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Avenanthramides as nutraceuticals, their physical-chemical-biological properties, and possibilities of their determination (Mini-review)

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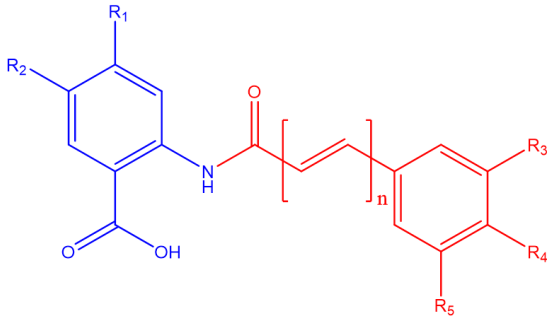
Abstract

Oats are considered a nutraceutical due to the content of biologically significant substances such as avenanthramides, from which are the most important avenanthramides A, B and C. These substances in terms of their physical and chemical properties meet Lipinski's criteria, which means that we can consider these substances as potentially pharmaceuticals. They could find their application due to their antioxidant, antitumor, anti-inflammatory, anti-obesity, vasodilating, and anti-diabetic effect, in the therapy of several diseases. Extraction of avenanthramides from oats for analytical purposes is most effectively done by using 70% methanol, while for commercial purposes, utilizing CO_2 in a supercritical state is preferred. Analyses of avenanthramides are mainly carried out by means of RP-HPLC with a UV/DAD detector, while currently there is a growing trend of determining avenanthramides by means of HPLC with MS for the unambiguous identification of avenanthramides and to reduce the LOQ.

Introduction

Oat (*Avena sativa*) is a cereal that is commonly grown mainly because of its rich starch content, which serves as a source of energy for heterotrophic organisms. In addition to the starch content, oats also contain other no less important molecules, such as proteins, fat, vitamin E, fiber, ß-glucans and polyphenols, while their content varies depending on the oat cultivar (Sterna *et al*. 2016). Polyphenols that are contained exclusively in oats include avenanthramides (AVNs) (Tripathi *et al*. 2018). AVNs are polyphenols consisting of a skeleton composed of anthranilic acid and cinnamic acid, which differ from each other mainly by the functional groups they have attached to each other. Around 40 of these derivatives have been identified (Collins 1989). However, the most represented derivates in oats are AVNs A, B and C (Figure 1) (Li *et al*. 2019). In this article, we will describe the physic-chemical properties of given AVNs, what their biological properties are, why oats produce them, in what quantity they are produced and how we can determine them.



Avenanthramide		n	Substituent				
Collins	Dimberg		R1	R2	R3	R4	R5
A	2p	1	H	OH	H	OH	H
B	2f	1	H	OH	OCH_3	OH	H
C	2c	1	H	OH	OH	OH	H
AA	5p	1	OH	OH	H	OH	H
G	4p	1	OH	H	H	OH	H
Y	3f	1	OCH_3	OH	OCH_3	OH	H
H	4f	1	OH	H	OCH_3	OH	H
O	$2P_d$	2	H	OH	H	OH	H

Figure 1. The structure of AVNs consists of a backbone shown in blue and red, with anthranilic acid depicted in blue and cinnamon acid in red. According to Dimberg's designations, such as 2p, 2c, and 2f, the number 2 indicates AVNs 5-hydroxyanthranilic acid, while the letters p, c, and f represent p-coumaric acid, caffeic acid, and ferulic acid, respectively.

Physicochemical properties of avenanthramides

The structure of AVNs relates to their physical-chemical-biological properties. The physicochemical properties of molecules can be used as a tool to search for potential new therapeutic drugs using the Lipinski criteria, which are as follows: molecular weight must be less than or equal to 500 g/mol, Log P must be less than or equal to 5, hydrogen bond donor number must be less than or equal to 5 and the number of hydrogen bond acceptors must be less than or equal to 10 (Lipinski 2004). As can be seen from Table 1, all the main AVNs found in oats meet the Lipinski criteria and are thus suitable candidates for pharmaceuticals.

Biological properties of avenanthramides

Since AVNs meet Lipinski's criteria for drugs, several studies have been carried out to reveal the biological properties of AVNs. It is described in the literature that AVNs have several effects on humans such as antioxidant effect, antitumor effect, anti-inflammatory effect, anti-obesity effect, vasodilating effect, and antidiabetic effect.

