

Full Length Research Paper

Evaluation of antioxidant capacity of the aqueous extract of *Cynara scolymus* L. (Asteraceae) *in vitro* and in *Saccharomyces cerevisiae*

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The present study evaluated the antioxidant capacity of the aqueous extract of the leaves of *Cynara scolymus* on *Saccharomyces cerevisiae* strains, proficient and deficient in antioxidant defenses, and by *in vitro* methods with 1,1-diphenyl-2-picrylhydrazyl (DPPH•), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•⁺), inhibition of hydrogen peroxide, lipid peroxidation, formation of nitric oxide, and removal of the hydroxyl radical. A simultaneous quantitative analysis to total phenolics, flavonoids and hydrolysable tannin was also performed, and according to the results obtained, phenolic compounds (661.15 ± 20.11 mg GAE/g of E), flavonoids (123.96 ± 11.47 mg R/g of E) and hydrolysable tannin (14.25 ± 3.18 mg GAE/g of E) were detected in the aqueous extract of *C. scolymus*. The aqueous extract at study showed high antioxidant capacity in all antioxidant methods *in vitro* and exhibited significant antioxidant capacity to protect strains of *S. cerevisiae* from oxidative damage induced by hydrogen peroxide. The analysis of the correlation made between the content of phenolic compounds and the different antioxidants *in vitro* methods, indicated that these compounds are mainly responsible for the antioxidant capacity of the aqueous extract of *C. scolymus*. Therefore, this study suggests that the aqueous extract of leaves of *C. scolymus* is a great natural source of compounds with antioxidant capacity.

Key words: Antioxidant capacity, *Cynara scolymus*, *Saccharomyces cerevisiae*, phenolic compounds.

INTRODUCTION

Reactive oxygen (ROS) and nitrogen (RNS) species, as well as other free radicals are potentially capable of producing damage in various biological macromolecules (DNA, proteins and lipids) playing an important role in the etiology of various diseases such as cancer, diabetes, cardiovascular diseases and premature aging (Kryston et al., 2011; Bhattacharya et al., 2011; Liochev, 2013). Some enzymatic antioxidant mechanisms promote the

protection of human cells against the harmful effects of free radicals, but these antioxidant mechanisms may not be enough to combat oxidative stress, which results from the imbalance between production and elimination of free radicals (Jensen, 2003). Accordingly, certain amounts of antioxidant supplements are necessary to ensure the balance of reactive species derived from oxygen and nitrogen produced by pathophysiological metabol

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(Dizdaroglu et al., 2002; Sailaja Rao et al., 2011).

Over the last decade, interest in the search for antioxidants from natural sources has increased and various studies by the scientific community have reported the importance of fruits and vegetables in the prevention of chronic diseases resulting from oxidative stress (Gulcin, 2012). The plants have a rich source of molecules with antioxidant potential, as phenolic compounds, carotenoids, vitamins, flavonoids and terpenoids (Kumar et al., 2012; Ranilla et al., 2010). Therefore, greater attention is turned to the antioxidants of natural origin, which may act by inhibiting lipid peroxidation and/or neutralizing reactive oxygen species and nitrogen, resulting in the modulation of oxidative stress (Fadel et al., 2011).

Cynara scolymus L. (Asteraceae) is a native plant of the Mediterranean (North Africa and southern Europe) and popularly known in Brazil as artichoke. Regardless of its origin in regions of subtropical climates, the *C. scolymus* is cultivated worldwide because of its nutritional benefits and medicinal properties (Lattanzio et al., 2009). Since ancient times, the *C. scolymus* has been cited as a drug in traditional medicine and in much of the world it is attached to beneficial effects in diseases of the biliary tract, digestive action, and help in the treatment of scurvy, anemia and antiesclerotic effect (Gebhardt, 1997; Kraft, 1997; Kucukgergin et al., 2010). The *C. scolymus* is a plant used for medicinal purposes and is considered a healthy food because of its nutritional composition and phytochemistry, which contain minerals, proteins, phenolic derivatives including caffeoylquinic acid (chlorogenic acid and 1.5 dicaffeoylquinic cinarina), tannins, flavonoids and sesquiterpenes in fewer quantities, and various aliphatic acids, especially hydroxy acids (lactic, malic, glycolic) (Pandino et al., 2011; Bundy et al., 2008). Several pharmacological and preclinical studies using the aqueous extract of leaves of *C. scolymus* demonstrated antibacterial, anti-HIV, hepato-protective, hepatostimulant, hypocholesterolemic, choleric, diuretic and antioxidant effects (Joy and Haber, 2007; Miccadei et al., 2008; Lutz et al., 2011; Qiang et al., 2012).

The interest in working with medicinal plants is the therapeutic value they have and because there are few studies on the antioxidant capacity of *C. scolymus*. Thus, this study aimed at providing scientific evidence of the antioxidant capacity of the aqueous extract of leaves of *C. scolymus* through various tests. Tests were conducted to study the antioxidant capacity in *Saccharomyces cerevisiae* proficient and deficient in antioxidant defenses, evaluation of antioxidant capacity *in vitro* method for 1,1-diphenyl-2-picrylhydrazyl (DPPH•), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+), inhibition of hydrogen peroxide and lipid peroxidation (TBARS) removal of the hydroxyl radical (OH•), and inhibition of nitric oxide (NO). Furthermore, a quantitative study on the content of total phenolics, flavonoids and hydrolysable tannin was conducted to assess the contribution of these phenolics to the antioxidant capacity

of the aqueous extract of *C. scolymus*.

MATERIALS AND METHODS

Reagents

Folin-ciocalteu reagent, aluminum chloride, potassium iodide, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hydrogen peroxide, thiobarbituric acid, 2,2'-azobis(2methylpropionamide) dihydrochloride (AAPH), trichloroacetic acid, n-butanol, 2-deoxyribose, malonaldehyde, ferrous sulfate, phosphate buffer, phosphoric acid, sodium nitroprusside, Griess reagent, rutin, quercetin, gallic acid were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Plant material and preparation of extract

The leaves of *C. scolymus* were provided by the processing industry of medicinal plants active life, located in the southeastern of Teresina city, Piauí, in August, 2012. The leaves of *C. scolymus* were dried at room temperature for 2 days and then crushed, milled in a knife mill to obtain 100 g and subsequently subjected to an extraction with water for 10 min by infusion (1/10, plant/solvent). After the treatment, the aqueous filtrate was frozen in liquid nitrogen and concentrated by lyophilization overnight for 3 days in order to obtain the aqueous extract of *C. scolymus* (AECS) in powder form. The yield of the extract under study was calculated by the following equation.

$$\text{Yield (\%)} = (A_1 \times 100) / A_2$$

Where A_1 corresponds to the AECS lyophilized and A_2 correspond to the powdered dried plant material used for extraction with water.

