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Evaluation of some *in vitro* bioactivities of sunflower phenolic compounds

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ABSTRACT

Phenolic compounds in crude extracts were obtained from defatted sunflower seed flour using sodium bisulfite and ethanol solutions as extracting agents. The antioxidant, antimicrobial, anti-proliferative, and DNA protective activities of the phenolic compounds in crude extract were analyzed. The phenolic compound contents were determined as chlorogenic acid (CGA) equivalent, presenting 11.57 and 15.44 g CGA eq/100g regarding the sodium bisulfite extract and ethanolic extract, respectively. The ORAC, DPPH, and ABTS methods were used to evaluate antioxidant activity. Both extracts presented antioxidant properties, considering that the ethanolic extract demonstrated higher values (EC_{50} 0.36 g extract/g DPPH•). The antimicrobial action was analyzed as to the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of 4 kinds of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*). The ethanolic extract was effective against all of these microorganisms, out of which *E. coli* was the most sensitive, with a MIC of 11.6 mg CGA/mL. The ethanolic extract presented DNA protective activity without cytotoxic activity concerning *in vitro* anti-proliferative assay. These findings can be considered as initial evidence of the potential use of phenolic compounds obtained from sunflower seed flour as natural additives in the food industry.

1. Introduction

The valorization of agro-industrial byproducts emerges as a worldwide trend in the context of sustainability, as a means of making agribusiness more profitable, increasing the supply of food in the world, and extracting bioactive compounds beneficial to health. The production processes generate solid residues and a large number of pollutants in the environment, which cause important climatic changes and damage to ecosystems (Murray et al., 2017). This scenario has initiated several international discussions that have holistically addressed issues related to the environment, society, the economy, and their interconnections (Geissdoerfer et al., 2017). The sunflower (*Helianthus annuus* L.) is one of the largest oilseed cultures in the world and the oil extraction process generates a byproduct (meal) with an elevated protein content, rich in phenolic compounds, its worldwide production reaches about 20 million metric tons (USDA. United States Department of Agriculture, 2021). This meal is not used for human consumption due to having a high content of shells and phenolic compounds, which give an undesirable greenish color with an astringent flavor. Currently, the meal is just employed in the animal feed. The phenolic compound content of the meal varies from 1 to 4 g/100 g, with a predominance of chlorogenic acid (Weisz et al., 2009).

There are two important complementary issues to be considered which involve the removal of phenolic compounds from sunflower seed meal and at the same time the use of natural plant compounds as antioxidants and antimicrobials in food and packaging. Therefore, the substitution of synthetic additives for natural ones consists of a market trend regarding the concept of natural food ingredients, sustainable means of production, and zero waste generation (Wildermuth et al., 2016).

The high antioxidant capacity of the phenolic compounds in

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sunflower seeds is already consolidated in the literature and some studies have evaluated their potential in biodegradable films made with isolates of sunflower proteins, naturally rich in phenolic compounds (Karakaya, 2004; Salgado et al., 2012b).

Currently, there is a growing interest in finding natural antioxidants capable of protecting the human body from free radicals (exogenous and endogenous) and delaying the onset of chronic non-communicable degenerative diseases. Thus, studies have been carried out to isolate, identify and quantify these natural compounds considering their future technological application in processed foods and nutraceuticals (Gunduc and El, 2003; Salgado et al., 2012a).

The phenolic compounds consist of flavonoids and phenolic acids, out of which benzoic acid derivatives can be found, such as gallic and cinnamic acid. Considering that cumarinic, caffeic, and ferulic acids are derivated from cinnamic acid (Karakaya, 2004). The most abundant phenolic compound in fruits and vegetables is caffeic acid, frequently found in the esterified form denominated as chlorogenic acid (D'Archivio et al., 2007).

Taking this into consideration, the literature presents experiments regarding the ability of fruit flour extracts to inhibit the proliferation of HT-29 colon cancer cells *in vitro*. This property was correlated to the content of phenolic compounds, suggesting that their presence could be an important indicator of anti-cell proliferation activity (Parry et al., 2006). Another study indicates chlorogenic acid as an excellent protector of gastric mucosa due to its antioxidant properties, which prevent cell damage due to DNA breakage induced by NH_2Cl, resulting from the ammonia production of *Helicobacter pylori* (Shibata et al., 1999).

Due to limited data found in the literature concerning bioactive characteristics of the sunflower seeds phenolic compounds extract, the present study proposed to extract the phenolic compounds with different solutions, as well as quantify and evaluate their *in vitro* antioxidant, antimicrobial, anti-proliferative, and DNA protective potentials.

2. Materials and methods

2.1. Materials

Dehulled sunflower grains (*Helianthus annuus* L.) of the Aguará 3 variety, used to obtain the flour, were provided by the company Giroil Agroindústria Ltda (Santo Ângelo, RS, Brazil). Chlorogenic acid, gallic acid, fluorescein, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and AAPH (2,2′-azobis (2-methylpropionamidine) dihydrochloride) were acquired from Sigma-Aldrich (St Louis, MO, USA). All other reagents were of analytical grade and purity.

