have happened to the avenanthramides during the drying process that could have caused the decrease. Drying may affect the compartmentalisation of the oat seed, which would lead to freeing of enzymes that may have degrading effects on the avenanthramides (Skoglund 2008b). The avenanthramides may act as antioxidants leading to consumption to a large extent. Two other possibilities are that the avenanthramides bind to structures in the oat groat which renders them impossible to extract or that the heat treatment leads to their destruction. The stability of the avenanthramides were investigated by Dimberg *et al.* (2001), showing that avenanthramides are quite stable to pH, UV-light and heat treatment, 2p and 2f were quite heat stable at pH levels ranging from 2 to 12. Treatment with neutral or alkali solutions together with heat treatment at 95-98°C lead to the most destruction, especially for 2c. Although the pH levels inside the oat groats are unknown, considering these results, the risk of destroying the

avenanthramides in the drying process at 105°C is relatively low. Naturally, more research is necessary to confirm or reject these explanations.

The decrease in avenanthramides due to drying is only visible in the untreated dried samples, but it is natural to assume that the drying has had similar effects on the steeped and germinated samples. For this study, the assumption has been that the decrease follows the same pattern in all the different samples, although for further experiments this needs to be investigated further.

5.3 Influence of pH and germination on avenanthramide amounts

Considering the insignificant difference in avenanthramide content between the untreated dried samples and the steeped samples, together with the increased amounts during germination compared with both dried and steeped samples, it is evident that the main increase in avenanthramide amounts occurs during the germination process. This matches the work of Bryngelsson *et al.* (2003) and Skoglund *et al.* (2008a), where germination had proven increasing effects on avenanthramides. The results from the present study showed that a higher enrichment of avenanthramides was obtainable using solutions with lower pH values, in this case pH 4.1 and the cultivar responding the most to the treatment was Matilda. The pH optimum for the avenanthramide transferase HHT is also of interest to obtain optimal enrichment of the avenanthramides.

One thing that needs to be addressed is that the pH 4.1 solution is a citrate-phosphate solution in tap water, while the pH 6 solution consists solely of tap water. The two solutions, therefore, differ in ion profile which may have affected the outcome of the experiment. Moreover, the levels 3 and 5 are not that far apart of the pH scale, it is therefore unclear how much of the enrichment effect is due to the low pH and not an effect from the citric acid itself.

In any case, lower pH levels mean lower enzyme activity, thus germination at lower pH levels should mean a desirable preservation of other compounds such as β-glucan, due to the inactivated β-glucanase (Skoglund 2008b). From a health perspective, preservation of beneficial compounds together with enrichment of avenanthramides is very interesting for

future applications. Naturally, extensive investigations regarding the profile of β-glucan and other compounds in the extracts are needed to support this theory.

5.4 Antioxidant activity of avenanthramides

The antioxidant activity is higher in the samples that have undergone the drying process, although it was shown that this treatment decreased the avenanthramide content. The higher antioxidant activity could therefore be due to other substances which display antioxidant properties, for instance Maillard reaction products that are formed through reactions between reducing sugars and amino acids, known to occur in dry environments at temperatures exceeding 50°C and slightly acidic pH (Capuano *et al.* 2008 and Rufián-Henares *et al.* 2009).

The results from the DPPH-radical scavenging system show that the antioxidant activity was lower in the steeped samples and the pH levels had no significant influence on the activity in this process. This absence of significance may be due to the fact that the steeping itself had no effect on the activity. During germination, however, the activity is strongly affected by pH levels, with the higher activities belonging to the pH 6 germination. A higher activity in the pH 6 germination is quite contrary to the enrichment of avenanthramides, which was considerably higher in the pH 4.1 germination. The correlation analysis performed indicated that there exists a significant positive correlation between antioxidant activity and the avenanthramides 2c and 2pd, both of which showed a pronounced enrichment in the pH 6 germination. Bratt *et al.* (2003) has proven the antioxidant properties of avenanthramides to decrease in the order of 2c > 2f > 2p and Xu *et al.* (2009) showed that the three major avenanthramides were significantly correlated to antioxidant activity, however the correlation between avenanthramides 2p and 2f and antioxidant activity was in this study found insignificant. The most likely explanation for the higher activity in the pH 6 germination samples is that this pH favours the enrichment of other antioxidants with exceedingly higher activities than the avenanthramides, overriding their antioxidant powers and obstructing the comparison between the pH 4.1 and the pH 6 samples. The pH 6 germination also had the highest germination frequency, and a correlation analysis confirmed that antioxidant activity correlates to germination frequency in a highly significant manner.

Additionally, the 1:2-dilution on the pH 6 samples needs to be addressed. No experiments concerning the linearity of the dilution curve was performed in this study, however previous findings have proven that dilutions have no effect on the outcome within the current boundaries, thus assuming that the dilution had no impact on the results.

