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AVENANTHRAMIDE-C, THE MAIN OAT POLYPHENOL, HAS ANTIOXIDANT AND ANTIHEMOLYTIC PROPERTIES

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Abstract: Free radicals are molecules with unpaired electrons and very unstable, which have the ability to interact with other compounds to transform them into reactive species. One of these molecules are reactive oxygen species (ROS), which have been directly involved in the generation of oxidative stress, characterized by a high production of ROS and by different antioxidant mechanisms in the organism. ROS have the capacity to react with different biomolecules such as lipids, proteins and nucleic acids, being the polyunsaturated fatty acids (PUFA) of cell membranes one of the most important targets of ROS. For this reason, the use of compounds capable of capturing ROS or of positively modulating the activity of the cell's antioxidant mechanisms has become an attractive strategy. Among these compounds are polyphenols, flavonoids and carotenoids present in natural products of plant origin. In this work, the antioxidant effect and potential protective action of avenanthramide C, a polyphenolic compound present in oat grain, on erythrocyte hemolysis induced by exogenous hydrogen peroxide (H_2O_2) was analyzed. Avenanthramide C showed more effective antioxidant properties than curcumin (polyphenol from *Curcuma longa*) in addition to a greater protective effect erythrocyte hemolysis.
Keywords: antioxidant, avenanthramide C, oat, reactive oxygen species, ROS.

INTRODUCTION

Our body is exposed to a wide variety of reactive oxygen species (ROS) and nitrogen species (RNS) that can be generated from endogenous sources, related to oxygen metabolism and the various defense reactions of our immune system, or from exogenous sources such as tobacco, air pollution, UV radiation, ozone and certain drugs. Although exposure to ROS from exogenous sources is extremely high, exposure to endogenous sources is much more important and extensive, due to the fact that it is a process that occurs continuously in the cells of our organism throughout life (Rodriguez *et al*., 2001). Among the main exogenous sources of free radicals are:

a) Nitrogen oxides from tobacco smoke
b) Iron and copper salts which promote the generation of oxidizing radicals from peroxide and
c) The foods we ingest through our diet, especially those of vegetable origin, oxidize to a greater or lesser degree, generating different types of oxidants such as peroxides, aldehydes, oxidized fatty acids and transition metals.

The damage caused by ROS and RNS attack can lead to DNA damage, loss of enzyme function, increased cell permeability, disruption of cell signaling, and sometimes cell death by necrosis or apoptosis (Kim *et al.*, 2016). Because of this, it is common to relate the damage caused by various reactive species, to the physiology of various diseases such as cancer and diabetes (Schieber and Chandel, 2014). In addition, lipoperoxidation is indicated as responsible for the degeneration of neurons causing some brain pathologies such as Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis and cerebral Ischemia (Ahmad *et al*., 2017). There are numerous consequences linked to the damage produced by free radicals, even the hypothesis of the aging process is strengthened by focusing it on the mitochondria, by the continuous action of free radicals and the selective accumulation of oxidative damage in particularly sensitive molecules. Thus, free radicals have a direct action on proteins, unsaturated lipids, nucleic acids and carbohydrates (Sena and Chandel, 2012). Halliwell and Gutteridge (2006) defined "antioxidant" as any substance that, when present in low concentrations relative to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. For an

antioxidant (AH) to have antiradical activity it must fulfill a basic characteristic which is to generate a more stable and less damaging radical (RH) after racination with the radical species (R*) (Carocho *et al*., 2013). This reaction is based on a redox transition in which the donation of an electron or a hydrogen atom to the radical species is involved (Figure 1).

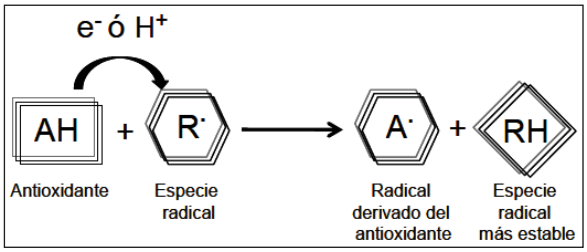


Figure 1. Mechanism of antioxidant molecules (Venereo Gutiérrez 2002).