2.2. Preparation of the sunflower flour

Defatted sunflower seed flour was obtained by extracting the oil from the grains in two steps, which involve the cold extraction in a mechanical press (Carver Press, USA), followed by the extraction of residual oil using hexane in a Soxhlet extractor. The defatted material was ground (Retsch ZM 200, Germany), homogenized, and used to extract the phenolic compounds.

2.3. Extraction of the phenolic compounds from the sunflower flour

The extraction production followed the protocol described by Alexandrino et al. (2017) that were based on the methodology of Salgado et al. (2011) with modifications. Two extraction solutions were used to obtain the crude extract of phenolic compounds: 0.1 g/100 mL (w/v) solution of sodium bisulfite and 70 mL/100 mL ethanolic solution (v/v) in water. The procedure consisted of dispersing the flour in the extraction solutions in the proportion of 1:10 (w/v), adjusting the pH value to 5 (HCl 1 mol/L), and keeping it in constant agitation for 1 h. The sample was then centrifuged at 11,000 × g for 20 min at 20°C (Sorvall RC-26 Plus, USA). For the ethanolic extract, the solvent was evaporated on a rotary evaporator (Logen Scientific, LS 540, United Kingdom) at 40°C. The extracts obtained were lyophilized and used in the tests. Extracts were performed in triplicate, in order to analyze the content of phenolic compounds and ORAC, DPPH, ABTS determinations, extracts with 1 mg/mL of concentration were used.

2.4. Determination of the total phenolic compounds and clorogenic acid contents

The total phenolic compound contents were determined in sunflower flour and in the freeze-dried extracts, expressed as chlorogenic acid (CGA) equivalents, according to the methodology of Kim et al. (2003) with modifications. An aliquot of diluted extract (1 mg/mL) was used for the reaction plus 9 mL distilled water and 1 mL of Folin-Ciocalteu reagent, and 10 mL of Na_2CO_3 solution (7 g/100 mL, w/v) added after 5 min. The absorbance values were determined at 750 nm (Varian Cary 50, USA) after 90 min at a temperature of 25°C in the absence of light, and used to calculate the phenolic compound contents using chlorogenic acid as the standard. The analyses were carried out in duplicate. The chlorogenic acid content was determined in sunflower flour by HPLC-DAD at 324 nm (Shimadzu Corporation, Tokyo, Japan), as described by Tfouni et al. (2012).

2.5. Oxygen radical absorbance capacity (ORAC)

The ORAC was measured by the peroxyl radical (ROO•) scavenger capacity to protect the fluorescein molecule from oxidation, as described by Chisté et al. (2011). The assay was carried out in a 96-wells microplate fluorescence reader (Synergy, BioTek®, software Gen5), with fluorescence filters for excitation at 485 nm and emission at 528 nm at 37°C. The reaction media contained 70 nmol/L fluorescein as the probe, 14 mmol/L AAPH as the radical generator, and antioxidants from the phenolic extract or the Trolox standard. Fluorescence emission was recorded for 2 h. Results were expressed in mmol of Trolox equivalent/g of freeze-dried extract.

2.6. DPPH radical capture capacity

The capacity of the antioxidant compounds presents in the extracts to sequester DPPH free radicals was determined according to the method described by Brand-Williams et al. (1995). Samples of 1 mg/mL of extracts diluted in water were mixed with an 80 mL/100 mL (v/v) solution of methanol in a 1:10 (v/v) proportion and with the DPPH•. For the analysis, the DPPH• had a concentration range from 13 to 53 μmol/mL and the total reaction volume was 4 mL. The extracts were tested regarding their EC_{50} concentrations after 60 min of reaction at 25°C and 515 nm of absorbance. The EC_{50} value is the extract concentration required to extinguish 50% of the DPPH radicals under the experimental conditions, in a predetermined time. The sample results were also expressed in TEAC (mmol of Trolox equivalent/g of freeze-dried extract) by comparing with the percentage of DPPH• inhibition on the Trolox curve.

2.7. ABTS radical capture capacity

This determination was based on the method described by Re et al. (1999), with modifications. The cationic radical ABTS (0.76 g/100 mL) was generated by the reaction with $K_2S_2O_8$ (0.13 g/100 mL), both dissolved in water at 25°C for 16 h. Afterward, the ABTS was diluted in methanol to reach an absorbance value of 0.70 ± 0.02 at 750 nm. Aliquots of the samples were diluted in water and subsequently in methanol (80 mL/100 mL) and then used for the reaction with $ABTS\bullet^{+}$. The mixture was incubated for 10 min in the dark at 25°C and the absorbance was measured at 750 nm. The results were expressed in TEAC

Table 1
Tumor cell lines and non-tumor a cell line used in the analysis of the anti-proliferative capacity of the sunflower phenolic extracts.