Determination of total phenols

The total phenolic content of AECS was determined according to Singleton et al. (1999) by Folin-Ciocalteu spectrophotometric method, which involves the reduction of Folin-Ciocalteu reagent by phenolic compounds with concomitant formation of a blue colored complex. An aliquot of 200 μ l of AECS at 1000 μ g/ml was mixed with 500 μ l of Folin-Ciocalteu reagent. Then the solution was stirred for 1 min and added 200 μ l of sodium carbonate (15%). The mixture was allowed to stand and the absorbance was measured after 2 h of reaction in spectrophotometer at wave length from 750 nm (nanometers). The analysis was performed in triplicate and results were expressed in mg gallic acid equivalents per gram of extract (mg GAE/g E) using the calibration curve of gallic acid

$$(y = 0.121x + 0.011, r^2 = 0.9819).$$

Determination of total flavonoid

Total flavonoid content was determined by the formation of a complex of aluminum flavonoid using the methodology of Oyedemi et al. (2012). An aliquot of 200 μ l of AECS (1000 μ g/ml) was mixed with 200 μ l of 20% aluminum chloride and two drops of acetic acid. The mixture was allowed to stand and the absorbance was measured after 40 min of reaction using spectrophotometer in length from 415 nm. The analysis was performed in triplicate and results were expressed as total flavonoid rutin milligram equivalent per gram of extract (mg R/g E) using the calibration curve of rutin

($y = 0.0182x - 0.004$, $r^2 = 0.9949$).

Determination of hydrolysable tannin

The hydrolysable tannin content was determined from AECS using the potassium iodide test (Saad et al., 2012). To 3 ml of the aqueous extract of AECS at 1000 µg/ml, 1 ml of a saturated solution of potassium iodide was added and the mixture left to stand at room temperature for 40 min. The absorbance was measured in a spectrophotometer at 550 nm. The analysis was performed in triplicate and results were expressed in mg equivalent of gallic acid per gram of extract (mg GAE/g E) using the calibration curve of gallic acid ($y = 0.121x + 0.011$, $r^2 = 0.9819$).

Antioxidant capacity by DPPH•

It was done as per the methodology described by Silva et al. (2005) with some modifications. Stock solution of AECS (250 µg/ml), of DPPH• (40 µg/ml), and of the standard solutions of gallic acid (250 µg/ml), quercetin (250 µg/ml) and rutin (250 µg/ml) were prepared. Concentrations of 25, 50, 100, 150 and 200 µg/ml of AECS and standards were prepared by dilution. The reaction mixture (0.3 ml of extract with 2.7 ml of a stock solution of DPPH•) was incubated at room temperature for 30 min and the absorbance was measured in a spectrophotometer at 517 nm. The antioxidant evaluation was performed in triplicate and the absorbance values were converted into percentage of antioxidant capacity (% AC) by the following equation.

$$\% \text{ AC} = [(\text{Control Abs.} - \text{Extract Abs.}) \times 100] / \text{Control Abs.}$$

Where, Control Abs. is the initial absorbance of the ethanol solution of DPPH•, and Extract Abs. is the absorbance of the reaction mixture (DPPH• + extract). The inhibitory effective concentration (EC_{50}) needed to extract the DPPH radical• reduced by 50% was determined by probit regression (Locatelli et al., 2009).

Antioxidant capacity by ABTS•+ method

In order to determine the antioxidant capacity by the method of ABTS•+, method described by Re et al. (1999) was used with modifications. Initially, a radical cation ABTS•+ was formed from the reaction of 5 ml of a 7 mM ABTS•+ solution with 88 µl of 2.45 mM potassium persulphate ($K_2S_2O_8$) solution and incubated at room temperature in absence of light for 16 h. After some time, a solution of ABTS•+ was diluted in ethanol to obtain a solution with absorbance of 0.70 (± 0.05) to 734 nm. The final concentrations of 25, 50, 100, 150 and 200 µg/ml of the AECS, gallic acid, quercetin and rutin were prepared. In dark environment, they were transferred at a rate of 40 µl of each sample for test tubes with 1960 µl of the radical ABTS•+. The absorbance readings, at times 1, 4 and 6 min, was done in a spectrophotometer at 734 nm and results were expressed in TEAC values (Trolox Equivalent Antioxidant Capacity) from a standard curve prepared with Trolox ($r^2 = 0.991$). The standard curve was prepared by measuring the decrease in absorbance of the solution ABTS•+ with different concentrations of the Trolox (0.1 and 2 mM).

Inhibition capacity of hydrogen peroxide (H_2O_2)

The ability to inhibit H_2O_2 was determined according to the method of Ruch et al. (1989) with some modifications. A solution of H_2O_2 (40 mM) and of AECS was formed in the same concentrations of DPPH• and ABTS•+ test in phosphate buffer (pH 7.4). 3.5 ml of AECS were added to 0.5 ml of H_2O_2 and the values of the absorbances

at time 10 min was determined in a spectrophotometer at 230 nm. The absorbance of the mixture of the extract with H_2O_2 was converted to percent inhibition of hydrogen peroxide by the following equation.

$$\% \text{ Inhibition of } H_2O_2 = [(\text{Control Abs.} - \text{Extract Abs.}) \times 100] / \text{Control Abs.}$$

Where, Control Abs. is the initial absorbance of the solution of H_2O_2 and Extract Abs. is the absorbance of the reaction mixture (H_2O_2 + extract).

Evaluation of the capacity to inhibit lipid peroxidation (TBARS)

The method used to evaluate the action of AECS in inhibiting lipid peroxidation, a major damage caused by reactive oxygen and nitrogen derivatives, was performed by the determination of substances reactive to thiobarbituric acid, known as TBARS method (Rosa et al., 2011). This method was used to measure the antioxidant capacity of AECS in a medium rich in lipids (Guimaraes et al., 2010). The lipid-rich substrate used was a homogenate of egg yolk 1% (w/v) in 50 mM phosphate buffer (pH 7.4). An aliquot of 0.5 ml of the substrate was sonicated and homogenized with 0.1 ml of AECS in different concentrations (25, 50, 100, 150 and 200 µg/ml). Lipid peroxidation was induced by adding 0.1 ml of AAPH (dihydrochloride of 2,2'-azobis-2 metilpropinamide 0.12 M). The reactions were performed for 30 min at 37°C. After cooling, samples (0.5 ml) were centrifuged in 0.5 ml of trichloroacetic acid (15%) at 1,200 × g for 10 min. An aliquot of 0.5 ml of the supernatant was added to 0.5 ml of thiobarbituric acid (0.67%) and heated at 40°C for 15 min. After heating, 2 ml of n-butanol were added to each test tube, then the tubes were shaken and centrifuged at 2000 rpm for 10 min. The absorbance of the samples was measured using a spectrophotometer at 532 nm. The results were expressed as a percentage of TBARS formed from AAPH only control (induced). Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Evaluation of the capacity of inhibiting the formation of hydroxyl radical (OH•)

