



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

Sarah F. Guimarães¹ , Inorbert M. Lima²  and Luzia V. Modolo^{1*} 

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ABSTRACT

Passiflora edulis Sims var. *flavicarpa* O. Deg. (Yellow-passion fruit) is the native plant species most used by juice industries in Brazil while its leaf extracts are widely employed in folk medicine. This study evaluated the phenolic content of leaves, roots, fruit shells and pulp of plants of *P. edulis* in juvenile, flowering and fruiting stages. The extent of scavenging and/or degradation of reactive nitrogen and oxygen species by plant extracts was also investigated. Leaves were the organs that most accumulated phenolics/flavonoids, regardless of plant developmental stage. Leaf extracts efficiently scavenged DPPH by up to 67 % while root and fruit shell extracts effectively captured up to 80 % O₂. Maximum activity of catalase (51.6 mmol H₂O₂ min⁻¹ mg prot⁻¹) and ascorbate peroxidase (2.2 mmol ascorbate min⁻¹ mg prot⁻¹) was recorded in leaf extracts from plants in the fruiting stage. Superoxide dismutase activity reached its highest levels (37.5 U min⁻¹ mg prot⁻¹, on average) in plant leaves of both juvenile and fruiting plants. Overall, these results suggest that, for therapeutic purposes, parts of *P. edulis* should be harvested when plants are in the fruiting stage due to the excellent antioxidant properties of their extracts and their accumulation of phenolic compounds.

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

Introduction

The genus *Passiflora* (family Passifloraceae) includes about 600 plant species (Ayres *et al.* 2015; Wosch *et al.* 2017) distributed in the tropical and subtropical regions around 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) and 60 species produce edible fruits (Pertuzatti *et al.* 2015). *Passiflora edulis* var. *flavicarpa* (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, purple corolla in the base and five stamens with large anthers

(Zibadi & Watson 2004). *Passiflora edulis* is the native species (Zibadi & Watson 2004) most cultivated and used in Brazil by juice industries (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* species leaves have been used in folk medicine for treating 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 and digestive stimulant and to treat asthma, bronchitis, whooping cough and urinary infections (Zibadi & Watson 2004). Indeed, *P. edulis* was included in 2009 by the Unified Health System (SUS; Brazil) in the Brazilian National List of Medicinal

¹ Grupo de Estudos em Bioquímica de Plantas, Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, MG, Brazil

² Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, 29900-970, Linhares, ES, Brazil

* Corresponding author: lvmodolo@icb.ufmg.br



Plants of Interest (MS 2009). A year later, *P. edulis* was also included in the Brazilian Pharmacopoeia (Brasil 2010).

Several secondary metabolites were reported to occur in leaves of *P. edulis*, but the phenolic contents in root, fruit shell and pulp remain to be evaluated. Besides this, the potential as antioxidant of extracts from different parts of such species will comprise valuable information to rationalize the folk use of *P. edulis* to treat several diseases. This work evaluated the accumulation of phenolic and polyphenolic (flavonoid) compounds in different parts of *P. edulis* plants in distinct development stages. The potential of ethanolic extracts of parts of *P. edulis* to scavenge free radicals were also investigated, together with the endogenous activity of antioxidant enzymes in plant leaves.

Materials and methods

Plant material and experimental design

Samples were harvested from plants cultivated in Pitangueiras Farm located in Sooretama, Espírito Santo, Brazil (19°12'05.6''S 40°03'38.5''W), unless otherwise stated. Plant parts were harvested during the phenological stages juvenile (Dec 2017; leaves and roots), flowering I (Feb 2017; leaves and roots), flowering II (Sep 2017; leaves and roots), fruiting I (Nov 2016; leaves, roots and fruits) and fruiting II (Dec 2017; leaves, roots and fruits) (Fig. 1). Shell and pulp were also separated from the fruits for subsequent

analyses. Plants in juvenile stage were characterized by those grown in a greenhouse at Incaper (19°25'01.2''S 40°04'44.1''W) at Tmin of 24.1 °C and Tmax 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.

