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**RED ONION (*Allium cepa* L.) METHANOLIC EXTRACT INCREASES
EXTRACELLULAR NUCLEOTIDE HYDROLYSIS IN RAT BLOOD SERUM**

**PATOS DE MINAS – MG
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Artigo Científico apresentado ao Instituto de Genética e Bioquímica da Universidade Federal de Uberlândia como requisito final para a obtenção do título de Bacharel em Biotecnologia.

**Orientadora: Profa. Dra. Cristina Ribas
Fürstenau**

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Extract of red onion (*Allium cepa* L.) increases extracellular nucleotide hydrolysis in rat blood serum

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Abstract

Aims: To evaluate the total phenolic content of the red onion (*Allium cepa* L.) extract, its antioxidant potential and its ability to interfere in extracellular hydrolysis of purine nucleotides in rat blood serum. **Materials and methods:** Firstly, red onion extract was obtained using methanol as the solvent. Phenolic content and antioxidant capacity of the extract were further analysed. Hydrolysis of ectonucleotidase substrates (AMP, ADP, ATP and *p*-Nph-5'TMP) were evaluated by colorimetric methods in the presence (or absence) of red onion extract at different concentrations (0, 125, 250, 500 e 1000 µg/mL). **Key findings:** Total phenolic content of red onion extract was 54.35 ± 0.32 mg GAE 100g⁻¹ sample in the crude extract, which demonstrated antioxidant capacity similar to the standard antioxidant (Trolox) at all tested concentrations (from 125 to 1000 µg/ mL). Correlation between extract total phenolic content and percentage of antioxidant activity was $R^2=0.9595$. Moreover, red onion extract increased ADP hydrolysis at 500 µg/mL ($p \leq 0.05$). The same occurred for *p*-Nph-5'TMP hydrolysis at 500 and 1000 µg/mL ($p \leq 0.05$). **Significance:** Our results confirm that red onion extract presents high levels of antioxidant molecules. Also, the red onion extract may be important to regulate vascular processes, such as decrease platelet aggregation since it increases ADP breakdown, which is an important platelet agonist in the vasculature. The increased ADP hydrolysis may be explained by the fact that ectonucleotidases have their activity favored when the reactive oxygen species (ROS) are removed by antioxidants of red onion extract.

Keywords: Red onion (*Allium cepa* L.), phenolic content, antioxidant activity, purinergic signaling, ectonucleotidases.

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1. Introduction

It is well established that ATP (adenosine triphosphate) is an intracellular energy source that is involved in several metabolic processes [1]. However, its role as a signaling molecule was proposed by Burnstock in 1972 [2]. Since then, signaling via extracellular nucleotides was discovered as a primitive and highly conserved pathway for communication between cells [1, 3].

Extracellular nucleotides and nucleosides of purines and pyrimidines (ATP, ADP, adenosine, UTP and UDP, respectively) are recognized as regulators of distinct biological processes in various tissues [4], including the cardiovascular system, where they can promote platelet aggregation, modify vascular tone [5], stimulate angiogenesis [6] and modulate cardiac functions [7]. Once they reach the extracellular space by stimuli such as cell lysis [8]; traumatic shock, organ injury and inflammatory processes [9]; shear stress and hypoxia [10]; or in a constitutive way [11], these molecules initiate signaling cascades binding to their specific membrane receptors, the purinoceptors, generally named P1 and P2 [12].

Purinergic signaling is inactivated through the nucleotide metabolization, performed by a multienzymatic complex, the ectonucleotidases [13]. These enzymes can be located on the cell surface or may also be in the interstitial medium or within body fluids and exhibit broad substrate specificity and tissue distribution [14]. The hydrolysis of nucleotides have included a role for ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase/ phosphodiesterases (E-NPPs), ecto-5'-nucleotidases, and alkaline phosphatases [10]. Depending on the family considered, ectonucleotidases typically hydrolyze nucleoside tri-, di-, and monophosphates and dinucleoside polyphosphates and produce nucleoside diphosphates, nucleoside monophosphates, nucleosides and inorganic phosphate (Pi) or pyrophosphate (PPi) [15].

A major functional role for extracellular nucleotide hydrolysis and production of extracellular nucleoside would thus relate to the control of ligand availability at nucleotide (P2) receptors and adenosine (P1) receptors [15]. These processes are highly relevant for purinergic transmission and can result in prevention of receptor desensitization or termination of receptor activation by ligand hydrolysis or also in receptor activation by the hydrolysis products generated [16].

It is known that reactive oxygen species (ROS) are atoms, molecules, or ions derived from oxygen metabolism, which in their great majority have high reactivity and can interact with DNA, lipids and proteins, causing tissue damage and risk of cardio-metabolic diseases if they are not neutralized by antioxidant compounds [17, 18]. It is known that inhibit the catabolism

of extracellular purines by the ectonucleotidases present in the endothelial cells, thus modifying the pathophysiology of cardiovascular diseases and, in chronic hypertensive individuals, favoring platelet aggregation [19, 20]. The control of cardiovascular functions is not only related to purinergic signaling, but the understanding of the mechanisms involved in this context are extremely important for the search of new drugs and therapies to treat cardiovascular diseases, such as systemic arterial hypertension (SAH), since this can occur after chronic imbalance in purinergic signaling and is in the group of cardiovascular diseases (CVD), which are currently the largest cause of death in the world [21].

