UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal
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SARAH FERREIRA GUIMARÃES

ACÚMULO DE METABÓLITOS SECUNDÁRIOS EM ÓRGÃOS DE *Passiflora edulis* AO LONGO DO DESENVOLVIMENTO VEGETAL

Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.

Área de Concentração Fisiologia Vegetal

BELO HORIZONTE – MG

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Área de Concentração Fisiologia Vegetal

Orientador: Profa. Dra. Luzia Valentina Modolo
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Dra. Luzia Valentina Modolo (orientadora)

Dra. Teresa Drummond Correia

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Dra. Maria do Carmo Batitucci

Dr. Cleber Cunha Figueredo

“Menina, amanhã de manhã

quando a gente acordar

quero te dizer que a felicidade vai

desabar sobre os homens, vai”

Menina, Amanhã de Manhã (o Sonho Voltou) – Tom Zé

À memória de minha mãe, Eunice, que me ensinou a colocar amor e dedicação em todas as coisas.

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Table of Contents

Resumo geral:

O gênero *Passiflora*, família Passifloraceae, inclui cerca de 600 espécies, distribuídas em regiões tropicais e subtropicais do mundo. Este trabalho analisou quais são os principais metabólitos secundários produzidos por *Passiflora edulis* em seus órgãos. Além disso, foi investigado se ocorre variação nas concentrações dessas substâncias em função dos órgãos analisados, e da fase do desenvolvimento vegetal (juvenil, floração e frutificação). As folhas foram os órgãos que mais acumularam fenólicos/flavonoides, independentemente do estágio de desenvolvimento da planta. Os extratos de folhas capturaram eficientemente as espécies reativas de nitrogênio (DPPH) em até 67%, enquanto os extratos de raiz e casca de fruta foram mais eficientes na captura de espécies reativas de oxigênio (em até 80% O_2^-). A atividade das enzimas antioxidantes catalase, ascorbato peroxidase e superóxido dismutase, em folhas de *P. edulis*, tiveram perfis semelhantes com atividade máxima na fase de frutificação. A análise por cromatografia em camada delgada indicou a presença de flavonoides em todos os extratos analisados, taninos em extratos de raízes e terpenos em folhas, casca de frutos e extratos de raízes. Extratos de folhas em qualquer estágio fenológico apresentaram teores de flavonoides 10 vezes superiores aos encontrados em outros extratos de acordo com protocolo da Farmacopeia Brasileira. A análise por Cromatografia Líquida de Alta Eficiência revelou que extratos de folhas e cascas de frutas apresentam nove flavonoides *C*-glicosídeos diferentes, glicoconjugados de luteolina, apigenina e crisina. Extratos de cascas de frutos na frutificação foram os mais potentes contra urease de *C. ensiformis*, pois inibem a atividade da enzima em mais de 43%, enquanto hidroxiuréia comprometeu a atividade da urease em 35,6%. Sendo assim, diferentes classes de metabólitos secundários presentes nas plantas de *P. edulis* variam em função das fases de desenvolvimento e dos órgãos analisados.

Palavras-chave: maracujá, metabólitos secundários, antioxidantes, compostos fenólicos, flavonoides, glicoconjugados da apigenina, glicoconjugados da luteolina, glicoconjugados da crisina.

General Abstract

The genus *Passiflora*, family Passifloraceae, includes about 600 species, distributed in tropical and subtropical regions of the world. This work analyzed which are the main secondary metabolites produced by *Passiflora edulis* in their different organs. In addition, it investigated whether there is variation in concentrations of these substances in function of the analyzed organ and the phase of the vegetal development (juvenile, flowering and fruiting). Leaves were the organs that most accumulated phenolics/flavonoids, regardless of the plant developmental stage. Leaf extracts efficiently scavenged reactive nitrogen species (DPPH) by up to 67% while root and fruit shell extracts were more efficient in capturing reactive oxygen species (by up to 80% O_2^-). The activity of antioxidant enzymes catalase, ascorbate peroxidase and superoxide dismutase in leaves of *P. edulis* had similar profiles with maximum activity in the fruiting phase. Thin layer chromatography analysis indicated the presence of flavonoids in all extracts analysed, tannins in root extracts, and terpenes in leaves, fruit shell and root extracts. Leaf extracts at any phenological stage presented flavonoid contents that were 10-fold higher than those found in other extracts according by Brazilian Pharmacopoeia protocol. Ultra High Performance Liquid Chromatography analysis, disclosed that leaves and fruit shell extracts present nine different flavonoid *C*-glycosides, likely luteolin, apigenin and chrysin glycoconjugates. Extracts of fruit shell on fruiting were the most potent against *Canavalia ensiformis* ureases, they inhibit the enzyme activity by over 43%, whereas hydroxyurea compromised the urease activity by 35.6%. The different classes of secondary metabolites present in *P. edulis* plants vary in fuction of the phases of development and the organs analyzed.

Keywords: passion fruit, secondary metabolites, antioxidants, phenolic compounds, flavonoids, apigenin glycoconjugates, luteolin glycoconjugates, chrysin glycoconjugates.

General Introduction

The genus *Passiflora*, family Passifloraceae, includes about 600 species (Wosch et al., 2017; Ayres et al., 2015) distributed in the tropical and subtropical regions of the world (Ayres et al., 2015). *Passiflora edulis* Sims f. *flavicarpa* O. Deg. (yellow passion fruit) is the native specie (Zibadi & Watson, 2004) most cultivated and used in Brazil by the juice industry (Meletti, 2011; Pertuzatti et al., 2015).

P. edulis f. *flavicarpa* O. Deg is popularly known as 'maracujá azedo' in Brazil, 'cocorilla' in Colombia, and 'passion fruit' in the USA and Europe (MOBOT, 2019). Many species of the genus *Passiflora* are traditionally considered as medicinal plants because of their sedative and anxiolytic properties (Freire et al., 2018), used for for the treatment of neurosystem disorders, such as anxiety, migraine and insomnia (Zibadi & Watson, 2004; Ayres et al., 2015). Ethnobotanical studies indicate that throughout the development of human species, there has been a number of times the of use plant-derived metabolites for medical treatment.

In plants, a wide variety of secondary metabolites are synthesized through the primary metabolites. Secondary metabolites are organic compounds of low molecular weight, which were initially defined as non-essential for plant growth and development (Deborde et al., 2017). These compounds are related to plant-environment interactions (defense against herbivores and pathogens, protection against biotic and abiotic stresses and allelopathy), and to plant reproductive strategies (colour of flowers, polen production, aromas, etc) (Ramakrishna & Ravishankar, 2011; Deborde et al, 2017).

Metabolites derived from *C*-glycosylated apigenin and luteolin (vitexin, isovitexin, orientin, isoorientin, schaftoside) (Zeraik & Yariwake, 2010; Li et al., 2011; Silva et al., 2013; Ayres et al., 2015; Costa et al., 2016; Wosh et al., 2017) and and harmane *β*-carbolinic alkaloids (Costa et al., 2016; Medina et al., 2017; Freire et al., 2018) are described as major

compounds in *P. edulis*. *C*-glycosylated flavones and *β*-carbolinic alkaloids acts in *P. edulis* plants as hydrophilic antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents, UV filters and can be substrate of polyphenol oxidase, protecting plant tissues after physical damage (Praveena et al., 2014). In addition, these metabolites are used medicinally to treat human diseases.

Despite the evidence of ethnobotanical studies about the presence of secondary metabolites of medicinal interest in other plant parts of *P. edulis*, besides leaves, there is a lack of studies in this sense (Zeraik & Yariwake, 2010; Li et al., 2011; Silva et al., 2013; Ayres et al., 2015; Medina et al., 2017). Therefore, this work investigated how the balance of secondary metabolites occurs during the development of *P. edulis*. In order to carry out this study, we followed the development of a passion fruit orchard during the juvenile (before the first flowering) and flowering and fruiting periods. Leaf, fruit shel, fruit pulp and roots extracts of *P. edulis* were prepared and analyzed.

Goals

This thesis sought to understand which are the main secondary metabolites produced by *P. edulis* in their different organs and developmental phases. In addition, it investigated whether there is variation in concentrations of these substances in function of the analyzed organ and the phase of the vegetal development. Biological assays of inhibition of *Canavalia ensiformis* urease enzyme and microbiological activity against phytopathogenic fungi were performed.

Specific aims:

Chapter 1

This chapter evaluated the accumulation of total phenolic compounds and flavonoids in extracts of *P. edulis* organs in distinct plant development stages. The corresponding ethanolic extracts were further used to determine the extent of the potential of *P. edulis* to scavenge free radicals while leaves were analyzed for the endogenous activity of antioxidant enzymes.

Chapter 2

This chapter evaluated qualitatively and quantitatively the classes of secondary metabolites of *P. edulis* in the leaves, fruit shell, roots and fruit pulp at different development phases using techniques of thin layer chromatography, quantification of flavonoids by the Brazilian Pharmacopoeia method, and High Performance Liquid Chromatography. In addition, biological assays were performed with *P. edulis* extracts, inhibition of urease enzyme activity and susceptibility testing against fungi *Fusarium solani* complex and *Colletotrichum gloeosporioides* complex.

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CHAPTER 1

Phenolic content and antioxidant activity of *Passiflora edulis* parts as a function of plant developmental stage

(Manuscrito em aprovado na Acta Botanica Brasilica)

Phenolic content and antioxidant activity of *Passiflora edulis* parts as a function of plant developmental stage

Running title: Phenolics accumulation in *P. edulis* organs

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Abstract. *Passiflora edulis* Sims var. *flavicarpa* O. Deg. (Yellow-passion fruit) is the native species most used by juice industries in Brazil while its leaf extracts are widely employed in popular medicine. This study focused on the evaluation of phenolic contents in leaves, roots, fruit shells and pulps of *P. edulis* plants at juvenile, flowering and fruiting stages. The extent of reactive nitrogen and oxygen species scavenging and/or degradation by plant extracts was also investigated and the activity of antioxidant enzimes. Leaves were the organs that most accumulated phenolics/flavonoids, regardless of plant developmental stage. Leaf extracts efficiently scavenged reactive nitrogen species (DPPH) by up to 67% while root and fruit shell extracts effectively captured reactive oxygen species (up to 80% O_2^-). Maximum activity of catalase (CAT; 51.6 mmol H_2O_2 min^{-1} mg $prot^{-1}$) and ascorbate peroxidase (APX; 2.2 mmol ascorbate min^{-1} mg $prot^{-1}$) was recorded in leaf extracts from plants at fruiting stage. Superoxide dismutase (SOD) activity reached the highest levels (37.5 U min^{-1} mg $prot^{-1}$, in average) in plant leaves at both juvenile and fruiting plants. Overall, these results suggest that, for therapeutic purposes, *P. edulis* parts should be harvested when plants are at fruiting stage due to the excellent antioxidant properties and accumulation of phenolic compounds.

Keywords: Passion fruit, secondary metabolism, DPPH, superoxide anion, antioxidant enzymes, flavonoids.

1. Introduction

The genus *Passiflora* (family Passifloraceae) includes about 600 species (Ayres *et al.* 2015; Wosh *et al.* 2017) distributed in the tropical and subtropical regions of the world (Ayres *et al.* 2015). Over 140 species were described to occur in Brazil, in which 83 of them are considered endemic (Gomes *et al.* 2017) whereas 60 species produce edible fruits (Pertuzatti *et al.* 2015). *Passiflora edulis* Sims var. *flavicarpa* O. Deg. (yellow passion fruit) is a perennial vine that bears trilobate, toothed-edged leaves. A lonely flower emerges from each node surrounded by three green bracts containing five sepals and white petals, corolla purple in the base, five stamens with large anthers (Zibadi & Watson 2004). *Passuiflora edulis* is the native species most cultivated and used in Brazil by the juice industry (Pertuzatti *et al.* 2015). Cultivation of *P. edulis* in Brazil for commercial purposes started in the early 1980s and was expanded to date by family farming (Meletti 2011).

Extracts of *Passiflora spp*. leaves have been used in popular medicine for the treatment of neurosystem disorders, such as anxiety, migraine and insomnia (Zibadi & Watson 2004; Ayres *et al.* 2015). Ethnobotanical studies show that the fruit pulp is used as cardiac tonic, moderate diuretic, digestive stimulant and for treatment of asthma, bronchitis, whooping cough and urinary infections (Zibadi & Watson 2004). Indeed, *P. edulis* was included in the Brazilian National List of Medicinal Plants of Interest to the Unified Health System and in the Brazilian Pharmacopoeia in 2009 and 2010, respectively (Ministério da Saúde do Brasil 2009; Brasil 2010).

