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Antioxidant activity of kafirins and procyanidins of sorghum against the superoxide anion radical
Actividad antioxidante de kafirinas y procianidinas de sorgo contra el radical anión superóxido

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Abstract: the objective of this research was to isolate, characterize, and determine the effects of kafirins and procyanidins of brown sorghum, against different concentrations of superoxide anion radical ($O_2^{\bullet -}$) chemically generated in an aprotic polar medium. Likewise (-)-epicatechin, which represents the most abundant monomer of sorghum procyanidins was included as a reference system. Studies in this context are scarce and are considered relevant due to the abundance of such compounds and their potential application as drugs or functional ingredients in foods. By the effect of the hydrophobicity of the kafirins, these were better inhibitors of $O_2^{\bullet -}$ in the aprotic polar medium (DMSO) in which they were analyzed, compared to procyanidins and (-)-epicatechin that have a hydrophilic character. This result is important since procyanidins are considered one of the bioactive compounds with the highest antioxidant ability against a considerable number of free radicals.
Keywords: Sorghum; kafirins; procyanidins; superoxide anion radical; antioxidant activity

Resumen: el objetivo de esta investigación fue aislar, caracterizar y determinar los efectos de kafirinas y procianidinas de sorgo café, contra diferentes concentraciones del radical anión superóxido ($O_2^{\bullet -}$) generado químicamente en un medio polar aprótico. Asimismo, se incluyó como sistema de referencia la (-)-epicatequina, que representa el monómero más abundante de las procianidinas del sorgo. Estudios en este contexto son escasos y se consideran relevantes debido a la abundancia de tales compuestos y su aplicación potencial como fármacos o ingredientes funcionales en alimentos. Por efecto de la hidrofobicidad de las kafirinas, estas fueron mejores inhibidores del $O_2^{\bullet -}$ en el medio polar aprótico (DMSO) en el que se analizaron, comparado con las procianidinas y la (-)-epicatequina que tienen un carácter hidrofílico. Este resultado es importante ya que las procianidinas son consideradas uno de los compuestos bioactivos con la más alta capacidad antioxidante contra un número considerable de radicales libres.
Palabras clave: Sorgo; kafirinas; procianidinas; radical anión superóxido; actividad antioxidante

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1. Introduction

Recently, interest in antioxidants from food protein sources has increased because these are not only safe and natural but also multifunctional due to their surface amphiphilicity and amino acid profile (Zamora, 2007, Neha *et al*., 2019, Pinto *et al*., 2020). Cereals are a source of proteins and peptide antioxidants (Ofosu *et al*., 2021) and sorghum deserves attention as is the fifth leading cereal crop worldwide, is environmental-friendly, and biologically efficient. Sorghum grain has 3.5-18% proteins, classified by their solubility in kafirins (prolamins), glutelins, albumins, and globulins (Balandrán-Quintana *et al*., 2019; Xu *et al*., 2019). Kafirins represent 70–80% of the total protein and are subclassified by molecular weight in α (25–23 kDa, 80% of the total); β (16, 18, and 20 kDa, 8-13%); γ (20-28 kDa, 9-21%), and δ (13 kDa, present in very low amounts). Kafirins have a significant influence on sorghum protein quality and digestibility (Badigannavar *et al*., 2016; Li *et al*., 2018; Castro-Jácome *et al*., 2020) but also possess antioxidant properties.

Agrawal *et al*. (2017) isolated two peptides from a kafirin hydrolysate which demonstrated high antioxidant capacity through scavenging of 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radicals, besides promoting metal chelation. Xu *et al*. (2019) reported a fraction of 5-10 kDa, upon kafirin hydrolysis, with high antioxidant capacity and a good inhibition effect against lipid oxidation. Castro-Jácome *et al*. (2020) predicted, through bioinformatic analysis, that bioactive peptides from sorghum kafirins hydrolysate could have, in addition to antioxidant activity, other biological benefits. Two peptide extracts, isolated from kafirin hydrolysis, were reported to reduce the UV damage to human skin cells by attenuating the depletion of the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as by maintaining or increasing the activity of catalase (CAT), all of them antioxidant enzymes (Castro-Jácome *et al*., 2020). Unhydrolyzed kafirins have also been tested for their therapeutical potential. Ortiz Cruz *et al*. (2015) measured the antioxidant capacity of extracts of kafirins by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox-Equivalent Antioxidant Capacity (TEAC) methods and reported significant increases compared to that of sorghum flour. Sullivan *et al*. (2018) found a correlation between anti-inflammatory properties and reduced production of intracellular reactive oxygen species (ROS) after administration of kafirins to THP-1 human macrophages.