Cell line	Name
Glioma	U251
Breast	MCF-7
Ovary (multiple drugs resistance)	NCI-ADR/RES
Kidney	786–0
Lung (non-small cells)	NCI–H460
Prostate	PC-3
Ovary	OVCAR-03
Colon	HT29
Leukemia	K562
Human keratinocytes (non-tumor)	HaCat

(mmol Trolox equivalent/g of freeze-dried extract) by comparing with the ABTS$\bullet^{+}$ inhibition percentage curve using the Trolox standard.

2.8. Evaluation of the antimicrobial activity

2.8.1. Minimal inhibitory concentration (MIC)

The antimicrobial activity of the sunflower phenolic compounds of ethanolic extract was analyzed against the following bacteria: *Escherichia coli* ATCC 10231, *Pseudomonas aeruginosa* ATCC 13388, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 5061. The bacteria were incubated at 36°C for 24 h in the Mueller-Hinton culture medium (Merck, Germany).

The inoculum was prepared by diluting the cell mass in 0.85 g/100 mL NaCl (w/v), adjusted to 0.5 on the McFarland scale, and confirmed by verifying the absorbance at 625 nm. For the analyses, the cell suspensions were diluted to 10^5 CFU/mL using Mueller-Hinton medium (Merck). The value for MIC was calculated in triplicate according to CLSI (2012) using 96-well cell culture microplates, each well containing 100 μL of culture medium. Based on its phenolic compounds concentration, the sunflower phenolic compounds extract was diluted in a culture medium (180 mg chlorogenic acid/mL) and 100 μL transferred to the first well of the microplate. Serial dilutions were then prepared and 100 μL of each transferred to a well, obtaining a concentration range of 45 to 0.04 mg/mL. The plates were incubated at 36°C for 24 h under anaerobic conditions. The MIC was defined as the smallest concentration sample capable of inhibiting microbial growth and was expressed as CGA equivalent in the extract according to the total phenolic compounds determination (item 2.4.).

2.8.2. Minimal bactericidal concentration (MBC)

Based on the results for MIC 10 μL of the cell suspensions from wells that did not show visible microbial growth and from the three wells above, were sub-cultured into Petri dishes containing nutrient agar (Difco®) and incubated at 36°C for 24 h. The MBC was defined as the lowest extract concentration that did not present microbial growth on the agar surface and was expressed as CGA equivalent in the extract (item 2.4.). The tests were carried out in triplicate.

2.9. Anti-proliferative activity

The phenolic compound extracts were evaluated *in vitro* for their anti-proliferative activity in human tumor cells and in one strain of non-tumor human cells (Table 1), according to the methodology recommended by the National Cancer Institute (NCI/NIH) (Frederick, WA, USA) (Monks et al., 1991; Shoemaker, 2006). The cell suspensions, cultivated in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 5% bovine fetal serum (BFS, Invitrogen) and 1% of a streptomycin/ penicillin solution (VitroCell®) were plated in triplicate in 96-well plates (100 μL/well) and incubated at 37°C for 24 h in a moist atmosphere with 5% CO_2. A control plate (T_0) was prepared in the same way, containing all the cell lines used in the experiment, the cells in this plate being fixed with 50 g/100 mL trichloroacetic acid (TCA) (w/v) until addiction of extract. After 24 h of incubation, the sunflower phenolic compounds extract was added to the plates in four concentrations (0.25; 2.5; 25; and 250 μg/mL), previously diluted in RPMI 1640 medium, 5% BFS and 0.2% dimethylsulfoxide (DMSO) (Merck®). The final DMSO concentration did not affect the cell viability. The chemotherapeutic agent doxorubicin was used as the positive control in concentrations of 0.025; 0.25; 2.5 and 25 μg/mL. After 48 h of incubation, all the treated cells were fixed with 50% TCA (w/v) and cell proliferation determined at 540 nm of the cell protein content using the dye sulforhodamine B (Monks et al., 1991). Thus three absorption measurements were taken, one at the start of incubation (zero time, T_0), one after 48 h in the presence of the extract (T), and another in the absence (T_1) of the extract. The cell growth was calculated from the mean absorbance values and considering the ratio between T and T_0. Thus when $T \geq T_0$, cell growth was determined according to the equation: $[(100 \times T\text{-}T_0)/(T_1\text{-}T_0)]$, whereas when $T < T_0$, equation $100 \times [(T\text{-}T_0)/(T_0)]$ was used. The results for each cell line were expressed as cell growth, expressed as a percentage, as a function of the concentration of the phenolic compounds in the extract, using the software Origin Pro® 8.0 (OriginLab Corporation).