Onion (*Allium cepa* L.) may help in the control of several pathologies due to its high concentration of phenolic compounds, which may have protective action against cardiovascular diseases [13]. It is known that red onion variety exhibits the highest content of these compounds, which can confer greater antioxidant activity and greater activity of reaction and elimination of ROS, consequently [22]. Previous studies have shown that phenolic compounds exert a number of important biological activities in the cardiovascular system, such as antioxidants, platelet aggregation and adhesion inhibitors, vasodilators and lipid metabolism modulators [23]. Phenolic compounds are substances generally found in natural products and have been well studied because of their antioxidant properties against oxidative damage [23, 24].

Although the phenolic compounds present in onion are studied and reported as modulators of enzymatic activity, their properties regarding the modulation of ectonucleotidases activities, and how they would be related to the activity of these enzymes in the cardiovascular system, have not yet been reported. Then, the objective of the present study was to investigate if the extract of red onion could modulate ectonucleotidase activities in rat blood serum.

2. Materials

2.1 Chemicals

Substrates ATP, ADP, AMP and *p*-Nitrophenyl thymidine 5'-monophosphate (pNph-5'-TMP), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 6-hidroxi-2,5,7,8tetramethylchrome-2-carboxylic acid (Trolox) and Folin-Ciocalteau reagent, were obtained from Sigma-Aldrich (St.Louis, MO, USA). All chemicals used in this study were of the highest purity.

2.2 Phenolic Compounds Extraction

For phenolic content extraction, red onions were purchased from a local supermarket in Patos de Minas, Minas Gerais, Brazil and handled as previously described by Souza (2009) [25], with minor modifications. Briefly, about 500 g of red onions were minced and dried in a drying oven (402-5D, Ethik Technology, SP, Brazil) at 60 °C for 48 hours. Dried contents were then crushed and the about 10 g of the powder was weighed and kept under magnetic stirring (MA-085/5L, Marconi, SP, Brazil) with 50 mL of methanol for 2 hours. Extracts were filtered through filter paper (C42, Unifil, SP, Brazil), dried in rotary evaporator (Model 802, Fisatom, SP, Brazil) and stored at -80 °C (Ultra freezer CLS20-86V, Coldlab, SP, Brazil) until use.

2.3 Measurement of Total Phenolic Content

Total phenolic content in red onion extract was analyzed spectrophotometrically (Spectrophotometer UV-340G, Gehaka, PR, Brazil) at 760 nm using the Folin– Ciocalteu reagent as already described by Wettasinghe et al. (1999) [26]. Gallic acid was used as a standard for the quantification and expression of the results. Phenolic content in onion was expressed as milligram gallic acid equivalents (GAE) per 100 gram of sample.

2.4 Measurement of Antioxidant Capacity by scavenging of 2,2- diphenyl-2-picrylhydrazyl (DPPH•)

Antioxidant capacity were determined according to Brand-Williams et al. (1995) [27], using 6-hidroxi-2,5,7,8-tetramethylchrome-2-carboxylic acid (Trolox) as a standard in the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•). The absorbance at 515 nm was measured after 30 min. The decline in the initial absorbance of the DPPH• (60 µM) concentration indicated the radical scavenging activity of the sample. Trolox (800 µmol/ L) was used as a reference corresponding to 100% radical scavenging activity. Radical scavenging activity percentage was calculated as $(A_0 - A_{\text{test}}) / (A_0) * 100$, where A_0 is the initial DPPH absorbance without antioxidant compounds and A_{test} is the absorbance after 30 min of reaction. Analyses were performed in triplicate. Trolox equivalent antioxidant capacity (TEAC) value was determined from a Trolox calibration curve (ranging from 100 to 800 µmol/ L final concentration, $R^2 = 0.979$). Results are expressed as 1 µmol of Trolox equivalents per liter (µmol/ L).

2.5 Animals

Male *Wistar* rats, weighing about 250 g, were maintained on a 12 h light/ 12 h dark cycle, at constant room temperature (25°C). They received water and rat chow *ad libitum*. All protocols were in accordance with the guidelines of the Ethics Committee on the Use of Animals (CEUA) at Universidade Federal de Uberlândia and all efforts were made to minimize the number of animals used in this study and their suffering.

2.6 Protein determination

Protein was measured by the Coomassie-Blue method according to Bradford (1976) [28], using bovine serum albumin as standard and used to calculate the enzymatic activities.