As far as we know, there are no more reports on the anti-radical activity of unhydrolyzed kafirins. In the study of Sullivan *et al*. (2018) the method for detecting intracellular ROS production is an indicator of hydrogen peroxide (H_2O_2), peroxyl, and hydroxyl radicals, according to the authors, but not of superoxide anion radical ($O_2^{\bullet-}$). It should be noted that $O_2^{\bullet-}$ is generated by enzymatic and non-enzymatic reactions under physiological and pathophysiological conditions, represents the first and most abundant product of molecular oxygen reduction and facilitates the propagation of the chain of lipid oxidation reactions, with similar damage to proteins and nucleic acids (Zamora, 2007; Mendoza-Wilson *et al*., 2020). For these reasons, is of outstanding interest to understand its behavior upon application of antioxidant proteins like kafirins.

On the other hand, sorghum is characterized as the cereal with the highest content of phenolic compounds, with phenolic acids, 3-deoxianthocyanidins, and particularly procyanidins, as the most abundant (Chiquito-Almanza *et al*., 2011). Due to the high antioxidant capacity of procyanidins, their activity against various free radicals has been evaluated, among these ABTS, DPPH, and also the $O_2^{\bullet-}$ enzymatically produced in polar protic media, such as buffer system and hydroalcoholic solutions (Hatano *et al*., 1989; Saint-Cricq *et al.,* 1999; Carmelo-Luna *et al*., 2020). However, the highest activity of $O_2^{\bullet-}$ can occur at the membrane interface, where reactions in polar aprotic environments could trigger. It should be mentioned that under these conditions, $O_2^{\bullet-}$ has a longer lifetime and allows the reaction to be followed. Some experimental and computational studies have reported that in polar aprotic solvents, such as dimethyl sulfoxide (DMSO) and acetonitrile, superoxide behaves like a strong base and reacts with flavonoids through mechanisms including proton abstraction or sequential proton-loss electron-transfer (Taubert *et al*., 2003; Mishra et al., 2004; Mendoza-Wilson et al., 2020). On this basis, the objective of the present research was to isolate, characterize and determine the effects of kafirins and procyanidins of brown sorghum, against different concentrations of superoxide anion radical ($O_2^{\bullet-}$) chemically generated in an aprotic polar medium. Likewise (-)-epicatechin, which represents the most abundant monomer of sorghum procyanidins was included as a reference system. A comparative study between these bioactive compounds of sorghum, under the same conditions, is considered relevant due to the abundance of such compounds and their potential application as drugs or functional ingredients in foods.

2. Methods, techniques, and instruments

2.1. Sorghum flour and bran

A variety of brown sorghum (SXR-19C) was used, produced in México, and provided by a feed supplier. A portion of clean grain was milled and sifted (0.5 mm) to obtain flour (CHRISTY & NORRIS, Pulverizers and grinders, Model 131345/1, UK). The other portion was decorticated for 1.5 min and passed through mesh No. 40 to obtain bran. Flour and bran were stored (–20 °C). Proximal characterization of flour was performed by AOAC (2005) methods for moisture (method 934.01), protein (method 960.52), fat (method 920.39), fiber (Methods 985.29, 991.43, 2001.03, and 2002.02), and ashes (method 945.05).

