Comparison of Antioxidant and Ant proliferative Effect among Four Passiflora Spp.

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Abstract

In the last years, increased the studies with phenolic compounds in the development of new natural drugs. The choice of solvent/method for extraction of phenolic compounds is crucial. The extraction of phenolic compounds with the solvents water, ethanol and methanol/acetone were used. The evaluation of the antioxidant capacity (DPPH;FRAP ;ABTS) and total phenolic was analysed in the four species of Pass flora. The inhibition cellular and apoptosis/necrosis analysis were studied on T lymphocytes after treatment with yellow P.edulis, purple P.edulis, P.alata and P.quadrangularis. Water was considered the best solvent to extract phenolic compounds. The yellow P.edulis was higher antioxidant activity. Already, the analysis of cell inhibition and apoptosis/necrosis on T lymphocytes showed that aqueous extract of P.alata has the highest ant proliferative effect. The results showed that different solvents can have different extraction efficiency of phenol compounds, antioxidant activity and cell activity, demonstrating the importance of a correct choice of extraction method.

Keywords: Antioxidant activity*; Passiflora edulis* Sims*; Passiflora alata Curtis; Passiflora quadrangulares*; T Lymphocytes.

1. Introduction

In recent years, substances derived from plants are described possessing characteristics antioxidants and antiproliferative (Atoui, 2005). One the most family compound involved with this property is the polyphenolic compounds.

Passiflora spp., popularly known as passion fruit, is internationally recognized as a medicinal plant by the Brazilian Pharmacopoeia (PharmacopeiaBrasileira, 2010), French (Santé, 1992), European (EDQM, 2007) and British Herbal (BritishHerbalMedicineAssociation, 1996).

Among many species, two of them are considered properly medicinal: *P.incarnata*, foundin Europe and North America, and *P.alata*, found in South America (Dhawan, 2004; Noriega, 2011).The chemical composition of *Passiflora* spp. is rich in flavonoids, phenols, alkaloids and cyanogenic compounds that act as an antioxidant leading to the hypothesis that dietary with these compounds could play a role as an antioxidant and anti-inflammatory, *in vivo*(Colomeu, 2014; Ferres, 2007). On the other hand, diseases such as cancer, cardiovascular disease and autoimmune disease as diabetes has been associated with uncontrolled cell proliferation and specific concentrations of the phenolic compounds may have a cellular ant proliferative activity (Sun, 2002). Specifically, during autoimmune diseases, the major cells involved in the majority of inflammatory process are T lymphocytes. The lymphocytes after activation produce cytokines, as tumor necrosis factor (TNF), interferon (IFN), among others, increasing the inflammatory process with the production of large amounts of free radicals (Gasteiger, 2014; Jatan, 2016).

In the present study, we compared the phenols extraction efficiency among three different solvents (water; ethanol; methanol/acetone) on yellow *P.edulis,* purple *P.edulis*, *P.alata*, and *P.quadrangularis* species considering total phenol content, antioxidant activity and antiproliferative effect on activated T lymphocyte.

2. Material and Methods

2.1 Processing leaves of *Passiflora* spp.

Passion fruit leaves (yellow *P.edulis*, purple *P.edulis*, *P.alata,P.quadrangularis)* were ceded by the Agronomic Institute of Campinas, dried in circulating air oven at 50°C/48 hours, crushed to a powder and stored refrigerated (8°C) (MARCONI, Piracicaba/SP, Brazil).

2.1.1 Extract of phenolic compounds (PC)

The extraction process was performed in dried leaves using three different solvents at the same concentration (40 mg/mL).

2.1.2 Methanol-acetone extract

For methanol-acetone extract, 1g of leaves were weighed and 15 mL of 50% methanol allowed resting for 60 minutes at room temperature. After this time, the sample was centrifuged at 9948xg for 20 minutes at 22 C°. The supernatant was transferred to a 25 mL volumetric flask and the residue suspended in 10 mL of 70% acetone for another 60 minutes at room temperature.

The sample was centrifuged again at 9948xg for 20 minutes and the supernatant was transferred to the same volumetric flask. The final volume was adjusted to 25 mL with distilled water and stored in amber glass, at 8°C, until analysis.