2.7 Assay of Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and Ecto-5nucleotidase activities

Assay of E-NTPDase and Ecto-5'-nucleotidase in serum samples were performed using a modification of the method previously described by Yegutkin (1997) [29]. Reaction mixture contained 112.5 mM Tris-HCl, pH 8.0, water, protein at 1.0 mg per tube and different concentrations (0, 125, 250, 500 and 1000 µg/ mL) of red onion extract. Extract was added during preincubation time that was performed for 10 minutes at 37 °C in water bath (CT-245-28, Cientec, MG, Brazil), prior to substrate addition. To start the reactions, substrates (ATP, ADP ou AMP) were added on the concentration of 3.0 mM in a final volume of 200 µL. After 40 minutes of incubation, 200 µL of TCA (5%, final concentration) was used to stop the enzymatic reactions. Incubation times and protein concentrations were chosen to ensure the linearity of the enzymatic reactions. The amount of inorganic phosphate (Pi) released was carried out using a colorimetric method as previously outlined [30] was measured at 630 nm (Spectrophotometer UV-340G, Gehaka, PR, Brazil). Controls to correct for non-enzymatic hydrolysis of substrates were performed by adding serum after the reactions had been stopped with TCA. All samples were performed in duplicate. Enzyme activities were generally expressed as nmol Pi released per minute per milligram of protein.

2.8 Assay of Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

E-NPP activity in serum samples was assayed according to the method described by Sakura et al. (1998) [31]. Reaction medium containing 100 mM Tris-HCl, pH 8.9, was preincubated for 10 minutes at 37°C in water bath (CT-245-28, Cientec, MG, Brazil), with approximately 1.0 mg of serum protein, water and different concentrations (0, 125, 250, 500 and 1000 µg/ mL) of red onion extract . Enzyme reactions were started by the addition of *p*-Nph-5'-TMP (5'TMP, an artificial marker substrate for E-NPP activity) to a final concentration of 0.5 mM. After the 8 minutes, 200 µL NaOH 0.2 N was added to the medium to stop the reactions. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm (Spectrophotometer UV-340G, Gehaka, PR, Brazil), using a molar extinction coefficient of $18.8 \times 10^{-3} \text{ M/cm}$. Controls to correct for nonenzymatic substrate hydrolysis were performed by adding serum after the reaction had been stopped with NaOH. All samples were performed in duplicate. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

2.9 Statistical Analysis

Statistical analysis was performed by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Data were analyzed by one-way analysis of variance (oneway ANOVA). Significance between means were calculated by Tukey or Newman-Keuls. Correlation index was determined by Pearson's Correlation. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1 Measurement of Total Phenolic Content

Total phenolic content of the crude extract was 54.35 mg GAE 100g⁻¹ sample (Table 1), being proportional and crescent according to the increment in the concentrations tested.

Table 1. Content of total phenolic compounds (mg GAE 100g⁻¹ sample).

Red onion extract	Phenolic content (mg GAE 100g ⁻¹ sample)
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125 µg/ mL	0.079 ± 4.7x10 ⁻⁴ ^a
250 µg/ mL	0.158 ± 9.5x10 ⁻⁴ ^a
500 µg/ mL	0.316 ± 1.9x10 ⁻³ ^a
1000 µg/ mL	0.632 ± 3.8x10 ⁻³ ^b
Crude extract	54.35 ± 0.32 ^c

Results are expressed as means ± SD, $n=3$. Comparison among groups was analyzed by oneway analysis of variance (ANOVA). Significance between means were evaluated by Newman-Keuls multiple comparison test. Different letters represent differences between means with $p \leq 0.05$.

3.2 Measurement of Antioxidant Capacity

Antioxidant capacity against the radical DPPH in Trolox equivalent antioxidant capacity (TEAC) is presented in Table 2. As verified for total phenolic content, increasing antioxidant capacity was proportionally observed at crescent concentrations of red onion extract.

Table 2. Trolox equivalent antioxidant capacity µmol/ L (TEAC).

Red Onion Extract	TEAC (µmol/L)
125 µg/ mL	115.35 ± 32.78 ^a
250 µg/ mL	232.66 ± 29.66 ^b
500 µg/ mL	436.95 ± 17.82 ^c
1000 µg /mL	691.77 ± 63.81 ^d

Results are expressed as means ± SD, $n=3$. Comparison among groups was analyzed by oneway analysis of variance (ANOVA). Significance between means were evaluated by Newman-Keuls multiple comparison test. Different letters represent differences between means with $p \leq 0.05$.

Percentage of antioxidant capacity by scavenging of 2,2- diphenyl-2-picrylhydrazyl (DPPH•) at different concentrations of red onion extract and the recognized antioxidant Trolox is shown in Figure 1.

