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# Enzymatic treatment improves the antioxidant and antiproliferative activities of *Adenanthera pavonina* L. seeds

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ABSTRACT

We evaluated the effect of enzymatic treatment by amylase, cellulase and protease on antioxidant and antiproliferative activities of *Adenanthera pavonina* L. seeds. The effect was evaluated by using an experimental mixture design. The results showed that the antioxidant activity of the seeds increased after the enzymatic treatment. The enzymatic treatment using protease and cellulase (1:1, v/v) showed the highest values of antioxidant activity due to the increase of phenolic compounds content, mainly phenolic acids. The antiproliferative activity of the extract obtained by optimum enzymatic treatment was evaluated. We found the antiproliferative activity against the tumour lineage of prostate (PC-3) and kidney (786–0) was improved after enzymatic treatment. This work provides information that may be useful in handling of functional foods and/or drugs, since the functional properties of seeds such as antioxidant and antitumor activities were improved by enzymatic treatment with protease and cellulase.

## 1. Introduction

In the last few decades, several scientific studies have shown that the daily intake of plant-foods with high content of bioactive compounds is a key factor to promote well-being and health. The antioxidant activity of these plant-foods have been associated with the presence of bioactive compounds such as phenolic compounds, which are also related to the prevention of cancer, inflammation, depression, cardiovascular and neurodegenerative diseases (Justino et al., 2016; Khalaj et al., 2013). The beneficial effects of phenolic compounds can be attributed to their antioxidant activity that protects cells against oxidative stress by neutralizing free radicals, hydrogen donating activities and electrons and chelating metal cations (Dolatabadi et al., 2016; Farzaneh and Carvalho, 2017). Recent studies have shown that anticancer drugs marketed between 1981 and 2010 were mainly produced from natural sources or their derivatives (Newman and Cragg, 2012), showing the importance of research about the anticarcinogenic activity linked to natural products. Therefore, there is great interest to discover new sources of natural bioactive compounds and their applications (Arruda et al., 2017).

*Adenanthera pavonina* L. is an exotic tree native from tropical Asia and it belongs to the family of Fabaceae. This plant is popularly known as red-bead tree, dragon's eye and coralwood (Jaromin et al., 2011). Previous studies have demonstrated several biological properties of the *A. pavonina* L. seeds, such as anti-inflammatory (Jayakumari et al., 2012), anticonvulsant (Oni et al., 2009), antihypertensive (Adedapo et al., 2009), antidiabetic (Pandhare and Sangameswaran, 2012), antioxidant (Mujahid and Mujahid, 2015), antimicrobial and anticancer activity (Chauhan et al., 2015). Although the seeds of *A. pavonina* L. have several popular medicinal uses, their bioactive compounds are not fully known.

The extraction procedure is one of the most important steps in the recovery of phenolic compounds from plant-based materials and it has an important effect on the yield of target compound, as well as on the

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functional potential of the extract (Azmir et al., 2013; Prasad et al., 2011). Normally, they are recovered by solid–liquid extraction employing solvents such as water, organic or hydroalcoholic (Boulila et al., 2015). However, new techniques have been developed to improve the recovery of bioactive compounds from plants, such as cold pressing, super-critical fluid, solvent extraction and enzyme-assisted extraction (EAE) (Puri et al., 2012).

EAE is an emergent extraction method in which enzymes are used to facilitate the recovery of bioactive compounds and as an alternative to replace conventional solvent-based extraction methods due to its low environmental impact, since non-toxic solvents are used. In addition, EAE has been demonstrating to be a faster extraction method with high recovery and lower energy consumption when compared to non-enzymatic methods (Liu et al., 2016; Puri et al., 2012). This process consists of the addition of specific enzymes in the system, under mild processing conditions, to catalyse reaction with specificity and regioselectivity, and increases the recovery of bioactive compounds from plant materials by the degradation or breaking down the structures of the cell walls components (cellulose, hemicellulose, lignin and pectin) of plants and, consequently, releasing bioactive compounds (Gardossi et al., 2010; Pinelo et al., 2006). Thus, after the degradation of the cell wall the intracellular materials become more accessible for extraction and recovery (Li et al., 2006).

Different enzymes such as cellulase, protease, amylase, pectinase and hemicellulase have been used to recovery of a variety of products (Puri et al., 2012). Researches have used α-amylase to degrade starch to release ferulic acid of barley (J. M. Zupfer et al., 1998) and the combination of the amylase, cellulase and protease enzymes improved the yield of oil extraction from rice bran and wheat (Fang and Moreau, 2014; Sharma and Gupta, 2001).

In this approach, this study aimed at evaluating the effect of enzymatic treatment on antioxidant and antiproliferative activities of *A. pavonina* L. seeds. In addition, the extracts were characterized by spectrophotometric and HPLC-ESI-(−)-MS/MS analysis.