2.10. DNA protective activity

The DNA protective activity was assessed using supercoiled plasmid DNA and reaction with the reactive oxygen species (ROS) generator AAPH, the methodology was based on Shahidi et al. (2007) and Kroth et al. (2020). The plasmid pcDNA 3.1 (Thermo Scientific, Rockford, IL, USA) used in the DNA analysis was amplified after transformation into *E. coli* (strain TOP10 - Thermo Scientific), growth in Luria-Bertani (LB) broth medium, and extracted using the QIAGEN Plasmid Midi Kit, following the manufacturer's instructions. The DNA solution (125 ng/μL) was incubated (37°C/1 h in the dark) in the presence of 1000 ppm phenolic extracts samples and 30 mmol/L AAPH. Afterward, each sample was mixed in the proportion of 1:5 with loading buffer (bromophenol blue in Tris EDTA (TE) buffer, pH 8.0) and 12 μL of DNA samples were separated into a 0.8% (m/v) agarose gel prepared using Tris-Acetate EDTA buffer (TAE) supplemented SyberSafe (Thermo Scientific, Rockford, IL, USA) (1:20,000). The molecular weight reference was GeneRuler 1 kb DNA Ladder (Thermo Scientific, Rockford, IL, USA). Electrophoresis was then run for 1 h at 80 mV and 1.5 h at 120 mV. The gels were analyzed using a molecular imager ChemiDoc Image System (Biorad, Hercules, CA, USA), and the resulting image was processed using the software ImageJ (NCBI, https://imagej.nih.gov/ij/). The supercoil band intensity for only DNA with no AAPH was considered as a positive control, and the relative percentage of this band intensity was considered as the protective effect of each sample upon the DNA treated with AAPH. The inhibition percentage was calculated as follows: inhibition of DNA strand breakage = [(intensity of supercoiled DNA in the presence of oxidant and extract/intensity of supercoiled DNA devoid of oxidant and extract) × 100]. Results were expressed as percentage retention of supercoiled DNA achieved with 1 μg/L of phenolic compound extract.

2.11. Statistical analysis

Results were expressed as mean ± standard deviation. The data were analyzed using analysis of variance (ANOVA) using XLSTAT (version 2012.6.03, Addinsoft, France) statistical program. For DNA protective capacity assay was applied ANOVA with Dunnett's post-test using GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA) statistical program. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Total phenolic compounds

The total phenolic content of the sunflower flour was 4.00 ± 0.01 g CGA eq/100 g (dry basis). The chlorogenic acid, determined by HPLC, corresponded to the predominant phenolic compound, representing approximately 62% of the total phenolic compounds (2.46 ± 0.11 g/100 g on dry basis) (Supplementary material 1). A study carried out by Weisz et al. (2009) using the HPLC-DAD/ESI-MS technique, confirmed that chlorogenic acid was the predominant compound amongst the phenolic compounds in samples of sunflower kernels and shells from several regions.

As expected, phenolic compounds were concentrated in 15.44 ± 0.65 and 11.57 ± 0.05 g CGA eq/100 g of extract to ethanolic and sodium bisulfite solution, respectively. The ethanolic solution was statically the most efficient regarding extraction. Other researchers have tested different extracting solutions and observed that the residue of phenolic compounds especially in sunflower seeds was smaller when an ethanol solution was used (Salgado et al., 2011; Alexandrino et al., 2017). During the sunflower protein isolation, the 70:30 mixture of ethanol and water was effective to remove the phenolic, resulting in a product with a low chlorogenic acid residual (<0.1%). However, the ethanolic solution resulted in an extract with higher content of phenolic compounds and a lighter colour protein isolate. The sodium bisulfite solution promoted one of the highest protein content in isolate and the best co-production of the fibrous concentrate (Alexandrino et al., 2017). Therefore, we found it interesting to use both solutions to evaluate their performance related to bioactivities.

Relevant references to the subject have shown that the efficiency of the extraction solution for plant phenolic compounds depends on both the solvent and the matrix. According to Moreira et al. (2014), the best solvents to extract the chlorogenic acids from coffee beans were ethanol or a ternary mixture of ethanol: ethyl acetate: dichloromethane (1:1:1 v/v/v). Another study extracting the phenolic compounds from defatted sunflower meal, using: water; 70 mL/100 mL methanol; 70 mL/100 mL acetone, and a 70:30 mixture of ethyl acetate and water showed the water as the best extractor solvent (Matthäus, 2002).

A method for the concentration of phenolic compounds was carried out by Karamać et al. (2012), using a preparative chromatography technique with a Sephadex LH-20 column and methanol as eluent. Under these conditions, they could separate 6 fractions from a crude sunflower seed extract, and the total phenolic compound contents of the fractions varied from 24.50 mg to 666.00 mg CGA eq/g of fractionated extract. The first fractions eluted presented the smallest phenolic compound contents probably due to the simultaneous extraction of sugars and other soluble compounds. Whereas the last fractions showed the highest contents due to the greater degree of purification. The values presented by the second fraction obtained by Karamać et al. (2012) (150 mg CGA eq/g) were equivalent to the obtained in this study using ethanol solution (154.35 mg CGA eq/g). These previous studies lead us to believe that the extraction of phenolics using ethanol solution was efficient considering the sunflower matrix, even though in the present study it carried many other soluble components, such as sugars and, to a lesser extent, proteins.