Table 1. Physicochemical properties of AVNs with the results of Lipinski's rules of five for these molecules. (Data obtained from https://pubchem.ncbi.nlm.nih.gov/)

	AVN A	AVN B	AVN C
Molecular weight (g/mol)	299.28	329.3	315.28
Log P (octanol/water)	2.91	2.5	2.1
Number of hydrogen bond donors	4	4	5
Number of hydrogen bond acceptors	5	6	6
Lipinski's rules of five	fulfills	fulfills	fulfills

Antioxidant effect of avenanthramides

Several studies (Peterson *et al*. 2002; Bratt *et al*. 2003; Fagerlund *et al*. 2009) confirms that AVNs have a direct antioxidant effect. Studies that confirm this claim also include a study by (Peterson *et al*. 2002). In this study, they investigated the antioxidant activity of AVNs A, B, C and found that all given AVNs exhibit antioxidant activity, with AVN C having the greatest antioxidant activity, AVN B having a slightly lower antioxidant activity, and AVN A having the least antioxidant activity. AVNs C and B at a lower concentration than TROLOX (reference antioxidant) reduced 50% of DPPH radical molecules.

In the study by Hou *et al*. (2021), however, the indirect effect of AVNs, which achieves an antioxidant effect, is also described. This effect consists in preventing the ubiquitination of the transcription factor NRF2, which is the main protein that, after binding to the element of the antioxidant response, ultimately causes the transcription of genes encoding antioxidant enzymes and proteins such as glutathione, catalase, and superoxide dismutase. As the study confirms, AVN C causes a significant increase in NRF2 protein (Figure 2).

It follows from the above that AVNs have potential in the prevention and treatment of diseases associated with oxidative stress, which include cancer, memory loss, depression, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease.

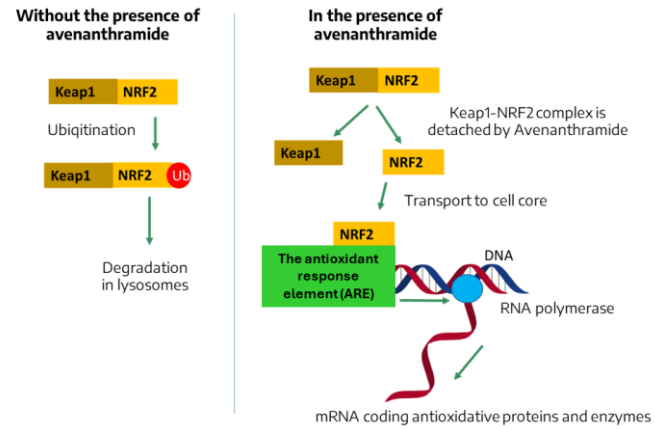


Figure 2. In the absence of AVNs, NRF2 is associated with Keap1, leading to the ubiquitination of this complex and subsequent degradation in lysosomes. However, in the presence of AVN, NRF2 is not bound to Keap1, allowing NRF2 translocation into the nucleus of the cell, where it binds to the antioxidant response element, resulting in the activation of RNA polymerase. This activation leads to the transcription of genes encoding antioxidant enzymes and proteins into mRNA. Subsequently, the mRNA undergoes post-transcriptional modifications (splicing, polyadenylation, and capping of exons) and is translocated to ribosomes for translation. Following translation and subsequent folding, the individual gains antioxidant enzymes and proteins, ultimately preventing damage to the body from reactive substances and thus preventing the development of diseases associated with oxidative stress.

Antitumor effect of avenanthramides

The anti-tumor effect of AVNs lies in their modulation of several signalling pathways in the human body associated with the cell cycle, angiogenesis, and apoptosis, as well as in the antioxidant effect of AVNs preventing mutations in DNA caused by oxidative substances (Figure 3).

Gately and Li (2004), in a study on the enzyme cyclooxygenase 2 (COX-2), reported that this enzyme is a key regulator of tumor angiogenesis. The activity of COX-2 mediates the increased production of vascular endothelial growth factor (VEGF) and eicosanoids (TXA2, PGI2, PGE2), which directly induce the growth of endothelial cells, migration, and blood vessel formation. For these reasons, COX-2 inhibition is a suitable inhibitor and a significant target for anti-angiogenic therapy. Lim and Kang (2020) provided evidence that AVNs can inhibit the activity of COX-2 and thus may be classified as molecules with anti-angiogenic effects and potentially used in the therapy of tumor progression.