The production of secondary metabolites in leaves of *P. edulis* has been disclosed but the phenolic contents in roots, fruit shells and pulps remain to be evaluated. This information, together with the potential of extracts from different parts of *P. edulis* as antioxidant will be valuable to rationalize the popular use of this species for the treatment of several diseases. This work evaluated the accumulation of total phenolic compounds and

flavonoids in different parts of *P. edulis* following distinct plant development stages. The corresponding ethanolic extracts were further used to determine the extent of the potential of *P. edulis* to scavenge free radicals while leaves were analyzed for the endogenous activity of antioxidant enzymes.

2. Materials and methods

2.1. Plant material and experimental design

Samples were harvested from plants field cultivated in the Pitangueiras Farm, located in Sooretama, Espírito Santo, Brazil (19°12'05,6''S 40°03'38,5''W), unless otherwise stated. A total of five harvestings took place in the phenological stages: juvenile (Dec 2017; leaves and roots), flowering I and II (Feb 2017 and Sep 2017, respectively; leaves and roots) and fruiting I and II (Nov 2016 and Dec 2017, respectively; leaves, roots and fruits) (Fig. 1). The shells and pulps were separated from the fruits for the analyses. Plants at juvenile stage were characterized by those grown in a greenhouse at Incaper Farm in Linhares, Espírito Santo, Brazil (T_{min} of 24.1 °C and T_{max} of 29.2°C) for 65 days before the first flowering event. The species was identified, and the voucher specimen deposited in the herbarium of the Department of Botany at the Federal University of Minas Gerais under the number BHCB 184739.

Sampling of plant parts of *P. edulis* was completely randomized in the orchard, with eight replicates per plant part collected, where collections were carried out in the juvenile, flowering I- II, and fruiting I – II phases.

Figure 1. Representative images of *Passiflora edulis* plants at the developmental stages investigated. Plants from the Pitangueiras Farm (Sooretama, Espírito Santo, Brazil) except for those at juvenile stage, which were grown in a greenhouse.

2.2. Ethanolic extracts preparation

The extracts were prepared using ethanol PA successively until exhaustion of samples (Brasil 2010; Shah *et al.* 2004; Shelar *et al.* 2018; Carvalho *et al.* 2019). The systems were filtered and the organic fraction evaporated at temperatures below 50 °C (Dai &

Mumper 2010; Shelar *et al.* 2018) to obtain the corresponding solid residues. The extracts yield was determined considering the plant material fresh weight and the mass of the dried organic fraction. One milligram of each dry extract was resuspended in 1 mL of ethanol absolute and the corresponding ethanolic extract used in the subsequent analysis.

2.3.Quantification of total phenolic compounds and total flavonoids in ethanolic extracts

The determination of phenolic compounds was performed according to else where (Murphy *et al.* 2002). Briefly, 1-volume of sample was mixed to 5-volume of 1X Folin Ciocalteu reagent and 4-volume of 7.5% sodium carbonate. Each system was incubated for 30 min at room temperature and 150 rpm for the measurement of absorbance at 765 nm. Experiments were done with four biological replicates and tannic acid was used as a standard (Box 1983; Blainski *et al.* 2013). The total amount of phenolic compounds was expressed as equivalents of tannic acid/dry extract.

The total flavonoid content was determined in the same samples following standard procedures (Jayaprakasha *et al.* 2001). One volume of ethanolic extract was added to 2.5-volume of 4% HCl and 2.5-volume of 10% vanillin. Each system was incubated for 30 min at room temperature and 150 rpm and analyzed at 460 nm. Quercetin was used as a flavonoid standard and the results were expressed as equivalents of quercetin/dry extract.

2.4. Scavenging of reactive nitrogen species

The potential of ethanolic extracts of *P. edulis* (1 mg mL^{-1}) to scavenge reactive species of nitrogen was investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (da Silva *et al.* 2012). One volume of ethanolic extract was added to equal volume of 200 μM DPPH and the systems were incubated for 30 min at room temperature followed by analysis at 517 nm. The percentage of DPPH scavenged by the ethanolic extract was

determined according to the formula DPPH $_{(\%)}$ = [1 - (As/Ac)] x 100, where Ac corresponds to the absorbance of systems containing DPPH only and As the absorbance of systems containing DPPH plus ethanolic extract.

2.5. Scavenging of Reactive oxygen species

The potential of ethanolic extracts of *P. edulis* (1 mg mL^{-1}) to scavenge reactive species of oxygen was investigated using anion superoxide radicals (da Silva *et al.* 2012). Superoxide anions were artificially generated in a system containing 1 µM EDTA, 17 µM L-methionine, 10 µM nitroblue tetrazolium (NBT) and 2 µM riboflavin in the presence or absence of plant extract (1:1) after incidence of fluorescent light for 10 min at 25°C. Control reactions were incubated for 10 min, at 25 °C in the dark. The absorbance was monitored at 525 nm and the polyphenol resveratrol was used as positive control.

2.6. Activity of antioxidant enzymes in leaves of P. edulis

The activity of ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) in leaves of *P. edulis* was assessed essentially as described (da-Silva *et al.* 2017). The molar extinction coefficients (ε) of 2.8 mM^{-1} cm^{-1} and 39.4 mM^{-1} cm^{-1} were used to determine oxidized ascorbate and H_2O_2 degradation for the estimation of APX and CAT activities, respectively. One unit of SOD refers to the amount of SOD necessary to inhibit the reduction of NBT by 50%. The total protein content in the leaf samples was determined according to the method of Bradford (Bradford 1976).

2.7. Statistical analyses

Each of the eight biological samples were analyzed in all tests in triplicate. Data were submitted to Shapiro-Wilk to check the normality and F test to verify the distribution using the ASSISTAT software (Silva & Azevedo, 2016). Data obtained were analyzed in two different ways, first by comparing the plant parts obtained at each stage of development individually, and then globally, by comparing all stages of development with each other. Data from were submitted to analysis of variance and mean test of Scott-Knott ($P < 0.01$) using ASSISTAT software (Silva & Azevedo, 2016).

3. Results

3.1. Total phenolics/flavonoids in P. edulis *extracts*

First, it was analyzed the extent of phenolic compounds accumulation in parts of *P. edulis* plants within a developmental stage and then all the results for the distinct plant developmental stages were compared to each other.

Leaves of *P. edulis* plants at juvenile (122.0 µg tannic acid mg^{-1} dry extract), fruiting I (710.0 µg tannic acid mg^{-1} dry extract) and flowering I (571.0 µg tannic acid mg^{-1} dry extract) stages presented the highest levels of phenolic compounds among the studied plant organs (Fig. 2). In all plant developmental stages analyzed, leaves from *P. edulis* plants presented the highest levels of flavonoids compounds when compared to the other organs (Fig. 2).

Comparison between the different phenological stages and plant parts showed that the greatest amounts of total phenolic compounds were recorded in leaves of plants at fruiting I (710.0 µg tannic acid mg^{-1} dry extract). Likewise, maximum accumulation of flavonoids occurred in leaves of plants at flowering I (161.2 µg quercetin mg^{-1} dry extract) and fruiting II (151.1 µg quercetin mg^{-1} dry extract) stages (Tab. 1). Pluviometric and

temperature variations during the 13 months in which *P. edulis* plants were monitored are shown in Fig. 3.

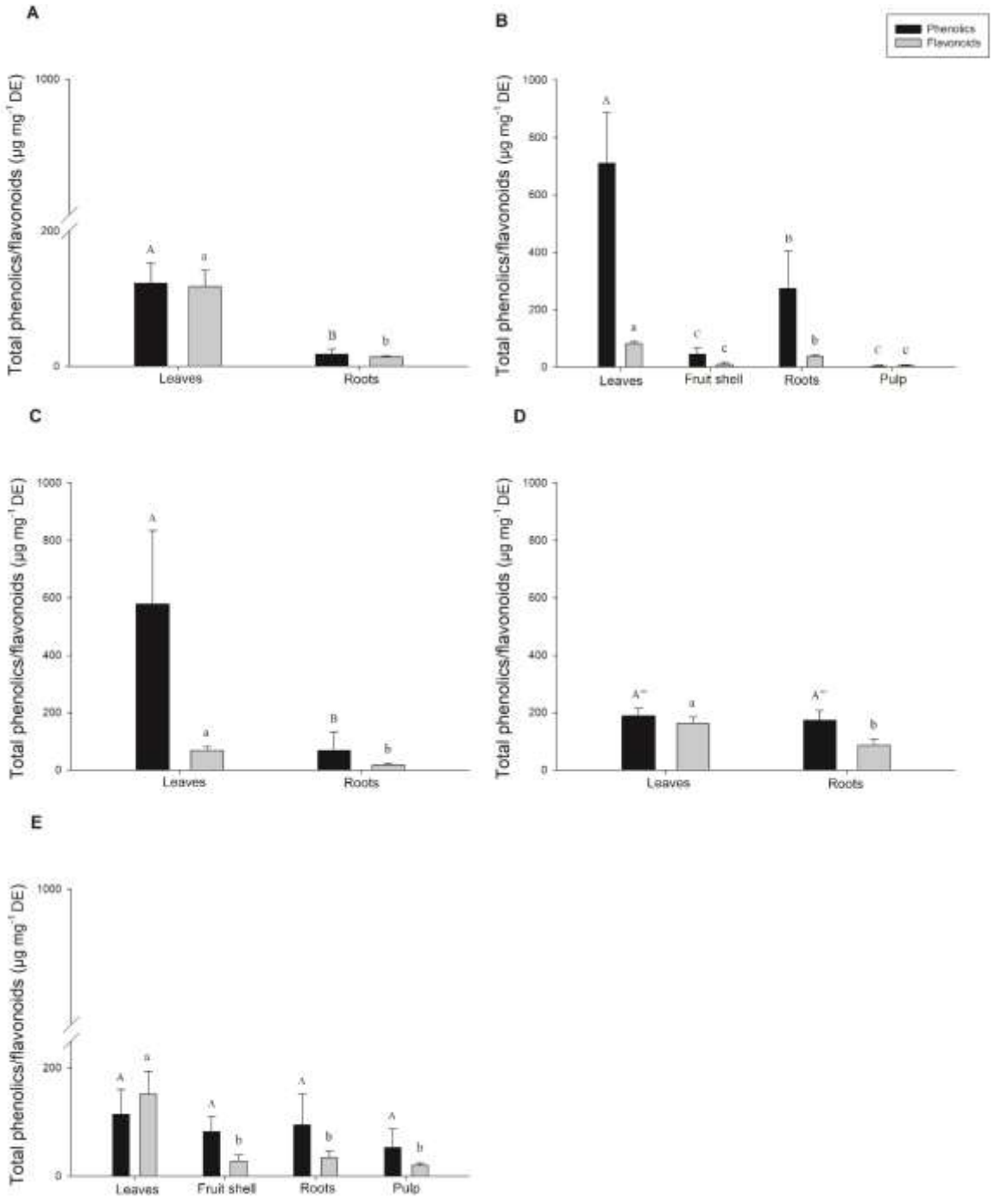

Figure 2. Total phenolic (black bars) and flavonoid (gray bars) contents in *Passiflora edulis* at various developmental stages. Ethanolic extracts (1 mg mL^{-1}) were prepared from plant samples harvested at juvenile (**A**), fruiting I (**B**), flowering I (**C**), flowering II (**D**) and fruiting

II (**E**) stages. Values correspond to the means + standard deviations of experiments done with four replicates. Distinct uppercase letters indicate significant differences in the phenolic contents while distinct lowercase letters indicate significant differences in flavonoid contents (Scott-Knott; $P < 0.01$) within a phenological stage. **DE**, dry extract.

Table 1. Total phenolic/flavonoid contents in *Passiflora edulis* organs according to phenological stages and plant organs.

Phenological phases	**Plant parts**	**Phenolics (mg tannic acid mg^{-1} DE)**	**Flavonoids (mg quercetin μg^{-1} DE)**
Juvenile	Leaves	122.2 d	117.5 b
	Roots	17.4 d	13.8 f
Fruiting I	Leaves	710.2 a	81.8 c
	Fruit shells	45.1 d	9.8 f
	Roots	273.9 c	38.0 e
	Fruit pulps	4.2 d	5.8 f
Flowering I	Leaves	578.1 b	67.7 d
	Roots	69.1 d	16.5 f
Flowering II	Leaves	188.4 c	161.2 a
	Roots	173.3 c	86.9 c
Fruiting II	Leaves	114.2 d	151.1 a
	Fruit shells	82.1 d	27.9 e
	Roots	94.2 d	33.7 e
	Fruit pulps	52.2 d	19.7 f
CV(%)		58.6	29.7

DE, dry extract; **CV%,** coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott; $P < 0.01$).