3.2. Antioxidant activity

The reactive oxygen species (ROS) elimination can be performed by electrons transfer (e.g., ABTS and DPPH) or a hydrogen atom (e.g., ORAC) (Granato et al., 2018). Therefore, distinct methods (ORAC, DPPH, and ABTS) were employed in the present study to measure the different abilities of antioxidant ROS eliminations. The antioxidant activities of natural fruit, vegetable, and grain extracts have been correlated with the presence of phenolic compounds, such as caffeic, chlorogenic, and ferrulic acids (Moure et al., 2001; Velioglu et al., 1998). Our values obtained for antioxidant activity (Table 2) are in agreement with the previous results (3.1 item), in which the most efficient extraction solution showed the higher antioxidant activity regarding all the analyses method carried out. The ethanolic extract presented the lower concentration required to reach the EC_{50}, and it had a higher value regarding the ORAC method (0.36 g extract/g DPPH•, 7.35 ± 1.11 mmol Trolox eq/g extract), such as in the DPPH• and ABTS•$^+$, in comparison to bisulfite extract (1.01 g extract/g DPPH•, 5.88 ± 0.66 mmol Trolox eq/g extract) (Table 2).

Table 2
ORAC (oxygen radical absorbance capacity), DPPH and ABTS radicals capture capacity expressed as the Trolox equivalent antioxidant capacity (TEAC) and DPPH• as the inhibition coefficient (EC_{50}) for the sunflower phenolic extracts.

Phenolic extraction solutions	TEAC (mmol Trolox eq/g)			EC_{50}[c] (g extract/g DPPH•)
	ORAC	DPPH•	ABTS•$^+$	
Sodium bisulfite[a]	5.88 ± 0.66[b]	0.40 ± 0.00[b]	0.41 ± 0.01[b]	1.01 ± 0.05[a]
Ethanol[b]	7.35 ± 1.11[a]	0.70 ± 0.01[a]	0.53 ± 0.01[a]	0.36 ± 0.00[b]

a,b Values in columns followed by different letters are significantly different ($p < 0.05$), according to ANOVA ($n = 2$).
a 0.1 g/100 mL sodium bisulfite solution in water (w/v).
b 70 mL/100 mL ethanolic solution in water (v/v).
c quantity in g of extract required to decrease the DPPH• concentration to 50% of the initial concentration.

In another study carried out from sunflower seeds homogenate in 50 mL/100 mL ethanol in water (v/v), 3 extracts were obtained using different extraction solutions (n-hexane, EtOAc, and water). They were evaluated by ORAC, and the EtOAc extract showed values comparable to the present findings. The authors suggest that the sunflower seed extract antioxidant capacity could be largely attributed to the presence of caffeic acid derivatives (Amakura et al., 2013). Extracts from the residues of different oilseeds such as sunflower, rapeseed, mustard, crambe, and others were evaluated by Matthäus (2002) using the DPPH method, in which the sunflower extract presented the greatest activity amongst the residues analyzed.

Karamać et al. (2012) used crude extract in the analysis of the antioxidant capacity of sunflower phenolic compounds, by the ABTS radical reduction technique, obtaining a result of 150 mg CGA eq/g and 0.51 mmol Trolox eq/g, which was very similar to the present study (Table 2). The synthetic antioxidant butylated hydroxyl toluene (BHT), in concentrations of 40 μg/mL, demonstrates 83.7% inhibition of the DPPH radical (Ghasemzadeh et al., 2010). The sunflower phenolic compounds extracts obtained by sodium bisulfite and ethanol in similar concentrations (40 μg/mL) presented 24.14% and 75.16% inhibition of DPPH, respectively. In order for the crude sunflower phenolic extract to produce an antioxidant effect similar to that of BHT, the extract concentrations needed to be adjusted. In addition, when formulating food products with the addition of the extract, possible interactions with proteins should be considered, and also organoleptic alterations due to the bitter taste of the polyphenols (Moure et al., 2001; Budryn and Rachwal-Rosiak, 2013).

The antioxidant action of the phenolic acids occurs due to the number of hydroxyl groups on the molecule, on account of their H-donating capacity and their stable intermediates, which also prevent oxidation (Brand-Williams et al., 1995; Rice-Evans et al., 1996). The antioxidant activity of the hydroxycinnamic acids (the class to which chlorogenic acid belongs) measured *in vivo* experiments, was capable of reducing the risk of various diseases related to oxidative stress, such as atherosclerosis, and also the inhibition of some types of carcinogens and neurodegenerative diseases (Cheng et al., 2007; Scalbert et al., 2005). In situations where there is an increase in the number of free radicals in the organism, reinforcement of the endogenous protection systems by the ingestion of dietary antioxidants can be of great importance, in order to

Table 3
Evaluation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the sunflower phenolic compounds from the ethanolic extract.