The formation of hydroxyl radical from Fenton's reaction was measured using oxidative degradation of 2-deoxyribose (Moravia et al., 2011). The principle of the test is to quantify the degradation product of 2-deoxyribose in malonaldehyde (MDA) by its condensation with thiobarbituric acid (TBA). The reaction was initiated by the addition of Fe^{2+} (6 mM $FeSO_4$) to the solution containing 5 mM of 2-deoxyribose, 100 mM of H_2O_2 and 20 mM phosphate buffer (pH 7.4). To determine the antioxidant AECS against the hydroxyl radical, the AECS different concentrations (25, 50, 100, 150 and 200 µg/ml) were added to the system before addition of Fe^{2+} . The reactions were performed for 15 min at room temperature and stationed by adding phosphoric acid, 4% (v/v) followed by TBA 1% (w/v) in 50 mM NaOH). The solutions were heated for 15 min at 95°C and then cooled to room temperature. The absorbance was measured at 532 nm and results were expressed as equivalents of MDA formed by Fe^{2+} and H_2O_2 . Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Evaluation of ability to inhibit formation of nitric oxide (NO)

In this test, the nitric oxide (NO) is produced from the decomposition of sodium nitroprusside (SNP) in 20 mM phosphate buffer (pH 7.4). Once generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Basu and Hazra, 2006). The reaction mixture (1 ml) containing 10

Table 1. *Saccharomyces cerevisiae* strains used in antioxidant evaluation.

Description	Genotype	Deficiency in enzymatic defenses	Origin
EG103 (Sod wt)	MATa leu2-3,112 trp1-289 ura3-52	None	Edith Gralla, Los Angeles
EG118 (Sod1Δ)	Sod1::URA3 all other markers as EG103	Cu-Zn superoxide dismutase (cystosolic)	Edith Gralla, Los Angeles
EG110 (Sod2Δ)	Sod2::TRP1 all other markers as EG103	Mn superoxide dismutase (mitochondrial)	Edith Gralla, Los Angeles
EG133 (Sod1ΔSod2Δ)	Sod1::URA3 Sod2::TRP1 double mutant all other markers as EG103	Superoxide dismutase cystosolic and mitochondrial	Edith Gralla, Los Angeles
EG223 (Cat1Δ)	EG103, except Cat1:: TRP1	Catalase cystosolic	Edith Gralla, Los Angeles
EG (Sod1ΔCat1Δ)	EG103, except Sod1:: URA3 and Cat1::TRP1	Cu-Zn superóxido dismutase e catalase citosólica	Edith Gralla, Los Angeles

mM of SNP in phosphate buffer and EACS at concentrations of 25, 50, 100, 150 and 200 µg/ml, was incubated at 37°C for 1 h. An aliquot of 0.5 ml was taken and homogenized with 0.5 ml of Griess reagent. The absorbance of the chromophore was measured at 540 nm in a spectrophotometer. The percentage inhibition of NO production was determined by comparing the absorbance values of the negative control (only 10 mM SNP) and preparations of substances used in the test. The results were expressed as percentage of nitrite formed by SNP. Rutin, quercetin, and gallic acid were used as positive control at concentrations of 200 µg/ml.

Antioxidant capacity in *S. cerevisiae* cells

To evaluate the antioxidant capacity of the AECS against oxidative damage induced by hydrogen peroxide, strains of *S. cerevisiae* proficient (Sod wt) and deficient (Sod1Δ, Sod2Δ, Sod1ΔSod2Δ, Cat1Δ, Sod1ΔCat1Δ) were used in antioxidant defense system. The methodology used was of the central disk, according to Fragoso et al. (2008), with modifications. The strains of *S. cerevisiae* proficient and deficient in superoxide dismutase (Sod) and catalase (Cat) were kindly provided by the research group in Genetic toxicology of Federal University of Rio Grande do Sul (UFRGS) and are described in Table 1.

Concentrations of 25, 50, 100, 150 and 200 µg/ml of AECS were used in this study. Three types of treatment for antioxidant evaluation were performed using the strains in the stationary phase (2×10^8 cells/ml) of *S. cerevisiae*: pre-treatment, co-treatment and post-treatment. In the pre-treatment, the concentrations of the extract were first added to a hard filter paper in center of plate yeast extract-peptone-dextrose (YEPD) and three hours later stressor hydrogen peroxide was added. In co-treatment the concentrations were added simultaneously to stressor

hydrogen peroxide in disk filter paper. In the post-treatment hydrogen peroxide was first added and three hours later, the drug concentration. After 48 h incubation at 37°C the halo of growth inhibition in millimeters (mm) was measured from the edge of the disc filter paper to the beginning of cell growth. The values can range from 0 mm (full growth) to 30 mm (no growth), these values being the size of a petri dish. All treatments were performed in triplicate. At each assessment, the strains were streaked on YEPD plate containing the center of a disc filter paper, to which 10 µl extract is added and hydrogen peroxide (40 mM). A control with only hydrogen peroxide was carried out for all strains of *S. cerevisiae*.

Statistical analysis

The antioxidant result is the value of mean \pm standard deviation (SD) of three experiments, and each experiment was performed in triplicate for each treatment. Statistical analysis was performed using analysis of variance (ANOVA) and significant differences between means were determined by Tukey's test, using the GraphPad Prism 5.01. The values of $p < 0.05$ were considered significantly different. To evaluate the relationship between total phenolic content and antioxidant capacity, Pearson correlation coefficient was calculated with 95% confidence using the GraphPad Prism 5.01.

RESULTS AND DISCUSSION

Determination of phenols, flavonoids and hydrolysable tannin

With 18% of yield, the results of the phytochemical

analysis showed the quantitative values of AECS 661.15 \pm 20.11 mg GAE/g E to phenolic compound, 123.96 \pm 12.47 mg R/g E to flavonoids and 14.25 \pm 3.18 mg GAE/g E to hydrolysable tannin. These results demonstrate that the AECS has high quantities of phenolic compounds. Other studies have performed the quantification of phenolic compounds and flavonoids in methanolic and ethanolic extract of leaves of *C. scolymus*, however, in smaller quantities compared to our results for both phenolic and flavonoids compounds (Llorach et al., 2002; Wang et al., 2003; Gouveia and Castilho, 2012; Nassar et al., 2013).