## 2. Material and methods

### 2.1. Chemicals, reagents, and enzymes

The enzymes, solvents and reagents used in this study were purchased from Sigma Aldrich (Steinheim, Germany) with analytical grade. Phenolic compound standards with a purity of ≥96% were purchased from Sigma-Aldrich (St. Louis, USA). Doxorubicin hydrochloride was purchased from Europharma (Sao Paulo, Brazil). All solutions were prepared with ultrapure water (18 MΩ $cm^{-1}$) obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

### 2.2. Plant material and sample preparation

The morphologically perfect seeds of *Adenanthera pavonina* L. were collected in November 2016. Seeds collection was carried out in natural areas located in the Fortaleza (03º43'02" south latitude, 38º32'35" west longitude and 21 m altitude), Ceará, Brazil. The seeds were transported to the laboratory up to 48 h after collection. The seeds free of defects were washed with distilled water, dried with paper towel and ground using a knife grinder (Marconi, model MA340, Piracicaba, Brazil). The powder obtained was placed in vacuum packs and stored at −20 °C until analysis. The Genetic Heritage Management Board (CGen) under number ADEFC9F, following the Law nº 13.123/2015 and its regulations, regimented the activity of access to Genetic Heritage.

### 2.3. Enzymatic treatment and experimental mixture design

#### 2.3.1. Enzymatic treatment

Aliquots (5 g) of the seed powder were mixed with phosphate buffer (100 mM pH 7, 45 mL) and different blends of enzymes (Table 1). The mixture was incubated for 2 h at 50 °C and 100 rpm using a water bath shaker, followed by rapid cooling to stop the enzymatic reactions, freeze-drying (LIOTOP, model L101, São Carlos, Brazil) and stored at −20 °C until analysis.

**Table 1**
- Matrix of experimental mixture design in *A. pavonina* L. seeds using different enzymatic preparations.

| Runs | Protease | α-Amylase | Cellulase | Protease volume (μL) | α-Amilase volume (μL) | Celulase volume (μL) |
|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 250 | 0 | 0 |
| 2 | 0 | 1 | 0 | 0 | 250 | 0 |
| 3 | 0 | 0 | 1 | 0 | 0 | 250 |
| 4 | 1/2 | 1/2 | 0 | 125 | 125 | 0 |
| 5 | 1/2 | 0 | 1/2 | 125 | 0 | 125 |
| 6 | 0 | 1/2 | 1/2 | 0 | 125 | 125 |
| 7 | 1/3 | 1/3 | 1/3 | 85 | 85 | 85 |
| Untreated | 0 | 0 | 0 | 0 | 0 | 0 |

#### 2.3.2. Experimental mixture design and statistical analysis

In order to evaluate the influence of enzymatic treatment on the seed powder characteristics, an experimental mixture design was proposed based on Neto et al. (2010). The enzymatic treatment process using the commercial protease (Neutrase® 0.8 L from *Bacillus amyloliquefaciens*), cellulase (Optimase® 56 L from *Bacillus subtilis*), α-amylase (Termamyl® 2X from *Bacillus licheniformis*) and the seed powder as substrate included seven assays with components evaluated at 4 levels: 0 (0%), 1/3 (33%), 1/2 (50%), and 1 (100%) (Table 1).

All the experiments performed were analysed comparatively with their respective enzymatically untreated sample. Special cubic or quadratic regression models were fitted for the variations of antioxidant activities studied as a function of significant ($p \leq 0.10$) interaction effects between the different proportions of protease, α-amylase and cellulase, thereby obtaining acceptable determination coefficients ($R^2 \geq 0.85$). Eq. (1) represents these models as follows:

$$Yi = \sum_{i=1}^{q} \beta_i X_i + \sum \sum_{i<j}^{q} \beta_{ij} X_i X_j + \sum \sum_{i<j<k}^{q} \sum \beta_{ijk} X_i X_j X_k$$

where $Y_i$ is the predicted response (ORAC, TEAC and DPPH); $q$ represents the number of components in the system; $X_i$, $X_j$, $X_k$ are the coded independent variables; $\beta_i$, $\beta_{ij}$ and $\beta_{ijk}$ are the model regression coefficients (linear, binary and ternary interaction, respectively) (de Castro et al., 20 + 17).

The statistical significance of each regression coefficient was determined using Student's *t*-test ($p \leq 0.10$) and the polynomial regression model was evaluated by analysis of variance (ANOVA) ($p \leq 0.05$) using STATISTICA software (Statsoft, Oklahoma, USA) version 13.3 (de Castro et al., 2017).

### 2.4. Screening of the extraction solvent

The antioxidant compounds from enzymatically treated and untreated seeds were extracted using different solvents, namely water, ethanol, methanol, ethanol:water (1: 1, v/v), and methanol:water (1: 1, v/v). The freeze-dried sample was mixed with solvent at the ratio of 1:10 (w/v). The mixture was incubated in a water bath shaker for 30 min at 25 °C and 150 rpm. The samples were centrifuged at 600 *g* for 6 min at 5 °C, and the supernatants were filtered with a 0.20 μm filter. The filtered extracts were analysed by antioxidant assays (Arruda et al., 2017).