Microorganisms	MIC[a] (mg eq CGA/mL)	MBC[a] (mg eq CGA/mL)
Bacteria Gram-positive		
Staphylococcus aureus	33.2	>39.8
Bacillus subtilis	26.5	>39.8
Bacteria Gram-negative		
Escherichia coli	11.6	>19.9
Pseudomonas aeruginosa	33.2	>39.8

[a] Values were presented according to the total phenolic compounds content expressed as chlorogenic acid (CGA) equivalent. ($n = 3$).

attenuate the cumulative effects of damage caused by the oxidative molecules (Silva et al., 2005).

Although the phenolic compound extracts obtained in the present study demonstrated antioxidant activity, as evaluated by the analyses with the ORAC, DPPH, and ABTS radicals, we consider it is important to carry out complementary tests with the employed food matrix. The action of antioxidant compounds depends on the medium where they are inserted and are passive to auto-oxidation, which generates reactive substances with pro-oxidant action (Moure et al., 2001). Frankel et al. (1997) using green tea in oil-water emulsions, observed a pro-oxidant effect of the compounds present in the tea in this kind of application. These divergences in action can occur due to differences in the relative partition between the phases in lipid systems, since the activity of natural antioxidants is greatly affected by complex interfacial phenomena, such as emulsions and multi-component foods (Frankel, 1996).

3.3. Antimicrobial activity

The ethanolic extract presented antimicrobial activity against all the bacteria tested (Table 3), in which *E. coli* was the most sensitive microorganism with MIC of 11.6 mg CGA/mL. Some authors working with commercial chlorogenic acid (98% pure) found values for MIC against *E. coli*, *P. aeruginosa*, and *S. aureus* of 1.0, 1.0, and 2.0 mg/mL, respectively (Fu et al., 2017). These values reflect a much higher antimicrobial capacity for chlorogenic acid than that found in the present study, probably due to the degree of purity of the compound. MIC values (mg CGA/ mL) were presented in Table 3 and demonstrate that the presence of other compounds, mainly soluble sugars have been extracted together with the chlorogenic acid, reducing its concentration and consequently decreasing the activity of the crude extract (Karamać et al., 2012). There was no MBC value for any of the microorganisms at the concentrations used.

The inclusion of sunflower protein concentrates at 5%, which is naturally rich in phenolic compounds, in the film preparation, conferred antioxidant properties to the film but showed no antimicrobial effect (Salgado et al., 2012a). On the other hand, Lou et al. (2011) found antimicrobial activity against both Gram-positive and Gram-negative bacteria in different burdock (*Arctium lappa*) leaf extracts containing chlorogenic acid. Whilst Puupponen-Pimiä et al. (2001) observed antimicrobial action only against Gram-negative bacteria in berry extracts. Chlorogenic acid can bind to and permeate the bacterial membrane, but cannot completely rupture it. However, the damage to the membrane integrity may result in bacterial death (Lou et al., 2011).

3.4. Evaluation of the anti-proliferative activity

Cancer is a complex pathology and its development involves several steps. Initially, the presence of free radicals can lead to DNA damage that results in changes in the normal gene functions or mutations (Sgarbieri and Pacheco, 2017). Due to their antioxidant effects, the phenolic compounds, in particular chlorogenic acid, have been reported as promising chemo-preventative agents, mainly in the prevention of