2.2. Total phenolic compounds extraction and determination

For phenolic extraction, 1 g of sorghum flour or bran was mixed with 10 mL of acetone/water/acetic acid (7ml:2.95ml:0.05ml) solution, which was vortexed for 30 s and then subjected to an ultrasound bath (37 °C, 10 min). The mix was standing for 25 min at 25 °C in the dark and centrifuged for 15 min at 3,500 rpm at room temperature in an Allegra X-30R centrifuge (Beckman Coulter Inc., Brea, CA). Eight ml of the supernatant were evaporated to dryness in a rotary evaporator at 37±2 °C. Finally, the sample was reconstituted with 6 ml of HPLC water and stored at -20 °C until use. The methodology of Gu *et al*. (2002) and Xu (2007), was used for phenolic determination with some modifications. Briefly, 50 µl of the reconstituted sample, 3 ml of HPLC water, and 250 µl of 1N Folin-Ciocalteu were added, and the sample was mixed manually and kept in darkness for 5 min. Subsequently, 750 µL of 20% Na_2CO_3 and 950 µL of HPLC water were added and kept at room temperature, for 40 min, in darkness. The samples were read at 765 nm in a spectrophotometer UV-VIS Cary 50 (Varian Inc., Palo Alto, CA). A gallic acid standard curve was used for phenol determination (r^2= 0.9918).

2.3. Kafirins fraction extraction and characterization

Kafirins fraction was obtained according to Espinosa-Ramírez and Serna-Saldívar (2016), with some modifications. One hundred g of sorghum flour was suspended in 900 ml of 70% ethanol and 0.3 g of sodium metabisulfite. The suspension was maintained for 1 h at 65 °C with partial stirring every 15 min, after which it was centrifuged at 3,300×g, 25 °C, for 15 min. The supernatant was separated and diluted to 60% with HPLC water and centrifuged at 1,900×g, 0 °C, for 30 min to eliminate lipids. The supernatant was diluted to 40% with HPLC water and kept at -20 °C for 24 h to precipitate proteins, and centrifuged at 3,300×g, 25 °C, for 15 min to recover the protein pellet. The supernatant was discarded and the precipitated was dried at room temperature for 24 h and then stored at -20 °C for further experiments. To determine the type of the extracted kafirins (α, β, γ, δ), the SDS-PAGE methodology was used. Kafirin sample was dissolved in 70% ethanol and heated for 15 min at 65 °C. Polyacrylamide gels were 15% for running gel and 4% for stacking gel. Electrophoresis was run under both reducing (mercaptoethanol) and no reducing conditions in a Mini Protean Tetra Cell (Bio-Rad Laboratories Inc., Hercules, CA) at 15 mA for 2 h. A molecular mass marker from BioRad was used. Gels were stained with Coomassie blue, destained in a solution of methanol-acetic acid, and documented in the Gel DocTM XR + System from Bio-Rad (Chaquilla-Quilca *et al.*, 2016).

2.4. Sorghum procyanidin extraction and characterization

A Sephadex LH-20 column (6 × 1.5 cm) was manually packed and equilibrated with methanol/water at 30% (v/v), for 4 h. The phenolic extract was loaded on the column and washed with 60 mL of HPLC water, pH=7.0, followed by 40 mL of 30% aqueous methanol (v/v) to remove sugars and other phenols; procyanidins were eluted with 80 mL acetone/water at 70% (v/v). This last fraction was concentrated in a BUCHI RE 121 rotary evaporator (Buchi Laboratoriums-Technik, CH) at 37±2 °C, reconstituted with 6 ml of HPLC water, frozen, lyophilized, and stored at -20 °C until use (Gu *et al.*, 2002). The fraction of PCs (oligomer mixture) was characterized in previous works in our research group through an ultra-high-performance liquid chromatography system (UHPLC) equipped with Ultraviolet–Visible diode array (DAD) and mass spectrometry (MS) detectors, including electrospray ionization (ESI) and quadruple time-of-flight (QTOF), as described by Carmelo-Luna *et al*. (2020).

2.5. Inhibition of superoxide anion radical ($O_2^{\bullet-}$)

For this analysis we used a reaction between dicyclohexane-18-crown-6-ether and potassium superoxide (KO_2) in DMSO as a source of $O_2^{\bullet-}$ and, to monitor the reaction of this radical with the antioxidants, the reduction of nitroblue tetrazolium (NBT) into formazan (560 nm) was examined according to the protocol established by Kładna *et al*. (2012).