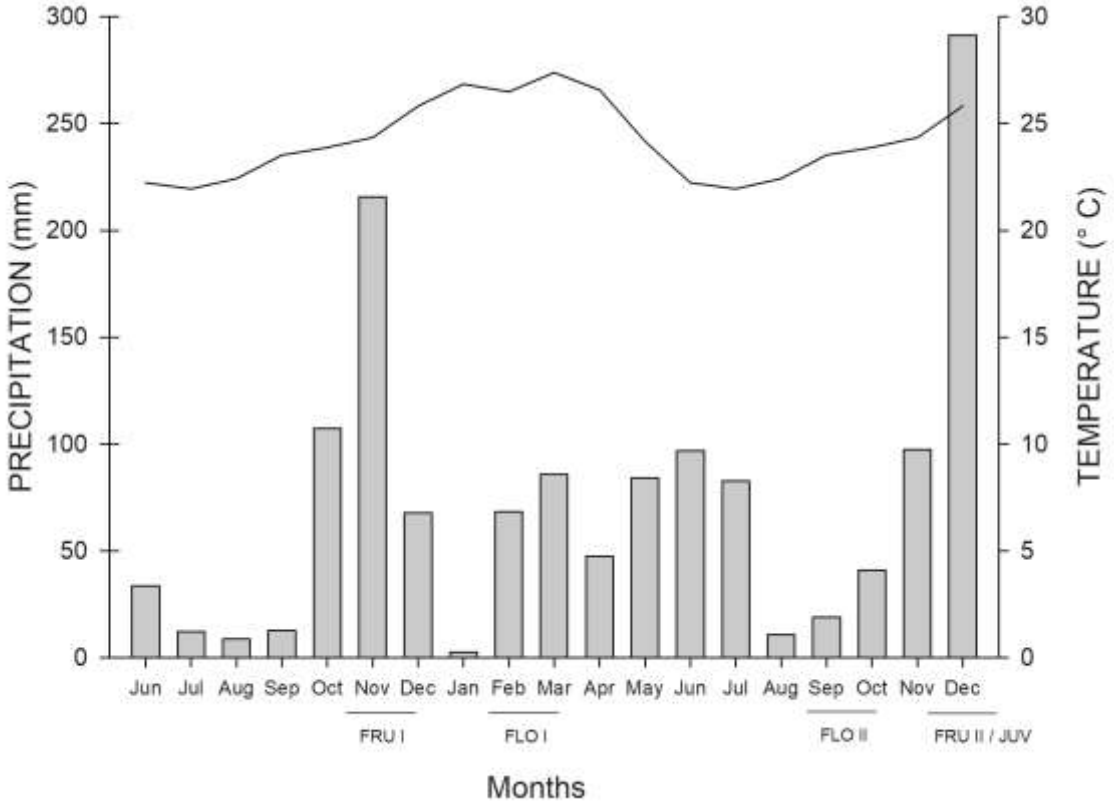

Figure 3. Accumulated precipitation (bar chart) and mean temperature (line chart) recorded from June 2016 to Dec 2017 at Pitangueiras Farm (Sooretama, Brazil), interval by which plant samples were harvested as indicated. **FLO I** and **FLO II**, plants at flowering developmental stage; **FRU I** and **FRU II**, plants at fruiting developmental stage; **JUV**, plants at juvenile developmental stage. Juvenile plants were grown at greenhouse at the same period that field plants were at fruiting stage II. Data from Incaper Weather Station – Linhares (Espírito Santo - Brazil).

3.2. Scavenging of reactive nitrogen and oxygen species by P. edulis *extracts*

Ethanolic extracts from leaves (1 mg mL^{-1}) at juvenile, flowering I and II and fruiting I and II stages effectively scavenged 53.7%, 66.3%, 50.8%, 64.8% and 50.2% of the reactive nitrogen species DPPH present in the reaction medium, respectively (Fig. 4). In contrast, root extracts were notable scavengers of the reactive oxygen species O_2^- as they capture 73.1% (juvenile plants), 61.3% (flowering I plants), 78.1% (flowering II plants), 75.0% (fruiting I plants) and 59.6% (fruiting II plants) of the free radical formed in the reaction medium (Fig. 5). The potential of extracts from fruit shells (fruiting I stage) to scavenge O_2^- was comparable to that of root extracts from the same plants while root and leaf extracts from plants at flowering II stage were equally effective in the capture of O_2^- (Fig. 5).

Among the plant parts and developmental stages investigated, leaf extracts from plants at flowering I and fruiting I stages exhibited the highest capacity to scavenge reactive nitrogen species (Tab. 2); Extracts from fruit pulp (fruiting II) were as efficient as extracts from roots (juvenile stage), fruit shells and roots (fruiting I) and leaves and roots (flowering II) with respect to the scavenging of reactive oxygen species (Tab. 2).

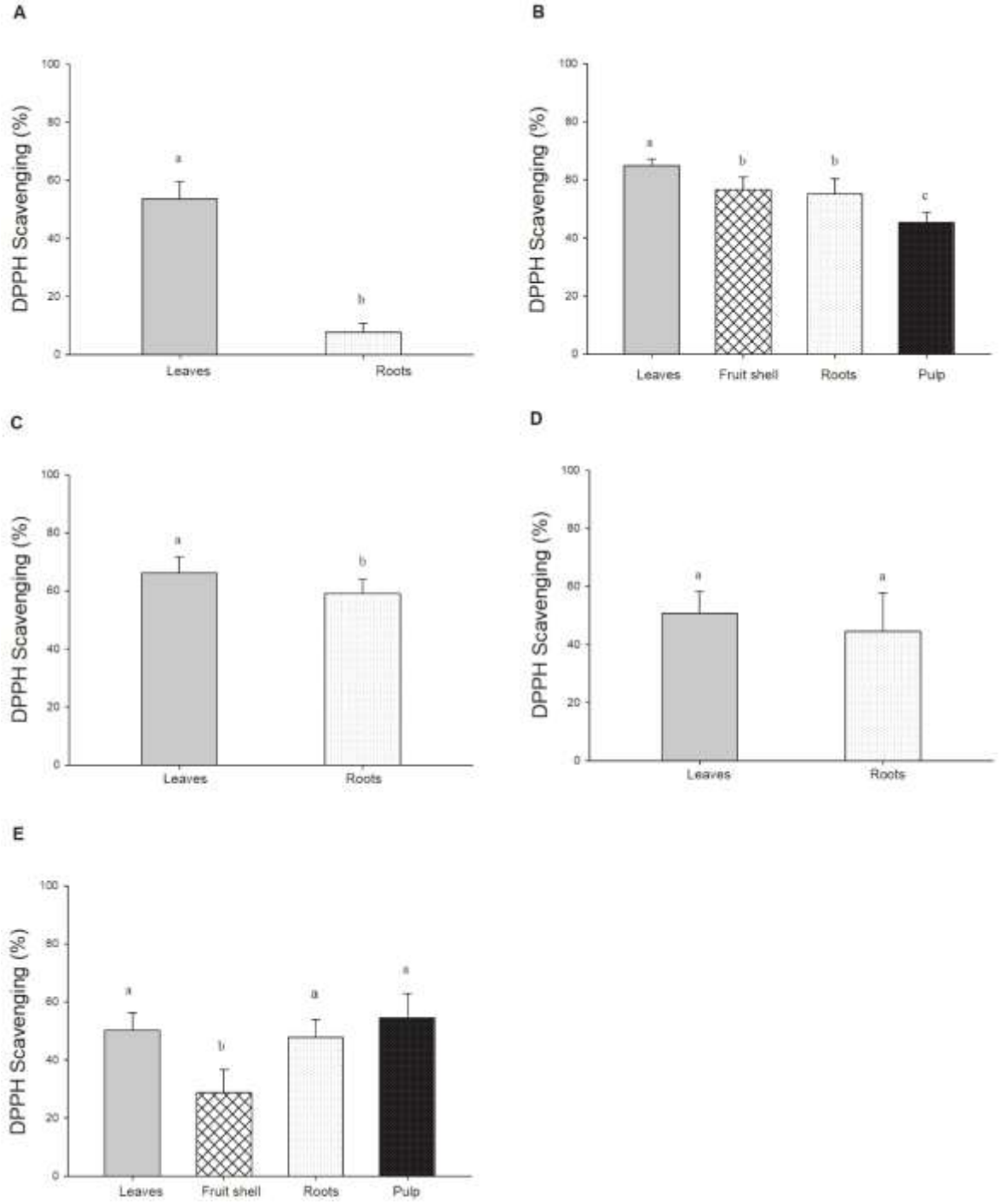

Figure 4. Scavenging of DPPH radicals by ethanolic extracts from *Passiflora edulis* plants at various developmental stages. Ethanolic extracts (1 mg mL^{-1}) were prepared from samples harvested at juvenile (**A**), fruiting I (**B**), flowering I (**C**), flowering II (**D**) and fruiting II (**E**) stages. Values correspond to the means + standard deviations of experiments done with four replicates. Distinct letters indicate significant differences among the plant parts (Scott-Knott; $P < 0.01$).

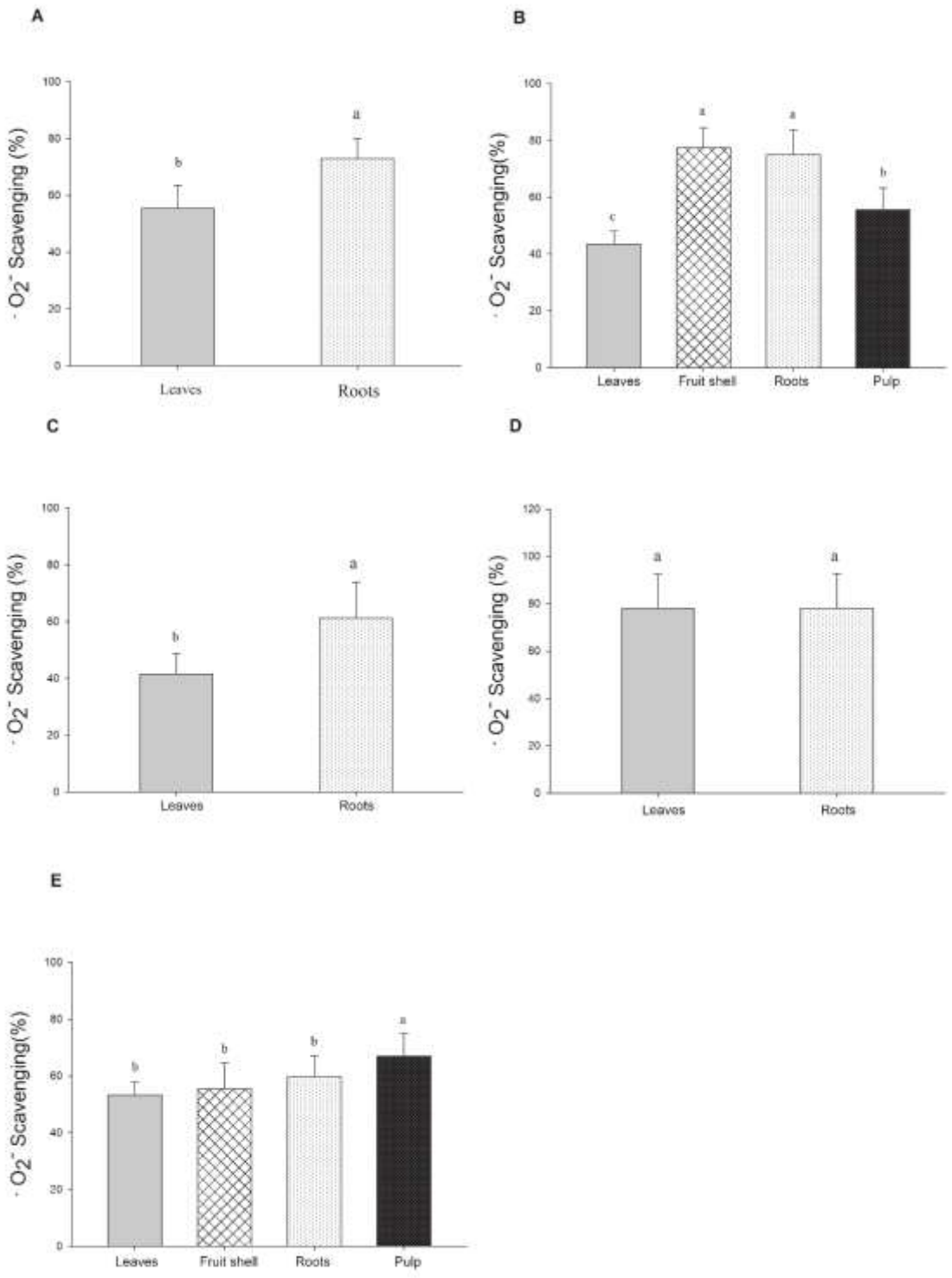

Figure 5. Scavenging reactive oxygen species by ethanolic extracts from *Passiflora edulis* plants at various developmental stages. Ethanolic extracts (1 mg mL^{-1}) were prepared from samples harvested at juvenile (**A**), fruiting I (**B**), flowering I (**C**), flowering II (**D**) and fruiting II (**E**) stages. Values correspond to the means + standard deviations of experiments done

with four replicates. Distinct letters indicate significant differences among the plant parts (Scott-Knott; P <0.01).