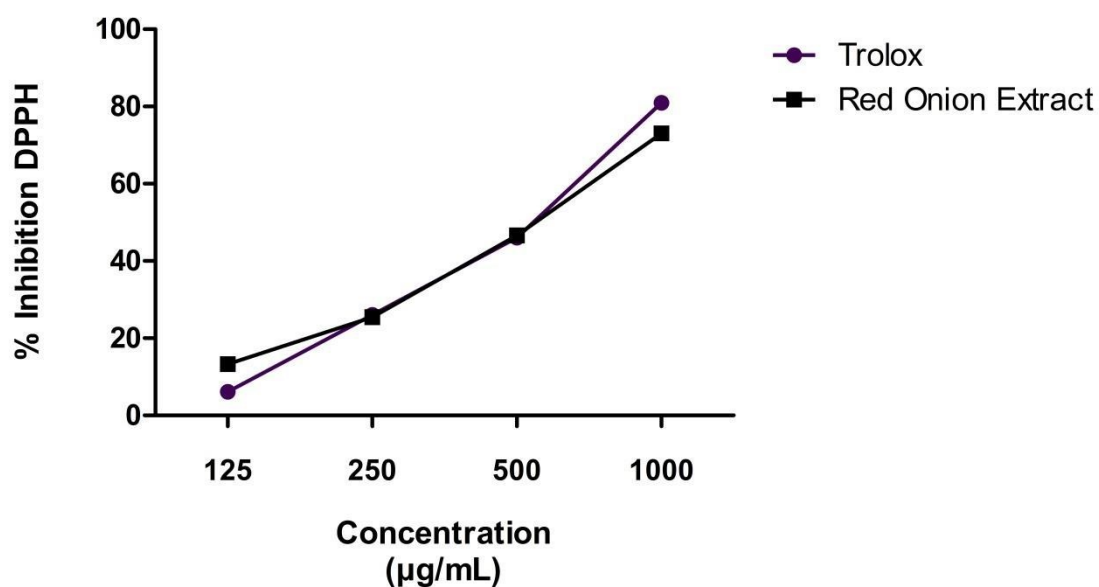


Figure 1. Percentage of inhibition of the DPPH radical by red onion extract and Trolox. Inhibition percentage increases with concentration elevation.

A very positive correlation between antioxidant activity and total phenolic content in red onion extract is shown in Figure 2.

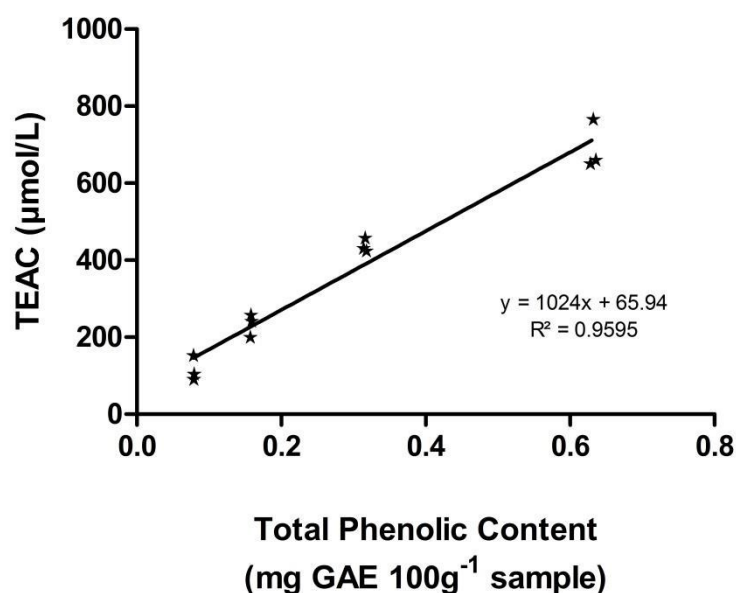


Figure 2. Correlation between total phenolic content and percentage of antioxidant activity. The correlation was analyzed by Pearson test, $R^2 = 0.9595$, $n=3$.

3.3 Assays of Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and Ecto-5nucleotidase activities

There was no statistically significant difference ($p \leq 0.05$) in the ATP hydrolysis in the absence (0 $\mu\text{g}/\text{mL}$ red onion extract, control group) or in the presence of red onion extract at any concentration tested: 125, 250, 500 and 1000 $\mu\text{g}/\text{mL}$ (Figure 3).

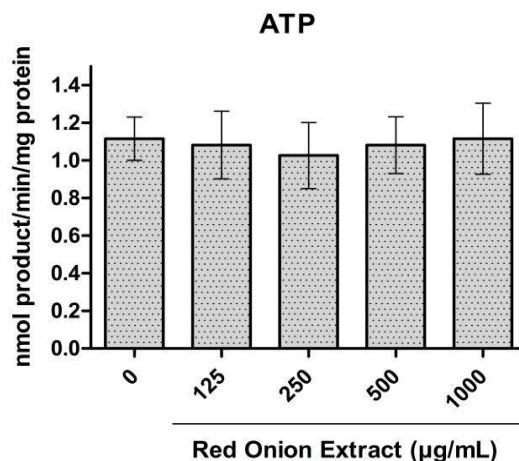


Figure 3. Effect of red onion extract treatment on ATP hydrolysis in blood serum of rats. Results are expressed as means $\pm$ SD, $n=4$. There were no significant differences between groups. Comparison among groups was analyzed by one-way analysis of variance (ANOVA). Significance between means were evaluated by Tukey test and $p \leq 0.05$.

ADP hydrolysis was significantly increased by 250 $\mu\text{g}/\text{mL}$ of red onion extract (Figure 4).

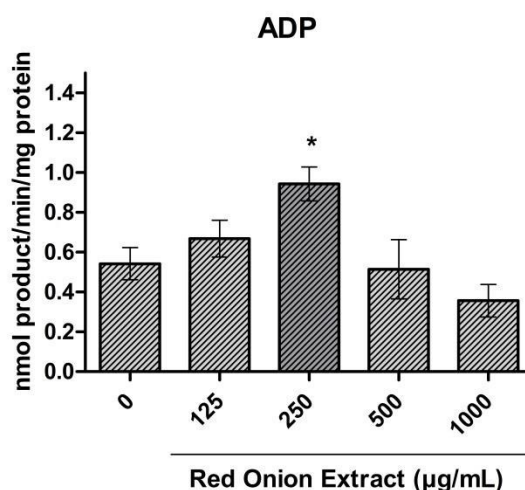


Figure 4. Effect of red onion extract treatment on ADP hydrolysis in rat blood serum. Results are expressed as means $\pm$ SD, $n=4$. Comparison among groups was analyzed by one-way analysis of variance (ANOVA). Significance between means were evaluated by Tukey test. * Represents statistical difference from control group, considering $p \leq 0.05$.