The experimental design was completely randomized, in which the indicated plant parts were harvested from eight individuals (each defined as a biological sample) in the following developmental stages: juvenile, flowering I and II and fruiting I and II. Pluviometric and temperature variations were monitored on a monthly basis by Incaper Weather Station (Linhares, Espírito Santo, Brazil) during the period by which *Passiflora edulis* Sims var. *flavicarpa* O. Deg plants were investigated (Fig. 2). Completely mature fruits were harvested immediately after natural detachment from the plants (Faleiro *et al.* 2005).

Ethanolic extracts preparation

The extracts were prepared by adding to intact tissues a volume of ethanol PA (in mL) correspondent to 10 times the organ weight in grams. After 72 h in sealed flasks in the absence of light Samples (Brasil 2010; Shah *et al.* 2004; Shelar *et al.* 2018; Carvalho *et al.* 2019), samples were filtered and the organic fraction dried at < 50 °C (Dai & Mumper 2010; Shelar *et al.* 2018) to obtain the corresponding solid

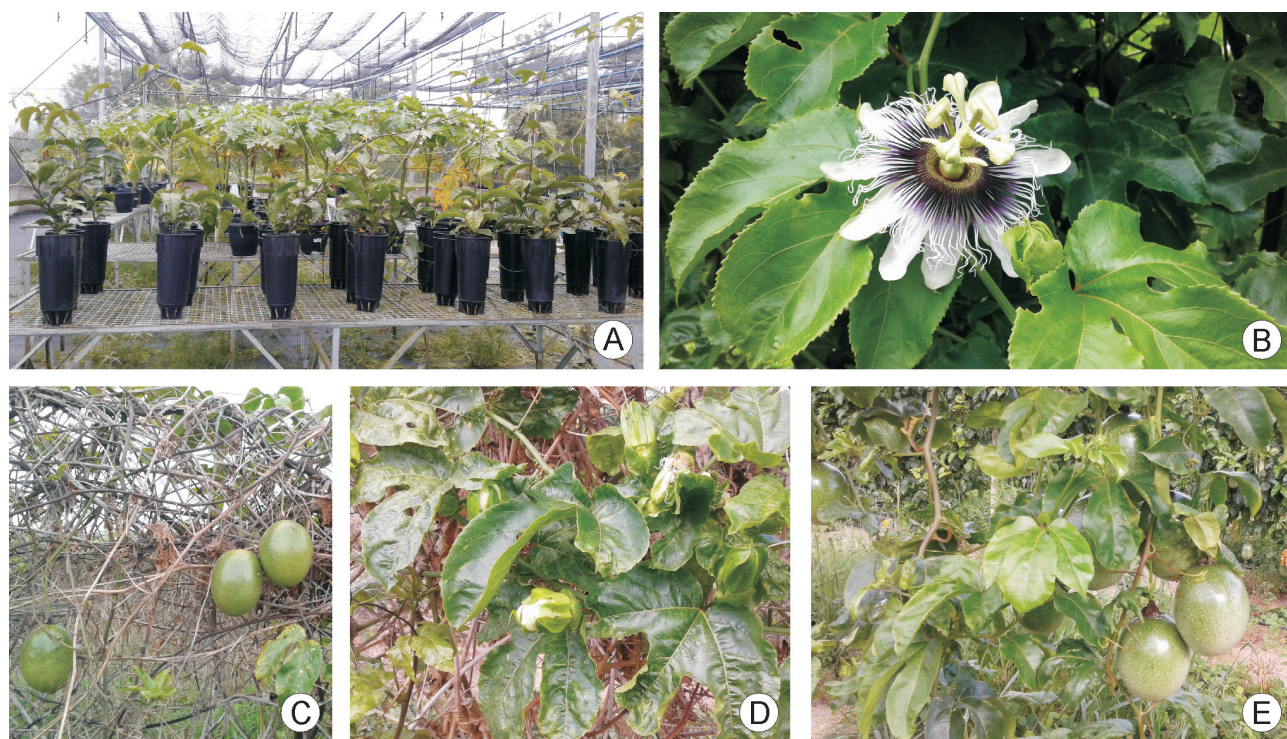


Figure 1. Representative images of *Passiflora edulis* plants in the developmental stages investigated. **A.** Juvenile; **B.** Flowering I; **C.** Fruiting I; **D.** Flowering II; **E.** Fruiting II; Plants are from the Pitangueiras Farm (Sooretama, Espírito Santo, Brazil) except for those in juvenile stage, which were grown in a greenhouse.