To standardize the inhibition method of $O_2^{\bullet-}$ to the conditions of our study, we used a standard of (-)-epicatechin with a degree of purity of 90%, which represents the most abundant monomer of sorghum procyanidins. In the first place, (-)-epicatechin standard, procyanidins, and kafirins, were solubilized in DMSO individually at different concentrations, 0.5, 1.0. 1.5, 2.0, 2.5, 5.0, and 10 mg/ml to obtain the antioxidant solutions. Superoxide radical ($O_2^{\bullet-}$) at a 10 mM concentration was formed by mixing dicyclohexane-18-crown-6-ether (60 mg) and KO_2 (7mg) complex in a volume of 10 ml of DMSO at a 2:1 mmol ratio, then the mix was sonicated for 0.5-3 hours until a pale-yellow solution was obtained. The inhibition reaction was carried out by mixing 500µl of $O_2^{\bullet-}$ solution (10mM) + 500µl NBT (0.05mM) + 50 µl of the antioxidant solution, vortexing, and reading absorbance at 560 nm after 90 s (Kładna, 2012; Famuwagun 2021). The percentage of inhibition was calculated by the equation (1):

$$\%\,Inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100 \qquad (1)$$

Where:
Abs control: absorbance of the $O_2^{\bullet-}$ solution (10 mM) + NBT (0.05 mM)
Abs sample: absorbance of the $O_2^{\bullet-}$ solution (10 mM) + NBT (0.05mM) + antioxidant solution at different concentrations.

The EC50 value of each sample was determined by nonlinear regression from a plot of the percentage of inhibition versus sample concentration (mg/mL).

2.6. Statistical analysis

Analytical determinations were performed in triplicate. Mean values and standard deviations are reported. In the case of superoxide radical anion inhibition, the differences or similarities between antioxidants and their concentrations were evident from the standard deviations, so there was no need to perform statistical analysis.

3. Results and discussion

3.1. Characterization of sorghum flour and bran

Flour production is almost always the first step in cereals processing. The chemical composition, nutritional value, and quality of sorghum flour depend on the sorghum grain (Palavecino *et al*., 2020**)**. The main components of sorghum flour are polysaccharides, followed by proteins, lipids, and fiber (Arouna *et al*., 2020). The most common method around the world to obtain flour from sorghum grain involves partial dehulling followed by dry milling, which allows for getting a low-fiber product (Palavecino *et al*., 2019, Tadesse *et al*., 2019).

The proximal composition of the flour obtained from the grain of brown sorghum in the present study is shown in Table 1. Similar results were found for carbohydrates (72%) and ashes (1–1.86%) in flour from red sorghum (Pezzali *et al*., 2020, Osibanjo *et al*., 2021). Lipid concentration was lower (2.55%) than previous works reported for flour of red sorghum from different species, such as those of Pezzali *et al*. (2020) (3.03%), Curti *et al*. (2022) (3.96%), and Rebellato *et al*. (2020) (3.44%). For protein concentration in this study (10.38%), the result is higher than flour from bicolor sorghum (8.97%), red sorghum (8.22%), and hybrid sorghum (8.78%) (Pezzali *et al*., 2020; Osibanjo *et al*., 2021; Curti *et al*., 2022). However, the fiber result (10.21%) is higher than red sorghum (1.79%); bicolor sorghum (1.60%) (Tadesse *et al*., 2019; Osibanjo *et al*., 2021), but lower than sorghum with no tannins (12.70%) (Sharanagat and Nema, 2021). The variety of sorghum (red, white, and brown) and the method of flour obtaining greatly affect the final flour characteristic (Tasie and Gebreyes, 2020; Rumler *et al*., 2021).

Table 1. Proximal composition of flour obtained from grain of brown sorghum on a wet basis.
Tabla 1. Composición proximal de la harina obtenida del grano de sorgo café sobre base húmeda.

Component	(g/100 g)
Moisture	2.55±0.01
Protein	10.38±0.45
Lipids	2.55±0.23
Total fiber	10.21±0.34
Ashes	1.50±0.05
Carbohydrates (by difference)	72.82±0.00

Mean ± standard deviation (n=3).