As observed for ATP hydrolysis, AMP hydrolysis was not modified by red onion extract at any concentration evaluated ($p \leq 0.05$).

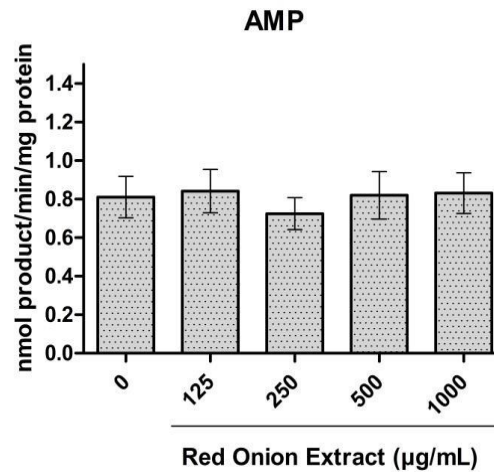


Figure 5. Effect of red onion extract treatment on AMP hydrolysis in rat blood serum. Results are expressed as means $\pm$ SD, $n=4$. Comparison among groups was analyzed by one-way analysis of variance (ANOVA). Significance between means were evaluated by Tukey test and $p \leq 0.05$.

3.4 Assay of Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

Hydrolysis of *p*-Nph-5'-TMP was significantly increased by 500 and 1000 µg/ mL of red onion extract ($p \leq 0.05$) (Figure 6).

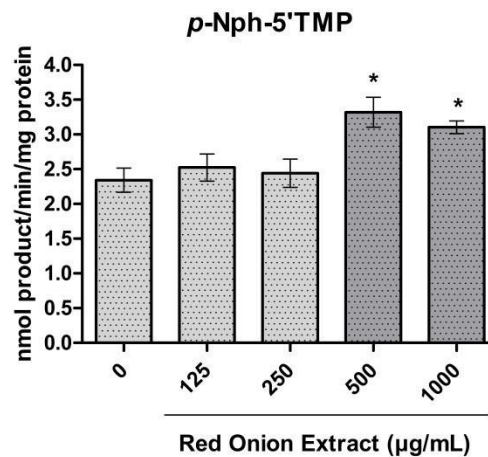


Figure 6. Effect of red onion extract treatment on the artificial substrate marker for E-NPP activity. *p*-Nph-5'TMP hydrolysis was increased in blood serum of rats at 500 and 1000 µg/mL. Results are expressed as means $\pm$ SD, $n=3$. Comparison among groups was analyzed by one-way analysis of variance (ANOVA). Significance between means were evaluated by Tukey test. * Represents statistical difference from control group for a $p \leq 0.05$.

4. Discussion

4.1 Phenolic Content

Red onion extract was chosen in the present study due to higher total phenolic content [22]. Total phenolic content observed in this work (Table 1) was comparable than data previously reported [32]. However, the use of different extraction protocols and as well as distinct methods to measure phenolic content complicates a comparison between data from different articles. It is noteworthy that we could extract a considerable amount of phenolics from red onion that was used to the functional tests upon serum ectonucleotidase activities.

4.2 Antioxidant Activity

Flavonoids are the main compounds responsible for the antioxidant activity in onion according with other authors, being the onion one of the major responsible for all the flavonoids consumed in the human diet [33, 34]. Oxidative damage is now considered to be a major event in disease processes such as cardiovascular diseases, consequently, much scientific investigation has now been focused on the possible role of natural antioxidants in delaying or suppressing oxidative stress and secondary effects like enzyme activities modulation [35]. The need for antioxidants, dietary or supplementary, to increase endogenous antioxidant activity has been well required and a wide variety of phenolic compounds and flavonoids present in vegetables are now experimentally investigated [35].

Analog to the phenolic content, the values found for percentual of inhibition of the DPPH• radical by red onion extract obtained in this study (Figure 1) are similar to those already reported in the literature [36]. Also, the inhibition presented by the extract is similar to that presented by Trolox, a well as recognized antioxidant compound (Figure 1), reaching around 80% of inhibition at 1000 µg/ mL. Trolox equivalent antioxidant capacity (TEAC) method is frequently used for determination of antioxidant activity [33]. However, the difference between the methodologies, experimental conditions used in the different studies and the units of expression of the results makes it difficult to compare the results obtained in this work (Table 2) with previous data [33, 34, 35]. Due to the wide variety of potential antioxidant compounds, such as vitamins, flavonoids, phenolic acids and sulfur compounds present in plants, differences in the method of sample extraction (e.g. solvent used) may result in a wide variation in the antioxidant activity of the extract [34]. In our study, there was an observable high correlation ($R^2= 0.9595$) between total phenolic content/ antioxidant activities (Figure 2), higher than the correlation

found in previous data [33]. This result thus confirm that phenols are mainly responsible for the antioxidant activity of extracts.