5.5 Physiological aspects

The empirics in this study examine the effects of avenanthramides both *in vivo* and *in vitro*, displaying a great diversity in the levels that have proven physiological effects. In this study, levels as high as 0.27, 0.3 and 0.55 mg/g were obtained for the major avenanthramides 2c, 2p and 2f, respectively (data not shown). These amounts correspond to 270, 300 and 550 ppm, i.e. exceedingly higher levels than the levels found effective in the mouse trial performed by Sur *et al.* 2008. Although no *in vitro* or *in vivo* studies have been performed on these extracts, the levels of avenanthramides achieved in the studied germination process with low pH suggests that similar beneficial physiological effects may be obtained. *In vitro* studies of the extracts are a necessary next step to confirm this theory.

5.6 Potential applications

Potential avenanthramide applications may consist of avenanthramide enriched mixtures, a few isolated avenanthramides, synthetically produced avenanthramides or synthetically produced avenanthramide derivatives. In an enriched mixture, there is a possibility of synergistic effects between the avenanthramides and other compounds in the mix, while synthetically produced avenanthramide derivates may possess higher activities compared to their natural counterparts (Guo *et al.* 2008). Furthermore the mode of uptake of an application can vary. Avenanthramides can be added to food products or in tablet form for oral consumption, while lotions of different compositions are an option for topical administration. Since the bioavailability of avenanthramides have yet to be fully unravelled, it is not clear what the most effective means of administration is. Furthermore it is important to adjust the avenanthramide composition and mode of uptake after the desired effects of the application and its therapeutic field.

For treatment or prevention of coronary heart diseases (CHDs), the applications need to be taken up in the blood stream for the avenanthramides to exert their therapeutic effects,

since these diseases is characterized by inflammation in the blood vessels, among other conditions. Administration suited for this purpose is either oral or topical administration. Topical administration means that effects from the gastro-intestinal system is avoided, however the compounds have to be able to penetrate the skin in order to reach their destined goal. For treatment of dermatological disorders, a topical administered application needs to be able to target the malfunctioning layer of the skin and not penetrate beyond this layer. The composition of the vehicle that carries the avenanthramides and other beneficial compounds affect their penetrating abilities and may be modified to suit different purposes (Heuschkel *et al.* 2008, 2009).

Together with avenanthramides other compounds may be included to enhance the therapeutic effects of the application. Menthol has cooling effects on irritated skin, which in combination with the effects from avenanthramides poses as a good candidate for dermatological therapy, as proven by Pacifico *et al.* (2005). Emollient qualities of the vehicle are an additional sought after effect for the restoration of skin barrier function and hydration. For an application in the CHD field, β-glucan is of interest since its ability of decreasing the risks of these diseases has been reported (Wood *et al*, cited in Skoglund 2008b:16). An application containing both avenanthramides and β-glucan would thus comprise not only the positive effects originating from the avenanthramides, but also the beneficial effects belonging to β-glucan. Furthermore, the cholesterol-reducing effects of both β-glucan and avenanthramides and the glucose-insulin regulating abilities of β-glucan, gives this combination an additional nutritional value.

Using the cultivar Matilda that contains high amounts of β-glucan and which responded the best to the germination treatment, extracts with high levels of both avenanthramides and β-glucan can be obtained. This extract could be formulated as a lotion together with hydrating and soothing compounds for dermatological use. For treatment of CHDs, the extract can be formulated as a topical administered lotion or an orally administered product. Moreover, grains from Matilda subjected to this method of germination could be processed into different oat products from a nutritional point of view.

6. Conclusions

This study has shown that enrichment of avenanthramides through germination is affected by the pH levels in the solutions used and that the highest enrichment is achieved when germinating in low pH levels. The highest antioxidant activity was obtained when germinating in pH 6, although the avenanthramide effect on these high values is questionable. The avenanthramide amounts obtained during germination are comparable to the levels found to have physiological effects in other studies, suggesting that this method of avenanthramide enrichment is sufficient for obtaining extracts with therapeutic potential. Furthermore, germination of oat grains at low pH levels potentially lead to preservation of β-glucan. An oat extract containing oat material subjected to this method, would thus comprise not only the positive physiological effects caused by avenanthramides but also the beneficial effects originating from β-glucan. This extract could, in turn, be included in a variety of therapeutic applications for treatment of e.g. coronary heart diseases, dermatological disorders, allergies or in products of nutritional value.

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Appendix

Glossary

Adhesion factors – Molecules responsible for transient adhesions between cells (Silverthorn 2007, p. 69).

Angiogenesis – The development of new blood vessels (Silverthorn 2007, p. 503).

Chemokine – Molecules that attract cells to a site of infection or inflammation (Silverthorn 2007, p. 786).

Cyclin dependent kinases, CDKs – Catalytical proteins that activates when they associate with cyclins, forming cyclin/CDK complexes that are involved in cell cycle progression (Lodish & Berk 2004). The cyclin D/CDK complex is responsible for the phosphorylation of Rb leading to cell proliferation (Nie *et al.* 2006b).