### 2.5. Antioxidant activity assays

The antioxidant activity was measured by DPPH, TEAC and ORAC assays according to Leite-Legatti et al. (2012a). The results were expressed as μmol TE/g freeze-dried extract (dw).

### 2.6. Phenolic compounds measurement

The total phenolic compound content (TPC), total flavonoid content (TFC), and condensed tannin content (CTC) were measured spectrophotometrically according to Arruda et al. (2017) from enzymatically treated and untreated seeds in the best enzymatic process conditions.

The phenolic compounds of freeze-dried samples were identified and quantified by HPLC-ESI-(−)-MS/MS using an HPLC system connected to a triple quadrupole mass spectrometer (LCMS 8040; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source according to the method described by Pereira et al. (2018). Quantification of individual phenolics was carried out by integrating the peak areas and using the calibration curves. The results were expressed as micrograms of each phenolic compound per gram of dry weight (μg/g dw).

### 2.7. Antiproliferative activity

The antiproliferative activity of the enzymatically treated seeds at the best enzymatic process conditions was investigated on ten human tumour cell lines [glioma (U251), breast (MCF-7), multidrug resistant ovary carcinoma (NCI-ADR/RES), kidney (786–0), non-small cell lung cancer (NCI-H460), prostate (PC-3), colon adenocarcinoma (HT-29), leukemia (K562), melanoma (UACC-62), ovarian (OVCAR-3)] provided by the Frederick Cancer Research & Development Center National Cancer Institute – Frederick, MA, USA. Samples were also evaluated against one spontaneously transformed keratinocytes from histologically normal skin (HaCaT cell line) kindly provided by Prof. Dr. Ricardo Della Coletta (University of Campinas, UNICAMP). Stock cultures were grown in 5 mL of RPMI-1640 supplemented with 5% fetal bovine serum (RPMI/FBS 5%) and penicillin:streptomycin mixture 1000 U/mL:1000 μg/mL (1 mL/L RPMI-1640), named complete medium (de Araujo et al., 2013).

Freeze-dried samples (10 mg) were diluted in DMSO (100 μL) at a concentration of 0.1 g/mL and then successively diluted in complete medium affording the final concentration 0.25, 2.5, 25 and 250 μg/mL (Leite-Legatti et al., 2012b). As a positive control we used the chemotherapeutic doxorubicin at concentrations of 0.025, 0.25, 2.5 and 25 μg/mL (100 μL/compartment) in triplicate.

Cells were exposed in 96-well plates (100 μL cells/well) to different concentrations of samples (0.25, 2.5, 25 and 250 μg/mL) in triplicate, for 48 h at 37 °C and 5% of $CO_2$. Final DMSO concentration (< 0.25%) did not affect cell viability. Before ($T_0$ plate) and after ($T_1$ plates) sample addition, cells were fixed with 50% trichloroacetic acid (TCA, Sigma-Aldrich®, EUA). Cell proliferation was evaluated by total protein content quantitation using sulforhodamine B and absorbance analysis at 540 nm (Monks et al., 1991). The $GI_{50}$ value (concentration that inhibit 50% of cell growth) was determined through non-linear regression, type sigmoidal, analysed using Origin 8.0® software (OriginLab Corporation).

### 2.8. Statistical analysis

The results were subjected to one-way analysis of variance (ANOVA) and differences between means were located using Tukey's HSD test. All statistical analyses were performed at a significance level of 5% ($p \leq 0.05$) using STATISTICA software (Statsoft, Oklahoma, USA) version 13.3. The data are presented as mean values with standard deviation.