4.3 Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and Ecto-5-nucleotidase activities

E-NTPDase and Ecto-5'-nucleotidase can be found anchored to the cell membrane and or in a soluble form circulating in the blood, being the activity of both families affected by the oxidative state of the medium [37, 19, 20]. Nucleotides preference is different for each ectoenzyme [37], and this may be the reason for the different responses of ATP, ADP and AMP hydrolysis to red onion extract (Figure 3, 4 and 5).

E-NTPDase hydrolyzes ATP and ADP and some molecules that inhibit its activity are known [37, 38, 39]. However, only few molecules able to increase its activity are reported. Red onion extract was precisely able to increase hydrolysis of ADP at 250 µg/ mL (Figure 4), but did not increase ATP hydrolysis at any concentration tested (Figure 3). It is known that in the blood circulation ADP is an important agent in platelet aggregation [37, 40]. Thus, the increase of its hydrolysis may suggest that the extract would be able to inhibit platelet aggregation process. ATP in the vasculature and kidneys may stimulate vasoconstriction or vasodilation [37, 41], depending on where it is released and which receptors it will sensitize. Ecto-5'-nucleotidase efficiently hydrolyzes AMP leading to the production of adenosine [42]. The coordinated action these enzymes is important for the efficient regulation of extracellular nucleotide and adenosine levels [43], which is an important vasodilator molecule in the vasculature and, as well as ATP, is known to participate in the regulation of renal microcirculation, renin secretion, and tubular function [37]. The onion extract, however, did not affect AMP hydrolysis (Figure 5) and probably does not change adenosine levels.

4.4 Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

E-NPPs hydrolyze nucleoside triphosphates and diphosphates, dinucleoside polyphosphates, ADP ribose, NAD⁺, and a variety of artificial substrates (e.g. *p*-Nph-5'TMP), but not AMP, and some members of this protein family hydrolyze phospholipids [15].

The use of artificial substrates is important for the differentiation between E-NPPs and ENTDPases because of the similarity between the substrates that both families hydrolyze. The

functional role of individual E-NPPs is very variable and depends on the specific catalytic properties and pattern of expression [44]. They are presumably the major enzymes responsible for the extracellular hydrolysis of dinucleoside polyphosphates, a widely distributed group of extracellular signal molecules [15]. A major function of NPP1 is in bone mineralization and also in vascular smooth muscle cell calcification [45, 46, 47]. By hydrolyzing extracellular ATP to AMP, it contributes to the formation of extracellular PPi, which can function as an inhibitor of calcification. NPP2 (Autotaxin) is a secreted protein catalytic activity present in various biological fluids, including serum, seminal plasma [48], or cerebrospinal fluid [49]. Its biological functions include cell migration, lymphocyte trafficking, angiogenesis [50, 51] and conversion of extracellular lysophosphatidylcholine (LPC) to lysophosphatidic acid in serum [15].

Enzyme activity is inhibited by EDTA [15], but red onion extract increased E-NPP activity at 500 and 1000 µg/ mL (Figure 6), suggesting that the extract could act physiologically increasing the previously mentioned effects of these enzymes in the serum. However, it is important to consider that NPP1 and NPP3 have a wide tissue distribution and their cell and tissue-specific expression may vary between rodents and humans [15].

5. Conclusion

Phenolic content present in the red onion extract is related to its antioxidant activity, which is in turn responsible for the enzymatic modulation of several classes of enzymes, including ectonucleotidases. Considering nucleotide hydrolysis, red onion extract showed the capacity to significantly increase ADP breakdown at 250 µg/ mL ($p \leq 0.05$) and the hydrolysis of *p*-Nph-5'-TMP at 500 and 1000 µg/ mL ($p \leq 0.05$). Finally, this study presents for the first time the effects of red onion phenolics on ectonucleotidase activities, evidencing the potential use of this vegetable as an adjuvant in the control and treatment of vascular diseases that include an imbalance in purinergic signaling, such as platelet aggregation disturbances or arterial hypertension, for example.

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Conflict of Interest Statement

The authors declare no conflict of interest.

References

- [1] G. Burnstock. Short and long-term (trophic) purinergic signalling. **Phil. Trans. R. Soc. B.** 371 (2016) 1-10.
- [2] G. Burnstock. Purinergic nerves. **Pharmacol Rev.** 24 (3) (1972) 509-581.
- [3] Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. **Physiol Rev.** 87 (2007) 659-797.
- [4] J.K. Angeli, D.B. Ramos, E.A. Casali, D.O.G. Souza, J.J.F. Sarkis, I. Stefanon, D.V. Vassalo, C.R. Fürstenau. Gadolinium increases the vascular reactivity of rat aortic rings. **Braz J Med Biol Res.** 44 (5) (2011) 445-452.
- [5] F.U. Fontella, A.N. Bruno, R.S. Balk, B. Rucker, L.M. Crema, M.D. Correa, A.M. Battastini, J.J. Sarkis, C.A. Netto, C. Dalmaz. Repeated stress effects on nociception and on ectonucleotidase activities in spinal cord synaptosomes of female rats. **Physiol Behav.** 85 (2) (2005) 213-219.
- [6] J. Spychala, A.G. Zimmermann, B.S. Mitchell, Tissue-specific regulation of the ecto-5'nucleotidase promoter: role of the camp response element site in mediating repression by the upstream regulatory region. **J Biol Chem.** 274 (32) (1999) 22705-22712.
- [7] D. Sandona, S. Gastaldello, T. Martinello, R. Betto. Characterization of the ATP hydrolyzing activity of α -sarcoglycan. **Biochem J.** 381 (2004) 105-112.
- [8] T.W. Traut, Physiological concentrations of purines and pyrimidines. **Mol Cell Biochem.** 140 (1) (1994) 1-22.
- [9] M.J. Bours, E.L. Swennen, F.D. Virgilio, B.N. Cronstein, P.C. Dagnelie. Adenosine 5'triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. **Pharmacol Ther.** 112 (2) (2006) 358-404.