Table 2. Scavenging of reactive nitrogen (DPPH) and oxygen (O_2^-) species by 1 mg mL^{-1} ethanolic extracts of *Passiflora edulis* at various phenological stages.

Phenological stages	**Plant parts**	**DPPH (%)[a]**	**O_2^- (%)[a]**
Juvenile	Leaves	53.7 c	55.3 b
	Roots	7.7 f	73.1 a
Flowering I	Leaves	66.3 a	41.5 c
	Roots	59.1 b	61.3 b
Fruiting I	Leaves	64.8 a	43.6 c
	Fruit shells	56.5 b	77.4 a
	Roots	55.1 c	75.0 a
	Fruit pulps	45.4 d	55.7 b
Flowering II	Leaves	50.8 c	78.1 a
	Roots	44.5 d	78.1 a
Fruiting II	Leaves	50.2 c	53.4 b
	Fruit shells	28.7 e	55.3 b
	Roots	47.8 d	59.7 b
	Fruit pulps	54.5 c	67.0 a
CV (%)		13.3	14.6

3.3. Activity of antioxidant enzymes in leaves of P. edulis

The activity of SOD was 2.3-fold higher in leaves of plants at juvenile and fruiting I stages (32.3 U min^{-1} mg $prot^{-1}$ in average) than that of leaves from flowering I and II and fruiting II plants (13.9 U min^{-1} mg $prot^{-1}$ in average) (Fig. 6). The APX and CAT activities showed similar profiles among the plant parts; The highest CAT and APX activities were observed in leaves from fruiting I (51.6 mmol H_2O_2 min^{-1} mg $prot^{-1}$ and 2.2 mmol ascorbate min^{-1} mg $prot^{-1}$) followed by juvenile (36.5 mmol H_2O_2 min^{-1} mg $prot^{-1}$ and 1.4 mmol ascorbate min^{-1} mg $prot^{-1}$) and the other stages (21.7 mmol H_2O_2 min^{-1} mg $prot^{-1}$ and 0.9 mmol ascorbate min^{-1} mg $prot^{-1}$, in average).

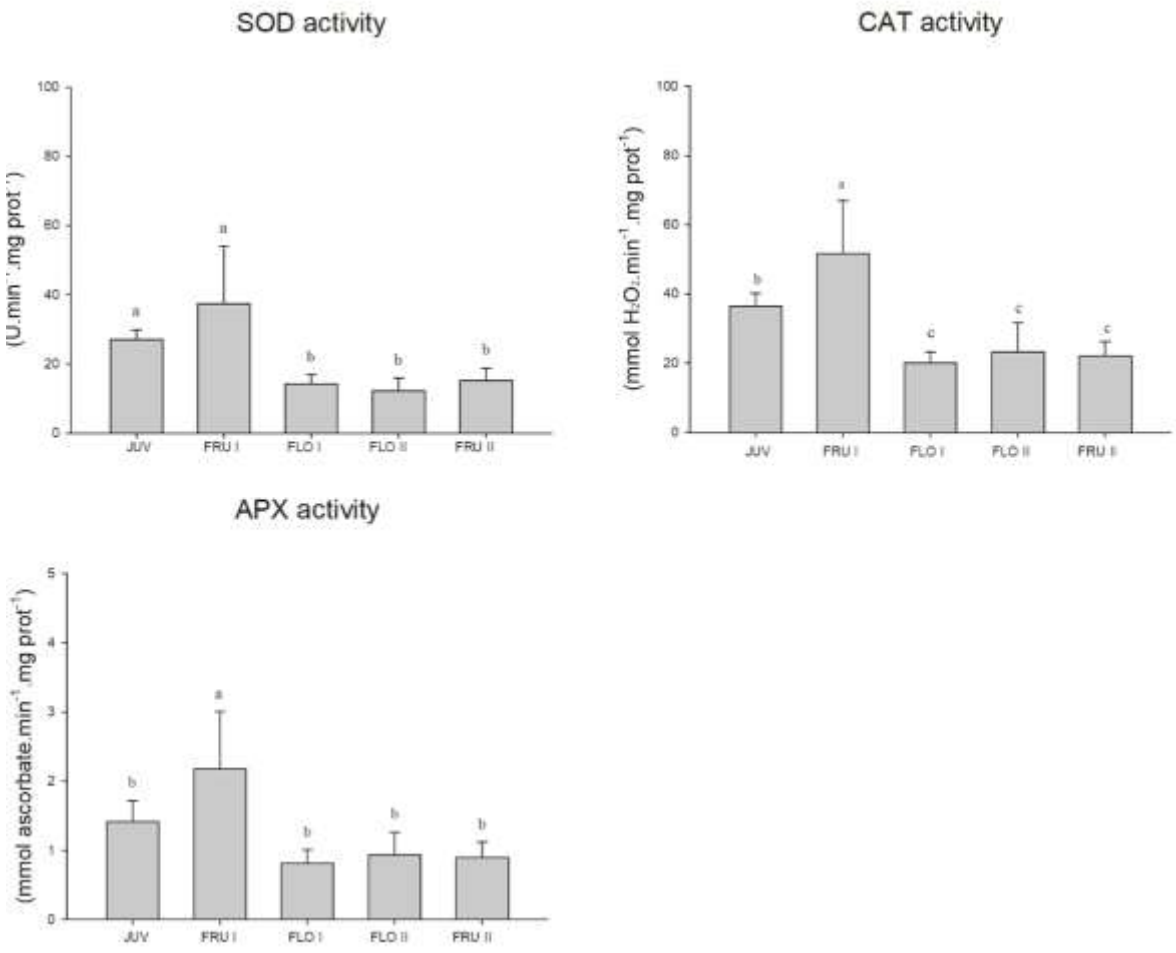

Figure 6. Activity of antioxidant enzymes in leaves of *Passiflora edulis* plants at various developmental stages. Leaves were harvested from plants at juvenile (**JUV**), flowering I and II (**FLO I** and **FLO II**) and fruiting (**FRU I** and **FRU II**) stages for the evaluation of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activities.

Values are the means ± standard deviations of experiments done with four replicates. Distinct letters indicate significant differences (Scott-Knott; $P < 0.01$).

4. Discussion

Leaves were the *P. edulis* part that accumulated the highest amount of total phenolics and flavonoids. Indeed, leaves are considered source organs, in which some of carbon fixed during photosynthesis are driven to produce energy and secondary metabolites (Ramakrishna & Ravishankar, 2011) and the remainder distributed to organs called sink via phloem (Dinant *et al.* 2010).

When comparing all the plant developmental stages, maximum accumulation of phenolic compounds occurred in leaves of plants at fruiting I stage while a peak of flavonoid levels was noticed in leaves during flowering I and fruiting II (Tab. 2). The level of secondary metabolites is reported to vary among the organs of a plant species and according to the environmental conditions and plant development stage (Deborde *et al.* 2017).

The low content of phenolic compounds in the juvenile stage may be attributed to the lower tissue lignification and therefore lower amounts of lignin precursors in cell wall when compared to mature plants (Ncube & Van Staden 2015). Likewise, lower levels of phenolic compounds were found in leaves of *Vaccinium macrocarpon* (cranberry) cv. 'Stevens' Ait. during budding stage than that of the same plants during flowering and fruiting stages (Berezina *et al.* 2017). The phenolic compounds levels diminished in *P. edulis* leaves and roots as plants began to flower until fruiting stage, a phenomenon that was also observed in cranberry leaves during flowering (Berezina *et al.* 2017). The lower levels of total phenolics and flavonoids in leaves and roots of flowering I *P. edulis* plants compared to the same organs from fruiting I stage plants are likely due to the reallocation of part of these metabolites to reproductive structures (Dinant *et al.* 2010; Ramakrishna & Ravishankar 2011). In fact, flavonoids can account for up to 4% of pollen dry weight, contribute to pollen

and petals coloration and, therefore attract pollinators (Theis & Lerdau 2003; Ferreyra *et al.* 2012). Also, the structure of *P. edulis* flowers is quite complex, whose crown's diameter can exceed 12 cm and present several pollen grains, various aroma and pollen grain (Jesus *et al.* 2017). Ecological studies have shown a positive correlation with respect to secondary metabolites among the different plant parts, in which increased amounts are found in the phloem, followed by leaves and nectar (Parachnowitsch & Manson 2015). In contrast, the lower levels of phenolics in leaves and roots of plants at fruiting II stage than those of flowering II stage may result from allocation of these secondary metabolites to fruits. Also, plants at flowering II and fruiting II phases were senescent, which may explain the highest levels of total phenolic compound/flavonoid in fruit shells and pulp. Atypical climate conditions, characterized by a 3.5-fold increase in rainfall in August/September 2017 in relation to the same period in 2016, were registered prior to plant harvesting at flowering II stage, which could lead to plant stress. The increase in the flavonoid contents in organs of senescent *P. edulis* is likely a response to oxidative stress generated during individuals aging (Ferreyra *et al.* 2012).

The differences in the performance of leaf and root ethanolic extracts from *P. edulis* to scavenge DPPH and O_2^- radicals can be explained by the fact that a given secondary metabolite may be chemoselective for reactive nitrogen species in detriment of reactive oxygen species. This has been demonstrated for resveratrol, a grape-accumulating polyphenol that is chemoselective to DPPH and, therefore, a poor O_2^- scavenger (da-Silva *et al.* 2012; Liberto *et al.* 2017; Das *et al.* 2018). The efficiency of *P. edulis* extracts to scavenge DPPH or O_2^- can be partly explained by the high phenolic content in the samples. Secondary metabolites other than flavonoids appear to contribute to the ability of *P. edulis* extracts to capture free radicals since extracts containing the highest levels of flavonoids were not necessarily the most efficient ones to scavenge DPPH or O_2^-. Expressive O_2^- scavenging

activity was observed for root and fruit shell extracts. Fruit shells, together with *P. edulis* seeds, account for up to 70% of fresh fruit weight that is disposed of by industries during the production of passion fruit juice (Oliveira *et al.* 2002). In this sense, the fruit shell, a juice industry by-product, could be considered for further use as free radical scavenger.

Similar profile of CAT, APX and SOD activities was observed in leaves of *P. edulis* in the all phenological stages investigated. The SOD catalyzes the dismutation of O_2^- to H_2O_2, whose excess is converted to H_2O by the action of CAT and APX, this latter assisted by ascorbate. Hence, *P. edulis* leaf extracts can effectively control the levels of reactive species via the activity of antioxidant enzymes and non-enzymatic oxidants such as phenolic compounds, which validates the use of *P. edulis* leaves for the treatment of oxidative-stress-driven diseases.

5. Conclusion

Passiflora edulis leaves were the parts with the highest phenolic compounds/flavonoids contents regardless of the plant developmental stage. The maximum accumulation of phenolics/flavonoids in in roots, leaves, fruit shells and pulp took place during the plants reproductive stage. Economical value is now given to the *P. edulis* fruit shell, a juice industries's waste, due to its notable ability to scavenge reactive oxygen species. Overall, the results suggest that one can most benefit from the antioxidant properties of *P. edulis* leaves if the harvesting is carried out during the plant fruiting stage before senescence.

Acknowledgments

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CHAPTER 2

Secondary metabolites profile in *Passiflora edulis* f. *flavicarpa* O. Deg organs according to plants phenological stages

(Manuscrito em processo de submissão à Scientia Horticulturae)

Secondary metabolites profile in *Passiflora edulis* f. *flavicarpa* O. Deg organs according to plants phenological stages

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ABSTRACT. Ethnobotanical studies revelead that *Passiflora* species exhibit medicinal properties. This work investigated the secondary metabolite profiles in ethanolic extracts of *Passiflora edulis* f. *flavicarpa* O. Deg organs originated from plants at juvenile, flowering and fruiting stages. Plant ethanolic extracts were tested for the ability to inhibit the ureolytic activity of *Canavalia ensiformis* urease and the growth of the phytopathogenic fungus *Fusarium solani* and *Colletotrichum gloeosporioides*. Thin layer chromatography (TLC) analysis indicated the presence of flavonoids in all extracts checked, while tannins were present in root extracts, and terpenes in leaf, fruit shell and root extracts. Using the Brazilian Pharmacopoeia protocol, leaf extracts of juvenile plants showed the highest flavonoid contents (3.5% apigenin equivalents in the dry mass) followed by leaf extracts of plant at fruiting II stage (2.7%) and leaf extracts at fruiting I and flowering I stages (2.3% on average). Indeed, leaf extracts of plants at any phenological stage presented flavonoid contents that were 10-fold higher than those found in other extracts. Ultra-High Performance Liquid Chromatography (UHPLC) analysis demonstred that leaves and fruit shell extracts present nine different flavonoid *C*-glycosides, likely luteolin, apigenin and chrysin glycoconjugates. A single type of condensed tannin was found in root extracts, regardless of the plant developmental stage. Fruit shell extracts of *P. edulis* in fruiting II stage were the most potent against jack bean urease as they inhibit the enzyme activity by over 43%; Hydroxyurea, a reference inhibitor, compromised the urease activity by 35.6%. *P. edulis* extracts stimulated the growth of *F. solani* and *C. gloeosporoides* complexes. The different classes of secondary metabolites present in *P. edulis* plants vary in fuction of the phases of development and the organs analyzed.