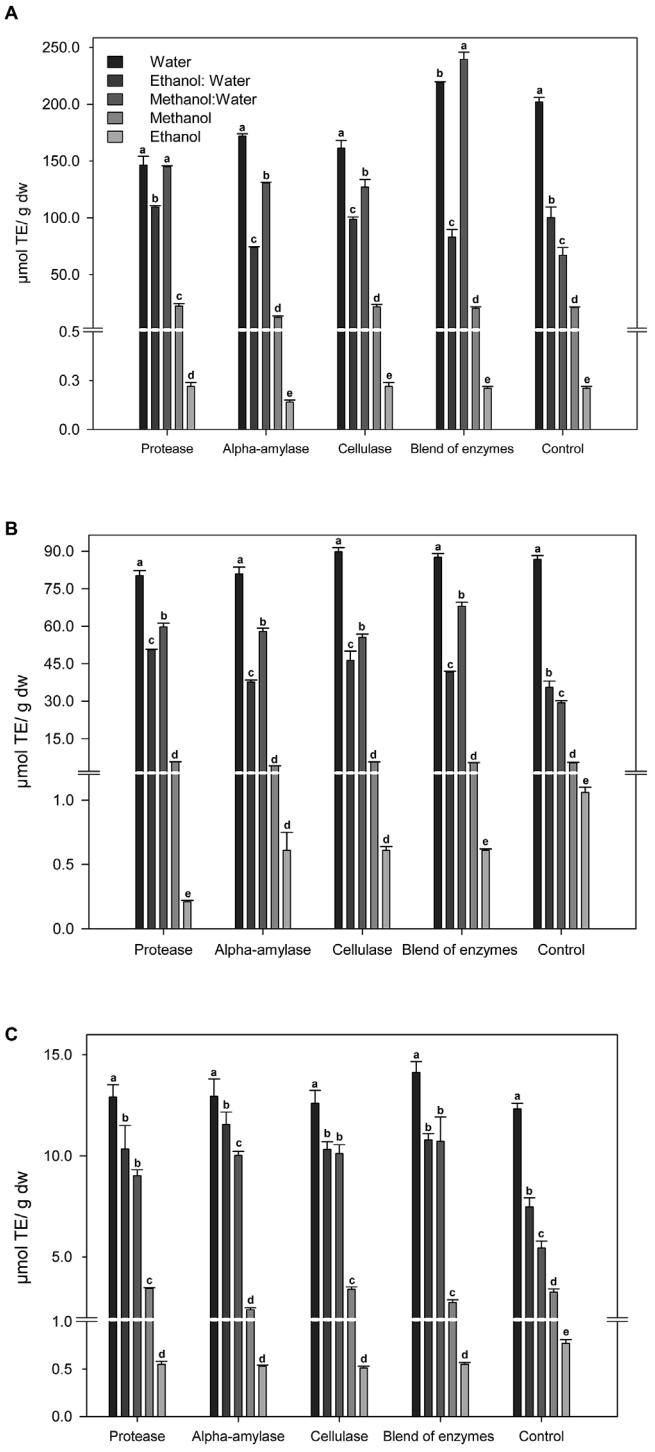


**Fig. 1. -** Antioxidant activity of different extracts from untreated and enzymatically treated seeds with α-amylase, protease and cellulase, and their blend (1:1:1) by using ORAC (a), TEAC (b) and DPPH (c) assays. Bars with same letters no differ significantly by Tukey's test ($p < 0.05$).

## 3. Results and discussion

### 3.1. Enzymatic treatment of seeds

#### 3.1.1. Selecting the best solvent to extract the antioxidant compounds

In the extraction process, the solvent selection is one of the most important parameters in the recovery of target compound. The extraction is based on the modification of the physicochemical properties

of the solvent to reduce the surface tension, increase the solubility of the target compound and promote higher diffusion rates (Takeuchi et al., 2009).

The solvent significantly affected the antioxidant compounds extraction of treated seeds (Fig. 1). In general, water was the better solvent to extract antioxidant compounds from treated seeds. Highly polar solvents, such as water, have been more efficient as extracting of phenolic antioxidant compounds from plant materials than low polarity solvents (Roby et al., 2013). The use of water as solvent can enhance the recovery of polar compounds, such as glycosylated phenolics, water soluble vitamins, and bioactive peptides (Puri et al., 2012; Weihofen and Martoglio, 2003). Interestingly, the enzymatic treatment was carried out in aqueous system and the better solvent to extract antioxidant compounds from treated seeds was water, which suggests that high polar compounds were released from seed after enzymatic treatment. Due to that, we are able to eliminate the extraction step with solvent, and the antioxidant compounds can be recovered after just a centrifugation step. It can minimise the costs, increase the efficiency of the process and reduce the environmental impact. Therefore, this procedure was employed in the following steps of the present study; that is mean, after the enzymatic treatment the mixture was centrifuged (1500 rpm, 6 min at 5 °C) and the extracts were evaluated for their antioxidant activity. The extraction of the antioxidant compounds from untreated seeds was carried out in aqueous system (*see 2.4*).

### 3.1.2. Effect of enzymatic treatment on antioxidants activity

The seeds enzymatically treated with protease and cellulase showed higher antioxidant activity in the TEAC and ORAS assays than the untreated sample, whereas the DPPH assay showed non-significant difference ($p > 0.05$) between the samples (Table 2). The enzymatic treatment increased the antioxidant activity of the seeds probably due to the release of polar antioxidant compounds bounded to the cell wall and/or by the hydrolysis of biopolymers, such as polypeptides and polysaccharides (Azmir et al., 2013).

The antioxidant activity of treated samples by ORAC assay (146.48–221.84 μmol TE/g) was higher than bean (10.00 μmol TE/g), black beans (21.40 μmol TE/g) and lentil (46.83 μmol TE/g), while DPPH (12.25–14.13 μmol TE/g) values were higher than chickpea (0.98 μmol TE/g) and lower than black bean (19.23 μmol TE/g) and lentil (19.61 μmol TE/g) (Xu and Chang, 2007).