2.1.3 Ethanol extract

Ethanol extract analysis, 1g of dried leaves was added to 15 mL of 60% ethanol, maintained at 70°C in water bath for 60 minutes. The extract was filtered through paper and stored at room temperature. Another 10 mL of 60% ethanol was added to the residue of leaves, maintained in the water bath at 70°C for 60 minutes and filtered through paper.

Ethanol extract obtained after two filtrations were transferred to volumetric flask and adjusted to a 25mL with distilled water. The sample was stored in amber glass, at 8°C, until analysis.

2.1.4 Aqueous extract

For aqueous extract, 1g of dried leaves and 25 mL of water, was autoclavedat 121°C for 20 minutes. The aqueous extract was filtered and transferred to 25 mL volumetric flask. The sample was stored in amber glass, 8°C until analysis.

2.2 Determination of total phenols

For total phenolic analysis, 50µL of each extract, 800µL distilled water, and 50µL Folin – Ciacalteu was transferred to 1.5 mL tube. After that, 100µL of 1N sodium carbonate solution was added to each flask, homogenized and incubated for 2 hours at dark ambient/room temperature. The reference curve of total phenols was made diluting gallic acid - 0.1 to 0.016 mg/mL. Quantification was performed using a spectrophotometer at 725 nm (SpectraMAx 190®, CA, USA), and the results were expressed as gallic acid equivalents (EAG/mg/100g sample).

2.3 Analysis of antioxidant activity

Methanol-acetone extract, ethanol and aqueous extract of passion fruit (yellow *Passiflora edulis,* purple *Passiflora edulis, Passiflora alata* Curtis *and Passiflora quadrangularis*) were carried out using 3 different antioxidant assays, DPPH, FRAP, and ABTS. The samples of three extracts were diluted in the following concentrations: 0.004, 0.01, 0.02 and 0.03 g/mL. Assays for determination of antioxidant activity are described below.

2.3.1 Sequestration of organic radical DPPH (2.2-diphenyl-1-picrylhydrazyl)

The assay DPPH dissolved 1.2 mg DPPH in 50 mL of 0.06mM methanol. The amount of 25 µL of leaf extracts was mixed with 1mL (0.06mM) DPPH solution and, after 30 minutes, read in the spectrophotometer (SpectraMax) at 515nm. The reading of the curve was linear between 0.004 and 0.024 mg/mL of DPPH. The results were expressed as the percentage of sequestration and calculated according to the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \right)$$

2.3.2 Metal reduction capacity - FRAP (*Ferric Reducing Antioxidant Power*)

Stock solutions were prepared in 300mM acetate buffer (3.1g - $C_2NaO_2 \cdot 3H_2O$ / 16 mL -$C_2H_4O_2$) pH 3.6; 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40nM hydrochloric acid, 20mM ferric chloride and 2mM ferrous sulphate solutions. The FRAP solution was prepared using 50 mL acetate buffer, 5 mL TPTZ and 5 mL ferric chloride. Each leaves extract (30 µL) was mixed with 90 µL water and 900 µL FRAP solution, homogenized and kept in the water bath, at 37°C, for 30 minutes. Reading at 595 nm was performed using a spectrophotometer (SpectraMax), and the curve was linear between 500 and 2000 mM of iron sulfate. The results were expressed as $FeSO_4$/g.

2.3.3 ABTS free radical (2,2´- AZINO-BIS(3-ethylbenzothiazoline-6-sulfonic acid)

The ABTS stock solution (192 mg ABTS+ 50 mL water) was mixed with 88 µL of 140mM potassium persulfate and kept standing for 16 hours in the dark, at room temperature. ABTS solution was diluted in ethanol until the absorbance at 0.70nm ± 0.05nm at 734 nm using the spectrophotometer (SpectraMax). Leaves extract (10 µL) were mixed with 1 mL of ABTS solution for 6 minutes, reading at 734nm, and the standard was linear between 100 and 2.000µM of 2mM Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid). The results were expressed as mM Trolox equivalents (TE/g).