As a result of the transfer, a radical derived from the antioxidant (A*) will be formed, which may be inert, stable or have a certain reactivity. The antioxidant defense system is constituted by a group of substances that, when present in low concentrations with respect to the oxidabel substrate, significantly delay or prevent its oxidation in a given microenvironment: plasma membrane, cytosol, nucleus or extracellular fluid. The following tables show the classification of antioxidants according to the microenvironment where they act or according to their origin.

Intracelular	Membrana	Extracelular
Superóxido dismutasa	Vitamina E	Ceruloplasmina
Catalasa	Betacarotenos	Transferinas
Peroxidasa	Ubiquinol-10	Lactoferinas
DT-deafarasa		Albúminas
GSH		Haptoglobinas
Proteínas que ligan metales		Vitamina C
Sistemas proteolíticos		Ácido úrico
Vitamina C		Vitamina E

Table 1. Classification of antioxidants according to their site of action

(Taken from Venéreo Gutiérrez, 2002)

Origen	Acción
1. Exógenos	
Vitamina E	- Neutraliza el oxígeno singlete - Captura radicales libres hidroxilo - Captura O_2 - Neutraliza peróxidos
Vitamina C	- Neutraliza el oxígeno singlete - Captura radicales libres de hidroxilo - Captura O_2 - Regenera la forma oxidada de la vitamina E
Betacarotenos	Neutraliza el oxígeno singlete
Flavonoides, Licopenos	
2. Endógenos	
Enzimáticos	Cofactor
Superóxido dismutasa (SOD)	Cobre, sodio, manganeso
Catalasa (CAT)	Hierro
Glutatión peroxidasa (GPx)	Selenio
3. No enzimáticos	
Glutatión	Barreras fisiológicas que enfrenta el oxígeno a su paso desde el aire hasta las células
Coenzima Q	
Ácido Tioctico	Transportadores de metales (transferrina y ceruloplasmina)

Table 2. Classification of antioxidants according to their origin

(Taken from Venéreo Gutiérrez, 2002)

Polyphenolic compounds constitute one of the most abundant and widely distributed natural antioxidant groups in the plant kingdom, with more than 8000 known polyphenolic structures. They are products of plant secondary metabolism and are found in roots, stems, trunks, leaves and fruits where they perform diverse functions ranging from protection against external influences such as UV radiation, animal bites, fungal and viral attacks to pollination mechanisms, odor, color and cold tolerance (Sindhi *et al*., 2018). We animals are not able to synthesize these types of compounds, so we rely primarily on the intake of plant-derived products to incorporate these substances into our organism. Regarding the metabolism of polyphenols, it is known that they are absorbed to varying de-

grees in the intestine in their native or modified form. These compounds are then metabolized into detectable products in the plasma, retain a large part of their antioxidant capacity and, finally, are excreted via the biliary or urinary tract. In general, polyphenol metabolites are rapidly eliminated from the plasma, which indicates that a daily consumption of plant products is necessary to maintain concentrations of these metabolites in the blood (Pisoschi and Pop, 2015). From a chemical point of view, polyphenols are characterized by containing an aromatic ring attached to two or more hydroxyl groups (phenol group). Their structure varies from simple molecules such as phenolic acids to complex structures such as condensed tannins. They are classified into four families (Tsao, 2010) according to the number of phenolic rings and the structural elements attached to these rings, these families are: flavonoids, phenolic acids, stilbenes and lignans (Figure 2).

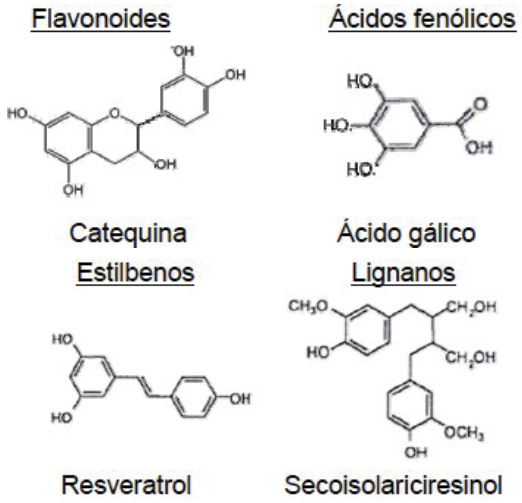


Figure 2. Classification of polyphenols according to their chemical structure.