Nie *et al*. (2006a), in an *in vivo* study, examined the inhibitory mechanism of the cell cycle by AVN C on A10 cell line, smooth muscle cells of rat embryonic aorta. Through flow cytometry, they found that exposure of cells to 80 µmol/L of AVN C halted the cell cycle in the G1 phase and reduced the number of cells in the S phase of the cell cycle. The modulation of the cell cycle by AVN C involves reducing the phosphorylation of retinoblastoma protein (pRb), as hyperphosphorylation of pRb is a typical feature of cell cycle progression from G1 to S phase. Additionally, they observed that AVN C reduced the expression of cyclin D1 and increased the expression of cyclin-dependent kinase inhibitor p21, while the exposure of cells to AVN C did not affect the expression of p27 protein. AVN C also increased the expression of p53, which is responsible for the increase in p21. Thus, by increasing the expression of p53, p21, and inhibiting the phosphorylation of pRb, AVN C can reduce the rate of cell proliferation.

Scarpa *et al*. (2018), in an *in vitro* study, examined the anti-tumor effect of AVNs on CaCo-2 and Hep3B tumor cells. They found that exposure of these cells to AVNs resulted in the activation of initiator caspase 8 and subsequent effector caspase 3 and downregulation of genes associated with cell survival hTERT, COX-2, and MDR1. Additionally, Hastings and Kenealey (2017), in an *in vitro* study, observed that AVNs, especially AVN C, reduced the viability of breast cancer cell line MDA-MB-231 by 25% at a concentration of 400 µmol/L. They attributed the decrease in cell viability to the activation of apoptosis, as they observed 97% positive staining for annexin V and 91% of cells showed positive caspase-3/7 activity. Caspases are proteases associated with apoptosis. Thus, if activated, they can safely eliminate the damaged cell. Therefore, from these findings, it can be concluded that AVNs can induce apoptosis in tumor cells.

Park *et al*. (2021), as well as Lee *et al*. (2011), in *in vitro* studies, investigated the effect of AVN C and dihydroavenanthramide D on the expression of matrix metalloproteinase MMP9. They found that AVNs successfully induced suppression of the

expression of this matrix metalloproteinase. Matrix metalloproteinases are key enzymes in the development of metastasis, indicating that AVNs may play a significant role in reducing the risk of metastasis development.

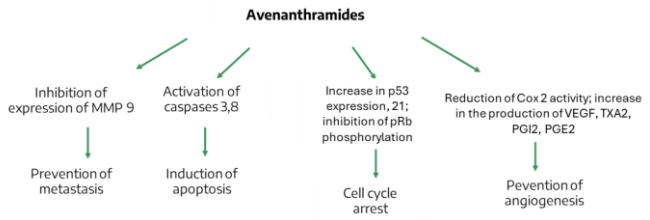


Figure 3. Overview of the effects of AVNs. Through modulation of specific signalling pathways, AVNs can prevent metastasis, induce apoptosis, halt the cell cycle, and inhibit angiogenesis.

Antidiabetic effect of avenanthramides

Zhouyao *et al*. (2022) recently published a study focusing on the inhibition of glucose transport proteins expressed in the small intestine as a strategy to reduce direct glucose absorption (Figure 4). Their findings suggest promising pharmacotherapeutic implications for diabetes management, potentially leading to substantial enhancements in the quality of life for individuals with diabetes mellitus.

In this experiment, they monitored whether AVNs cause inhibition of glucose transport proteins, which are mainly found in the small intestine, i.e. glucose transporter 2 and sodium-glucose transport protein 1. They found that the presence of AVNs reduces glucose absorption, while they did not note a significant difference in the inhibition of glucose transport proteins between AVNs C and B. From the above, it follows that oats are a promising nutraceutical that should be included among foods suitable for diabetics.