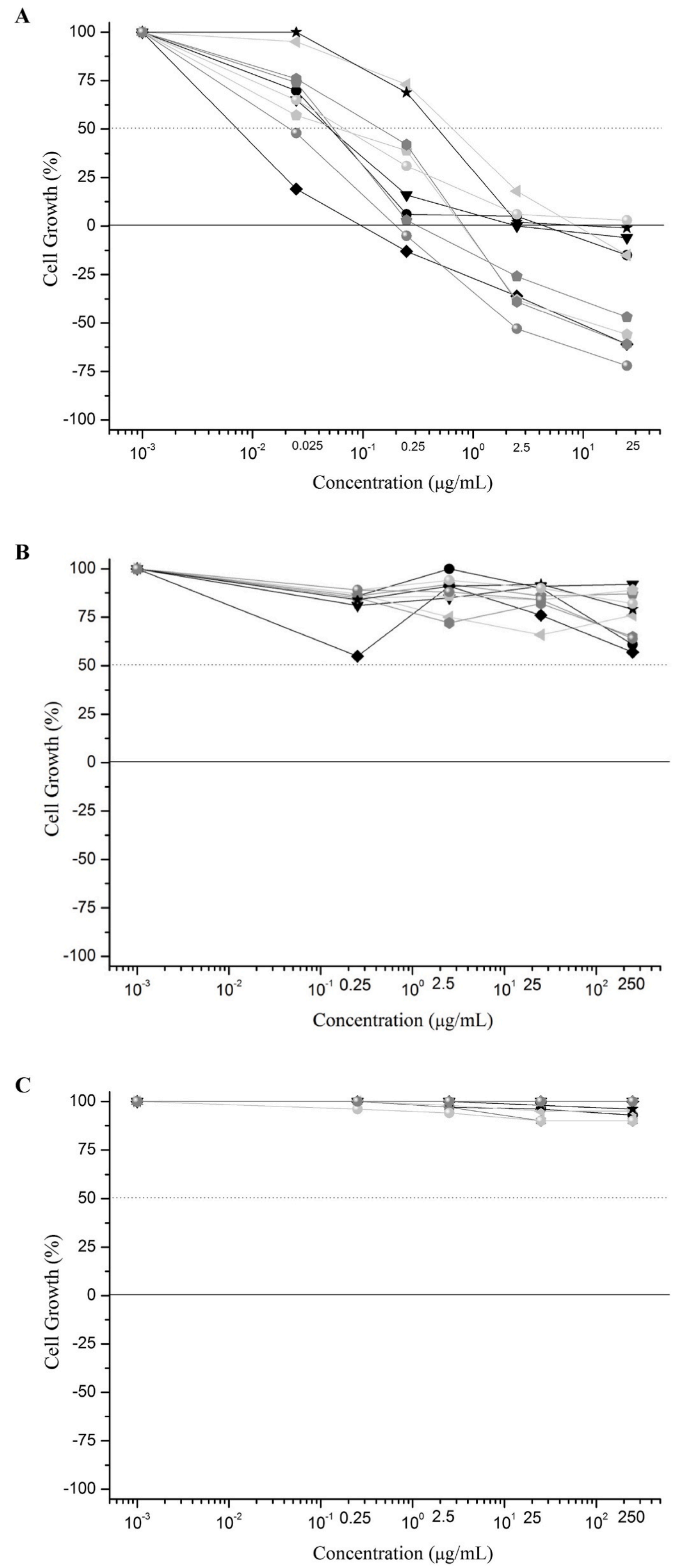

Fig. 1. Anti-proliferative activity of sunflower phenolic compound extract in a panel of human tumor and non-tumor cell lines after 48 h of treatment. Lines: = U251 (glioma), = MCF-7 (breast), = NCI-ADR/RES (ovary with multiple drugs resistance), = 786–0 (kidney), = NCI–H460 (lung), = PC-3 (prostate), = OVCAR-03 (ovary), = HT29 (colon), = K562 (leukemia) and = HaCat (human non-tumor keratinocytes). Chemotherapeutic agent doxorubicin (positive control) **(A)**, sunflower phenolic compound extract elaborated with 0.1 g/100 mL sodium bisulfite **(B)**, sunflower phenolic compound extract elaborated with 70 mL/100 mL ethanol **(C)**.