Keywords: Passion fruit, ethanolic extracts, secondary metabolism, C-glicosilated flavonoids, tannins, urease enzyme.

1. Introduction:

The genus *Passiflora* (Passifloraceae) is known by the medicinal properties attributed to the different plant parts. Several species are reported as antispasmodic, sedative, anxiolytic (Wosh et al., 2017; Freire et al., 2018) and are used for the control of cardiac arrhythmias, as muscle relaxants and antidepressants and for the treatment of neuralgias, pains (Frye & Haustein, 2008). Their differents uses in therapeutics may indicate that specie likely exhibit distinct chemical profiles (Wosh et al., 2017).

Passiflora edulis f. *flavicarpa* O. Deg (passion fruit) is popularly known as 'maracujá azedo' in Brazil and 'cocorilla' in Colombia (MOBOT, 2019). Flavone *C*-glycosydes such as vitexin, isovitexin, orientin, isoorientin and schaftoside are described as most abundant secondary metabolites in *P. edulis* (Zeraik et al., 2010; Li et al., 2011; Silva et al., 2013; Ayres et al., 2015; Costa et al., 2016; Wosh et al., 2017). Harmane β-carbolinic alkaloids are also reported as main compounds of alkaloids family in *P. edulis* extracts (Costa et al., 2016; Medina et al., 2017; Freire et al., 2018). Flavonoid glycosydes are hydrophilic antioxidants, antimicrobials, that can also work as repellents, and UV filters (Praveena et al., 2014). β-carbolinic alkaloids are pyridoindole alkaloids produced from indoleamines and aldehydes or α-keto-acids (Herraiz & Galisteo, 2015). They have a broad spectrum of psychopharmacological effects as they bind to benzodiazepine, imidazoline, serotonin, and opioid receptors, besides functioning as monoamine oxygenase inhibitors (Patel et al., 2012).

The biosynthesis of these secondary metabolites is affected by environmental conditions, which consequently can change the therapeutic effect (Wosh et al., 2017), and nutraceutical properties of the plants as well (Freire et al., 2018). The type and concentrations of secondary metabolites vary according to the plant organ and developmental stage (Liu et al., 2010).

Urease is a enzyme that catalyzes the hydrolysis of urea to ammonia (NH_3) and carbono dioxide (CO_2). The use of urease inhibitors is one of the strategies adopted to improve urea performance in agriculture and mitigate urea driven emission of pollutants like NH_3, CO_2, N_2O and NO (Modolo et al., 2018). Urease inhibiting substances are used to treat infections caused by the bacterium *Helicobacter pylori*, this bacteria colonizes the human stomach wich involves the production of urease that increases the pH around bacterial microenvironment (Lage et al., 2018). Studies have been looking for new substances with the potential inhibitor of the enzyme urease, so plant secondary metabolites can be used for this purpose.

Diseases caused by fungi affect *Passiflora* sp. from the germination phase to the adult plant, damaging the roots, branches, leaves, flowers and fruits (Pires et al., 2011; Embrapa 2016). The fungi that cause the greatest economic damage to the *P. edulis* crop are *Colletotrichum gloeosporioides* causing anthracnose and *Fusarium solani* causing root rot (Embrapa, 2016).

Brazil is the number one producer of *P. edulis*. The juice, jellies, sweets and drinks prepared from fruit pulp are much appreciated worldwide. Due to the growing interest in the medicinal and nutritional properties of *P. edulis*, knowledge about the chemical composition of secondary metabolites is extremely relevant for its safe use as a phytotherapic (Freire et al., 2018). This work evaluated qualitatively and quantitatively the classes of secondary metabolites of *P. edulis* in the leaves, fruit shell, roots and fruit pulp at different development stages. It was verified the biological action of *P. edulis* extracts against *Canavalia ensiformis* urease enzyme, as well as the effect of the extracts on the growth of phytopathogenic fungi.

2. Materials and methods

2.1. Plant material and experimental design

Plants were harvested from plants cultivated in the Pitangueiras Farm, located in Sooretama, Espírito Santo, Brazil (19°12'05,6''S 40°03'38,5''W), unless otherwise stated. The management of Fazenda Pitangueiras orchard including propagation, nutrition and fertilization, irrigation and disease control occurred according to Embrapa recommendations (Embrapa, 2005).

A total of five harvestings took place in the phenological stages: juvenile – greenhouse grown in Farm Incaper, Linhares, Espírito Santo, Brazil (Dec 2017; leaves and roots), flowering I and II – farm cultivation (Feb 2017 and Sep 2017, respectively; leaves and roots) and fruiting I and II – farm cultivation (Nov 2016 and Dec 2017, respectively; leaves, roots and fruits) (Figure 1). Whenever applicable, fruit shell and pulps were separated from the fruits for the analyses. Plants at juvenile stage were grown in a greenhouse (T_{min} of 24.1 ° C and T_{max} of 29.2 °C) for 65 days before the first flowering event. The voucher specimen (BHCB 184739) was deposited in the herbarium of the Department of Botany at the Federal University of Minas Gerais.

The experimental design was completely randomized, with eight replicates per treatment, in which each developmental stage was defined as a treatment. Data obtained were analyzed in two different ways, first by comparing the plant parts obtained at each stage of development individually, and then globally, by comparing all stages of development with each other.

Figure 1. Representative images of *Passiflora edulis* plants at the developmental stages investigated. Plants are from the Pitangueiras Farm (Sooretama, Espírito Santo, Brazil) except for those at juvenile stage, which were grown in a greenhouse.

2.2. Ethanolic extracts preparation

Ethanolic extracts were prepared until samples' exhaustion (Shah et al., 2004; Brasil, 2010; Shelar et al., 2018; Carvalho et al., 2019). Systems were filtered and the organic fraction evaporated at temperatures bellow 50 °C (Dai & Mumper, 2010; Shelar et al., 2018) to obtain the corresponding solid residues. The extracts yield was determined based on the plant fresh weight and organic fraction dry weight. One milligram of each dry extract was

resuspended in 1 mL of ethanol and the corresponding ethanolic extract used in the subsequent analysis.

2.3.Characterization of secondary metabolites by thin layer chromatography:

Metabolite profiles were obtained by thin layer chromatography using the dried extracts of the several *P. edulis* plant parts of development. One milligram of the dried plant extracts was resuspended in 0.5 mL of acetone and applied in silica gel plates. Chloroform/acetic acid/methanol/water (60:32:12:8) was used as mobile phase for the substances separation. Plates were developed with a methanolic solution of aluminum chloride 5% (Wagner & Bladt, 2001) while vitexin was used as a standard to investigate the presence of flavonoids in the ethanolic extracts. Plates were analyzed at 365 nm for the detection of spots typical of flavonoids presence.

A mobile phase constituted of ethyl acetate/acetic acid/formic acid/water (10:1.3:1.3:2) (modified from Wosch et al., 2017) was used to investigate the presence of alkaloids. Dragendorff reagent was used to develop the plates and harmane and harmine used as alkaloid standards. Ethyl acetate/methanol/water (100:13.5:10) was used as mobile phase for the identification of terpenes. Plates were developed under Liebermann-Burchard solution (Wagner & Bladt, 2001).

The identification of tannins was done as describe elsewhere (Wagner & Bladt, 2001), with modification. Ethyl acetate/methanol/water (100:18.5:5) containing 2 drops of acetic acid was used as mobile phase. Plates were developed in the presence of a methanolic solution of ferric chloride 2% and the monomer unit epicatechin was used as standard.

2.4.Quantification of flavonoids by the Brazilian Pharmacopoeia method:

Total flavonoids were calculated as apigenin equivalents according to a method developed for *P. edulis* and reported in the Brazilian Pharmacopoeia (Brasil, 2010). Four hundred milligrams of dried extract was resuspended in 20 mL of 50% (v/v) ethanol and the system kept under reflux for 30 min. The mixture was filtered using cotton and the cotton transferred to a reflux flask together with other 20 mL of 50% (v/v) ethanol. After 30 min reflux, the system was filtered using paper filter, both fractions combined in a volumetric flask and the volume completed to 50 mL with 50% (v/v) ethanol to yield the stock solution of plant extract. The stock solution was diluted 12.5 times in 2% (w/v) aluminum chloride prepared in 50% (v/v) ethanol. Plant extracted diluted 12.5 times in 50% (v/v) ethanol was used as the reaction blank. The absorbance (A) was recorded at 397 nm, 30 min after samples preparation and the total flavonoids calculated as apigenin equivalents (%) according to the formula:

$$TFT = \frac{A\ x\ DF\ x\ 100}{S_{1cm}^{1\%}\ x\ m\ (100)}$$

Where, TFT indicates total flavonoid content, DF is the dilution factor, $S_{1cm}^{1\%}$ is the molar absorptivity for apigenin and m corresponds to the extract dry weight.

2.5. UHPLC Analysis of the metabolic profiles in P. edulis *extracts according to plants developmental stages:*

Leaf and root dried extracts (5.0 mg) and fruit shell and fruit pulp dried extracts (10 mg) were added to methanol (1.0 mL) and the systems sonicated for 15 min for complete dissolution. The solutions were filtered in a 0.22 μm PVDF membrane prior to the analysis on a UHPLC (Ultra High Performance Liquid Chromatography) Acquity System® equipped with an VanGuard™ C18 pre-column (0.5×0.21 cm). Acquity BEH C18 column (10.0×0.21

cm) and coupled to UV/DAD detector. The oven temperature was 30 °C. Filtered samples (2.0 µL) were loaded on the column and subjected to the conditions described in Table 1. A flow of 250 µL min^{-1} was applied and the chromatograms obtained at 335 nm were recorded.

Flavonoids were quantified by UHPLC using standard addition method, based on analytical curves (y = 94980 x-161763; R^2= 0.9979) prepared from samples spiked with a stock, methanolic solution of vitexin (1 mg mL^{-1}), to reach a final concentration ranging from 7 µg mL^{-1} to 420 µg mL^{-1}. Tannins were quantified using standard addition method, based on analytical curves prepared (y = 16463x+60965; R^2= 0.9858) from samples spiked with a stock, methanolic solution of the tannin monomer epicatechin (1 mg mL^{-1}), to reach a final concentration ranging from 7 µg mL^{-1} to 420 µg mL^{-1}. The standard curves were perfomed in triplicate. Vitexin, isovitexin, orientin, isoorientin, schaftoside and vicenin were used as flavonoid standards. Harmane, harmine and harmaline were used as *β*-carbolinic alkaloid standards. Commercial tannins and/or respective monomer units (ellagic acid, gallic acid, epicatechin, epigallocatechin, epigallocatechin gallate, gallocatechin, procyanidin a_2, procyanidin b_2 and catechin) were also used.

Table 1. UHPLC method used for the analysis of *Passiflora edulis* extracts.

Time (min)	0.1% formic acid (%)	Methanol in 0.1% formic acid (%)	Acetonitrile (%)
0.0	90	10	0
1.0	75	25	0
18.0	65	35	0
18.1	5	0	95
20.0	5	0	95
21.0	90	10	0
25.0	90	10	0

2.6. Biological activity:

2.6.1 Inhibitory effect of P. edulis *ethanolic extracts on the ureolytic activity of jack bean urease:*

The extracts that contained the highest amounts of secondary metabolites as determined by UHPLC analysis were selected for the biological assays. Reactions were constituted of 20 mM phosphate buffer (pH 7.0), 1 mM ethylene diamine tetra acetic acid (EDTA), 10 mM urea, 12.5 mU jack bean type III urease and 1 mg mL^{-1} *P. edulis* extract. After 10 min incubation at 25 °C, reactions were stopped by addition of 0.5 volume of 1% (m/v) phenol in 5 mg L^{-1} sodium nitroprusside (SNP) followed by 0.7 volume of 0.5% (m/v) NaOH in 0.1% (v/v) NaOCl. The mixtures were incubated at 50 ºC for 5 min and the absorbance was measured at 630 nm to determine the amount of ammonium (NH_4^+) formed

from urea hydrolysis (Weatherburn, 1967). Hydroxyurea (HU; 500 μM) was used as a reference of urease inhibitor. Urease inhibition was determined in terms of percentage of NH_4^+ formed in plant extract-containing reactions in relation to the total urease activity in reactions devoid of inhibitors. Three independent experiments were performed, each with four replicates.