Anti-inflammatory effect of avenanthramides

AVNs and their anti-inflammatory effect were discussed in a study by Landberg *et al*. (2020). In the given study, it is stated that the essence of the anti-inflammatory effect of AVNs lies in the inhibition of lipoxygenase 2. In addition to this study, the issue of the anti-inflammatory effect of AVNs was also addressed by Lim and Kang (2020). In the given study, they found that AVN C exhibits an inhibitory effect against cyclooxygenase 2 (Figure 5). If the anti-inflammatory effect of AVNs is confirmed by further studies, AVNs could be used for the therapy and prevention of diseases such as colon cancer (Araújo *et al*. 2020) and against diseases related to chronic inflammation, such as depression and schizophrenia (Bando *et al*. 2017; Müller 2019), autism (Saha *et al*. 2015), poor memory, Alzheimer's disease (Giridharan *et al*. 2019), multiple sclerosis (Palumbo *et al*. 2012), impairment of function thyroid hormones (Li *et al*. 2013; Nam *et al*. 2018), asthma (Li *et al*. 2022), inflammatory bowel disease (McCartney *et al*. 1999), edema (Wilgus *et al*. 2000; Ayer *et al*. 2011), hypertension (Izhar *et al*. 2004), dermatitis (Liana *et al*. 2024), osteoporosis (Richards *et al*. 2006), carpal tunnel syndrome (Talmor *et al*. 2003), diabetes (Nasrallah *et al*. 2009).

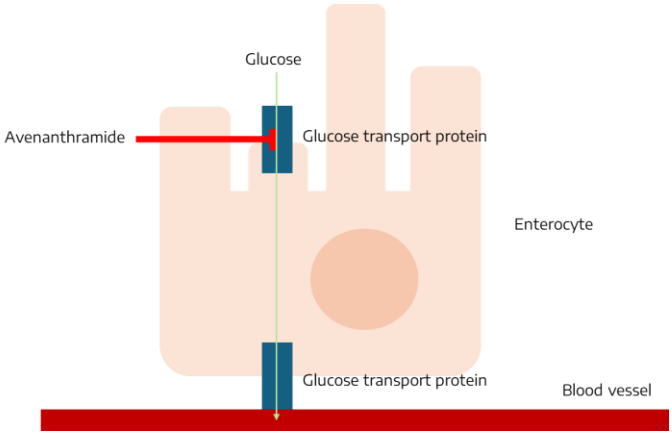


Figure 4. Ingested AVN blocks glucose transport proteins in enterocytes, thereby preventing the entry of glucose into the bloodstream.

Anti-obesity effect of avenanthramides

Obesity is a disease that has been affecting an increasing number of people in recent decades, representing a significant global issue with consequences that lead to the death of many individuals each year. This condition is closely associated with other chronic diseases, such as type 2 diabetes mellitus (Bhupathiraju and Hu 2016), liver steatosis (Fabbrini *et al*. 2010), cardiovascular diseases (Thomas *et al*. 2018; Powell-Wiley *et al*. 2021), reproductive disorders (Norman and Clark 1998), as well as gastrointestinal (Emerenziani *et al*. 2019) and cancerous diseases (Pati *et al*. 2023).

Current pharmacotherapy principles for obesity focus on two major approaches: suppressing appetite and preventing the absorption of nutrients from the small intestine into the bloodstream (Brandfon *et al*. 2023). Currently known anti-obesity drugs include tetrahydrolipstatin, known as orlistat (a lipase inhibitor that prevents the breakdown of triacylglycerols, thus inhibiting lipid absorption through the small intestine), which is available over the counter, and lorcaserin (a serotonin receptor 5-HT2C activator known to suppress appetite by activating the receptor) (Hogan *et al*. 1987; Malin and Kashyap 2016). However, AVNs can treat obesity in a different way.

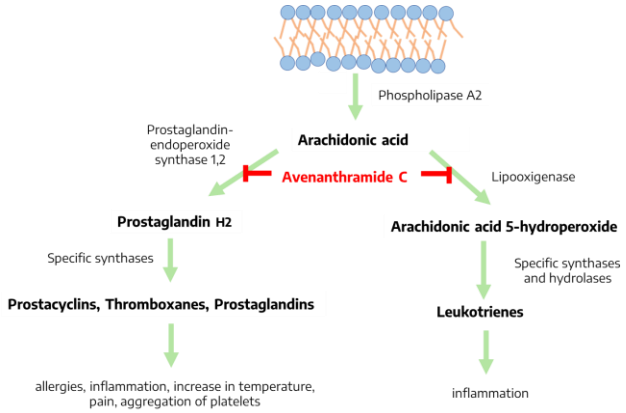


Figure 5. AVN C acts as an inhibitor of cyclooxygenases (prostaglandin-endoperoxide synthase) and lipoxygenase, ultimately reducing inflammation in the human body.