2.4 Animal model

BALB/c mice (n=5) were obtained from Multidisciplinary Centre for Biological Research- University of Campinas. The animals with 30 weeks of life were used to the experimental assays. All procedures were approved by Ethics committee on the use of animals. The animals were sacrificed by heart puncture under anesthesia (Ketamine hydrochloride 100-200mg and Xylazine hydrochloride 5-16mg / kg body weight, Dopalen, Vetbrands, Paulínia, SP, Brazil). The spleens were aseptically removed and the T lymphocytes were separated through the adhesion protocol on nylon wool fibber columns as described by Henry et al. (Henry C, 1980).

2.5 T Lymphocytes proliferation assay

T lymphocytes proliferation was analysed in aqueous extract of four species of Passiflora. The total lymphocytes were adjusted to $2{,}5x10^5$ cells/200µL density in RPMI 11mM of glucose (Gibco, Invitrogen Corporation, Massachusetts, USA) containing 10% bovine fetal serum (Hyclone, Thermo Scientific, South Logan UT, USA) and 5µg/mL of Concanavalin A (ConA) (Sigma, St.Louis, USA) antigen-independent mitogen. The lymphocytes were stained with 1,25 µg CFSE dye (Biolegend, CA, USA) and were treated with different concentration of aqueous extract of yellow *Passiflora edulis* (2.0, 2.5, 3.0, 4.0, 5.0 e 10 mg/mL), purple *Passiflora edulis* (0.8, 1.0, 2.0, 2.5, 3.0 e 4.0 mg/mL), *Passiflora alata* Curtis (0.5, 0.8, 1.0, 2.0, 2.5 e 3.0 mg/mL) and *Passiflora Guadrangularis* (0.5, 1.0, 2.0, 2.5, 3.0 e 4.0 mg/mL). The culture was plated in 96 bottom plates, for 96 hours in cell incubators at 37°C with 5% of CO_2.

After 96 hours, the culture of T lymphocytes was washed with 1M PBS centrifuged at 300 xg for 5 minutes and fixed in 1% formaldehyde until flow cytometry analysis (Guava easycyte, Millipore®, Bellirica, EUA).

2.7 Apoptosis and Cell Necrosis

The culture of T lymphocytes was analysed to apoptosis and necrosis by FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend CA, USA). The isolated lymphocytes were stained with 2 µl of Annexin V and 2 µl of 7-AAD, for 15 minutes in the dark at room temperature. After this time added 400µl of Annexin V Biding Buffer, according to manufacture instruction. The analysis was performed by flow cytometry (Guava easycyte).

2.8 Statistical analysis

Statistical analysis of data was performed using ANOVA, followed by Kruskal-Wallis and Dunn´s post-test. Significant differences were set at P values lower than 0.05. Analysis software by GuavaSoft (Millipore,USA).

3. Results

3.1 Total Phenols contents

The concentrations of total phenols in obtained extracts showed similar values in aqueous and ethanolic extracts of the four species of *Passiflora* sp. (Table.1), while methanol solvent showed the lowest concentration of total phenols (Fig. 1).

Table 1: Analysis of Total Phenols. Statistics by ANOVA Kruskal-Wallis test and Dunn´s test. *p<0.05;p<0.01; ***p<0.001**

	Total Phenol- mgEAG/g sample		
	Aqueous Extract	Ethanol Extract	Methanol Extract
P.edulis (yellow)	0,228±0,009	0,217±0,007	0,174±0,006
P.edulis (purple)	0,099±0,007	0,098±0,007	0,089±0,003
P.alata	0,096±0,003	0,071±0,027*	0,056±0,002
P.quadrangularis	0,052±0,002***	0,046±0,002***	0,023±0,002**

3.2 DPPH

The technique DPPH showed that among the three solvents analysed, the aqueous and ethanolic extract showed the highest antioxidant activity (Table.2). The analysis of antioxidant activity, *Passiflora edulis* (yellow and purple) showed a higher activity compared with the others two species *Passiflora alata* and *Passiflora quadrangularis*. *Passiflora quadrangularis* showed the lowest antioxidant activity (Fig.2).