2.6.2 Effect of P. edulis *ethanolic extracts on the growth of* Fusarium solani *complex and* Colletotrichum gloeosporioides *complex:*

F. solani complex was grown on malt agar at 35 °C for 48 h and afterwards kept in at 25 °C for 10 days when it reached sporulation stage. Oat medium was used to grow *C. gloeosporioides* complex in a germination chamber at 25 °C, 12 h photoperiod, for 17 days.

The fungus susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI, 2002). Tests were performed in Sabouraud dextrose broth. *P. edulis* ethanolic dried extracts were re-suspended in 1% dimethylsulfoxide (DMSO). One volume of 1 mg mL^{-1} of *P. edulis* extracts (from leaf, fruit shell, fruit pulp or root) were added to wells of sterile 96-well microtiter plates together with equal volume of the microorganism suspension to yield 1.5 x 10^5 CFU/mL per well. The plates were incubated at 25 °C for 48 h. Controls were also prepared to check microorganisms viability and medium culture cross contamination. Each well was analyzed at 625 nm to verify fungus growth, which was calculated as relative percentage based on optical density. All tests were performed in triplicate.

2.7. Statistical analyses

Data were submitted to Shapiro-Wilk to check the normality and F test to verify the distribution using the ASSISTAT software (Silva & Azevedo, 2016). Data were also subjected to analysis of variance and mean (P <0.05) and Scott-Knott (P <0.05) tests using ASSISTAT software (Silva & Azevedo, 2016).

3. Results:

3.1. Characterization of secondary metabolites by thin layer chromatography (TLC):

Flavonoids, terpenes and tannins, but not alkaloids, were detected in the passion fruit extracts (Figure 2). The profile of flavonoids, as analyzed by TLC, was very similar for *P. edulis* leaf and fruit shell extracts, with formation of bands characterists of glycosylated flavones (Wagner & Bladt, 2001; Wosh et al., 2017). One can assume that the extracts of fruit shell, root and fruit pulp had lower concentration of flavones glycosides because such extracts require four times application to the TLC plates in comparison to the others. Root extracts showed a bright blue band typical of carboxylated phenolic acids (Wagner & Bladt, 2001).

TLC analysis also revelead similarity of terpene profiles among extracts of leaf, fruit shell and roots, mostly terpene glycosides, while compounds from this class were absent in fruit pulp extracts, regardless of the plant developmental stage. It is evident in these extracts the formation of brown and reddish-brown bands, indicating the presence of glycosidic terpenes (Wagner & Bladt, 2001). Surprisingly, tannins were detected in root extracts and this class of secondary metabolites has not yet been reported in *P. edulis* ethanolic extracts.

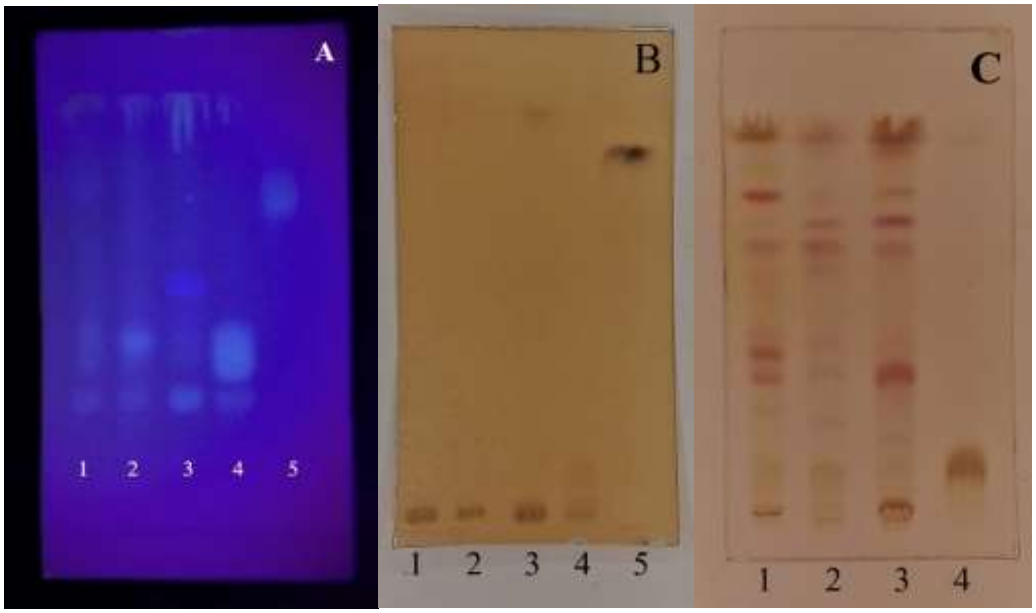

Figure 2. Thin layer chromatography of *P. edulis* ethanolic extracts. **A)** Profile of flavonoids in thin-layer chromatography, vitexin is reference substance. Mobile phase: chloroform/acetic acid/methanol/water (60:32:12:8). **B)** Profile of tannins in thin-layer chromatography, epicatechin is reference substance. Mobile phase: ethyl acetate/methanol/water (100:18.5:5). **C)** Profile of terpenes in thin-layer chromatography. Mobile phase: ethyl acetate/methanol/water (100:13.5:10).

3.2. Quantification of flavonoids according to the Brazilian Pharmacopoeia:

The highest concentration of flavonoids (expressed as apigenin equivalents) was verified in dried extracts of *P. edulis* leaves at juvenile stage (3.5% of the flavonoid mass; Table 2). Leaf extracts of fruiting II plants had 2.7% while leaf extracts (fruiting I and flowering I stages) showed on average 2.3% flavonoids. Regardless of the plant developmental stage, leaf extracts had flavonoid contents that are ten times higher than those of fruit shell extracts (Table 2). The flavonoid contents in root and fruit pulp extracts are likely below the detection limit of spectrophotometer used.

Table 2. Total flavonoid contents (apigenin equivalent) in *Passiflora edulis* organs according to phenological stages. Brazilian pharmacopoeia method (Brasil, 2010).

Phenological phase	Plant organ	Flavonoids (% apigenin)[a]
Juvenile	Leaves	3.5 a
Fruiting I	Leaves	2.3 c
	Fruit shells	0.3 d
Flowering I	Leaves	2.3 c
Flowering II	Leaves	2.3 c
Fruiting II	Leaves	2.7 b
	Fruit shells	0.3 d
CV(%)		12.68

[a] Percentage of apigenin in dried extract of *P. edulis*. CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott, P <0.05).

3.3. UHPLC Analysis of the metabolic profiles of P. edulis *throughout plant developmental stages:*

The different colors represented in the chromatograms refer to the replicates of the samples collected in the orchard (Figures 3, 4, 5 and 6). Nine different flavonoids were identified in leaf extracts of *P. edulis* plants at all investigated developmental stages (Figure 3). They are all flavones, which include five apigenin derivatives, a chrysin derivative and three luteolin derivatives (Table 3). The highest flavonoid concentration was detected in leaf extracts of juvenile *P. edulis* plants (Table 4). Fruit shell (fruiting I or II) extracts presented chromatographic profiles similar to those of leaf extracts (Figure 4), except that the flavonoid

concentrations in fruit shell extracts were much lower (Table 4). No flavonoids were detected in root extracts at the experimenta conditions tested while low amounts (near the detection limit) were recorded fruit pulp extracts.

Presence of a single peak relative to a condensed tannin (t_R ~ 4.7 min, λ_{max} = 196.3/279.1) was observed in chromatograms of root extracts (Figure 5). Roots had the highest tannin concentration, with 212.6 μg mg^{-1} dried extract at fruiting I stage and 180.1 μg mg^{-1} dried extract at flowering I stage (Table 5). Tannins were also observed in fruit pulp extracts (4.7 μg mg^{-1} at fruiting I and 29.0 μg mg^{-1} at fruiting II; Table 5). Leaf and fruit shell extracts also presented tannins (t_R ~ 3.6 min, λ_{max} = 218.7/279.1) (t_R ~ 3.4 min, λ_{max} = 218.7/279.1) but at concentrations near the dection limit of the equipment used. For this reason, the tannins quantification by UHPLC was not feasible.

At the experimental conditions tested, no alkaloids were detected in *P. edulis* extracts by UHPLC.

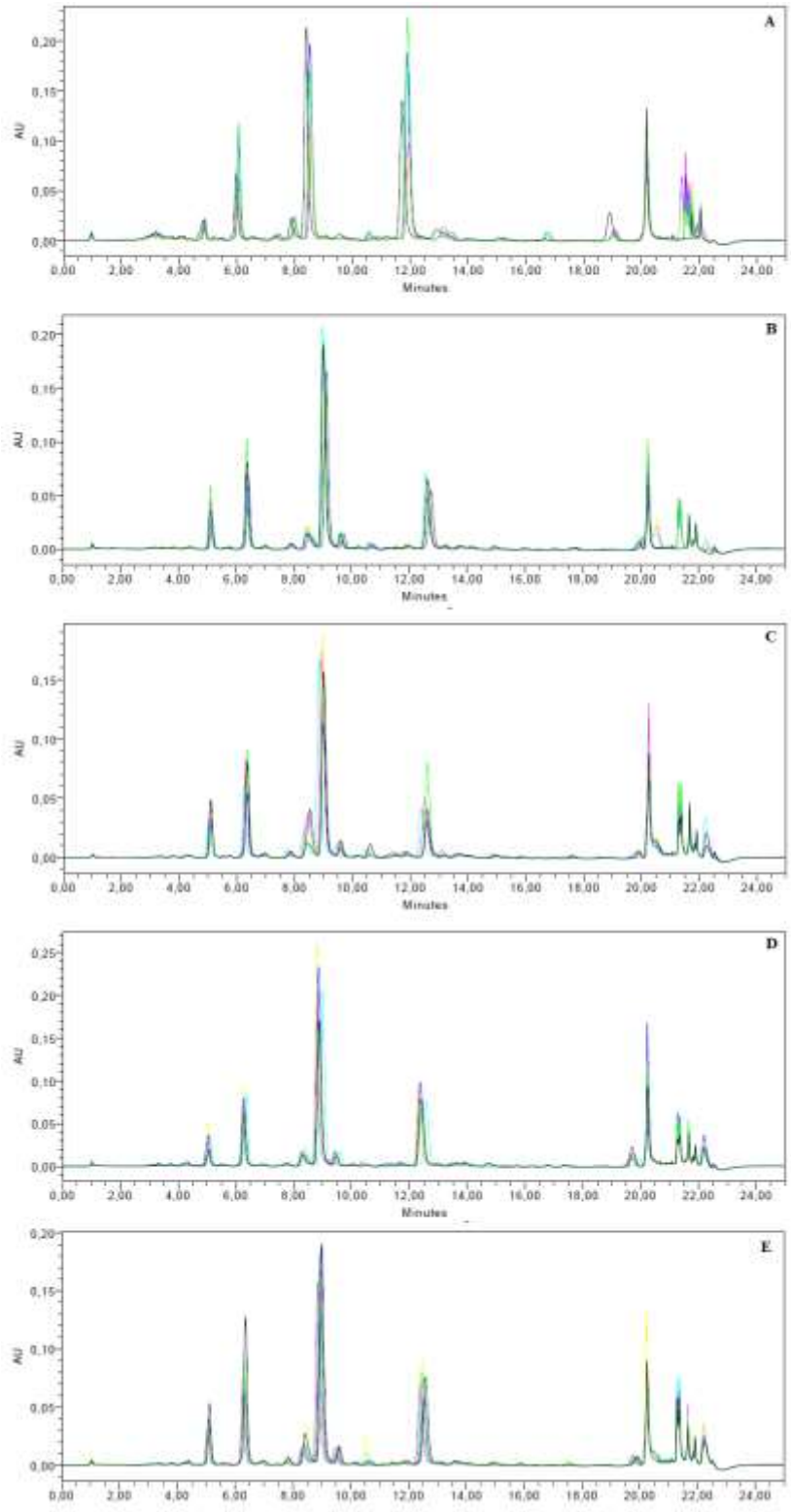

Figure 3. Chromatograms of *P. edulis* leaves extract at 335 nm **A)** Leaves at juvenile stage; **B)** Leaves of in fruiting I; **C)** Leaves in flowering I; **D)** Leaves in flowering II; **E)** Leaves in fruiting II.

Table 3. Classification of flavonoids detected in *P. edulis* leaves and fruit shell extracts by UHPLC.