Zhang *et al*. (2020) found that the consumption of AVNs at doses of 100 and 300 mg/kg per day significantly reduced body weight gain by 9.6% to 14.8%. The study also reported that consumption of AVNs improved lipid profile, reduced serum glucose levels, increased the activity of superoxide dismutase, glutathione peroxidase, and catalase, and downregulated the expression of tumor necrosis factor, interleukin-6, and nuclear factor-kB genes. Furthermore, consumption of AVNs led to the regulation of intestinal flora and inhibition of harmful microbiota growth. Thus, the results of this study suggest that AVNs may alter body weight by alleviating oxidative stress, inflammation, and regulating intestinal microbiota. In addition to this effect, as mentioned earlier, AVNs block glucose absorption across inhibitions glucose transport proteins, which could also contribute to weight reduction in individuals. Therefore, in summary, AVNs represent a new approach in the pharmacotherapy of obesity.

Vasodilating effect of avenanthramides

The vasodilatory effect of AVNs was observed in a study by (Nie *et al*. 2006b). In that study, they investigated the impact of AVNs on smooth muscle cell proliferation and the induction of nitric oxide (NO) production. They discovered that AVN C significantly suppressed the serum-induced proliferation of smooth muscle cells. Specifically, at a concentration of 120 μM, AVN C inhibited over 50 % of smooth muscle cell proliferation. Furthermore, at a concentration of 120 μM, AVN C increased NO production threefold in smooth muscle cells and ninefold in human aortic endothelial cells. These increases were paralleled by up-regulation of endothelial NO synthase mRNA expression in both vascular smooth muscle cells and human aortic endothelial cells. The results of this study suggest that AVNs found in oats may help prevent atherosclerosis by inhibiting smooth muscle cell proliferation and increasing NO production (Figure 6).

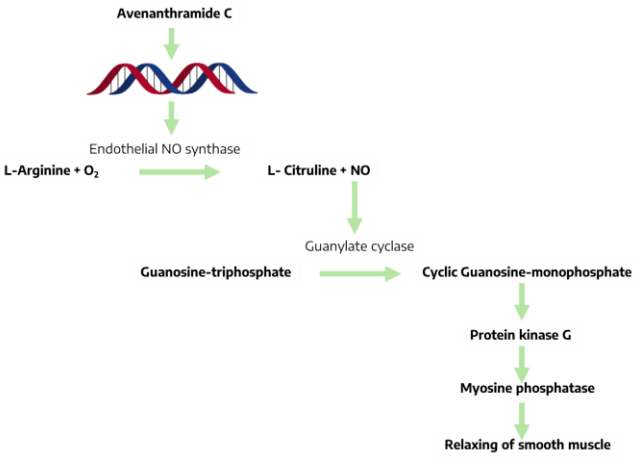


Figure 6. AVN C acts as an inhibitor of cyclooxygenases (prostaglandin-endoperoxide synthase) and lipoxygenase, ultimately reducing inflammation in the human body.

The role of avenanthramides in oats

AVNs in oats are biosynthesized as response to biotic stressors, such as pathogen attacks, particularly from fungi like Puccinia coronata, which causes crown rust in oats. This production of AVNs is part of the plant's systemic acquired