Table 2:Analysis of DPPH. Statistics by ANOVA Kruskal-Wallis test and Dunn´s test. *p<0.05; ***p<0.001

	DPPH % of Scavering		
	Aqueous Extract	Ethanol Extract	Methanol Extract
P.edulis (yellow)	54,27±3,11	53,21±4,61	40,88±5,41
P.edulis (purple)	31,30±6,22	38,81±1,57	32,04±1,18
P.alata	23,07±3,96*	30,57±1,29*	4,31±1,33***
P.quadrangularis	29,08±2,68***	33,20±0,70***	11,11±1,79*

3.3 FRAP

The FRAP analysis, showed a higher antioxidant activity in aqueous extract when compared with ethanol and methanol/acetone extract (Table.3). The yellow *Passiflora edulis* showed the highest antioxidant activity and the *Passiflora quadrangularis* showed the lowest activity (Fig.3), similarly found in DPPH analyses (Fig.2).

Table 3:Analysis of FRAP. Statistics by ANOVA Kruskal-Wallis test and Dunn´s test. *p<0.05; ***p<0.001

	FRAP- µM FeSO3/g sample		
	Aqueous Extract	Ethanol Extract	Methanol Extract
P.edulis (yellow)	8452±583	6262,9±452	4893,6±152
P.edulis (purple)	4489±265	2917,4±154	3453,4±229
P.alata	4675±350	2306,8±160*	1995,4±142*
P.quadrangularis	1295±49***	1915,2±126***	1155,8±39***

3.4 ABTS

ABTS analysis showed similar results to the DPPH, aqueous and ethanol extract showed the higher antioxidant activity when compared to methanol/acetone extract (Table.4). The *Passiflora edulis* (yellow and purple) showed the highest antioxidant activity when compared to *Passiflora alata* and *Passiflora quadrangularis* (Fig.4).

Table 4:Analysis of ABTS. Statistics by ANOVA Kruskal-Wallis test and Dunn´s test. *p<0.05;p<0.01; ***p<0.001**

	ABTS- µM Trolox/gsample		
	Aqueous Extract	Ethanol Extract	Methanol Extract
P.edulis (yellow)	2778,3±216	2546,6± 169	2038,3±239
P.edulis (purple)	2160±191	1996,6±154	2090±173
P.alata	1963,3±329*	1698±271***	1138,3±196
P.quadrangularis	1637±304***	1816,5±207*	903±211**

3.5 Lymphocytes Proliferation

The analysis of antioxidant activity demonstrated that aqueous extract was the best solvent for extraction of the phenolic compounds from Passiflora spp. T lymphocyte proliferation assay was performed to analysis in a dose response effect of the aqueous extracts on inhibition cell proliferation. The analysis of T lymphocytes proliferation showed the reduction of 50% (IC50) when treated with yellow *Passiflora edulis* at the concentration of 2mg/mL, purple *Passiflora edulis* in2.5 mg/mL, *Passiflora alata* in 0,8mg/mL and *Passiflora quadrangularis* in2mg/mL (Fig.5). The comparison among IC50 of four species of *Passiflora*, showed that *P.alata* have the highest activity in cell inhibition proliferation at lowest extract concentration (0,8mg/mL).

3.6 Apoptosis and Necrosis

The apoptosis/necrosis analysis, at IC50 concentration for all aqueous extracts, showedyellow *Passiflora edulis* with late apoptosis and necrosis cells (25% in both); purple *Passiflora edulis* 33% in late apoptosis and 36% in necrosis.

Both *Passiflora alata* e *Passiflora quadrangularis* showed similar results; the majority cells are found in late apoptosis (40% and 44% respectively), and in necrosis (38% and 31%) (Fig.6).

Thus, the differences among species of *Passiflora* suggest that although the aqueous extract from species *Passiflora edulis* (yellow and purple) exhibited the highest antioxidant activity, the effect on cell proliferation of isolated T lymphocytes was the lowest. Nonetheless, the aqueous extract of *Passiflora alata* presented low antioxidant activity when compared to *Passiflora edulis*, but a higher inhibitory activity on T lymphocytes proliferative assay.

4. Discussion

Solvents as methanol, ethanol, acetone, and propanol are commonly used for phenolic extraction from plants.The choice of the best solvent will depend on solubility and solvent polarity of these compounds (Larrauri, 1997; Rizvi, 2010). Usually, solvents with low polarity are used to extract lipophilic phenols and with high polarity for hydrophilic phenols. The difference in solubility of the phenolic compounds, the correct choice a suitable solvent is extremely important during an analysis (Azmir, 2013; Sibul, 2016)

Phenolic compounds are ubiquitously present in plants with antioxidant properties. The activity of total phenols is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Thaipong, 2006).