### 3.1.3. Analysis of variance (ANOVA) and interpretation of contour plots

According to the Table 2, it is possible to note that the enzymatic treatment affected the antioxidant activity of the seeds. The experimental design data were used to construct contour plot by regression model analysis, which allow us to discover the enzymatic process condition that improve the recovering of antioxidant compounds from seeds.

**Table 2**
- Matrix of experimental mixture design and experimental values for antioxidant activities (ORAC, TEAC and DPPH methods) in *A. pavonina* L. seeds using different enzymatic preparations.

| Runs | ORAC (μmol TE/g dw) | TEAC (μmol TE/g dw) | DPPH (μmol TE/g dw) |
|---|---|---|---|
| 1 | 146.48 ± 7.84 ** | 80.24 ± 2.04 * | 12.91 ± 0.61 ns |
| 2 | 172.07 ± 1.86 ** | 80.92 ± 2.72 * | 12.94 ± 0.86 ns |
| 3 | 161.36 ± 6.77 ** | 89.75 ± 1.71 ns | 12.60 ± 0.64 ns |
| 4 | 191.47 ± 1.24 * | 84.22 ± 0.91 ns | 12.25 ± 0.51 ns |
| 5 | 221.84 ± 5.46 ** | 100.08 ± 0.54 ** | 13.49 ± 1.70 ns |
| 6 | 184.32 ± 4.88 ** | 78.67 ± 1.33 ** | 12.46 ± 0.37 ns |
| 7 | 219.13 ± 0.51 ** | 87.59 ± 1.46 ns | 14.13 ± 0.53 ** |
| Untreated | 201.99 ± 3.94 | 86.69 ± 1.55 | 12.33 ± 0.27 |

Data represent mean values for each sample ± standard deviations (n = 3). Symbols in the same column indicate statistical difference (**p ≤ 0.01, *p ≤ 0.05, and ns is not significant) between untreated sample and hydrolysates by the Student's *t*-test.

The Table 3 shows the ANOVA table including regression models, $R^2$, *F*-test and probability values of the antioxidant activity by TEAC and ORAC of treated samples. ANOVA indicated that proposed models for antioxidant activities by ORAC and TEAC methods provided a good fit to the data, since they were able to explain approximately 96% and 98% of the total variation of the results, respectively, besides presenting small *p*-values (*p*-value < 0.0001). The regression model for DPPH assay showed $R^2$ value less than 85% ($R^2 = 0.43$), the minimum suggested value for a good fit, suggesting that the model for DPPH is not well adjusted to the experimental data (Bae et al., 2015; Mašković et al., 2016). Therefore, the model obtained for the DPPH assay was not used to estimate the antioxidant activity of enzymatically treated seeds.

The two-dimensional (2D) interaction contour plots are given in Fig. 2. It shows the interaction effect of α-amylase, cellulase and protease on antioxidant activity of seeds. In these figures, each enzyme is represented at the vertices of an equilateral triangle, the midpoints of the three sides of the triangle represent the results for binary mixtures of enzymes and the central point indicates the variation of the results when ternary mixtures of enzymes were applied (de Castro et al., 2017). The interpretation of contour plots demonstrated that the enzymatic treatment using the combination of protease and cellulase resulted in the highest antioxidant activities (Table 2), which can confirm a synergistic effect between these two enzymes, in which the interaction between them demonstrates a better result than their isolated action.

The cell wall of plants is composed of carbohydrates (cellulose, hemicelluloses, and pectic polysaccharides) as well as proteins (peripheral and integral proteins), and the blend of enzymes cellulase and protease hydrolyse the cell wall components thereby increasing cell wall permeability, leading to higher extraction yields of bioactive compounds (Passos et al., 2009; Puri et al., 2012).

To facilitate the recovery of bioactive compounds it is important that the enzyme is able to cross several barriers: first the extra cellular walls that are constituted of cellulose, pectins, hemicelluloses and arabinogalactans and then the cell wall that contains glycoproteins (Domínguez et al., 1995; Ricochon and Muniglia, 2010). Thereby, the enzymatic treatment with cellulase may improve the access of proteases to proteins of cell wall.

The α-amylase hydrolyses alpha-bonds from polysaccharides, such as starch that is found in most reserve tissues (Okita et al., 1979; Yu et al., 2005), this suggests that this enzyme was not efficient in increasing bioactive compounds recovery because they are found primarily in the cell wall of the plants (Wallace and Fry, 1994).

## 3.2. Phenolic compounds from enzymatically treated seeds

### 3.2.1. Total phenolic content (TPC), total flavonoid (TFC) and condensed tannins (proanthocyanidins) (CTC) contents

The enzymatic treatment improved the recovery of total phenolic compounds from seeds (Table 4). After the enzymatic treatment, the TPC, TFC and CTC increased 34%, 47%, 22%, respectively.