resistance (SAR) mechanism, which enhances its ability to fend off infections and stress (Izumi *et al*. 2009; Wise 2011). The biosynthesis of AVNs is catalyzed by specific enzymes, including hydroxyanthranilate N-hydroxycinnamoyltransferase (HHT), which facilitates the conjugation of anthranilic acid with hydroxycinnamic acids (Wise 2018; Li *et al*. 2019). This enzymatic pathway is upregulated in response to various elicitors, such as benzothiadiazole (BTH), which are known to stimulate plant defence responses (Wise 2011; Wise *et al*. 2016). The accumulation of AVNs in oat tissues not only acts as phytoalexins, compounds that inhibit pathogen growth, but also contributes to the overall resilience of the plant against environmental stresses (Izumi *et al*. 2009; Reich *et al*. 2020). Research has shown that the levels of AVNs can be influenced by several factors, including the developmental stage of the plant, environmental conditions, and agricultural practices such as fertilization and crop management (Shewry *et al*. 2008; Perrelli *et al*. 2018). For instance, the application of elicitors like methyl jasmonate and abscisic acid has been found to significantly enhance the production of AVNs during the germination of oats, indicating that these compounds are integral to the plant's adaptive responses (Thomas *et al*. 2018; Kim *et al*. 2021). Furthermore, the presence of AVNs in oats may also play a role in attracting beneficial organisms or deterring herbivores, thereby contributing to the plant's ecological fitness (Lee-Manion *et al*. 2009). The antioxidant properties of AVNs not only protect the plant from oxidative damage due to environmental stressors but may also enhance the nutritional value of oats, making them more appealing to herbivores and humans alike (Bratt *et al*. 2003; Perrelli *et al*. 2018).

Possibilities of extraction and determination of avenanthramides from oats

For commercial utilization of AVNs derived from their biological benefits for human health, as well as for selecting the most resilient oat cultivar, which also serves as the optimal nutraceutical, it is essential to extract AVNs from oats and determine the quantity of AVNs in oats.

Extraction of avenanthramides for analytical uses

Solvent extraction is one of the most common methods for isolating AVNs. AVNs present in the solid phase of the oat matrix can be extracted into a solvent. AVNs are polar molecules, because they have got polar functional groups such as OH, and NH which that will allow to interact with water through dipole dipoles and hydrogen bridges interactions. Therefore, the solvent that is suitable for the extraction of AVNs should be polar. Hence, ethanol and methanol are frequently used solvents due to their ability to dissolve phenolic compounds effectively (Fontes-Candia *et al*. 2018; Woolman and Liu 2022). Other factors that should be considered include the fact that phenolic compounds in plants are usually bound to cell wall components from where they can be released either by heating or pH adjustment (Multari *et al*. 2018; Xie *et al*. 2021). However, the stability of AVNs must be considered. In a study by (Dimberg *et al*. 2001), it is stated that an increase in temperature to 98°C together with an increase in pH leads to the degradation of AVN C. The optimal extraction conditions of AVNs from oats were investigated by (Maliarova *et al*. 2015). They observed the effect of extraction time of AVNs from oats in methanol, ethanol and isopropanol solvents and temperature on the yield of AVNs from oats. For optimization, they used the response surface methodology (RSM), through which they found that the best yield of AVNs is achieved when, among the given solvents, 70% methanol is used, in which oats will be extracted for 165 minutes at a temperature of 55°C. However, the disadvantage of this method is the high toxicity of solvent - methanol, which is undesirable from the point of view of preparative use, but fully sufficient for analytical purposes and the best choice among the listed solvents.

Solvent extraction may be improvement by ultrasound. Ultrasound extraction (UAE) has ability to enhance extraction efficiency by using ultrasonic waves to agitate the solvent, which increases the mass transfer of the target compounds from the solid matrix into the solvent (Streimikyte *et al*. 2022). Research by (Lee *et al*. 2019) and (Streimikyte *et al*. 2022) has shown that UAE can

significantly increase the total phenolic content in oat extracts, thereby enhancing the yield of AVNs.

Extraction of avenanthramides for commercial uses

For preparative purposes, a non-toxic extraction method of AVNs, such as using CO_2 in a supercritical state, would be preferable. Supercritical fluid extraction (SFE) technique offers advantages such as reduced solvent usage and the ability to extract thermally sensitive compounds without degradation (Fontes-Candia *et al*. 2018). SFE is commonly used to extraction phenolic compounds (Tyśkiewicz *et al*. 2018). This technique, known as eco-green extraction, is considered "solvent-free" and non-toxic, making it more acceptable for use. (Escobedo-Flores *et al*. 2018) published a study in which they focused on the optimization of the extraction of polyphenols from oats precisely through CO_2 in a supercritical state. In this study, they stated that the optimal conditions for extraction using CO_2 in a supercritical state are CO_2 under a pressure of 30-40 MPa, co-solvent ethanol (80% v/v), and an extraction temperature in the range of 50-60°C. This method enables the extraction of AVNs from oats in significant quantities suitable for commercial purposes, with minimal toxicity and environmental impact.