- [10] G.G. Yegutkin. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. **Biochim Biophys Acta**, 1783 (5) (2008) 673694.
- [11] E.R. Lazarowski, R.C. Boucher, T.K. Harden. Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. **J Biol Chem**. 275 (40) (2000) 31061-31068.
- [12] G. Burnstock. Intracellular expression of purinoceptors. **Springer Science**. 11 (2015) 275–276.
- [13] R. Schmatz, T.R. Mann, R. Spanevello, M.M. Machado, D. Zanini, V.C. Pimentel, N. Stefanello, C.C. Martins, A.M. Cardoso, M. Bagatini, J. Gutierrez, C.A.M Leal, L.B. Pereira, C. Mazzanti, M.R. Schetinger, V.R. Morsch. Moderate Red Wine and Grape Juice Consumption Modulates the Hydrolysis of the Adenine Nucleotides and Decreases Platelet Aggregation in Streptozotocin-Induced Diabetic Rats. **Cell Biochem Biophys**. (2013) 1-15.
- [14] H. Zimmermann. Ectonucleotidases: Some Recent Developments and a Note on Nomenclature. **Drug Development Research**. 52 (2001) 44-56.
- [15] H. Zimmermann, M. Zebisch, N. Sträter. Cellular function and molecular structure of ecto-nucleotidases. **Purinergic Signalling**. 8 (2012) 437–502.
- [16] G. Burnstock. Pathophysiology and therapeutic potential of purinergic signaling. **Pharmacol Rev** 58 (2006) 58–86.
- [17] A.S. Catania, C.S. Barros, S.R.G. Ferreira. Vitamins and minerals with antioxidant properties and cardiometabolic risk: controversies and perspectives. **Arq Bras Endocrinol Metab**. 53 (5) (2009) 550-559.
- [18] F. Martelli, F.M.F. Nunes. Radicais livres: em busca do equilíbrio. **Ciência e Cultura**. 66 (3) (2014) 54-57.
- [19] T.K. Aalto, K.O. Raivio. Metabolism of extracellular adenine nucleotides by human endothelial cells exposed to reactive oxygen metabolites. **Am J Physiol**. 264 (2) (1993) 282286.
- [20] F. Krötz, H.Y. Sohn, M. Keller, T. Gloe, S.S. Bolz, B.F. Becker, U. Pohl. Depolarization of endothelial cells enhances platelet aggregation through oxidative inactivation of endothelial NTPDase. **Arterioscler Thromb Vasc Biol**. 22 (12) 2002 2003-2009.
- [21] C.A.T. Radovanovic, L.A. Santos, M.D.B. Carvalho. Hipertensão arterial e outros fatores de risco associados às doenças cardiovasculares em adultos. **Rev. Latino-Am. Enferm**. 22 (4) (2014) 547-553.
- [22] U.P. Onyeoziri, E.N. Romanus, U.I. Onyekachukwu. Assessment of antioxidant capacities and phenolic contents of nigerian cultivars of onions (*Allium cepa* L.) and garlic (*Allium sativum* L.). **Pak J Pharm Sci**. 29 (4) (2016) 1183-1188.