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 ethanolic extract used in the subsequent analysis.

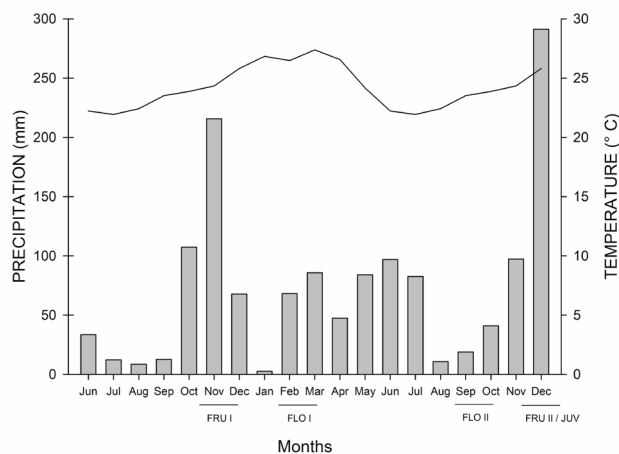


Figure 2. Cumulative precipitation (bar chart) and mean temperature (line chart) recorded on a monthly basis from June 2016 to Dec 2017 at Pitangueiras Farm (Sooretama, Brazil), interval by which plant samples were harvested as indicated. **FLO I** and **FLO II**, plants in flowering developmental stage; **FRU I** and **FRU II**, plants in fruiting developmental stage; **JUV**, plants in juvenile developmental stage. Juvenile plants were grown at greenhouse during the same period that field plants were in fruiting stage II. Data were gathered by Incaper Weather Station located in Linhares (Espírito Santo, Brazil).

Quantification of total phenolic compounds and total flavonoids in ethanolic extracts

The determination of phenolic compounds was performed as previously described (Murthy *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 following analysis at 765 nm. Tannic acid (0 – 800 $\mu\text{g mL}^{-1}$) was used as a standard (Box 1983; Blainski *et al.* 2013) and the total phenolics compounds 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 (0 – 800 $\mu\text{g mL}^{-1}$) was used as a flavonoid standard and the results were expressed as equivalents of quercetin/dry extract.

Scavenging of reactive nitrogen species

The potential of ethanolic extracts of *P. edulis* (1 mg mL^{-1}) to scavenge reactive nitrogen species was investigated

against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Silva *et al.* 2012). One volume of ethanolic extract was added to an 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 $\text{DPPH}(\%) = [1 - (\text{As}/\text{Ac})] \times 100$, where Ac corresponds to the absorbance of systems containing DPPH only and As the absorbance of systems containing DPPH plus ethanolic extract.

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 against anion superoxide radicals (Silva *et al.* 2012). Superoxide anions were artificially generated in a system containing 1 μM EDTA, 17 μM L-methionine, 10 μM 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 resveratrol was used as positive control.

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

The activity of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) in leaves of *P. edulis* was assessed essentially as previously reported (Silva *et al.* 2017). The molar extinction coefficients (ϵ) of 2.8 $\text{mM}^{-1} \text{cm}^{-1}$ and 39.4 $\text{mM}^{-1} \text{cm}^{-1}$ were used to determine the amount of oxidized ascorbate and degraded H_2O_2 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 nitroblue tetrazolium (NBT) by 50%. The total protein content in leaf samples was determined according to the method of Bradford (Bradford 1976).

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 from organs of plants in each developmental stage were individually submitted to analysis of variance and mean test (Scott-Knott; $P < 0.01$) using ASSISTAT software (Silva & Azevedo 2016).