Enzymatic treatment of plant-based materials is a technique suitable to the liberation of phenolic compounds because the using of combined enzymes is able to disintegrate the cell wall, which can enhance the phenolic extraction (Khoddami et al., 2013). A combination of cellulase, protease, xylase and pectinase was used to improve the recovery (17.5%) of bioactive compounds from grape seeds (Passos et al., 2009). Previous study showed enzyme-assisted extraction with cellulase improved the extraction of essential oils from coriander (*Coriandrum sativum* L.) seeds by 44.2%, besides increasing the recovery of polyphenols (%) and their antioxidant activity (%) (Abbassi et al., 2018). Latif and Anwar (2011) reported the enzyme-mixtures (Protex 7L, Alcalase 2.4L, Viscozyme L, Natuzyme, and Kemzyme) increased the antioxidant activity and effectiveness in extracting the oil and protein recovery from sesame seeds.

**Table 3**
- Analysis of variance (ANOVA) including regression models, $R^2$ and probability values for antioxidant activities of freeze-dried samples.

| Response | Model | Equations | $F_{calculated}$ | $F_{tabulated}$ | $R^2$ | *p*-values |
|---|---|---|---|---|---|---|
| ORAC | Cubic | $Y = 146.48x_1 + 172.071x_2 + 161.36x_3 + 128.77x_1x_2 + 271.67x_1x_3 + 70.39x_2x_3 + 184.92x_1x_2x_3$ | 100.58 | 2.243 | 0.98 | < 0.0001 |
| TEAC | Quadratic | $Y = 80.33x_1 + 81.01x_2 + 89.85x_3 + 12.66x_1x_2 + 58.42x_1x_3 - 28.57x_2x_3$ | 69.95 | 2.273 | 0.96 | < 0.0001 |

The coded values in equations represent the components in the mixture and their interactions: $x_1$ = Protease (Neutrase® 0.8 L); $x_2$ = α-Amylase (Termamyl® 2X) and $x_3$ = Cellulase (Optimase® 56 L).

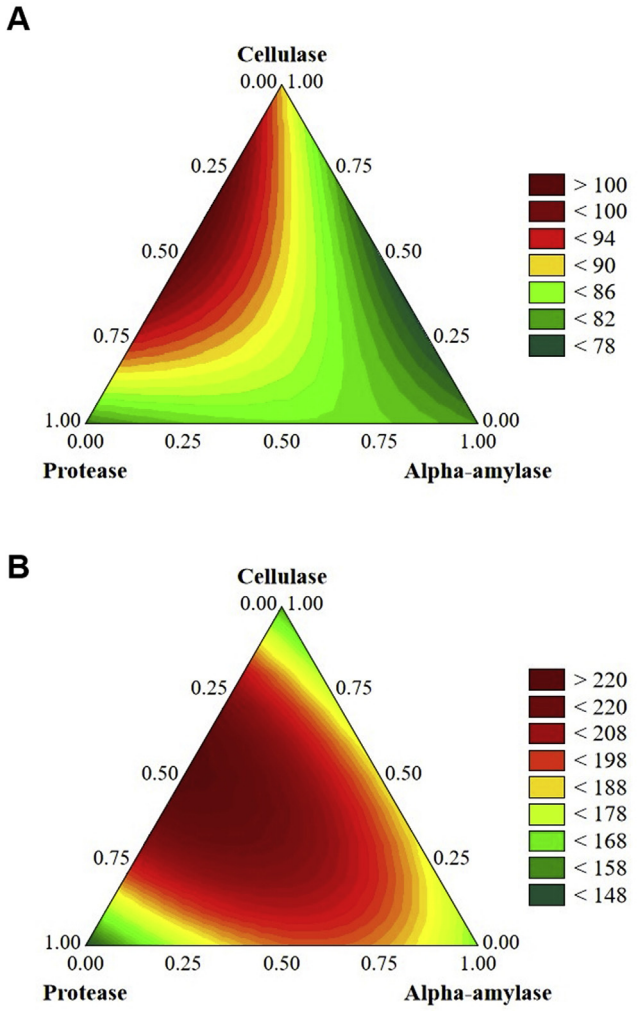


**Fig. 2.** – Experimental mixture designer showing the interactive effect of enzymes on antioxidant activities: TEAC (A) and ORAC (B).

**Table 4**
– Phenolic compounds contents, flavonoids and condensed tannins from enzymatically treated (protease and cellulase, 1:1, v/v) and untreated seeds of *A. pavonina* L.

| Analysis | Untreated | Enzymatically Treated* |
|---|---|---|
| Phenolic compounds content (mg GAE/g dw) | 884.82 ± 2.68 $^{b}$ | 1183.20 ± 2.30 $^{a}$ |
| Flavonoid (mg CE/g dw) | 249.69 ± 6.36 $^{b}$ | 366.98 ± 1.66 $^{a}$ |
| Condensed tannins (mg CE/g dw) | 180.67 ± 2.94 $^{b}$ | 220.09 ± 7.07 $^{a}$ |

The phenolic contents were presented as mean values with standard deviations ($n = 3$).
Different lowercase letters in the same line indicate statistical difference ($p \leq 0.05$) between the results by Student's *t*-test.