- [23] Y.S. Velioglu, G. Mazza, L. Gao, B.D. Oomah. Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. **J. Agric. Food Chem.** 46 (1998) 4113-4117.
- [24] C.A. Calliste, D.P. Allais, A. Simon, A. Marfak, C. Delage, J. Luc. Antioxidant, antiinflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. **Food Chemistry.** 80 (3) (2003) 399-407.
- [25] M.M. Souza, V.M. Recart, M. Rocha, E.P. Cipolatti, E. Badialefurlong. Estudo das condições de extração de compostos fenólicos de cebola (*Allium cepa* L.). **Rev. Inst. Adolfo Lutz**; 68 (2) (2009) 192-200.
- [26] M. Wettasinghe, F. Shahidi. Evening primrose meal: a source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. **Journal of Agricultural and Food Chemistry.** 47 (1999) 1801-1812.
- [27] W. Brand-Williams, M.E. Cuvelier, C. Berset. Use of a free radical method to evaluate antioxidant activity. **Food Science and Technology.** 28 (1995) 25-30.
- [28] M.M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248-254.
- [29] G.G. Yegutkin. Kinetic analysis of enzymatic hydrolysis of ATP in human and rat blood serum. **Biochemistry (Mosc).** 62 (6) (1997) 619-622.
- [30] K.M. Chan, D. Delfert, K.D. Junger. A direct colorimetric assay for Ca²⁺ -stimulated ATPase activity. **Anal Biochem**, 157 (2) (1986) 375-380.
- [31] H. Sakura, S. Nagashima, A. Nakashima, M. Maeda. Characterization of fetal serum 5'nucleotide phosphodiesterase: a novel function as a platelet aggregation inhibitor in fetal circulation. **Thromb Res.** 91 (2) (1998) 83-89.
- [32] B.R. Galdón, E.M.R. Rodríguez, C.D. Romero. Flavonoids in onion cultivars (*Allium cepa* L.). **Journal of Food Science.** 73 (8) (2008) 599-605.
- [33] J. Santas, R. Carbó, M.H. Gordon, M.P. Almajano. Comparison of the antioxidant activity of two Spanish onion varieties. **Food Chem.** 107 (2008) 1210–1216.
- [34] A.M. Nuutila, R. Puupponen-Pimiä, M. Aarni, K.M. Oksman-Caldentey. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. **Food Chem.** 81 (2003) 485–493.
- [35] K. Srinivasan. Antioxidant Potential of Spices and Their Active Constituents. **Critical Reviews in Food Science and Nutrition.** 54 (3) (2014) 352-372.
- [36] S.M. Shim, H.L. Yi, Y.S. Kim. Bioaccessibility of flavonoids and total phenolic content in onions and its relationship with antioxidant activity. **International Journal of Food Sciences and Nutrition.** 62 (8) (2011) 835–838.

- [37] C.R. Fürstenau. Participation of Purinergic Signaling in L-NAME Induced Arterial Hypertension. (Doctoral Thesis) (2010) 1-172.
- [38] M.N. Munkonda, G. Kauffenstein, F. Kukulski, S.A. Levesque, C. Legendre, J. Pelletier, E.G. Lavoie, J. Lecka, J. Sevigny. Inhibition of human and mouse plasma membrane bound NTPDases by P2 receptor antagonists. **Biochem Pharmacol.** 74 (10) (2007) 1524-1534.
- [39] S.C. Robson, J. Sevigny, H. Zimmermann. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. **Purinergic Signal.** 2 (2) (2006) 409-430.
- [40] J.P. Maffrand, A. Bernat, D. Delebassee, G. Defreyn, J.P. Cazenave, J.L. Gordon. ADP plays a key role in thrombogenesis in rats. **Thromb Haemost.** 59 (2) (1988) 225-230.
- [41] D. Erlinge, G. Burnstock. P2 receptors in cardiovascular regulation and disease. **Purinergic Signal.** 4 (1) (2008) 1-20.
- [42] H. Zimmermann. 5'-Nucleotidase: molecular structure and functional aspects. **Biochem J.** 285 (1992) 345-365.
- [43] H.J. Agteresch, P.C. Dagnelie, J.W. van den Berg, J.H. Wilson. Adenosine triphosphate: established and potential clinical applications. **Drugs.** 58 (2) (1999) 211-232.
- [44] C. Stefan, S. Jansen, M. Bollen. Modulation of purinergic signaling by NNP-type ectophosphodiesterases. **Purinergic Signal.** 2 (2006) 361–370.
- [45] K. Johnson, M. Polewski, D.van Etten, R. Terkeltaub. Chondrogenesis mediated by PPi depletion promotes spontaneous aortic calcification in NPP1^{-/-} mice. **Arterioscler Thromb Vasc Biol.** 25 (2005) 686–691.
- [46] R. Villa-Bellosta, X.N. Wang, J.L. Millán, G.R. Dubyak, W.C. O'Neill. Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. **Amer J Physiol Heart Circ Phy.** 301 (2011) H61–H68.
- [47] D.A. Prosdocimo, S.C. Wyler, A.M. Romani W.C. O'Neill, G.R. Dubyak. Regulation of vascular smooth muscle cell calcification by extracellular pyrophosphate homeostasis: synergistic modulation by cyclic AMP and hyperphosphatemia. **Amer J Physiol Cell Physiol.** 298 (2010) C702–C713
- [48] J. Aoki. Mechanisms of lysophosphatidic acid production. **Semin Cell Dev Biol.** 15 (2004) 477–489.
- [49] K. Sato, E. Malchinkhuu, T. Muraki, K. Ishikawa, K. Hayashi, M. Tosaka, A. Mochiduki, K.Inoue, H. Tomura, C. Mogi, H. Nochi, K. Tamoto, F. Okajima. Identification of autotaxin as a neurite retraction-inducing factor of PC12 cells in cerebrospinal fluid and its possible sources. **J Neurochem.** 92 (2005) 904–914.

- [50] L.A. van Meeteren W.H. Moolenaar. Regulation and biological activities of the autotaxin-LPA axis. **Prog Lipid Res.** 46 (2007) 145–160.
- [51] W.H. Moolenaar, A. Perrakis. Insights into autotaxin: how to produce and present a lipid mediator. **Nat Rev Mol Cell Biol.**12 (2011) 674–679.