The extraction of phenolic compounds showed effectiveness by three solvents (water, ethanol and methanol/acetone), but water demonstrated to be the best solvent for extracting total phenol and facility of preparation in four species of *Passiflora* spp. The *Passiflora* spp. has been used as medicinal plants in several countries. Epidemiological studies have shown that consumption of antioxidant-rich food and beverages can reduce the risk of heart disease, diabetes, inflammatory process, cancer, among others (Noriega, 2011; Pulido, 2000). The variety of fruits with bioactive compounds has been increasingly investigated. Antioxidant consumption is important for health because of the ability of redox molecules in scavenging free radicals. There is a strong evidence that free radicals are responsible for cell damage, leading to various chronic diseases, including diabetes mellitus (Nimse, 2015; Valko, 2016).Chamarro *et al.*(Chamarro, 2012) and our work group showed that extraction using water, at high temperature under controlled pressure, extract the phenolic compounds more efficiently. There are different methods to evaluate the antioxidant capacity of plants extracts, and at least three standards assays are necessary to prove the efficiency, such as ABTS, DPPH, FRAP and ORAC (Brand-Willians, 1995; Thaipong, 2006).

In this work, we analysed the extracts of *Passiflora* spp. leaves using DPPH, FRAP and ABTS, demonstrating that antioxidant capacity depends on the efficiency of the method and the solvent used for extraction. Our results showed that Passiflora spp. species presented different antioxidant capacity, which may be related to the variation of concentration among compounds present in each species and to the solvent used. The antioxidant methods have shown different results among previous reports, but with a high correlation between ABTS and DPPH methods (Schaich, 2015). Similarly, we found a correlation between DPPH and ABTS in yellow *Passiflora edulis* extracts comparing the three studied solvents.

P.quadrangularis leaf extract showed similar results to the study of Contreras-Calderón,*et al*.,(Contreras-Calderón, 2011), with lower antioxidant activity, when compared to yellow and purple *P.edulis* and *P.alata*. Moreover, similarly to Patel study (Patel, 2009), the leaf extract of *Passiflora edulis* showed a high antioxidant activity. However, despite the antioxidant activity found in the extracts of four *Passiflora* spp, the determination of the potential of oxidation reduction can vary depending on compounds present in the species analysed (Colomeu, 2014; Ramaiya, 2012).

The evaluation of the *in vivo* and *invitro* antioxidant activity is an important tool in the investigation of antioxidant properties. Even though the *P.edulis* shows a high antioxidant activity, this extract presents low inhibition of activated lymphocytes in cell proliferation assay. However, *P. alata* leaves extract showed more efficiency to promote cell inhibition. These differences on T lymphocytes proliferation, among species of *Passiflora*, may be related to the class of phenolic compounds content in each species (Atoui, 2005; Zucolotto, 2012).

Among the polyphenols found in *Passiflora sp*, the main compounds found were Vitexin, Isoorientin, Catechin, Rutin, Orientin, Isovitexin and others (Figueiredo, 2016). The polyphenols act as an anti-inflammatory, but in specific concentration can act as antimicrobial and anti-tumor, leading to cell death (Saravanan, 2014) . Our results suggest that the polyphenols present in aqueous extract of *P.alata* have dose dependent properties on T lymphocytes proliferation with apoptosis/necrosis induction. Previous studies suggested that polyphenols, such as Isoorientin, Vitexin, Rutin and Catechin are related to decreasing of inflammatory cells by anti-proliferative action (Tang, 2014).

Moreover, our results suggest that effect of Passiflora spp. leaves extract at IC50 concentration inhibit the proliferation of activated T lymphocytes and induces to apoptosis/necrosis, properties that could be benefits to the treatment of inflammatory diseases as a natural anti-inflammatory and antiproliferative. However, the clinical trial must be established to confirm these activity properties in human diseases.

5. Conclusion

Our results showed that leaves extract of *Passiflora* spp is an excellent source of the antioxidant compound, but the different compounds extracted depended on solvents and the applied methods. Besides that, we verify differences between extraction of phenolic compounds *in vitro* effect on T lymphocytes suggesting that different phenolic compounds may play a role on inhibiting T lymphocyte proliferation by assisting during an inflammatory process.

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