The content of phenolic compounds found in plants may vary during processing steps such as growing, harvesting, storage and technological procedures used (Gil-Izquierdo et al., 2001; Fratianni et al., 2007; Lombardo et al., 2010). The *C. scolymus* is a plant rich in phenolic compounds among which are caffeic, coumarin, hydroxybenzoic and hydroxycinnamic, ferulic and gallic (Hammouda et al., 1991; Fratianni et al., 2007; Seneviratne and Kotuwagedara, 2009; Nassar et al., 2013; Abu-Reidah et al., 2013; Zan et al., 2013; Palermo et al., 2013). The phenolic compounds present in plants have received great attention because of their antioxidant properties and they can potentially interact with biological systems and play an important role in anticancer, anti-inflammatory and antimicrobial activity (Abu-Reidah et al., 2013; Wang et al., 2003). The

Table 2. Antioxidant capacity of the aqueous extract of *Cynara scolymus*, rutin, quercetin and gallic acid by the DPPH• method.

Concentration (µg/ml)	<i>Cynara scolymus</i>	Antioxidant capacity (%)		Gallic acid
		Rutin	Quercetin	
25	11.26±1.07 ^{abc}	25.37±2.15	55.99±2.15	89.87±0.83
50	27.02±1.75 ^{bc}	32.65±1.77	79.59±0.97	92.95±0.10
100	49.69±1.85 ^{bc}	37.96±0.36	81.50±0.25	93.19±0.06
150	72.10±1.06 ^{bc}	60.85±6.33	81.87±0.21	93.71±0.15
200	83.40±0.19 ^c	64.22±1.76	82.18±0.15	93.95±0.47
CE ₅₀	100.70	111.96	10.25	1.20

^asignificant ($P < 0.05$) in relation to rutin. ^b significance ($P < 0.05$) in relation to quercetin. ^csignificance ($P < 0.05$) in relation to gallic acid.

Table 3. Trolox equivalent antioxidant capacity (TEAC) of the aqueous extract of *Cynara scolymus*, rutin, quercetin and gallic acid.

Sample	TEAC, mM of Trolox		
	1 min	4 min	6 min
<i>Cynara scolymus</i>	3.45±0.26 ^c	4.53±0.15 ^c	5.88±0.11 ^c
Rutin	1.45±0.10	1.75±0.10	1.89±0.05
Quercetin	3.76±0.11	5.01±0.23	6.05±0.25
Gallic acid	8.03±0.13	12.84±0.08	13.21±0.03

^asignificance ($P < 0.05$) in relation to rutin. ^bsignificance ($P < 0.05$) in relation to quercetin. ^csignificance ($P < 0.05$) in relation to gallic acid.

antioxidant properties are attributed to flavonoids due to their hydroxyl groups that can act as free radical scavengers, reducing agents and metal chelation (Agati et al., 2012). Flavonoids are natural chemical constituents which may be found in larger quantities and play an important role in the pharmacological properties attributed to *C. scolymus* (Häusler et al., 2002; Zhu et al., 2004; Pandino et al., 2011). Among the flavonoids already found in extracts of *C. scolymus* may be cited luteolin, apigenin, myricetin, quercetin and rutin (Zan et al., 2013; Palermo et al., 2013).

Antioxidant capacity by DPPH• method

The DPPH• is a stable organic radical which has been widely used in studies to evaluate the antioxidant capacity of plant extracts or pure compounds. In this method, the antioxidant capacity is determined by the analysis of the decrease of absorbance of DPPH• solution at 517 nm in a spectrophotometer, in which the DPPH radical of purple color is reduced to form DPPH₂ of yellow (Mishra et al., 2012). Therefore, the AECS showed good antioxidant capacity by reducing DPPH• radical in a concentration-dependent manner (Table 2), wherein the values in percent of the antioxidant capacity of AECS at

concentrations of 100, 150 and 200 µg/ml were significantly superior ($p < 0.05$) when compared to the values of antioxidant capacity of rutin at the same concentrations. When the antioxidant capacity of AECS is compared with the positive control quercetin at the concentrations of 25, 50, 100 and 150 µg/ml, it is observed that the values were significantly lower ($p < 0.05$). In contrast, at the concentration of 200 µg/ml, the AECS presented antioxidant capacity higher than quercetin. Compared to the control gallic acid, the values of antioxidant capacity at the concentrations of 25, 50, 100, 150 and 200 µg/ml were significantly lower ($p < 0.05$). The results of the EC₅₀ of AECS, rutin, quercetin and gallic acid are shown in Table 2, being observed that the AECS was more effective in reducing of DPPH• radical than the control rutin.

The evaluation of antioxidant by DPPH• method has already been accomplished by Llorach et al. (2002) with the AECS using only the concentration of 10 µg/ml and with the reaction time of 1 h. In another study by Wang et al. (2003), it was shown that the extracts of three different varieties of *C. scolymus* presented good antioxidant capacity against DPPH• radical, which was attributed to the phenolic compounds with correlation $r^2 = 0.96$. Zan et al. (2013) using the AECS also achieved satisfactory results for the antioxidant capacity by the method of DPPH•.

Antioxidant capacity by the method of ABTS•+

Like DPPH•, the method ABTS•+ or TEAC is widely used to evaluate the antioxidant capacity of a variety of substances including plant extracts. The TEAC values of the AECS were obtained at three different times and compared to the TEAC of positive control rutin, quercetin and gallic acid (Table 3). As shown in Table 3, the values of TEAC of the AECS for the times of 1, 4 and 6 min were significantly ($p < 0.05$) higher when compared with the TEAC of the positive control rutin. Regarding the TEAC values of quercetin and gallic acid for the times 1, 4 and 6 min, it was observed that the TEAC values of the AECS

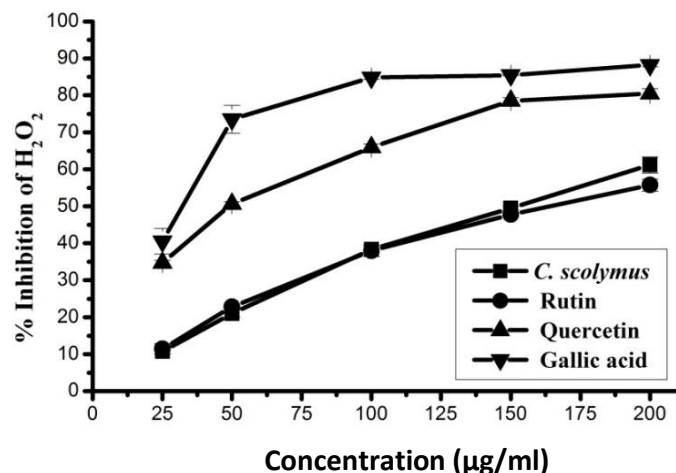


Figure 1. Percent inhibition of hydrogen peroxide from the aqueous extract of *Cynara scolymus* of positive controls rutin, quercetin and gallic acid. Each value represents the mean \pm standard deviation ($n = 3$).

were lower, however, only relative to gallic acid was observed significant difference ($p < 0.05$). The TEAC value takes into account the capacity of a substance to react with ABTS^{•+} radical (Arts et al., 2004). Thus, this study demonstrated that the AECS was capable of reacting with the ABTS^{•+} radical, showing antioxidant capacity better than rutin. Wang et al. (2003) using only the concentration of 10 $\mu\text{g/ml}$ also showed antioxidant activity against ABTS^{•+} radical. Betancor-Fernández et al. (2003) also evaluated the antioxidant capacity of the AECS by the TEAC method and the results obtained were similar to those in this study.