Phenolic compounds show antioxidant ability providing health benefits to the consumer, such as anti-cancer, anti-diabetic, anti-obesity, and anti-aging, in addition to being excellent antimicrobials**.** Sorghum typically presents higher phenolic content than other cereals like rice, wheat, barley, maize, rye, and oats. (Oladele *et al*., 2019). In the sorghum grain, the highest content of phenols is concentrated in the pericarp, and, in flours, it is usually less than other products such as bran, since they are made from the whole grain or from the decorticated grain (without pericarp) (Palavecino *et al*., 2020, Unate-Fraga *et al*., 2022). In the present work, the flour of brown sorghum obtained by dry milling and 0.5 mm sieving, had a total phenolic content of 6.49±0.99 mg GAE/g extracted with a mixture of solvents (acetone/water/acetic acid, 7:2.95:0.05). This result is higher than the value of 0.86–1.34 mg GAE/g reported for flour made from sorghum bicolor sifted through a mesh of 0.355 mm and extracted with another mixture of solvents (formic acid, ethanol, water, 50:48:2) (Dia *et al*., 2016), and the value of 0.763 mg GAE/g of flour prepared from bicolor sorghum with water extraction (Kamath *et al*., 2004). Unlike previous values, Moraes *et al*. (2015) found a higher phenolic content of 8.63 mg GAE/g in flour of brown sorghum using a sieve of 0.850 mm and extraction with a distinct solvent mixture (HCl-methanol). Both results suggest that the total phenolic content is affected by the sieve used to obtain flour, the solvents employed for the extraction, as well as the variety of sorghum from which the flour is obtained (Moraes *et al*., 2015; Salazar-López *et al*., 2018; Wu *et al*., 2017; Kumari *et al*. 2021).

Polyphenols are abundant in plants like sorghum, they are aromatic polyhydroxylated compounds that include several classes, such as phenolic acids and flavonoids; among the latter procyanidins stand out. Sorghum phenolic compounds are conventionally obtained through refluxing extraction, water extraction, maceration extraction, soxhlet extraction, and organic solvent extraction (Oladele *et al*., 2019; Xu *et al*. 2021; León-López *et al*., 2022). The procyanidins are oligomers and polymers formed by units of (-)-epicatechin and (+)-catechin, joined by interflavan bonds. Particularly, sorghum procyanidins are B-type, that is, they contain C4-C8 and/or C4-C6 bonds (Mendoza-Wilson *et al*., 2020). The total phenolic compounds of sorghum bran in the present study were 15.44±2.19 mg GAE/g, using the acetone/water/acetic acid extraction system. This result is in the range obtained by Awika *et al*. (2004), who reported values of 4.8–22.4 mg GAE/g for black sorghum extracted with 1% HCl in methanol and 70% aqueous acetone. However, Choi *et al*. (2019) reported lower values (7.68 mg GAE/g) in brown sorghum by solvent extraction (acidified methanol), whereas Barros *et al*. (2013) obtained higher values of phenolic compounds (45 mg GAE/g) for red sorghum using ethanol (50% or 70%) and high temperature (120–150°C).

In general, the different concentration of phenolic compounds in sorghum bran and flour depends on grain characteristics, pericarp thickness, and color, as well as the production environment (Wu *et al*. 2017; Kumari *et al*., 2021). Specifically, the content of phenols in the bran also is related to the process of decortication of the grains because a high proportion of phenolic compounds can be lost depending on the method used to decorticate them (Awika *et al*. 2004; Xu *et al*., 2021).

3.2. Characterization of sorghum kafirins

Kafirins are related to the digestibility and quality of both grain and sorghum-based foods. Sorghum kafirins, especially those belonging to classes β- and γ-, are one of the least digestible food proteins because of their high content of non-polar aminoacids and cysteine, which favors the formation of disulfide bonds and therefore the crosslinking between proteins (Sullivan *et al*., 2018; Badigannavar *et al*., 2018; Li *et al*., 2018; Balandrán-Quintana *et al*., 2019; Castro-Jácome *et al*. 2020; Xu *et al.*, 2021).