The antioxidant activity of polyphenolic compounds is based on their ability to scavenge free radicals and chelate metals. Their chemical structure is ideal for reacting with free radicals to form a more stable and less reactive intermediate radical because the presence of aromatic rings and hydroxyl groups allows electron delocalization (Tsao, 2010). More than 5000 different structures of polyphenolic compounds present in fruits, vegetables and cereals as well as in foods and beverages obtained from plants such as olive oil, tea and red wine are known (Pareja *et al.*, 2016). These biologically active substances, have been studied in *in vivo* and *in vitro* models indicating that these compounds provide a benefit to the organism against various diseases such as cell injuries, inhibition of tumor growth, activation of hepatic detoxification systems and blocking of pathways that can cause carcinogenesis (Mercado *et al.*, 2013). Polyphenolic compounds in cereals include phenolic acids, flavonoids and in the case of oats, a unique group of amides known as Avenanthramides. More than 25 avenanthramide isoforms have been identified in oat extracts that vary in the substituents on the cinnamic acid and anthranilic acid rings, with avenanthramides A, B and C being the three most abundant forms with the difference of a hydrogen, hydroxyl or methoxy at carbon 3 of cinnamic acid. These three isoforms exhibit antioxidant, antiproliferative, antihistamine, and anti-inflammatory activity in coronary heart disease, colon cancer, and skin and skeletal muscle inflammation (Ortiz *et al.*, 2014). Of the three isoforms, the most abundant in oat grain is avenanthramide-C (Figure 3), which is why in this work its antioxidant and antihemolytic power was evaluated using the erythrocyte as a membrane model.

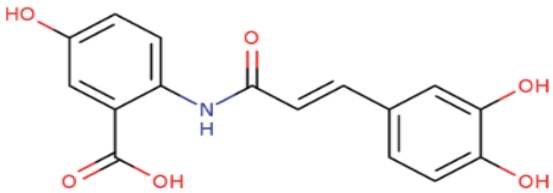


Figure 3. Chemical structure of avenanthramide-C

METHODOLOGY

BLOOD SAMPLES

A total of 12 heparinized peripheral blood samples were obtained from healthy volunteers in a lilac tube and centrifuged at 2500-3000 rpm for 10 minutes to separate plasma and leukocytes from erythrocytes. The erythrocyte pellet was washed twice with 5% phosphate buffered saline (PBS) pH 7.4 and centrifuged at 2500-3000rpm for 7 minutes at room temperature. Erythrocytes were resuspended in 5% PBS pH 7.4 to a final volume of 4ml (Pareja *et al.* 2016).

INCUBATION WITH THE PROBE

The stock solution of 2uM DCFH-DA dissolved in dimethyl sulfoxide was stored at 4°C in the dark (Vuorte *et al.* 2000) and 10ul was added to the erythrocyte solution and incubated under constant agitation at 37°C in the dark for 30 minutes. After incubation, the samples were washed by centrifugation at 1900 rpm for 5 minutes and resuspended in PBS to remove those that were not incorporated into the erythrocytes.

ANTIOXIDANT INCORPORATION

The antioxidant prepared in a stock solution at 1mM in methanol was incorporated at the required concentration into the erythrocyte suspensions and incubated in agitation for 15 min at 37°C.

FLUORESCENCE SPECTROSCOPY

Incubated cells were subjected to fluorescence analysis by spectrophotometer at the maximum fluorescence exhibited by DFC oxidized by ROS, said excitation wavelength was 485nm in an emission band from 500 to 600nm.

REACTIVE OXYGEN SPECIES REDUCING MATERIAL

Erythrocytes were stimulated for 2 minutes with hydrogen peroxide at different concentrations in order to determine the optimal stimulus concentration for the probe. The concentration of 100 m M was the optimal concentration because it showed the highest fluorescence.