Cytokines – Proteins that are released by one cell affecting another cell in various manners (Silverthorn 2007, p. 786).

Endothelium – The inner lining of cells in blood vessels (Silverthorn 2007, p. 502).

Epidermal barrier – The outermost layer of the skin functioning as a physical barrier against pathogens (Silverthorn 2007, p. 784).

E-selectin – See Adhesion factors

Exocytosis – The fusion of intracellular vesicles to the cell membrane, resulting in release of the content in the extracellular fluid (Silverthorn 2007, p. 150).

Histamine – Powerful substance released from the granules in e.g. Mast cells, affecting blood vessels and airways. Involved in inflammation and allergies (Silverthorn 2007, p. 786).

Interferone-γ, IFN-γ – See Cytokines

Interleukin-1α, IL-1α – See Cytokines

Interleukin-1β, IL-1β – See Cytokines

Interleukin-6, IL-6 – See Cytokines

Interleukin-8, IL-8 – See Cytokines

Intimal layer – A layer in the vascular wall, normally containing only a few vascular smooth muscle cells (Nie *et al.* 2006b).

Intracellular Adhesion factor-1, ICAM-1 – See Adhesion factors

Keratinocyte – Skin cells (Silverthorn 2007, p. 83).

Leukocytes – White blood cells, immune cells circulating in the blood, including lymphocytes, monocytes, neutrophils and mast cells (Silverthorn 2007, p. 538).

Low density lipoprotein, LDL – Lipoprotein associated with cholesterol. Elevated levels of this complex are found in blood plasma in patients suffering from coronary heart disease (Silverthorn 2007, p. 525).

Lymphocyte – The white blood cells responsible for antigen-specific responses in the immune system. The majority of these cells are found in lymphoid tissues and they produce cytokines, affecting both other immune cells and non-immune cells (Silverthorn 2007, p. 784 and 787).

Macrophage – An immune cell that engulfs and ingest particles like pathogens or dead cells (Silverthorn 2007, p. 782).

Mast cell – An immune cell residing in various tissues. Contains granules with histamine and cytokines, contributing to allergic and immune responses (Silverthorn 2007, p. 782).

Monocyte – Precursor cells of tissue macrophages (Silverthorn 2007, p. 783).

Monocyte-chemoattractant protein-1, MCP-1 – See Cytokines

Neutrophils – Immune cells that ingest and kill bacteria and foreign particles and release cytokines and other inflammatory mediators (Silverthorn 2007, p. 783).

NF-κB – A transcription factor responsible for regulating the transcription of compounds such as pro-inflammatory cytokines and adhesion factors (Kulms and Schwarz 2006 and Pasparakis 2009).

p21cip1 – A cyclin-dependent kinase inhibitory protein (CIP), inhibits CDKs and must be degraded before DNA replication can begin, i.e. inhibits cell proliferation (Lodish & Berk 2004).

p27kip1 - Another cyclin-dependent kinase inhibitory protein (CIP), inhibits CDKs and must be degraded before DNA replication can begin, i.e. inhibits cell proliferation (Lodish & Berk 2004).

p53 – A transcription factor responsible for inhibiting proliferation in cells with DNA damage and activating programmed cell death. A tumor suppressing protein (Lodish & Berk 2004).

PAMPs – Pathogen associated molecular patterns, structural patterns that are conserved among microorganisms rendering them to be recognised by the immune system (Kulms *et al.* 2006).

Phytoalexins – Antimicrobial compounds in plants, produced as a response to pathogens (González-Lamothe *et al.* 2009).

Proteasome – A cylindrical enzyme complex located in the cytoplasm of cells which degrades proteins (Kulms *et al.* 2006 and Silverthorn 2007, p.122).

Retinoblastoma protein, Rb – A protein that inihibits the cell cycle progression until the cell is ready to divide. It is in its phosphorylated form (pRb) is involved in the progression into the proliferating S-phase and loss of function in this protein is associated with cancer (Lodish & Berk 2004).

Synergy – The event where two compounds work together resulting in a greater effect than the sum of the effects from the two compounds (Silverthorn 2007, p. 231).

Transcription factor – A compound that has DNA-binding activities and can by binding to the DNA activate or inactivate one or more genes, regulating the production of the respective protein (Silverthorn 2007, p. 221).

Tumor necrosis factor-α, TNF-α – See Cytokines

Ubiquitylation – The process in which a protein gets marked with a protein called ubiquitin, destining it for proteasomal degradation (Kulms *et al.* 2006 and Silverthorn 2007, p. 122)

Vascular cell adhesionmolecule-1, VCAM-1 – See Adhesion factors

Vascular endothelial cells – See Human aortic endothelial cells

Vascular smooth muscle cells, VSMC – Smooth muscle cells of blood vessels (Silverthorn 2007, p. 502).

Vasodilation – Widening of the vessel diameter (Silverthorn 2007, p. 502).