Peak	Retention time (min)	$\lambda_{máx}$ (nm)	Category
1	5.108	270.8/346.0	Luteolin derivative
2	6.359	270.8/335.1	Apigenin derivative
3	6.997	269.6/348.9	Luteolin derivative
4	7.868	269.6/342.0	Luteolin derivative
5	8.537	267.2/347.8	Apigenin derivative
6	9.006	269.6/347.8	Apigenin derivative
7	9.602	271.9/313.6	Chrysin derivative
8	10.61	267.2/337.4	Apigenin derivative
9	12.593	269.6/337.4	Apigenin derivative

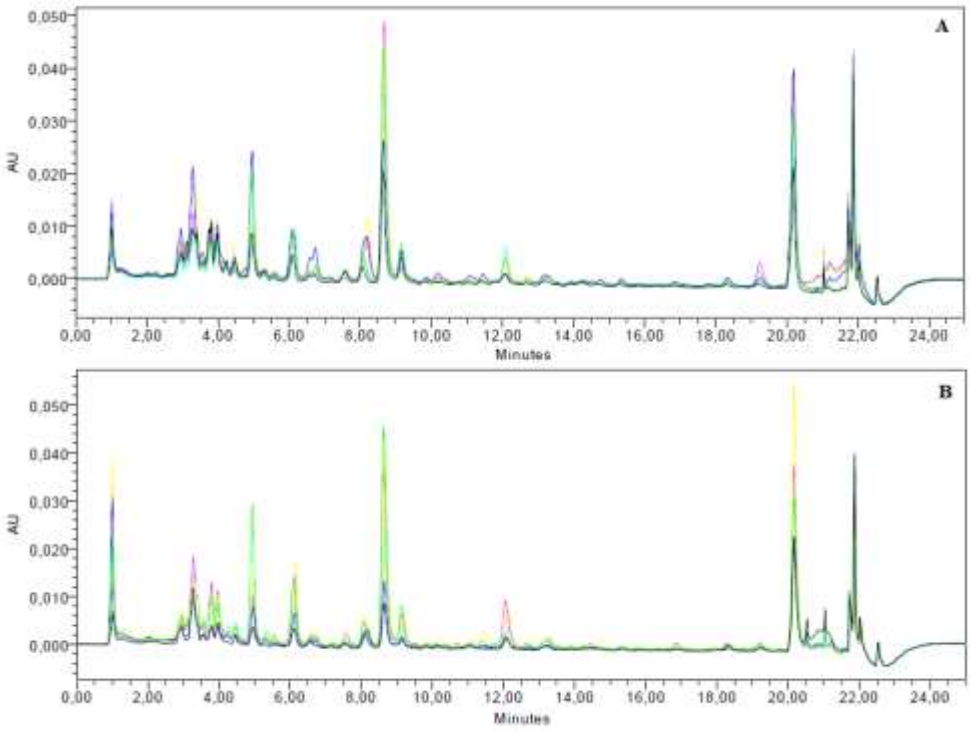

Figure 4. Chromatograms of *P. edulis* fruit shell extract at 335 nm. **A)** Fruit shell in fruiting I; **B)** Fruit shell in fruiting II.

Table 4. Total flavonoid contents (vitexin equivalent) in *Passiflora edulis* organs according to phenological stages determined by UHPLC analysis.

Phenological phase	Plant organ	Flavonoids (%)[a]
Juvenile	Leaves	3.9 a
Fruiting I	Leaves	3.6 a
	Fruit shells	1.1 b
Flowering I	Leaves	3.4 a
Flowering II	Leaves	3.8 a
Fruiting II	Leaves	3.1 a
	Fruit shells	0.9 b
CV(%)		20.1

[a] Percentage calculated as vitexin equivalent in dried extract of *P. edulis*. CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott, $P < 0.05$).

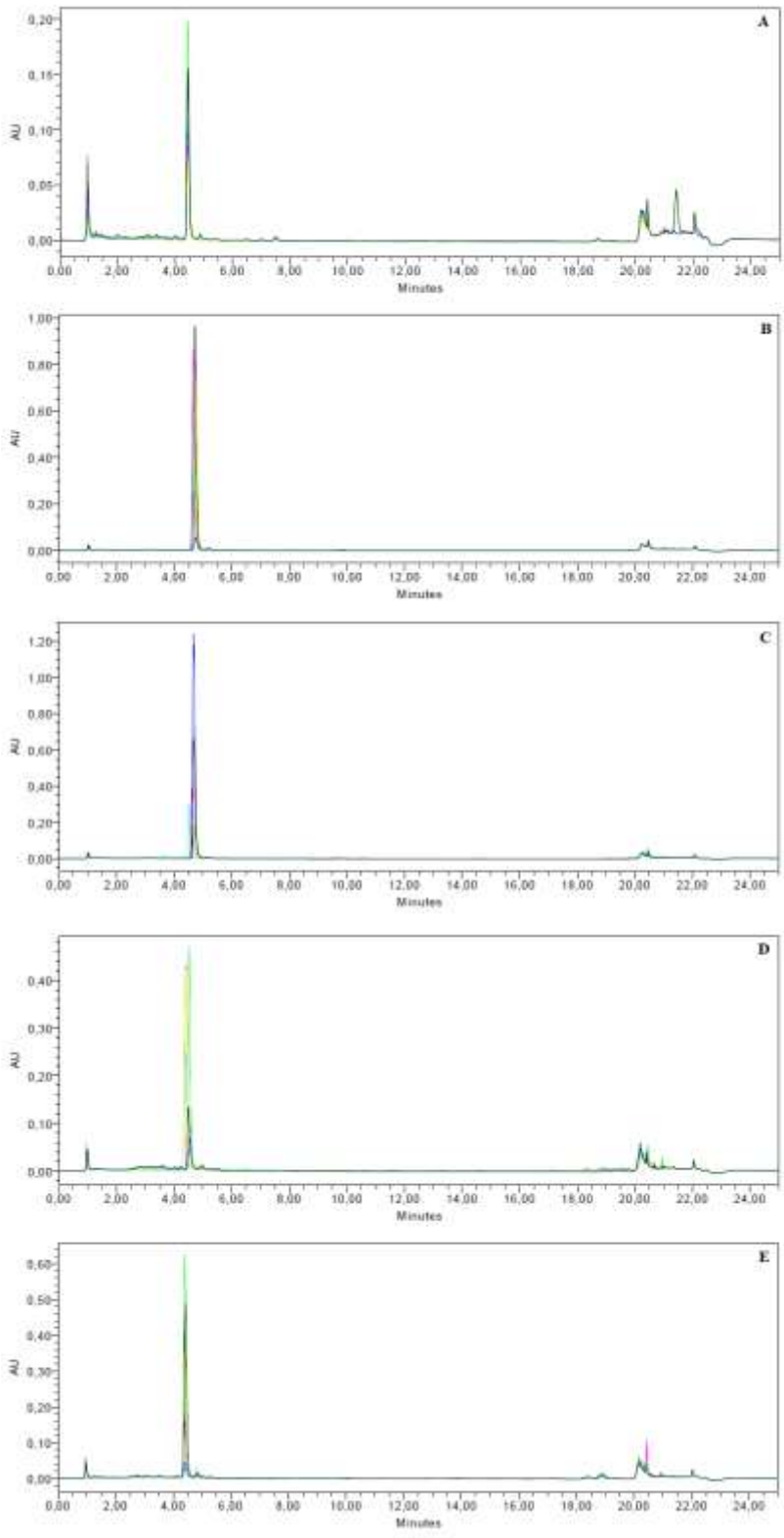

Figure 5. Chromatograms of *P. edulis* roots extracts at 279 nm. **A)** Roots in juvenile phase; **B)** Roots in fruiting I; **C)** Roots in flowering I; **D)** Roots in flowering II; **E)** Roots in fruiting II.

Table 5. Total tannin contents (epicatechin equivalent) in *Passiflora edulis* organs according to phenological stages determined by UHPLC analysis.

Phenological phases	Plant parts	Tannins (µg/mg)[a]
Juvenile	Roots	41.8 b
Fruiting I	Roots	212.6 a
	Fruit pulps	4.7 b
Flowering I	Roots	180.1 a
Flowering II	Roots	102.2 b
Fruiting II	Roots	100.0 b
	Fruit pulps	29.0 b
CV(%)		86.1

[a] Percentage calculated in terms of epicatechin in dry extract of *P. edulis*. CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott, P <0.05).

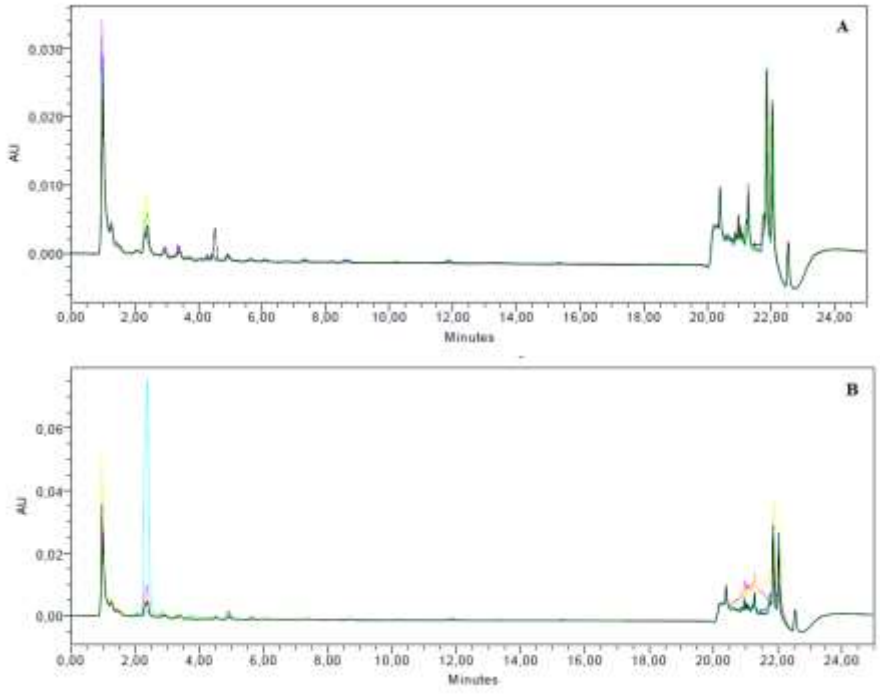

Figure 6. Chromatograms of *P. edulis* fruit pulp extracts at 279 nm. **A)** Fruit pulp in fruiting I; **B)** Fruit pulp in fruiting II.

3.4. Biological activity:

3.4.1. Inhibitory effect of P. edulis *extracts on jack bean urease:*

Fruiting II-derived *P. edulis* fruit shell extracts at 1 mg mL^{-1} inhibited the jack bean type III urease by over 40% and was more potent than hydroxyurea, the reference of inhibitor (Table 6; $P < 0.05$). Leaf (juvenile stage), root (fruiting I stage) and fruit shell (fruiting I stage) extracts were less efficient than hydroxiurea, but registered inhibitions as high as 30% on average (Table 6).

Table 6. Inhibition of jack bean urease by *P. edulis* extracts of plants at different stages of development.

Plant extract	**Urease inhibition (%)**
Leaves (juvenile)	31.6 c
Roots (fruiting I)	32.7 c
Fruit shells (fruiting I)	29.1 c
Fruit shells (fruiting II)	43.5 a
Hydroxyurea	35.6 b
CV (%)	16.7

CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott, $P < 0.05$). Hydroxiurea was used as a reference of urease inhibitor.

3.4.2. Test of susceptibility of F. solani *complex and* C. gloeosporioides *complex in crude extracts of* P. edulis*:*

All plant extracts analyzed in this study promoted an increase in the growth of *F. solani* complex (Table 7). The highest increase in the growth of *F. solani* growth occurred in leaf extracts in the juvenile phase (22.2%), leaves in fruiting I (16.6%), roots in flowering II (17.9%) and leaves in fruiting II (23.1%) (Table 7).

The *P. edulis* extracts also stimulated *C. gloeosporioides* complex growth; Leaf extracts (juvenile stage) promoted the fungus growth by over 70.7% (Table 7). The extracts that less stimulated *C. gloeosporioides* growth were those from roots (7.2%) and pulp (4.8%) at fruiting I stage and roots and furit pulps of plants at flowering II stage (9.7% and 7.7%, respectively).

Table 7. Effect of *P. edulis* extracts on the growth (sporulation induction) of the fungi *F. solani* complex and *C. gloeosporioides* complex.

Phenological phases	**Plant parts**	***F. solani* growth (%)**	***C. gloeosporioides* growth (%)**
Juvenile	Leaves	22.2 a	70.7 a
	Roots	10.3 b	5.3 d
Fruiting I	Leaves	16.6 a	16.6 c
	Fruit shells	8.7 b	4.6 d
	Roots	1,3 b	7.2 d
	Fruit pulps	2.6 b	4.8 d
Flowering I	Leaves	12.9 b	41.1 b
	Roots	13.5 b	22.3 c
Flowering II	Leaves	10.5 b	40.1 b
	Roots	17.9 a	9.7 d
Fruiting II	Leaves	23.1 a	35.7 b
	Fruit shells	5.6 b	24.1 c
	Roots	6.6 b	16.2 c
	Fruit pulps	1.0 b	7.7 d
CV(%)		95.3	78.4

CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott, P <0.05).