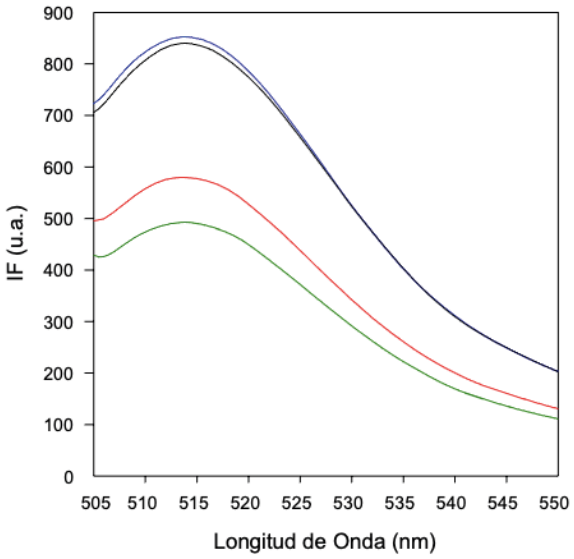
ACTIVITY ANTIHEMOLYTIC

It was evaluated from 250 m l of the erythrocyte suspension plus 100 m M of H_2O_2 with the addition of the antioxidant extract at the chosen concentrations. The samples kept in an incubator at 37°C for 3 hours and then centrifuged at 5000 rpm for 15min, the supernatants were taken and their absorbance was recorded at 540nm, as a positive control a sample of erythrocytes treated with hydrogen peroxide at 20mM was used. The percentage of hemolysis inhibition was calculated using the equation: % hemolysis inhibition= [(Ac-A)/Ac] x100. Where Ac is the absorbance of the negative control and A is the absorbance of the sample (Pareja *et al.* 2016).

RESULTS

The diacetate dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) probe is the most commonly used probe for the detection of cellular hydrogen peroxide oxidative stress, therefore, in order to determine the optimal concentration of exogenous hydrogen peroxide stimulus) capable of causing an increase in fluorescence intensity due to the formation of the compound dichlorofluorescein (DCF) in erythrocytes, a dose-response curve was performed using different concentrations of $H_2O_{(2)}$ (10, 50, 100 and 300m M) and incubating the cells for 30 minutes at 37ºC. Figure 4 shows that fluorescence is dependent on the stimulus concentration, with 100m M being the concentration of H_2O_2 at which a maxi-

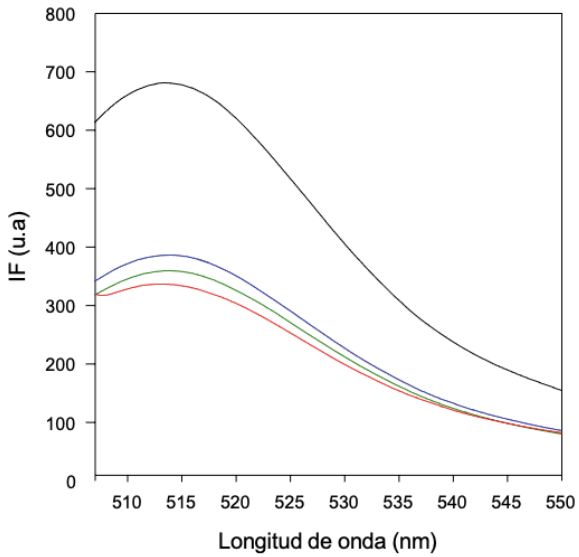
mum in fluorescence intensity is obtained, so this concentration was selected for subsequent experiments.



Fluorescence emission spectra of the dose- response curve of hydrogen peroxide (H_2O_2) performed on erythrocytes. The H_2O_2 concentrations tested were 10 (green line), 50 (red line), 100 (blue line) and 300 (black line)m M pre-labeled with 0.02 m M DCFH- DA probe and incubated for 30 min at 37°C. The spectra correspond to the average of 3 experiments performed independently subtracting the corresponding controls using an excitation wavelength of 485 nm. The concentration of erythrocytes used was 1.84 x 10^6 cells/mL.