Results

Total phenolics/flavonoids in *P. edulis* extracts

Leaves of *P. edulis* plants in 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

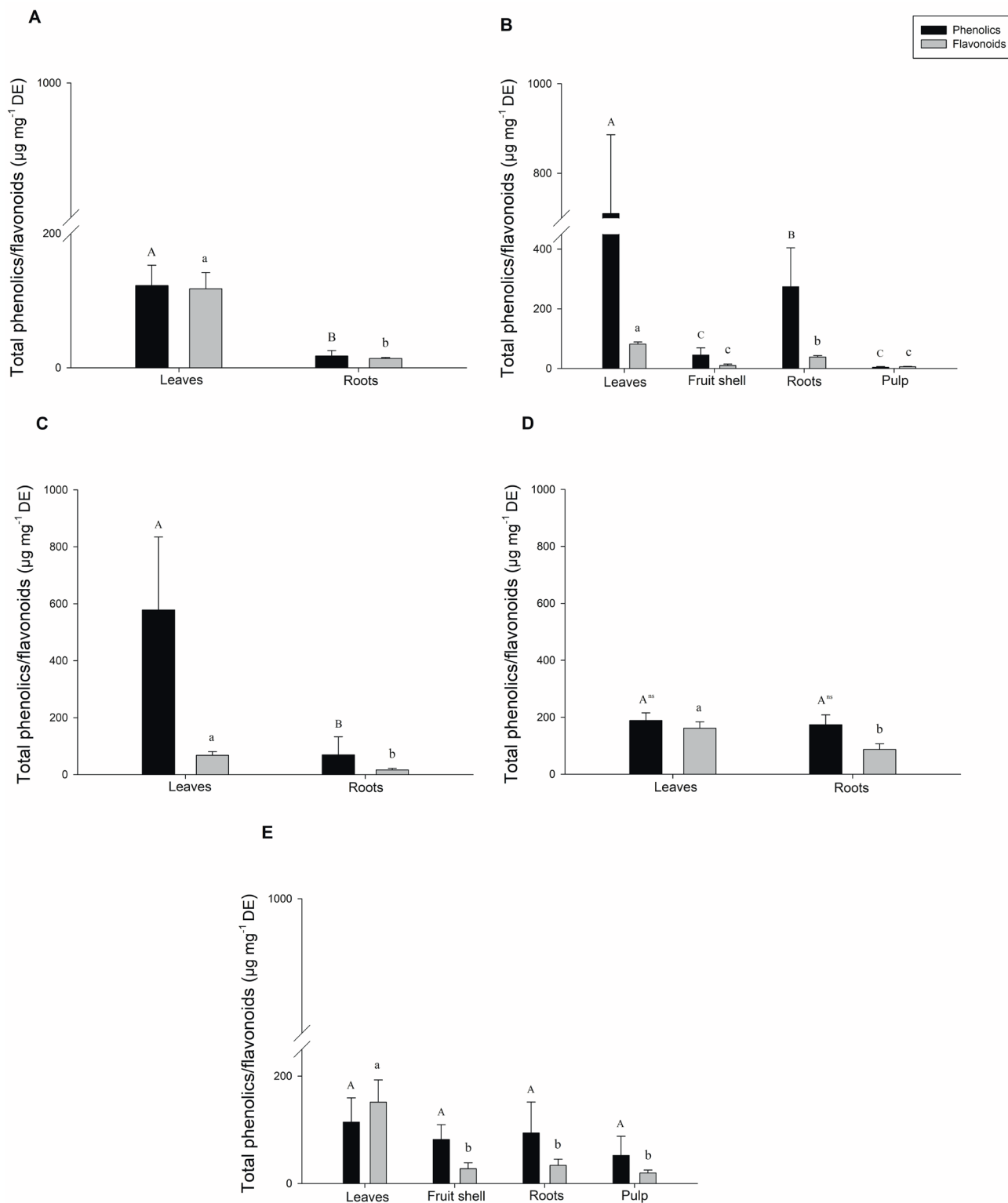


Figure 3. Total phenolic (black bars) and flavonoid (gray bars) contents in *Passiflora edulis* in various developmental stages. Ethanolic extracts (1 mg mL⁻¹) were prepared from plant samples harvested in juvenile (A), fruiting I (B), flowering I (C), flowering II (D) and fruiting II (E) stages. Values correspond to the means (n = 8) + standard deviations. Total phenolics are expressed as tannic acid equivalents while total flavonoids as presented as quercetin equivalents. 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.



extract) stages presented the highest levels of phenolic compounds among the studied plant organs, regardless of the plants development stage (Fig. 3). Leaves from *P. edulis* plants presented the highest levels of flavonoids compounds when compared to the other organs (Fig. 3).