Inhibition capacity of H₂O₂

Hydrogen peroxide is a type of ROS which can be formed *in vivo* by many oxidizing enzymes, through the membranes and slowly oxidize a large number of biological molecules (Halliwell, 1991). The capacity of AECS to inhibit the hydrogen peroxide was determined according to the method of Ruch et al. (1989) and the results were 10.75 ± 0.61 , 21.0 ± 0.97 , 38.34 ± 0.89 , 49.50 ± 0.46 and $61.18 \pm 2.20\%$ for concentrations of 25, 50, 50, 100, 150 and 200 $\mu\text{g/ml}$, respectively (Figure 1). The AECS was able to inhibit the hydrogen peroxide in a concentration-dependent manner, and the results of AECS were significantly lower ($p < 0.05$) in relation to results of quercetin and gallic acid. Furthermore, the results of AECS at concentrations higher than 100 $\mu\text{g/ml}$ were superior to those of rutin, which showed values 11.47 ± 1.42 , 22.83 ± 0.54 , 37.98 ± 1.57 , 47.78 ± 0.33 and $55.73 \pm 1.60\%$ of inhibition of hydrogen peroxide in concentrations of 25, 50, 50, 100, 150 and 200 $\mu\text{g/ml}$,

respectively. No other study that addresses the action of AECS in the inhibition of the hydrogen peroxide was found, which makes this study the first.

Inhibition of lipid peroxidation (TBARS)

Free radicals are responsible for the chain reactions that cause lipid oxidation, inducing functional loss of biomembranes and various types of damage to essential biological molecules such as proteins and nucleic acid bases and, thus, having been implicated in the pathogenesis of various diseases (Niki, 2009). The TBARS method has been widely used to evaluate the extent of the *in vitro* lipid peroxidation, in which the oxidation of unsaturated fatty acids occurs from a source rich in lipids (Niki, 2010). Thus, the AECS at concentrations of 25, 50, 50, 100, 150 and 200 $\mu\text{g/ml}$ was able to prevent lipid peroxidation by reduction of the levels of TBARS in 83.5, 83.95, 84.57, 86.56 and 87.6% (Figure 2). Similar results were obtained with the controls rutin, quercetin and gallic acid, which decreased the levels of TBARS in 85.25, 87.13 and 85.15%, respectively (Figure 2). As the positive controls, rutin, quercetin and gallic acid, the AECS significantly ($p < 0.05$) reduced the levels of TBARS formed by AAPH.

The percentage of inhibition of peroxidation shown in this study decreased according to the increase in the concentration of AECS. Besides, the concentration of 200 $\mu\text{g/ml}$ presented a result of inhibition of lipid peroxidation greater than the controls rutin, quercetin, and gallic acid. Thus, the AECS may be considered as a good inhibitor of lipid peroxidation in this experimental model. In addition, studies that have used the TBARS method for evaluation of the inhibition of lipid peroxidation by AECS were not found. However, one study was conducted with AECS, whose capacity to inhibit lipid peroxidation was demonstrated through ferric thiocyanate method (Wang et al., 2013).

Inhibition of hydroxyl radical (OH[•])

Among the ROS, hydroxyl radicals are the more reactive, being that these radicals can react with a wide variety of biomolecules and consequently induce severe cell damage and mutation, cell death, carcinogenesis and aging (Li et al., 2013). In the present study, the hydroxyl radicals were generated from the Fenton reaction, and as shown in Figure 3, the AECS at concentrations of 25, 50, 100, 150 and 200 $\mu\text{g/ml}$ was able to significantly remove the hydroxyl radical ($p < 0.05$) (Figure 3). At the concentration of 200 $\mu\text{g/ml}$, the positive controls rutin, quercetin and gallic acid presented the values 59.02, 52.77 and 64.75% of inhibition of the hydroxyl radical, respectively (Figure 3). According to these results, the AECS may be considered a good hydroxyl radical inhibitor.

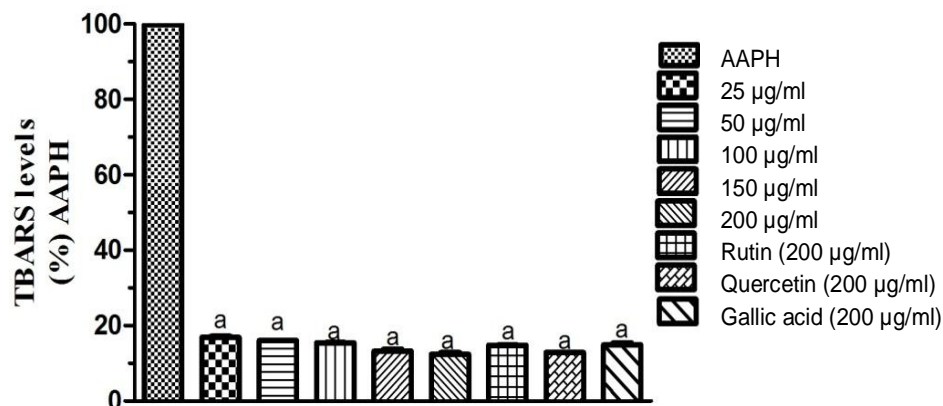


Figure 2. Inhibition of TBARS formed *in vitro* by AECS, rutin, quercetin, and gallic acid. Each value represents the mean \pm standard deviation ($n = 3$). The significance ($P < 0.05$) is relative to vehicle AAPH.

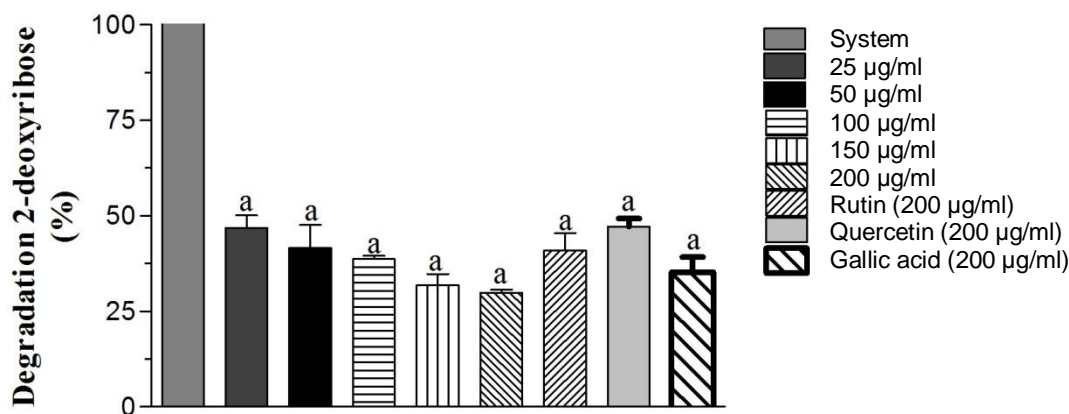


Figure 3. Inhibiting the formation of hydroxyl radical by AECS, rutin, quercetin and gallic acid. Each value represents the mean \pm standard deviation ($n = 3$). The significance ($P < 0.05$) compared to the System (does not contain the AECS).

inhibitor. The innovator character of this study can be expressed by the absence of reports that evaluate the antioxidant capacity of AECS in the inhibition of the hydroxyl radical.