Now, given that erythrocyte assays allow characterizing free radical-induced oxidative processes and assessing the nature and extent of protection exerted by antioxidant compounds, we proceeded to use curcumin, a polyphenol with a wide range of potential therapeutic effects due to its high antioxidant power and its ability to bind to different molecules such as glutathione and lipoxygenase, among others (Pareja *et al.*, 2016). The conversion of the non-fluorescent $DCFH_2$-DA compound to the fluorescent DCF compound occurs in several steps: $DCFH_2$-DA is transported across the cell membrane and deacetylated by cellular esterases forming the non-fluorescent compound $DCFH_2$. This compound remains inside the cells. Subsequently, $DCFH_2$ is converted to DCF through the action of intracellular oxidants such as H_2O_2, HO^*, ROO^*. Curcumin combats the intracellular generation of ROS (H_2O_2, HO^*, ROO^*) leading to a reduction in DCF levels and therefore to a decrease in the fluorescence intensity of the probe.

Figure 5 shows how the decrease in the fluorescence intensity of the DCF compound is directly proportional to the increase in the concentration of curcumin present in the erythrocytes, being the concentration of 20m M, with which significant reduction in fluorescence intensity is obtained.



Analysis of the concentration of curcumin capable of inhibiting hydrogen peroxide produced in erythrocytes. Erythrocytes at a concentration of 1.84 x 10^6 cells/m L were incubated with 0.02m M DCFH-DA probe at 37°C for 30 min at 300rpm and subsequently stimulated with 100m M H_2O_2 in the absence (black line) and presence of 5 (blue line), 10 (green line) and 20 (red line)m M curcumin. The graph shows the average of three independent experiments using a l_{exc} of 485 nm.

When comparing the antioxidant power of avenanthramide C using the same concentrations as in the previous experiment, it can be observed that, from the lowest concentration (5m M) used, a drastic reduction in the fluorescence intensity is obtained as a consequence of the H_2O_2 sequestering capacity Figure 6), which demonstrates that Avenanthramide C not only has greater antioxidant power than curcumin (approximately 3 times more), but also that the polyphenol has the ability to cross the erythrocyte cell membrane and attack with high scavenging power the hydrogen peroxide in the intracellular polar environment.

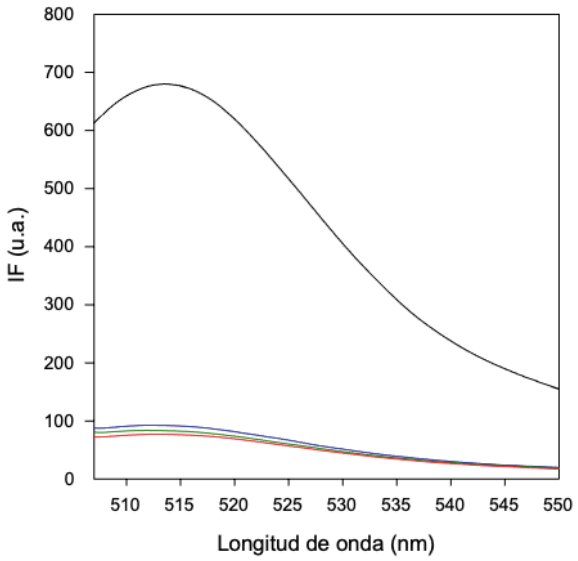


Figure 6. Evaluation of the antioxidant power of Avenanthramide C in erythrocytes. Erythrocytes at a concentration of 1.84 x 10^6 cells/m L were incubated with 0.02m M of DCFH-DA probe at 37°C for 30min at 300rpm and subsequently stimulated with 100m M H_2O_2 absence (black line) and presence of 5 (blue line), 10 (green line) and 20 (red line)m M of Avenanthramide C. Spectra from three independent experiments were recorded and averaged using al $_{exc}$ of 485nm.