The cumulative precipitation and temperature during the harvesting of plants in fruiting I stage (Nov 2016) averaged 215.8 mm and 24.3 °C, respectively (Fig. 2). Plants in flowering I stage (Feb 2017) experienced pluviometric and temperature conditions of 68.2 mm and 26.5 °C. The cumulative precipitation and temperature in Sep, 2017, when plants in flowering II stage were harvested, were on average 18.8 mm and 23.5 °C (Fig. 2). The cumulative precipitation and temperature in Dec 2017 (period of harvesting of plants in juvenile and fruiting II) were 291.4 mm and 25.8 °C, respectively (Fig. 2).

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

Ethanollic extracts from leaves (1 mg mL⁻¹) in 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 O₂⁻ 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 O₂⁻ was comparable to that of root extracts from the same plants while root and leaf extracts from plants in flowering II stage were equally effective against O₂⁻ (Fig. 5).

Among the plant parts and developmental stages investigated, leaf extracts from plants in flowering I and fruiting I stages exhibited the highest capacity to scavenge reactive nitrogen species (Tab. 1); 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. 1).

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

The activity of SOD was 2.3-fold higher in leaves of plants in juvenile and fruiting I stages (32.3 U min⁻¹ mg prot⁻¹ on 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 of fruiting I plants (51.6 mmol H₂O₂ min⁻¹ mg prot⁻¹ and 2.2 mmol ascorbate min⁻¹ mg prot⁻¹) followed by juvenile plants (36.5 mmol H₂O₂ min⁻¹ mg prot⁻¹ and 1.4 mmol ascorbate min⁻¹ mg prot⁻¹) and the

remainder stages (21.7 mmol H₂O₂ min⁻¹ mg prot⁻¹ and 0.9 mmol ascorbate min⁻¹ mg prot⁻¹, on average).

Discussion

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 plants 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 & 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 cranberry plants in flowering and fruiting stages (Berezina *et al.* 2017). The levels of phenolic compounds 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). In fact, flavonoids can account for up to 4 % of pollen dry weight, provide color to pollen and petals and contribute to the attraction of pollinators (Theis & Lerda 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 and aromas (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

Table 1. Scavenging of reactive nitrogen (DPPH) and oxygen (O₂⁻) species by 1 mg mL⁻¹ ethanollic 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
Flowering II	Fruit pulps	45.4 d	55.7 b
	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
CV (%)	Fruit pulps	54.5 c	67.0 a
		13.3	14.6

^a Percentage relative to the total amount of free radical in the reaction medium. CV%, coefficient of variation. Means followed by distinct letters in each column indicate significant differences (Scott-Knott; P < 0.01) between plant parts and phenological stages. Resveratrol (200 μM), tannic acid (200 μM) and quercetin (200 μM) were used as positive controls yielding the scavenging of DPPH radicals by 52.3, 65.5 and 68.3 %, respectively.