Inhibition of nitric oxide (NO)

Nitric oxide is considered species reactive derivative of nitrogen, which if produced in excess can contribute to the development of pathologies such as chronic inflammation, cancer and autoimmune diseases (Muntane et al., 2013). The measure of antioxidant capacity by the elimination of nitric oxide was based on the principle that the sodium nitroprusside (SNP) in aqueous solution at physiological pH spontaneously generates nitric oxide,

which interacts with oxygen to produce nitrite ions (NO_2^-), which can be calculated by means of the Griess reagent. Thus, the AECS showed inhibitory capacity of nitric oxide generated by sodium nitroprusside, as for the concentrations of 25, 50, 100, 150 and 200 $\mu\text{g/ml}$, the values in percentage were 73.17, 77.46, 78.95, 79.42 and 80.18%, respectively (Figure 4). These outcomes also demonstrate a concentration-dependent and significantly ($p < 0.50$) different inhibitory capacity of AECS relative to SNP. When the inhibition of nitric oxide is compared with the controls rutin (79.25%), quercetin (76.8%) and gallic acid (74.62%), the concentrations of 150 and 200 mg/ml of AECS exhibited superior results than the positive controls. Thus, the extract of the *C. scolymus* at study can be considered as a good inhibitor of nitric oxide. Like the methods for evaluating of inhibition of

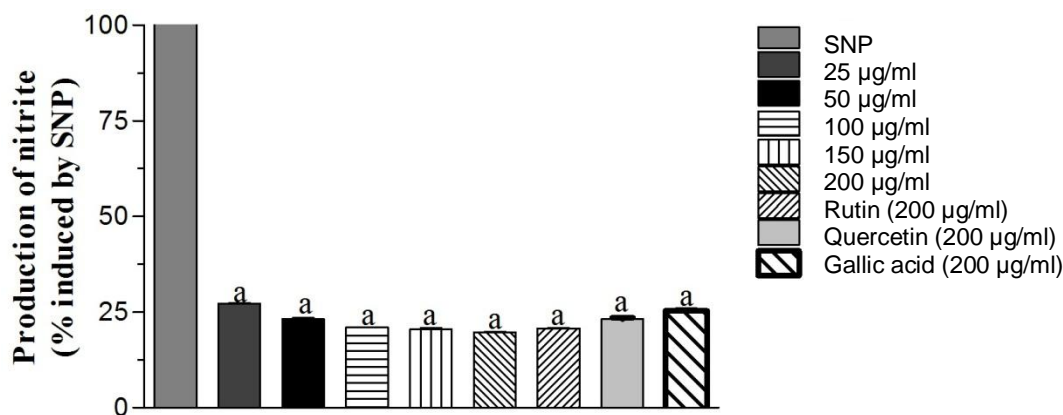


Figure 4. Inhibition of nitric oxide by AECS, rutin, quercetin, and gallic acid. Each value represents the mean \pm standard deviation ($n = 3$). The significance ($P < 0.05$) compared to SNP.

Table 4. Pearson correlation coefficient (r) between the content of phenolic compounds and antioxidant capacity.

Correlation	r	r (%)
Total phenols vs. DPPH•	0.989	98.9
Total phenols vs. ABTS•+	0.992	99.2
Total phenols vs. Inhibition of H_2O_2	0.983	98.3
Total phenols vs. TBARS	0.984	98.4
Total phenols vs. Hydroxyl	0.960	96.0
Total phenols vs. Nitrite	0.854	85.4

Table 5. Pearson correlation coefficient (r) between the different evaluation methodologies for antioxidant capacity.

Correlation	r	r (%)
DPPH• vs. ABTS•+	0.999	99.9
DPPH• vs. Nitrite	0.899	89.9
DPPH• vs. TBARS	0.966	96.6
DPPH• vs. Hydroxyl	0.971	97.1
DPPH• vs. Inhibition of H_2O_2	0.996	99.6
ABTS•+ vs. Nitrite	0.943	94.3
ABTS•+ vs. TBARS	0.996	99.6
ABTS•+ vs. Hidroxila	0.875	87.5
ABTS•+ vs. Inhibition of H_2O_2	0.991	99.1
Nitrite vs. TBARS	0.785	78.5
Nitrite vs. Inhibition of H_2O_2	0.925	92.5
Nitrite vs. Hidroxila	0.893	89.3
Hydroxyl vs. TBARS	0.964	96.4
Hydroxyl vs. Inhibition of H_2O_2	0.960	96.0

hydrogen peroxide and hydroxyl radical, the study presents itself as the first report on the evaluation of AECS in the inhibition of nitric oxide.

Correlation between the total phenolic content and antioxidant capacity

Phenolic compounds represent an important class of antioxidant compounds which are found in many plants. In this context, the correlation between the concentration of total phenolics and antioxidant capacity has been widely studied involving extracts of plants (Mulinacci et al., 2004; Wang et al., 2003; Llorach et al., 2002; Toma et al., 2013). Studies involving extracts of *C. scolymus* demonstrated that there is a linear correlation between phenolic content and antioxidant capacity (Mulinacci et al., 2004; Toma et al., 2013). In order to determine the contribution of the phenolic content in AECS on antioxidant capacity, the Pearson correlation coefficient (r) was determined. According to the results, strong positive correlations were observed between the concentration of phenolic content and antioxidant capacity of all antioxidants *in vitro* methods (Table 4). Therefore, the results suggest that phenolic compounds may exert an important contribution to *in vitro* antioxidant capacity of the AECS. The highest correlations were observed for the *in vitro* antioxidant methods DPPH• and ABTS•+, in which the two organic radicals are easily reduced in the presence of phenolic compounds. Phenolic compounds have been mainly responsible for the antioxidant capacity of natural extracts and probably due to their redox properties, they act as reducing agents, hydrogen donors and singlet oxygen scavengers (Letelier et al., 2008).