4. Discussion:

As for the quantitative method suggested by the Brazilian Pharmacopoeia (Brasil, 2010), the highest concentration of flavonoids was verified in leaves of *P. edulis* at juvenile stage, prior to the first reproductive cycle. Berezina et al., 2017 describe similar accumulation of flavonoids in leaves of cranberry (*Vaccinium macrocarpon* Ait., Ericaceae Juss.), cv. 'Stevens' in the budding phase, before the reproductive stages (flowering and fruiting).

UHPLC analyse indicated the presence of vicenine in *P. edulis* extracts from plants at various developmental stages, although the presence of other flavonoids (vitexin, isovitexin, orientin, isoorientin, sccafoside) are also described in *P. edulis* plants (Grice et al., 2006; Frye & Haustein, 2007; Machado et al., 2010; Zeraik et al., 2010; Li et al., 2011; Ayres et al., 2015; Medina et al., 2017; Araújo et al., 2017). No *β*-carbolin alkaloids were detected in *P. edulis* extracts at the present study, even though these substances are described in *Passiflora* spp. (Frye & Haustein, 2007; Machado et al., 2010; Araújo et al., 2017). One should note that the current study was based on ethanolic extracts of *P. edulis* parts. The basic character of *β*-carbolinic alkaloids, might be the reason for not detecting such compounds in the extracts. Freire et al., 2018 recommends a specific type of extraction using two SBSE (stir bar sorption extraction using polydimethylsiloxane as stationary phase), at pH 10 and pH 13, to successfully extract *β*-carbolinic alkaloids from plant tissues. The UHPLC results for extracts of various *P. edulis* parts indicates variation in the quality and quantity of secondary metabolites accumulated as a function of the organs analyzed and the plant developmental stage (Figures 3-6).

The leaves of *P. edulis* are the plant parts with the highest concentration of flavonoids (Table 4). Nine distinct flavones glycosides were detected in *P. edulis* ethanolic leaf extracts (apigenin, chrysin and luteolin derivatives). Glycosylated flavones are an important group of flavonoids that plays an important role in a wide range of physiological processes, protecting tissues from oxidative damage (Mekawy et al., 2018). Apigenin (Figure 8) is widely produced in plant tissues as glycoconjugates with higher water solubility (Madunic et al., 2018). Luteolin (Figure 8), a flavone found in pollen and honey, is an antioxidant and antimicrobial that can also form complex with metals (Wang et al., 2019). Chrysin (Figure 8), on the other hand is a flavone with no substitutions at ring B (Mani & Natesan, 2018). From the physiological point of view, leaves are photosynthetic organs that function as carbon source for sink, developing organs. Despite extensive research on carbon allocation in plants, it is still not well understood how plants direct metabolites other than sucrose to sink tissues (Huang et al., 2019). The luteolin glycosides content in juvenile plants was found to be lower than that of mature plants at flowering and fruiting stages (Figure 7). Flavones like luteolin and apigenin and respective glycoconjugates are notable for protecting plant cells against UV-B radiation (Harborn & Williams, 2000). The contrasting results for apigenin glycoside contents – lower amounts in flowering I and fruiting I plants with considerable increment in the second (II) episode of flowering and fruiting stage – is explained by the fact that plants at a second reproductive stage are senescent. The increase in apigenin derivatives levels in plants during flowering II and fruiting II stages may comprise a strategy to attenuate the oxidative stress intensified during plant senescence (Figure 1). Apigenin and derivatives are reported to inhibit lipid peroxidation, scavenge free radicals and protect DNA from oxidation (Mekawy et al., 2018). The chrysin glycoconjugates levels in *P. edulis* plants remained unchanged and relatively low (<15 μg mg^{-1}) during plant's development likely because such flavone is a poor antioxidant due to

the reduced number of phenolic hydroxyl groups when compared to apigenin and luteolin. Fruit shell extracts presented flavone profile similar to that of leaf extracts, except that the levels of such flavonoids were much lower in the former (Figures 3 and 4). Then, it is possible that leaves exported flavones to fruit shell in a source-sink manner as it occurs for sucrose.

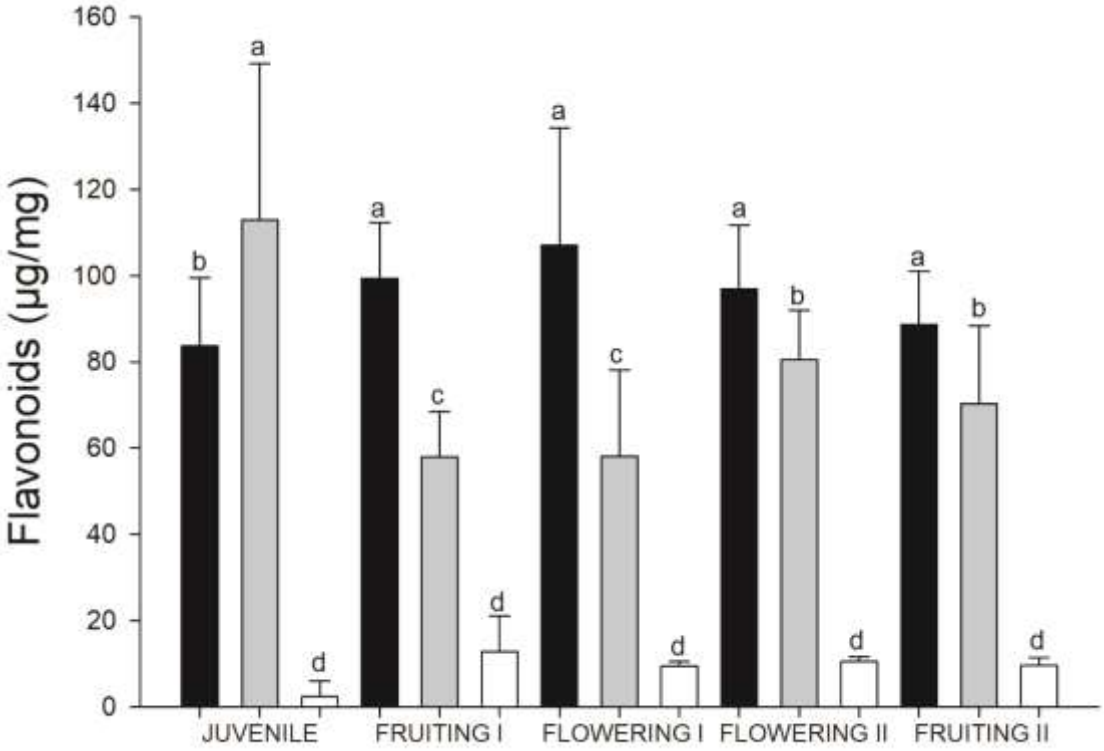

Figure 7. Proportion of flavonoids derived from luteolin (black bars), apigenin (gray bars) and chrysin (white bars) in leaf extracts of *P. edulis* throughout development.

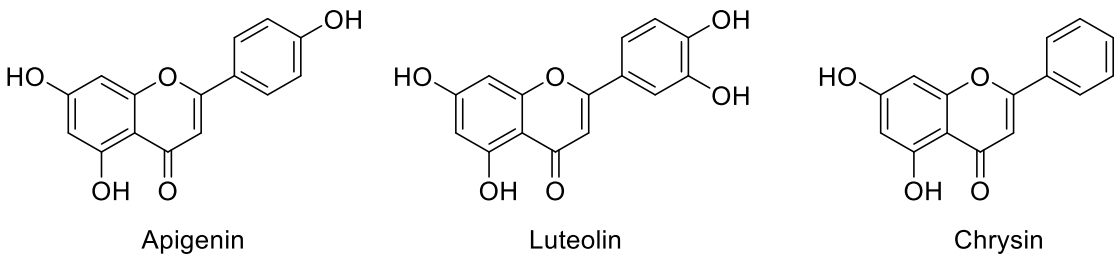

Figure 8. Structure of the flavones aglycones apigenin, luteolin and chrysin.

Only roots extracts presented condensed tannins (Figure 5), high molecular weight polyphenols derived from the flavonoid catechin. Tannins occurrence in roots may be related to the exudation of these substances for signaling purposes to estimulate plant association with microorganisms (Chin et al. 2009). The highest concentration of condensed tannins in *P. edulis* roots occurred during the first episode (I) of fruiting and flowering, a condition where the orchard was healthy and quite productive (Table 5). Condensed tannins exuded from roots are known to play important roles in soil mineralization and plant defense (Solaiman & Senoo, 2018). As expected, the tannins levels in roots of senescent plants dropped when compared to the other developmental stages and senescent plants are usually more susceptible to pathogen attacks.

Biological assays indicated that *P. edulis* fruit shell extracts (fruiting II) are the most promising as urease inhibitors (Table 6). Soil ureases negatively impact nitrogen nutrition in plants because they catalyze the hydrolysis of urea (fertilizer used worldwide) to ammonia (NH_3) and carbon dioxide, which in turn causes environmental and economical losses due to lower crop productivity (Modolo et al., 2015). The use of urease inhibitors as additive in urea-based formulations is a common practice used to overcome nitrogen losses in agriculture.

The *P. edulis* fruit shell, a by product of juice industries could offer a sustainable alternative to mitigate nitrogen losses particularly in family agriculture. Processing for juice production generates thousands of tons of residues, composed of seeds and fruit shells, which can be considered co-products derived from agricultural activity (López-Vargas et al., 2013). Ureases also impact human and animal health (Modolo et al., 2015). This hydrolase is the virulent factor of *Helicobacter pylori*, bacteria that cause gastrointestinal ulcer due to the increment of stomach pH. The inhibitory activity of fruit shell extracts towards jack bean ureases are encouraging for further tests against *H. pylori* ureases specially because the

active site is conserved among the ureases from various organisms. Then, *P. edulis* fruit shells could be used in infusion or jam preparations as functional food for the prevention of *H. pylori* gastric infection.

There was significant growth of *F. solani* complex in root extracts in flowering II (Table 7), even though this tissue is rich in tannins (Table 5). Leaf extracts of juvenile plants boosted the growth of *C. gloeosporoides* complex (Table 7), even though they had the highest flavone glycoside levels (Table 4). When infection occurs, due to coevolutionary mechanisms, some fungi can circumvent plant defense systems causing diseases (Pearson et al., 2009). Pathogen-driven damage to plant tissues can cause variation in the plant metabolic profile, affecting the production of plant organic volatile and nonvolatile compounds production and nutrients profile in addition to visual cues (Franco et al., 2017). Any plant-fungal interaction is preceded by a physical encounter between the organisms followed by several physical and chemical barriers that must be overcome to successfully establish an association (Kusari et al., 2012).

5. Conclusion:

The different classes of secondary metabolites present in *P. edulis* plants vary in fuction of the phases of development and the organs analyzed. Flavones derived from apigenin, chrysin and luteolin are the main secondary metabolites found in ethanolic extracts of leaves and fruit shell of *P. edulis*. Major secondary metabolites in ethanolic root extracts are tannins condensed. In Brazil, the cultivations of *P. edulis* are aimed at the commercialization of the fruits, which are destined to the industry for the production of juices and drinks. However, processing for juice production generates thousands of tons of residues, composed of seeds and fruit shells, which can be considered co-products derived

from agricultural activity. Is possible use leaves and fruit shell, with appropriate technology, as a nutraceutical food in prevention of bacterial gastric infections and source of flavones for the pharmaceutical industry.

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Conflict of interest

The authors declare no conflict of interest.

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General Conclusions

- The different classes of secondary metabolites present in *P. edulis* plants vary in fuction of the phases of development and the organs analyzed.
- *P. edulis* leaves were the plant parts with the highest phenolic compounds/flavonoids contents regardless of the plant developmental stage. This evidence has been demonstrated through various techniques such as Brazilian Pharmacopoeia Method and high efficiency liquid chromatography.
- Flavones derived from apigenin, chrysin and luteolin are the main secondary metabolites found in ethanolic extracts of leaves and fruit shell of *P. edulis*. Major secondary metabolites in ethanolic root extracts are tannins condensed.
- Extracts of leaves and roots/fruit shells efficiently scavenged reactive nitrogen and oxygen species, respectively. This distinct profile denotes differences in the chemical diversity in extracts from *P. edulis* parts, verify by ultra high efficiency liquid chromatography analyses.
- Passionfruit processing for juice generates residues, composed of seeds and fruit shells. From the results obteined, we suggest use levaes and fruit shell, with appropriate technology, as a nutraceutical food in prevention of bacterial gastric infections and source of flavones for the pharmaceutical industry.