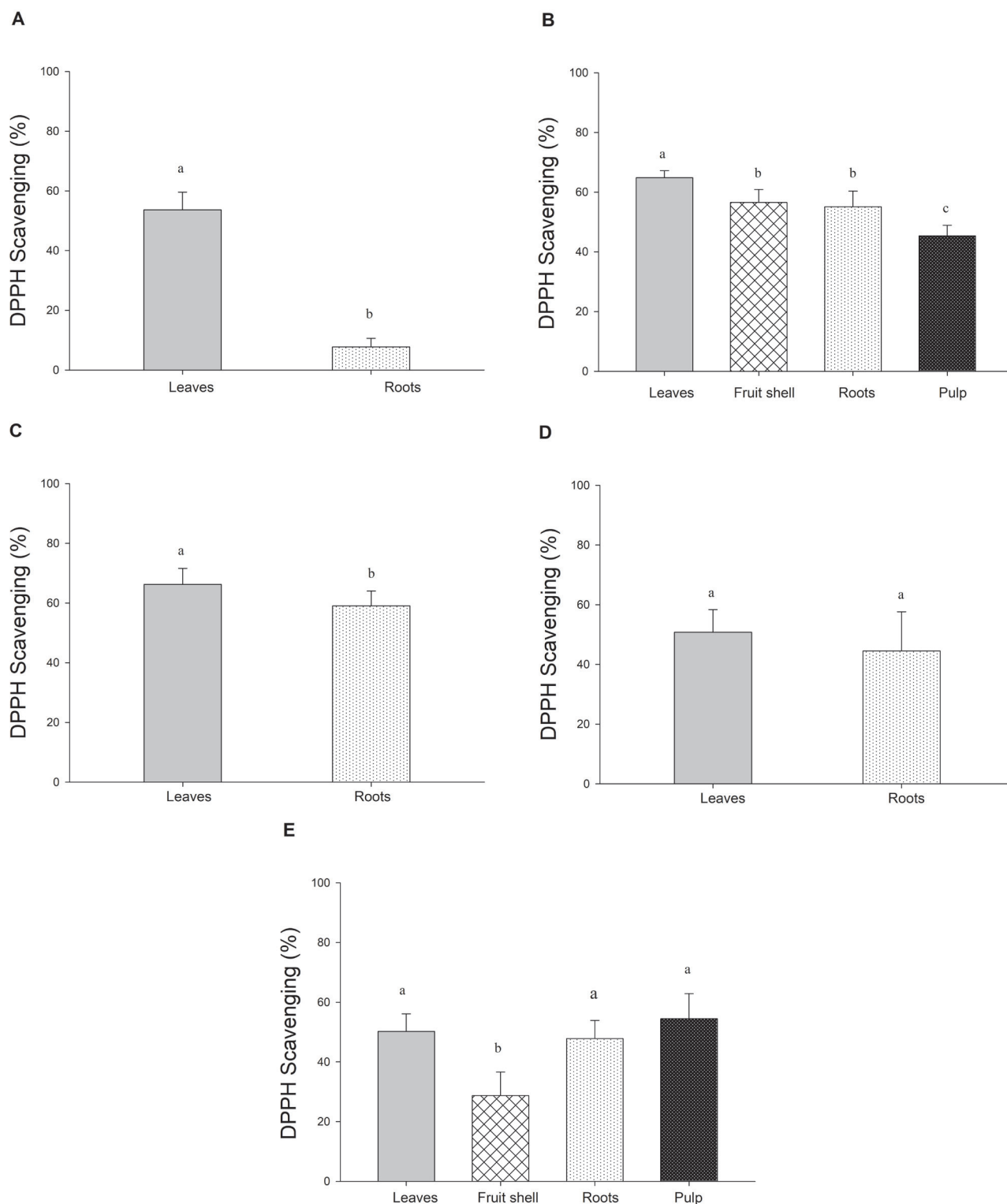


Figure 4. Scavenging of DPPH radicals by ethanolic extracts from *Passiflora edulis* plants in various developmental stages. Ethanolic extracts (1 mg mL⁻¹) were prepared from samples harvested in juvenile (**A**), fruiting I (**B**), flowering I (**C**), flowering II (**D**) and fruiting II (**E**) stages. Values correspond to the means (n = 8) + standard deviations. Distinct letters indicate significant differences among the plant parts (Scott-Knott; *P* < 0.01).