Although the mechanisms of reactions are different for the antioxidant evaluation methods employed in this work, a correlation between the *in vitro* results for the antioxidant capacity was determined and it is shown in Table 5. Positive correlations ($r > 0$) were observed between the antioxidants results, indicating that the antioxidants *in vitro* methods provide comparable values when they are used to determine the antioxidant capacity

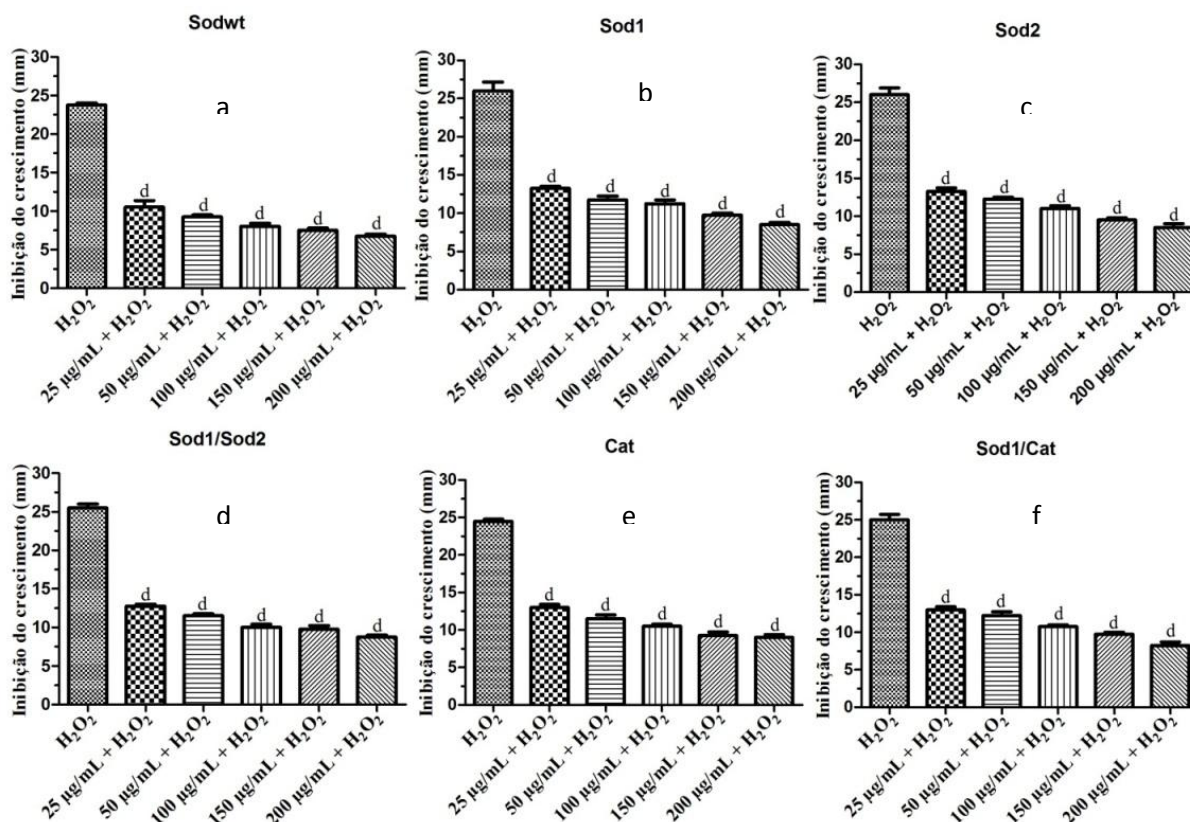


Figure 5. Antioxidant capacity of the aqueous extract *Cynara scolymus* against oxidative damage induced in strains of *Saccharomyces cerevisiae* pre-treatment. Significance ($P < 0.05$) compared to the stressor (H_2O_2).

of AECS. The best correlation was for DPPH• vs. ABTS•+ in which the two tests have the same mechanism in a reaction with a substance which has antioxidant properties. The lowest correlation was found for inhibition of nitric oxide vs. TBARS, however, the results on AECS antioxidants are still considered a good correlation.

Antioxidant capacity in strains of *S. cerevisiae*

In the pre-treatment, co-treatment and post-treatment (Figures 5 and 6), it was possible to observe a trend to decrease in growth inhibition and the consequent increase in survival of strains of *S. cerevisiae* (wt Sod, Sod1 Δ , Sod2 Δ , Sod1 Δ Sod2 Δ , Cat1 Δ , Sod1 Δ Cat1 Δ) at all concentrations of AECS was statistically significant ($p < 0.05$) compared to the results on stressor hydrogen peroxide. In the pre-treatment and co-treatment, the AECS showed significant antioxidant capacity for all strains of *S. cerevisiae* in a dose-dependent manner, inhibiting the oxidative effect of hydrogen peroxide and allowing the enhancement of the survival of strains tested. The concentration of 200 $\mu\text{g}/\text{ml}$ was the most protective having the greatest antioxidant capacity, indicating that higher concentrations of AECS are required to nullify

required to nullify the oxidizing effects of hydrogen peroxide. The strain of *S. cerevisiae* wild (Sodwt) showed a higher level of survival in the pre-treatment and co-treatment, while the strains of *S. cerevisiae* deficient in antioxidant defenses were more sensitive to hydrogen peroxide, suggesting the importance of superoxide dismutase in cellular protection against oxidative stress. Importantly, antioxidant capacity, was observed on AECS Sodwt lineage as well as in strains deficient in superoxide dismutase (Sod1 Δ , Sod2 Δ and Sod1 Δ Sod2 Δ), which suggests that the protective effect may increase the activity of superoxide dismutase, demonstrating the key role of the aqueous extract of the leaves of *C. scolymus* in cellular antioxidant defense. In addition, the protection afforded against hydrogen peroxide seems to be also required for the double mutant strain and mutant Cat1 Δ Sod1 Δ Cat1 Δ because they were able to acquire tolerance to oxidative stress with decreasing inhibition of growth in pre-treatment and co-treatment.