Determination of avenanthramides

Currently, AVNs are separated and subsequently determined mainly by means of high-performance liquid chromatography (HPLC). This method is preferred for its ability to analyze AVNs accurately and reliably in a variety of matrices. HPLC enables the separation of AVNs from other components of the matrix. It also has high sensitivity, which enables the identification and quantification of AVNs even at low concentrations. Therefore, this technique is currently preferred mainly for research purposes.

Reverse phase high performance liquid chromatography with a UV/DAD detector can be used for the determination of AVNs. Through this technique, it is now possible to effectively separate the 3 mains AVNs A, B, C found in oats. The advantages of this determination are that the sample does not need to be derivatized, as AVNs can be detected at wavelengths around 340 nm, availability and price of the device. One of the disadvantages of determining AVNs using this technique is the higher limit of quantification, which ranges from 100 to 400 ng/mL (Jastrebova *et al*. 2006). Another disadvantage of the RP-HPLC-UV/DAD determination lies in the indirect qualitative identification of the separated fractions of the oat extract by comparing the chromatogram of the retention times of the separated fractions of the sample with the chromatogram of the reference retention times of AVNs standards.

Therefore, there is currently a growing trend to determine AVNs by means of RP-HPLC with MS detection. Such a connection has several benefits. It will cause a reduction of the detection limit as well as obtaining qualitative and quantitative information about individual separation fractions. Through this technique, AVNs were determined, for example, in a study by (Xie *et al.* 2017), where they used high-performance liquid chromatography coupled with a triple quadrupole mass spectrometer for the quantitative-qualitative determination of the three main AVNs found in oats. This caused a reduction the LOQ to the level of 0.42-2.2 µg/L and to the correct qualitative determination of the AVN molecule found in a particular separated fraction.

New method for the determination of AVNs using ultra-performance liquid chromatography coupled with high-resolution high-field quadrupole orbitrap mass spectrometry (UHPLC-QE-HF-HRMS) is described in the study by (Feng *et al*. 2022). Using this method, they were able to effectively separate 3 mains AVNs (A, B, C) and reduce the quantification limit to 5.1-11.1 µg/L. The disadvantage of determining AVNs using UHPLC-MS mainly lies in the availability of the instrument.

Conclusion

AVNs are promising nutraceuticals with significant health benefits. Research has demonstrated that AVNs have antioxidative, anti-inflammatory, anti-cancer, anti-obesity, vasodilator, and anti-diabetic effects, mediated by various biochemical pathways, such as COX and lipooxygenase inhibition, cell cycle modulation, apoptosis induction and

regulation of glucose and lipid metabolism. AVNs significantly inhibit the activity of COX-2, suppress the expression of MMP9 and increase the production of NO, which contributes to their anti-inflammatory, anti-cancer and vasodilating effects. Inhibition of COX-2 and lipooxygenase points to their potential in reducing chronic inflammatory diseases. The antiobesity properties of AVNs, which reduce body weight gain and improve lipid profiles, offer a new approach to the treatment of obesity. Regulation of intestinal flora and reduction of oxidative stress and inflammation support the therapeutic potential of AVNs anti-obesity and related metabolic disorders. The antidiabetic effect of AVNs, mainly through the inhibition of glucose transport proteins, makes them suitable candidates for the management of diabetes mellitus. By reducing glucose absorption, AVNs contribute to better glycemic control, which is crucial for diabetics. The overall potential of AVNs as therapeutic agents is great. Future research should include clinical trials to validate these findings and optimize extraction methods for commercial use. AVNs represent an important field of research with the potential to improve human health and wellness. For future applications of AVNs as nutritional supplements or therapeutic agents, UAE using a methanol-water extraction solution appears to be optimal for analytical purposes. For preparative applications, SFE proves to be the most suitable method. When monitoring AVNs in drugs, nutritional supplements, or nutraceutical products, HPLC methods offer the highest accuracy and reliability.

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