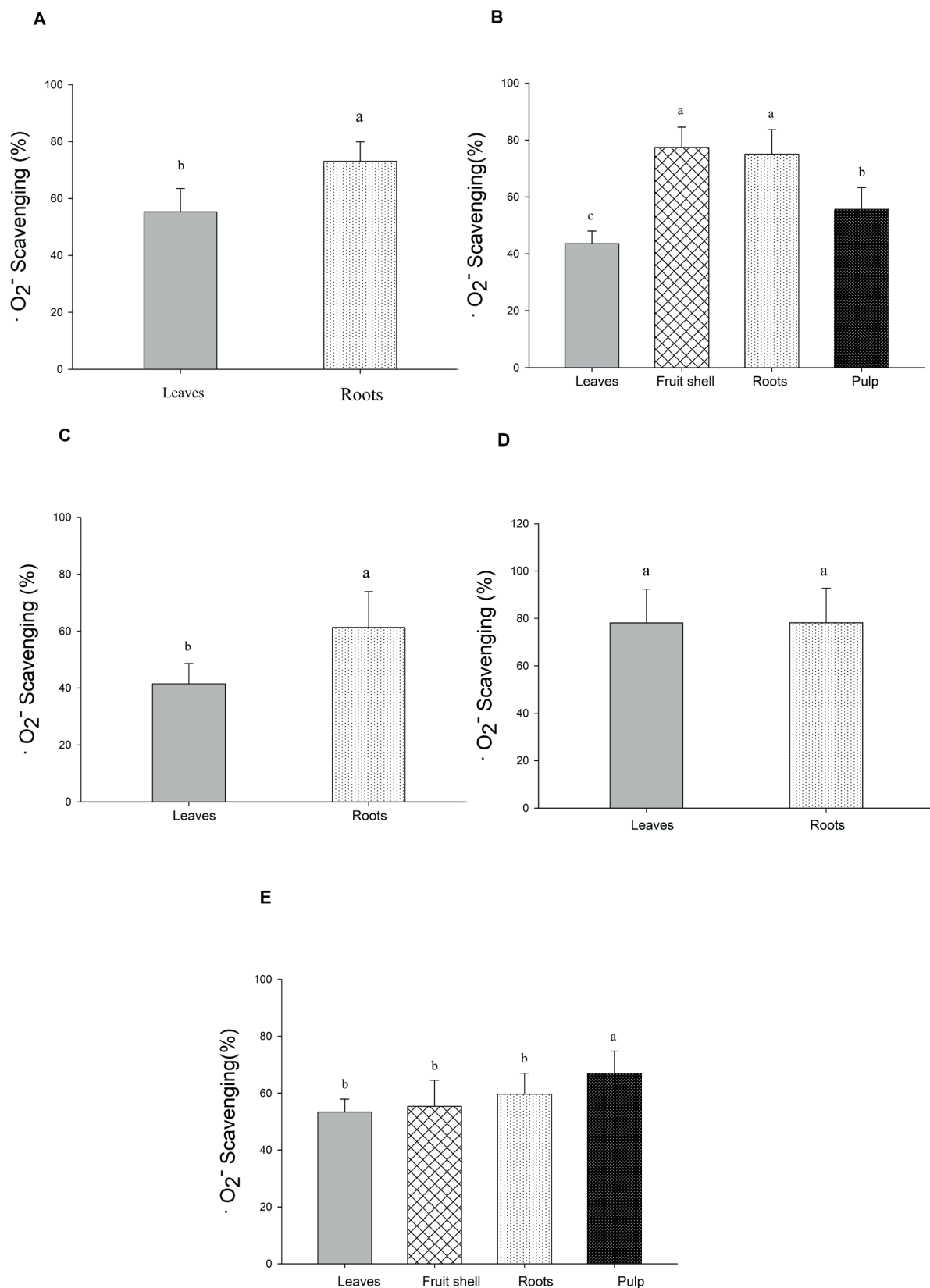


Figure 5. Scavenging reactive oxygen species by ethanolic extracts from *Passiflora edulis* plants in various developmental stages. Ethanolic extracts (1 mg mL⁻¹) were prepared from plant samples harvested in juvenile (**JUV**), flowering I and II (**FLO I** and **FLO II**) and fruiting (**FRU I** and **FRU II**) stages. Values correspond to the means (n = 8) + standard deviations. Distinct letters indicate significant differences (Scott-Knott; P < 0.01).



& Manson 2015). In contrast, the lower levels of phenolics in leaves and roots of plants in fruiting II stage in comparison with those of plants in flowering II stage may result from allocation of these secondary metabolites to fruits. Also, plants in flowering II and fruiting II stages 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 the harvesting of plants in flowering II stage. Such condition 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 plants aging (Ferreira *et al.* 2012).

The differences in the performance of ethanolic extracts of leaf and root 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 (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 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 and they are 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.