The method used to extract kafirins from sorghum flour was carried out including the reducing agent sodium metabisulfite (food-grade). It is used to break the disulfide bonds within and between kafirin molecules, increasing the solubility of the kafirin in the aqueous ethanol and, consequently, increasing the amount of extractable protein (Taylor and Taylor, 2018). Representative bands of β, γ, α1, and α2 kafirins are seen in the SDS-PAGE gel in Fig. 1**,** indicating that kafirin fraction contained the most abundant classes of kafirins. The electrophoretic profile is consistent with previous reports (Li *et al*., 2018; Dianda *et al*., 2019; Shah *et al*., 2021). The band with a molecular weight of around 16 kDa is representative of β-kafirin and is only visible in reducing conditions. It should be mentioned that, since sodium metabisulfite was used in the extraction, it could be expected that the 16 kDa band would also be seen in lane 3, as b-kafirin is reported to be associated with oligomers (Espinosa-Ramírez and Serna-Saldivar, 2016). However, the disulfide bridges are not always accessible to the reducing agent, besides that non-disulfide crosslinks could form at 65 °C, with the subsequent formation of resistant dimers or even oligomers (Doudu *et al*., 2003). This may be the reason why the 16 kDa band is only visible when electrophoresis was performed under reducing conditions since mercaptoethanol facilitates the complete denaturation and unfolding of protein molecules. In addition, protein subunits joined by disulfide would separate (Chiquito-Almanza *et al*., 2011; Castro-Jácome *et al*., 2020; Ioerger *et al*., 2020). The bands of 20 kDa and 22 kDa are representative of α1 and α2 kafirins, respectively. The result is like Li *et al*. (2018) and Espinosa-Ramírez and Serna-Saldívar (2016), who reported molecular weights of around 20–24 kDa for this type of kafirins. The band around 25 kDa is assigned to γ-kafirins, very similar to Dianda *et al*. (2019), who observed γ bands between 25–26 kDa.

SDS-PAGE electrophoretic profile of different types of kafirins, under reducing and non-reducing conditions, showed essentially the same bands. The band around 50 kDa under reducing conditions suggests the presence of stable dimers. However, under no reducing conditions (lane 3), the bands around 48 kDa, 74 kDa, and 250 kDa suggest the presence of no reduced fractions, trimers, and oligomers, respectively (Xiao *et al*., 2015; Espinosa-Ramírez and Serna-Saldívar, 2016; Cabrera-Ramírez *et al*., 2020; Castro-Jácome *et al*., 2020). Not observing additional bands in the gel is related to the method of extraction and indicates a successful method without contamination and with good concentration and purity, but also about the composition of kafirins (Pontieri *et al*., 2019; Castro-Jácome *et al*., 2020).

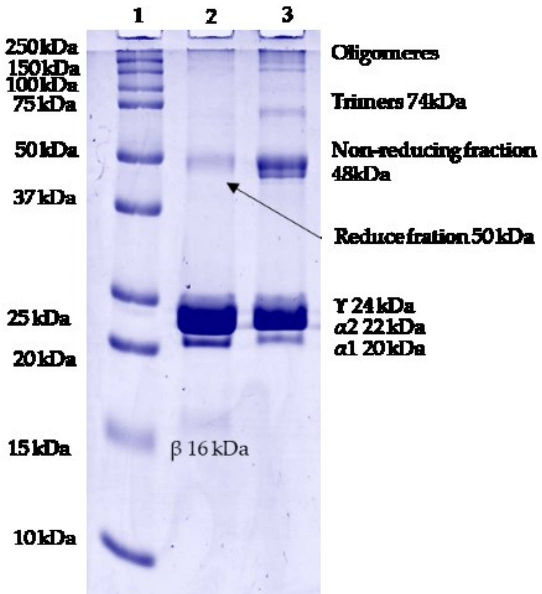

Figure 1. Electrophoretic profile of kafirins isolated from flour of brown sorghum.
Line 1: MW standard; Line 2: reducing conditions; Line 3: non-reducing conditions.
Figura 1. Perfil electroforético de las kafirinas aisladas de la harina de sorgo café.
Linea 1: PM estándar; Linea 2: condiciones reductoras; Linea 3: condiciones no reductoras.

3.3. Ability of kafirins and procyanidins isolated from sorghum to scavenge $O_2^{\bullet-}$

The $O_2^{\bullet-}$, is known as the initiator of oxidative reactions and consequently leads to cell damage that promotes chronic diseases in the human body, among these, cancer, arthritis, hypertension, atherosclerosis, and cardiovascular and neurodegenerative diseases, among others (Costa, 2021; Dubois, 2020; Mendoza-Wilson *et al*., 2020; Wen *et al*., 2020; Yang *et al*., 2020; Verma *et al*., 2021; Ifeanyi, 2018). On the other hand, in food products, it affects the physicochemical and sensorial properties because of the generation of undesirable odors and flavors, or even toxic compounds (Hellwig 2019; Zhang, 2019).