Once the antioxidant power of Avenanthramide C was analyzed in this membrane model, we proceeded to evaluate its hemolytic inhibition capacity, since oxidizing agents at high concentrations cause the rupture of the erythrocyte membrane to the point of triggering cell lysis and originating the release of hemoglobin (Pandey and Rizvi, 2011). When H_2O_2 was added to the erythrocyte suspension under the tested test conditions, a high degree of hemolysis (100%) was observed. Curcumin and Avenanthramide C varied in their ability to limit such behavior. The percentage of hemolysis is lower in the case of Avenanthramide C for all the concentrations tested, obtaining at the maximum concentration used, a protection close to 99%. While the hemolysis obtained in the presence of curcumin at the same concentration was around 4%. These results demonstrate a slightly greater protective effect of Avenanthramide C on curcumin against hemolysis induced by hydrogen peroxide.

DISCUSSION

The components of the cell membrane that are responsible for its stability, such as phospholipids, cholesterol and transmembrane proteins, are affected under conditions of oxidative stress. The alteration of the erythrocyte structure derives from the loss of membrane integrity and can originate various disorders at the cellular and organism level, favoring the appearance of numerous diseases such as Diabetes mellitus, Cancer, Alzheimer, among others (Schieber and Chandel, 2014;Kim *et al*., 2006). For this reason, it is important to adopt strategies that enhance the antioxidant capacity of our body. Thus, the consumption of plant products such as fruits, vegetables, whole grains and legumes, capture ROS and/or positively modulate the activity of cellular antioxidant systems and biotransformation of xenobiotics (Kasote *et al*., 2015). Although the *in vitro* antioxidant activity of Avenantramides has been extensively studied and found to be 10 to 30 times more effective than other phenolic antioxidants present in oats, such as vanillin and caffeic acid, no study has investigated the antioxidant capacity of Avenantra-

mide C associated with the scavenging power of different types of free radicals or even the analysis of its protective activity against ROS--induced oxidative stress. The results obtained in this work show that Avenanthramide C is three times more effective as an antioxidant than Curcumin demonstrating the capacity of the polyphenol to cross the erythrocyte cell membrane and to scavenge hydrogen in the intracellular environment. As already noted, H_2O_2 is not a free radical, however, it is a toxic oxidant for cells, as it can injure DNA, cause membrane rupture or release calcium ions by activating calcium ion-dependent proteolytic enzymes (Pisoschi and Pop, 2015). Recent studies show that the antioxidant power of polyphenolic compounds such as Curcumin and Avenanthramide C depends on the number and position of OHs in the phenolic groups (Barzegar *et al*., 2011). Curcumin has 2 omethoxyphenolic groups and it is thought that the subtraction of the H atom from these groups are responsible for the remarkable antioxidant activity of Curcumin.

Although it has also been reported that the methylene group present in its structure could be the one that dissociates the hydrogen atom. So in an oxide-reduction reaction, curcumin participates in the reaction by either "transferring electrons" or "donating protons" to the free radical. Avenanthramide C has 4 OH groups in its structure, which increases its antioxidant activity. Moreover, recent studies with other polyphenols such as quercetin have suggested that the presence of the C=O group also enhances the antiradical activity and Avenanthramide C shows 2 C=O groups in its structure, which favors free radical scavenging. Now, the evaluation of erythrocyte membrane stability is a cellular model that has been widely used as an indicator of oxidative damage (Pandey and Rizvi, 2011). When the erythrocyte membrane loses its integrity it is more susceptible to hemolysis, for this reason, hemoglobin is released to the medium after centrifugation, so it can be used as an indicator of erythrocyte damage. The mechanism of erythrocyte hemolysis induced by the pro-oxidant agent H_2O_2, occurs due to the degradation of the heme group, resulting in the release of catalytically active Fe ions that enhance the production of free radicals, membrane lipoperoxidation and the generation of hydroxyl radical (Pareja *et al*., 2016). In the present study, the antioxidant and protective effect of Avenanthramide C on hydrogen peroxide-induced erythrocyte hemolysis was evaluated. The results suggest that Avenantramide C has a greater protective effect on erythrocyte hemolysis than Curcumin, evidenced by the reduction in the percentage of hemolysis in a dose-dependent manner. These results allow suggesting Avenantramide C as a possible drug for the treatment of some pathologies in which oxidative stress plays a relevant role.

CONCLUSION

Avenanthramide C shows greater antioxidant power and protective effect on hemolysis than curcumin in the erythrocyte model used.

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