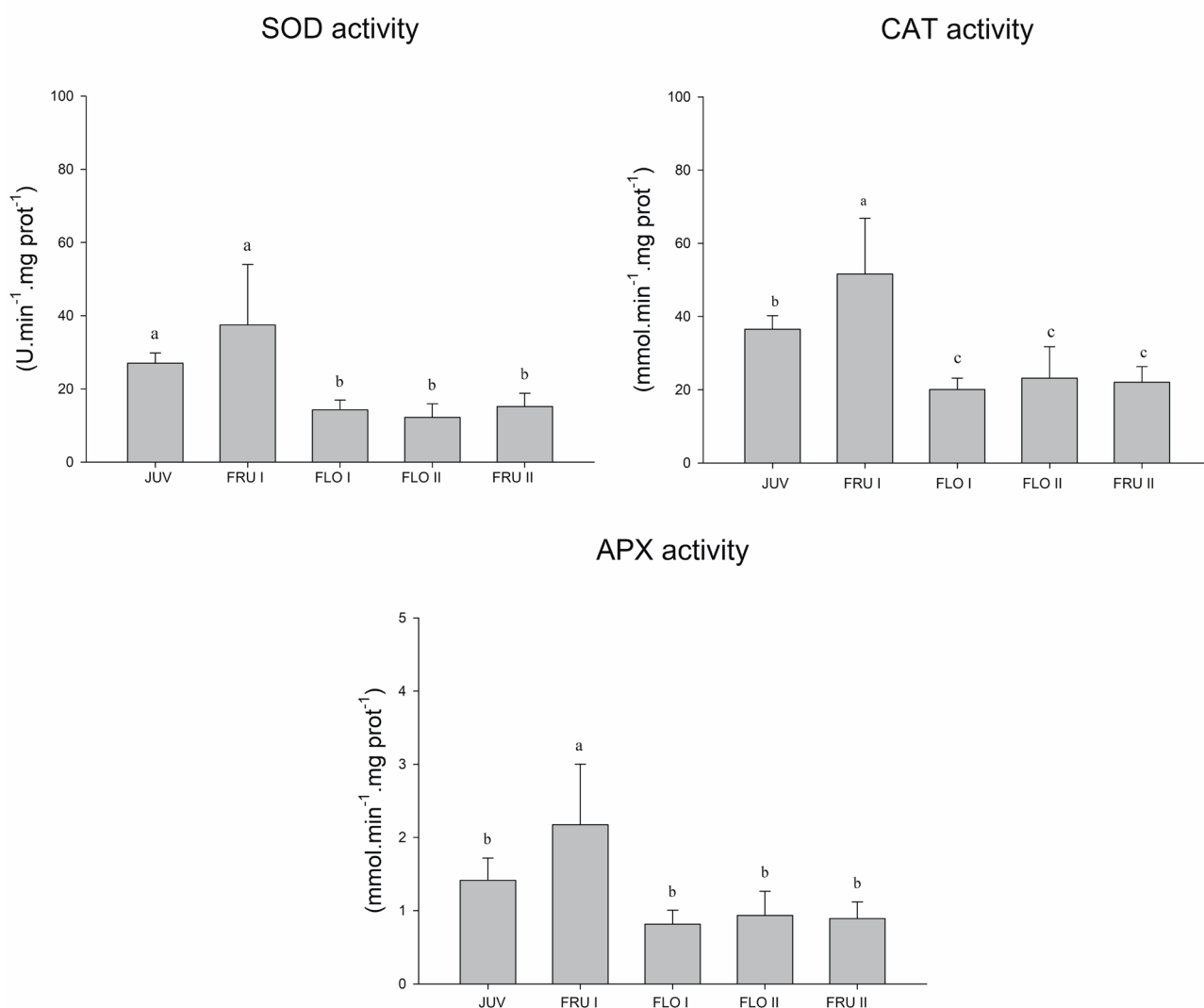


Figure 6. Activity of antioxidant enzymes in leaves of *Passiflora edulis* plants in various developmental stages. Leaves were harvested from plants in juvenile (**JUV**), flowering I and II (**FLO I** and **FLO II**) and fruiting (**FRU I** and **FRU II**) stages for the evaluation of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) activities. Values are the means (n = 8) + standard deviations. Distinct letters indicate significant differences (Scott-Knott; $P < 0.01$).



The increment in CAT, APX and SOD activities in leaves of *P. edulis* in fruiting I stage indicated that plants were coping well with adverse environmental conditions since the individuals from the orchard were visibly healthy. 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, in which this latter is 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.

Conclusion

Leaves of *P. edulis* presented the highest phenolic compounds/flavonoids contents regardless of the plant developmental stage. The maximum accumulation of phenolics/flavonoids in roots, leaves, fruit shells and pulp occurred when plants were in the reproductive stage. Economical value is now given to fruit shells of *P. edulis*, a juice industries's waste, due to its notable antioxidant property 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 fruiting stage.

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