Antioxidant compounds can inhibit or delay undesired oxidation reactions caused by radicals such as $O_2^{\bullet-}$. Antioxidant therapy, based on dietary antioxidants, has emerged as a useful approach to preventing or minimizing the progression of chronic diseases. Likewise, antioxidants from natural sources may be useful to stop the deteriorating reactions of foods, in addition to acting as functional ingredients by providing an extra benefit to consumers (Huang, 2018; Ozkan et al., 2019; Rendra et al., 2019; Pinto et al., 2020). Undoubtedly, among these compounds, kafirins and procyanidins from sorghum can be listed, which have already shown their antioxidant power against different oxidative systems, but as indicated above, more specific studies on their activity against biologically produced radicals as $O_2^{\bullet-}$, are still lacking.

In this study, as shown in Fig. 2, the inhibition of $O_2^{\bullet-}$ was greater as the concentration of both kafirins and procyanidins isolated from sorghum increased. In (-)-epicatechin the same trend was also observed, although on a smaller scale, since its percentages of inhibition of $O_2^{\bullet-}$ were significantly lower compared to procyanidins and kafirins. In the 0.5–5 mg/ml concentration range of kafirins and procyanidins, they showed very similar superoxide inhibition percentages, which fluctuated from 41.3–60% and 42.8–59.2%, respectively. From the overlapping of standard deviations observed in Fig. 2 is easy to corroborate that there are no significant differences between kafirins and procyanidins at the mentioned concentrations. However, at the concentration of 10 mg/ml, significant differences were observed (no overlap in standard deviations), since in this case, kafirins showed 66.4% inhibition of $O_2^{\bullet-}$ and procyanidins 60%. In relation to (-)-epicatechin, it always showed significantly lower inhibition percentages of $O_2^{\bullet-}$ (14.8–45.1% in the range of 0.5–10 mg/ml) compared to kafirins and procyanidins, and therefore, no overlap was observed with the standard deviations of these compounds. It is important to mention that in the concentration range of 2.5–10 mg/ml, the percentage of inhibition of $O_2^{\bullet-}$ achieved by (-)-epicatechin showed little changes, so no significant differences were found in the corresponding standard deviations.

When comparing with other similar investigations, it was found that some peptides obtained from the hydrolysis of zein, a maize prolamin analogous to sorghum kafirins, showed to be effective in inhibiting $O2^{\bullet-}$ up to 69% at a concentration of 50 mg /ml in an aqueous medium (Tang et al., 2010). Contrary to this result, in a very recent study, it was reported that prolamines obtained from sesame seeds showed prooxidant effects when reacting with $O2^{\bullet-}$ in an aqueous medium at a concentration of 1 mg/ml (Idowu *et al*., 2021). These results show the importance of the reaction medium and the concentration in the behavior of prolamins against superoxide, as well as the source from which these proteins come. It is known that sorghum kafirins are the most hydrophobic prolamines (Balandrán-Quintana, 2019; Castro-Jácome, 2020), therefore, polar aprotic reaction media, as DMSO, could favor their direct reaction with free radicals, achieving better percentages of inhibition to lower concentrations, compared to polar protic media such as water.