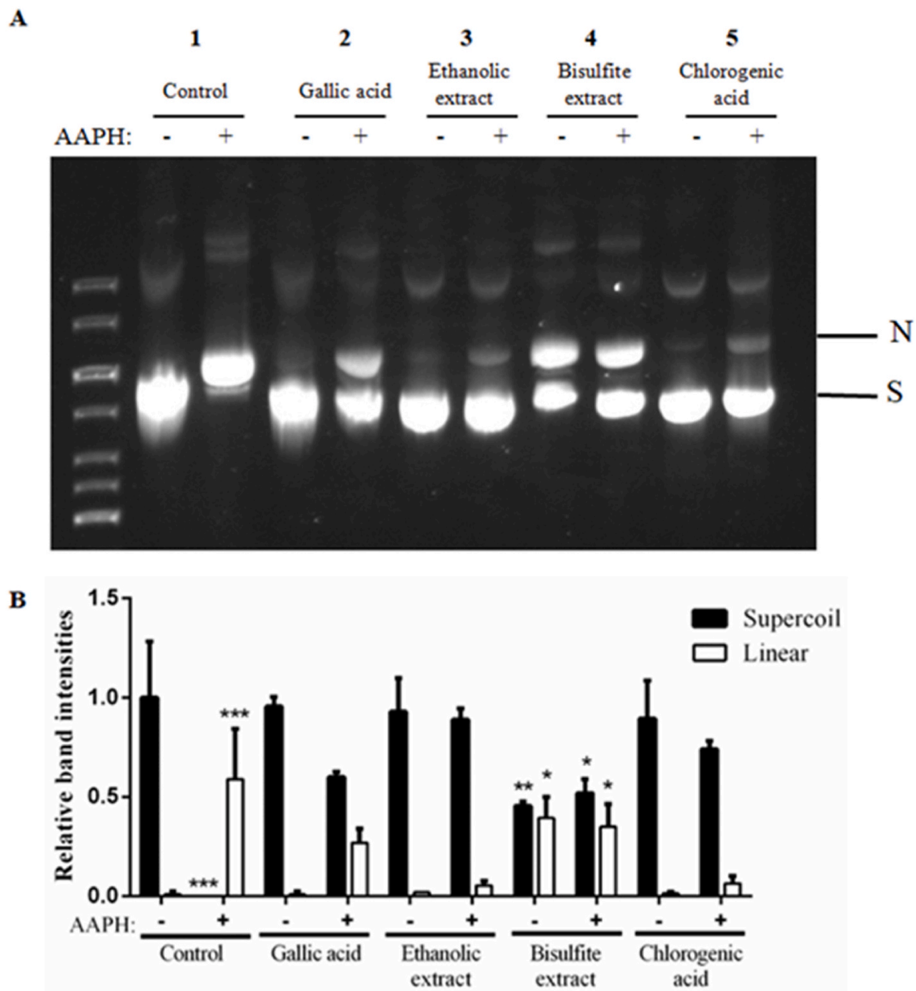

Fig. 2. DNA protective activity of the gallic acid and chlorogenic acid (main phenolic compound present in sunflower) standard, and sunflower phenolic compound extracts elaborated with 70 mL/100 mL ethanol and 0.1 g/100 mL sodium bisulfite in the concentration of 1 μg/L. Lanes: Control (DNA) (1); Gallic acid (60 ± 2% DNA retention) (2); Ethanolic extract (89 ± 6% DNA retention) (3); Bisulfite extract (52 ± 7% DNA retention) (4); Chlorogenic acid (74 ± 4% DNA retention) (5). Data represent the mean ± standard deviation of each sample ($n = 3$). Means with different symbols indicate significant differences ($p < 0.05$). S and N are supercoiled and nicked plasmid DNA strands, respectively.

damage caused to the DNA by carcinogenic agents and also in the control of chronic inflammatory processes (Lewandowska et al., 2016).

The anti-proliferative profiles of the sunflower phenolic compound extracts were evaluated against human tumor lines and one human non-tumor line, at concentrations of 0.25; 2.50; 25.00 and 250.00 μg/mL. Under these experimental conditions they did not present anti-proliferative activity (Fig. 1) since the count remained above the cell death point delimited by the line (zero). Fig. 1A shows the anti-proliferative action of the chemotherapeutic agent doxorubicin employed as the positive control in the experiment.

The results obtained in the anti-proliferative evaluation (Fig. 1B and C) for the sunflower phenolic compounds extracts, showed that the phenolic compounds present in these extracts did not directly inhibit cell proliferation, but since they present antioxidant activity, this suggests that they could act in tumor chemoprevention. *In vivo* carcinogenesis studies could verify this hypothesis.

3.5. DNA protective activity

Both sunflower phenolic extracts showed a DNA protective effect. The highest protection against the DNA damage by AAPH was by ethanolic extract (89% DNA retention), while bisulfite extract presents lower protection (52% DNA retention) (Fig. 2). The antioxidant activity of this extract was also higher (Table 2), which probably resulted in better protection against AAPH radical. The present study used two standards as a positive control (chlorogenic acid and gallic acid). The ethanolic extract had the same protection as the chlorogenic acid (74% DNA retention) and gallic acid (60% DNA retention) standard. Indicating that the chlorogenic acid, the major phenolic compound present in the ethanolic extract, contributed to the DNA protection exhibited. The phenolic compounds present in juice- and winemaking byproducts, that possess gallic and caffeic acids as major phenolic acids, also demonstrated a DNA protective action against peroxyl radical (de Camargo et al., 2014). Further, chlorogenic acid, the main phenolic compound in sunflower, is known for the capacity of inhibiting DNA damage *in vitro* (Shibata et al., 1999). Studies carried out with pro-oxidant agents have shown that they are responsible for the greatest sources of mutagenic DNA alterations, therefore compounds capable of preventing these damages may be preventing the initiation of cancer and other non-transmissible chronic degenerative diseases (Kay et al., 2019).

4. Conclusion

The evaluation of the phenolic compound extracts demonstrated that they can be applied as food additives, exploiting their antioxidant, antimicrobial, and DNA protective activities. These properties make this extract attractive for current desirable and important health benefits regarding several chronic diseases. In general, the ethanolic extract possesses superior activities. The lack of anti-proliferative activity showed that these compounds were not cytotoxic and that the extract should therefore be safe for consumption, although complementary *in vivo* studies must be carried out to provide evidence of the non-occurrence of any toxic effects. The present study aimed to contribute to the future application of a sunflower byproduct, the phenolic compounds, little explored up to now, and hence add value to the productive chain.

CRediT authorship contribution statement

Thaís Dolfini Alexandrino: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Marta Gomes da Silva:** Investigation, Resources, Writing – original draft. **Roseli Aparecida Ferrari:** Conceptualization, Resources. **Ana Lúcia Tasca Gois Ruiz:** Investigation, Resources, Writing – original draft. **Renata Maria Teixeira Duarte:** Investigation, Resources, Writing – original draft. **Fernando Moreira Simabuco:** Investigation, Resources, Writing – original draft. **Rosângela Maria Neves Bezerra:** Investigation, Resources. **Maria Teresa Bertoldo Pacheco:** Conceptualization, Validation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2021.09.007.

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