In the post-treatment (Figure 7), the strains of *S. cerevisiae* were first exposed to oxidative damage caused by hydrogen peroxide, and after three hours the AECS was added at various concentrations for the verification of the behavior of strains of *S. cerevisiae*. The data obtained in the post-treatment showed a behavior similar to the pre-

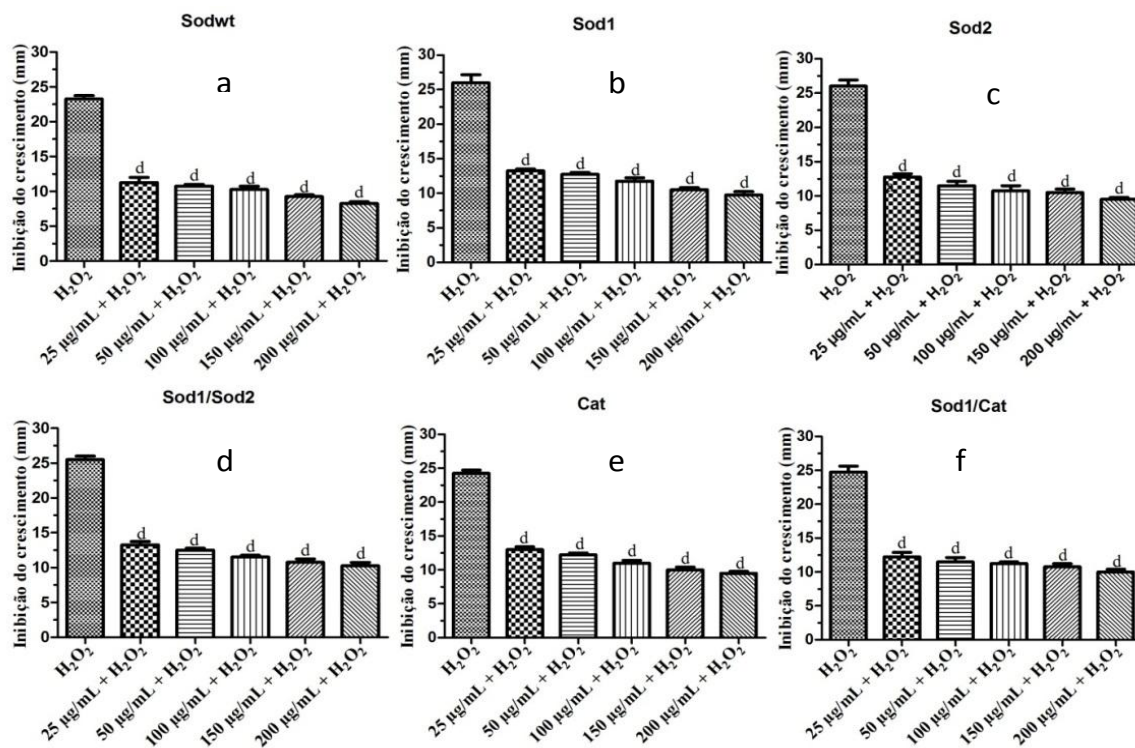


Figure 6. Antioxidant capacity of the aqueous extract of *Cynara scolymus* against oxidative damage induced in strains of *Saccharomyces cerevisiae* co-treatment. Significance ($P < 0.05$) compared to the stressor (H_2O_2).

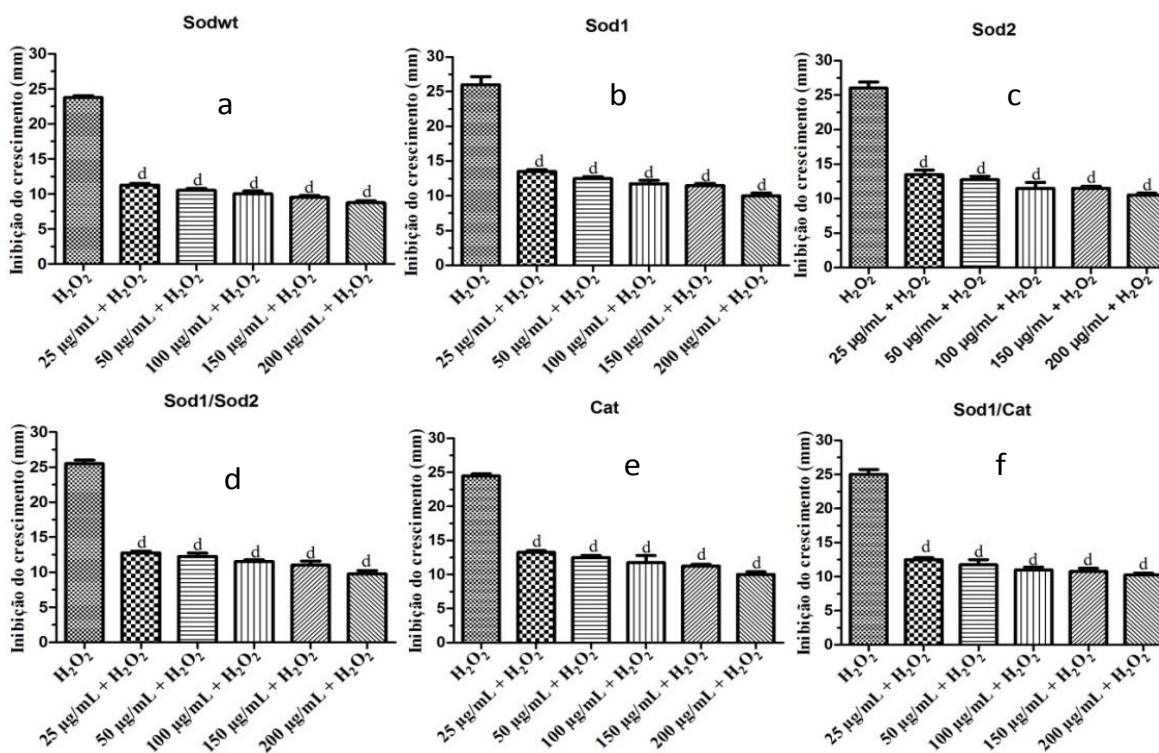


Figure 7. Antioxidant capacity of the aqueous extract of *Cynara scolymus* against induced oxidative damage in lines *Saccharomyces cerevisiae* after treatment. Significance ($P < 0.05$) compared to the stressor (H_2O_2).

treatment and co-treatment, where the strains of *S. cerevisiae* in the presence of AECS showed an increase in survival rate with a decrease in growth inhibition at all concentrations tested as compared with the results of stressor hydrogen peroxide. It can also be observed that the strain of *S. cerevisiae* wild (Sodwt) was the one with the lowest rate of growth inhibition when compared with the mutant strains. The strains Sod1 Δ and Sod2 Δ Sod1 Δ Sod2 Δ also had an increased survival with decreased growth inhibition when post-treated with the AECS at concentrations of 25, 50, 100, 150 and 200 μ g/ml. This antioxidant effect is also evident for the mutant and double mutant Cat1 Δ and Sod1 Δ Cat1 Δ at all concentrations tested significantly ($p < 0.05$) in comparison to the results on hydrogen peroxide. In addition, all strains of *S. cerevisiae* showed a lower rate of inhibition at a concentration of 200 μ g/ml compared with the inhibition induced by hydrogen peroxide.

Conclusion

The results obtained in the present work clearly indicate that AECS has considerable antioxidant capacity *in vitro* and in strains of *S. cerevisiae*. The antioxidant capacity of this extract can be attributed to the phenolic compounds in accordance with the correlations made with the *in vitro* evaluation of antioxidant capacity. Consequently, our results suggest that the aqueous extract may be used as a natural source of antioxidants, since there is a current need for the provision of new bioactive molecules derived from plants to the development of new drugs with antioxidant.

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