**Table 5**
– Profile of phenolic compounds from untreated and enzymatically treated (protease and cellulase, 1:1, v/v) seeds of *A. pavonina* L.

| Compound | Concentration (μg/g dw) | |
|---|---|---|
| | Untreated | Enzymatically Treated* |
| Catechin | 52.61 ± 1.24 $^{aA}$ | 6.08 ± 0.36 $^{bB}$ |
| Quercetin | n.d. | 0.18 ± 0.04 $^{d}$ |
| Naringenin | 1.09 ± 0.05 $^{cdA}$ | 1.31 ± 0.17 $^{cA}$ |
| Protocatechuic acid | 2.77 ± 0.28 $^{bA}$ | 3.35 ± 0.33 $^{bA}$ |
| Gentisic acid | 2.34 ± 0.25 $^{bcA}$ | 2.81 ± 0.24 $^{bA}$ |
| 4-Hydroxybenzoic acid | 0.43 ± 0.04 $^{dA}$ | 0.53 ± 0.03 $^{dA}$ |
| Ferulic acid | 0.28 ± 0.10 $^{dB}$ | 0.45 ± 0.08 $^{dA}$ |
| **Total phenolic compounds** | **59.52 ± 1.25** $^{A}$ | **14.71 ± 0.28** $^{B}$ |

Different lowercase letters in the same column indicate statistical difference ($p < 0.05$) between the results by paired Student's t-test.
Different capital letters in the same line indicate statistical difference ($p < 0.05$) between the results by Tukey test.
n.d.: not detected.
*A. pavonina* L. seeds enzymatically treated with the protease and cellulase enzymes (1:1, v/v).

### 3.2.2. Individual phenolic compounds by HPLC-ESI-(−)-MS/MS

The flavonoid quercetin was identified only after enzymatic treatment (Table 5), suggesting that the use of protease and cellulase improved the recovery of this phenolic compound. Catechin was the main phenolic compound identified in *A. pavonina* seeds and the only one that decreased after enzymatic treatment. This reduction may have occurred due to oxidative degradation or other mechanism, for example, polymerization and precipitation by proteins (Martins et al., 2016). However, the enzymatic treatment improved the recovery of phenolic acids, such as protocatechuic, gentisic, 4-hydroxybenzoic and ferulic acids. The enzymatic treatment has been used to improve the release of phenolic acids from foods, especially the phenolics linked to the cell wall of plant-based materials, such as ferulic acid (de Camargo et al., 2016).

Although the total phenolic compounds from enzymatically treated sample was lower than in the untreated sample, the antioxidant activity was higher for the enzymatically treated sample (Tables 2 and 5). This can be explained by the presence of phenolic acids and flavanoids, and also of other antioxidant compounds that may have been released after enzymatic treatment, such as bioactive peptides. In addition, several studies have demonstrated that the antioxidant capacity of phenolic compounds is influenced by interaction with other compounds or with themselves, and these interactions may be synergistic or antagonistic depending on the conditions and compounds (Paz et al., 2015).

### 3.3. Antiproliferative activity

Previous studies have showed the use of natural foods, such as plant residues, for the chemoprevention of cancer and also the relationship between antioxidant and antiproliferative activities of phytochemicals (Deng et al., 2012; Lee et al., 2012). The chemotherapeutic drugs have the capacity to promote metabolic alteration in tumour cells by several mechanisms, acting primarily on cell signalling (Hoshino et al., 1991).

To achieve greater amplitude for the antiproliferative activity of

**Table 6**
– $GI_{50}$ values (μg/mL) of doxorubicin and extracts from *A. pavonina* L. (untreated and enzymatically treated) in human tumour cell lines.

| | 2 | u | m | a | 7 | 4 | p | o | h | k | q |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Doxorubicin | < 0.025 | < 0.025 | 0.025 | 0.1 | 21.8 | < 0.025 | < 0.025 | 0.053 | 0.19 | 0.16 | 0.034 |
| Untreated | > 250 | > 250 | > 250 | > 250 | 67.5 | > 250 | 250 | > 250 | > 250 | > 250 | > 250 |
| Enzymatically Treated* | > 250 | > 250 | > 250 | > 250 | 29.9 | > 250 | 2.5 | > 250 | > 250 | > 250 | > 250 |

Tumour cell lines: 2 = U251 (glioma), u = UACC-62 (melanoma); m = MCF-7 (breast); a = NCI-ADR/RES (multidrug resistant ovary carcinoma); 7 = 786–0 (kidney); 4 = NCI-H460 (non-small cell lung cancer); p = PC-3 (prostate); o = OVCAR-03 (ovarian); h = HT-29 (colon adenocarcinoma); k = K562 (leukemia). Non-tumour cell lines: q = HaCaT (keratinocyte).
Doxorubicin: Chemotherapeutic; $GI_{50}$: *Growth Inhibition 50* – concentration that inhibit 50% cell growth.
*A. pavonina* L. seeds enzymatically treated with the protease and cellulase enzymes (1:1, v/v).