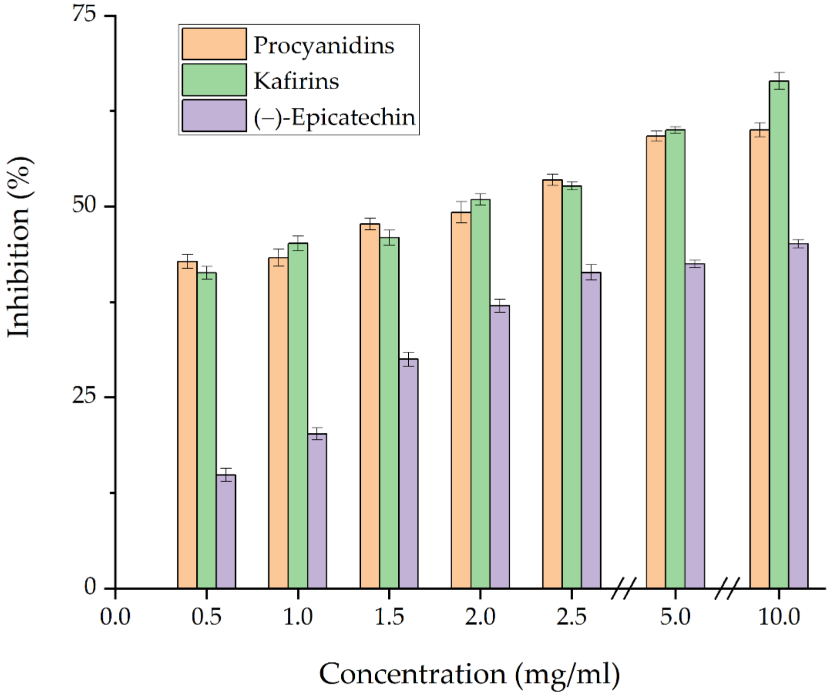

Figure 2. Antioxidant activity of kafirins and procyanidins isolated from brown sorghum and the (-)-epicatechin standard at different concentrations against superoxide radical anion ($O_2^{\bullet-}$) in DMSO.
Figura 2. Actividad antioxidante de las kafirinas y procianidinas aisladas del sorgo café y el estándar de (-)-epicatequina a diferentes concentraciones contra el radical anión superóxido ($O_2^{\bullet-}$) en DMSO.

Another way of expressing the ability of an antioxidant to inhibit free radicals is the EC50, which is defined as the concentration of a substance that produces 50% of the maximal effect, for this case 50% inhibition of $O_2^{\bullet-}$ (Xu, 2020). The results obtained in this study for EC50 of kafirins, procyanidins, and (-)-epicatechin in DMSO, were 1.72 mg/ml, 2.02 mg/ml, and 10.04 mg/ml, respectively. These results suggest the following decreasing order of effectiveness in inhibiting the $O_2^{\bullet-}$: kafirins > procyanidins > (-)-epicatechin. Results fluctuating in these EC50 ranges were obtained for proteins from different vegetable sources, such as eggplant whose EC50 value was 1.21 mg/ml (Famuwagun, 2021), and corn protein with an EC50 value of 12.8 mg/ml (Esfandi, 2019). Regarding procyanidins and (-)-epicatechin, EC50 values of 0.03 mg/ml and 0.06 were reported to inhibit $O_2^{\bullet-}$ in an aqueous medium (Saint-Cricq *et al*., 1999), respectively. This result is consistent with our results in the sense that procyanidins showed greater ability than (-)-epicatechin to inhibit $O_2^{\bullet-}$. On the other hand, it is expected that in an aqueous medium (protic polar) procyanidins and (-)-epicatechin show greater ability than in a non-protic polar medium (DMSO) to trap free radicals due to their hydrophilic character (Mendoza-Wilson *et al*., 2016; Yanagida *et al*., 2007).

4. Conclusions

In general, the brown sorghum flour obtained in our study showed a proximal composition comparable to that reported for red, bicolor, hybrid, and white sorghum varieties. As expected, the content of total phenols was notably higher in the bran than in the flour. The electrophoretic profile of the kafirin fraction isolated from brown sorghum flour showed the characteristic bands of the $\alpha 1$, $\alpha 2$, β, and γ types. Due to the hydrophobicity of the kafirins, these were better inhibitors of the $O_2^{\bullet -}$ in the aprotic polar medium (DMSO) in which they were analyzed, compared to procyanidins and (-)-epicatechin that have a hydrophilic character. The decreasing order of effectiveness in inhibiting $O_2^{\bullet -}$ of these bioactive compounds of sorghum in DMSO was: kafirins > procyanidins > (-)-epicatechin. This result is important since procyanidins are considered one of the bioactive compounds with the highest antioxidant ability against a considerable number of free radicals.

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Arely León López performed the experimental part and contributed to the writing of the article. Ana María Mendoza Wilson is the author of the project, she provided advice to the postdoctoral student in the extraction and characterization of procyanidins and phenols and in the writing and editing of the article. René Renato Balandrán Quintana provided advice to the postdoctoral student on electrophoretic techniques and participated in the writing and editing of the article. José Ángel Huerta Ocampo provided advice to the postdoctoral student on electrophoretic techniques, identification of bands and gel documentation.

Interest conflict

The authors declare that there is no conflict of interest.

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