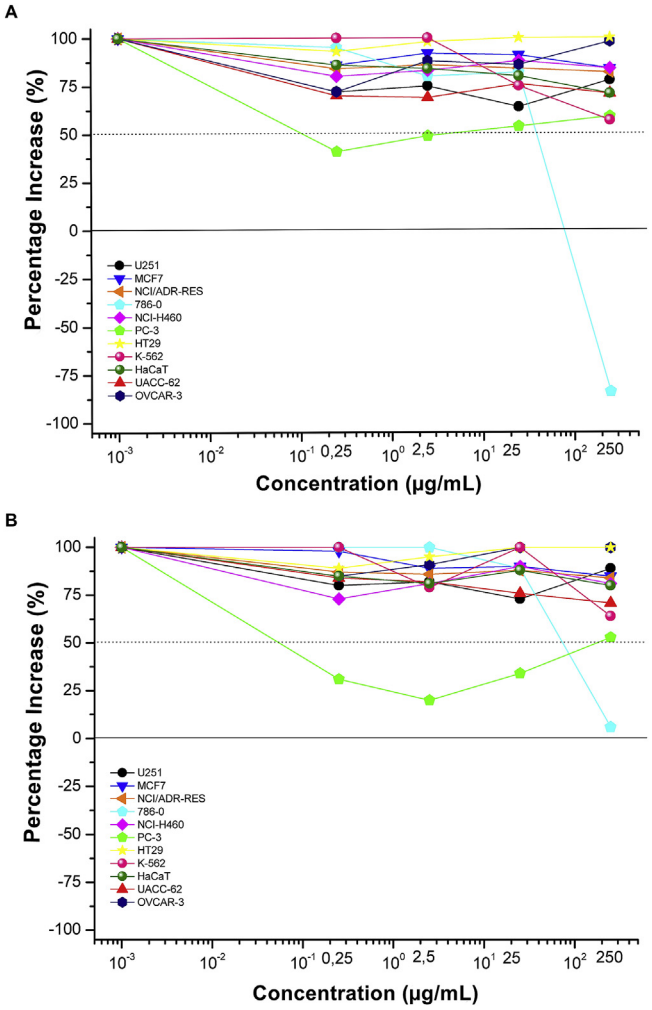


**Fig. 3. -** Antiproliferative activity of enzymatically treated (protease and cellulase) (A) and untreated (B) seeds from *A. pavonina* L. seeds on the cell lineages, correlating the percentage increase *versus* the sample concentration (U251 – glioma, UACC-62 – melanoma, MCF-7 – breast, NCI-ADR/RES - multidrug resistant ovary carcinoma, 786–0 – kidney, NCI-H460 – non-small cell lung cancer, PC-3 – prostate, OVCAR-3 – ovarian, HT29 – colon, K562 – leukemia, HaCaT – keratinocyte).

vegetable matrices, we tested *A. pavonina* seed extracts (enzymatically treated and untreated seeds) against human tumour cell lines of different histological and genetic origins. Table 6 summarises the concentrations of extracts from *A. pavonina* L. (enzymatically treated and untreated) required for 50% ($GI_{50}$) cell growth inhibition.

Fig. 3A and B correlate the cell growth as a function of the concentration of the samples tested. The untreated sample showed weak cytostatic activity for 786–0 (kidney, $GI_{50}$ = 67.5 μg mL) cell line, while enzymatically treated seeds showed strong antiproliferative activity against PC-3 (prostate, $GI_{50}$ = 2.5 μg mL) e 786–0 (kidney, $GI_{50}$ = 29.9 μg mL) cell lines. Therefore, the enzymatic treated improved the antiproliferative activity of *A. pavonina* seeds.

The increase in antiproliferative activity may be partially related to the increase of the phenolic acids content after the enzymatic treatment and/or the release of other unidentified compounds. Previous study related that polyphenols, such as gallic acid, would be responsible for antiproliferative activity from *Vicia* seed (Fabaceae) (Megías et al., 2018).

## 4. Conclusion

The enzymatic treatment of *A. pavonina* L. seeds with protease and cellulase improved the phenolic compounds extraction, and antioxidant and antiproliferative (against the prostate and kidney tumour cell lines) activities. The enzymatic treatment released antioxidant compounds that may explain the improvement of tumour-cell growth inhibition. However, the mechanisms of action of extract from *A. pavonina* L. seeds against human tumour cell lines still unclear and need to be further explored to assist in providing useful information for its application in functional foods and/or drugs for cancer chemotherapy and/or chemoprevention.

## Conflicts of interest

The authors in this manuscript have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bcab.2019.01.040.

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