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Guava flavonoids and the effects of industrial air pollutants

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ABSTRACT

Saplings of *Psidium guajava* (guava, Myrtaceae), a tropical tree species, were exposed to industrial air pollutants at Cubatão, the largest industrial complex of Latin America, along two periods, each one comprising one-year: period I, July/2000 - June/2001; period II, December/2000 - November/2001. Saplings were exposed in two experimental sites: Pilões River Valley (PV), reference site, with low contamination by air pollutants; and Mogi River Valley (MV), a site severely affected by pollutants from chemical, fertilizer, ceramic, iron and steel industries. At both sites, the main flavonoids found were guaijaverin, quercitrin and two quercetin diglycosides. No interactions among factors were found as well as no significant differences were found among periods and among sites. However, total foliar flavonoid amounts showed the tendency of decrease after 12 months of experimentation. Cubatão industrial air pollution, with high concentrations of NO_2, SO_2 and particulate matter, plus climatic conditions of the initial months of exposure seem that does not influence flavonoid composition and quantities.

Keywords:
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1. Introduction

Environmental factors such as water deficiency, pathogen attack, increased UVB-radiation; and air pollutants such as SO_2 or ozone may generate oxidative stress in plants (Grassmann et al., 2002; Monaghan et al., 2009). A strict control of Reactive Oxygen Species (ROS) levels is essential to prevent toxicity. Plant cell protection depends on antioxidant systems, a pool of enzymatic and non-enzymatic molecules that react with ROS, turning them inactive. Enzymatic defenses, such as antioxidative enzymes (peroxidase, catalase, superoxide dismutase), are the most important components of ROS scavenging systems (Grassmann et al., 2002; Monaghan et al., 2009).

Non-enzymatic defenses are also important in antioxidant scavenging systems. Glutathione (GSH), ascorbic acid (vitamin C) and α-tocopherol prevent free radical formation and scavenge ROS already accumulated, by breaking chain reactions and eliminating oxidized molecules, thus repairing oxidative damage (Grassmann et al., 2002). Other non-enzymatic antioxidants are xanthophylls, photo-protective carotenoids (violaxantine, zeaxantine and anteroxantine), and flavonoids (Dellapenna and Pogson, 2006).

Flavonoids are polyphenolic compounds ubiquitous in vascular plants. They were shown to have health benefits related to antioxidant effects, thus attracting attention as possible therapeutic drugs against free radical mediated diseases such as some cancers, parkinson and alzheimer (Harborne and Williams, 2000; Pietta, 2000; Havsteen, 2002). The basic flavonoid structure is the flavan nucleus with 15 carbon atoms arranged in three rings (C_6-C_3-C_6). Free radicals scavenging activity of flavonoids depends on the substitution pattern of hydroxyl groups, availability of phenolic hydrogens and possibility of stabilization of the resulting phenoxyl radicals. According to Amic et al. (2003) the number and location of phenolic hydroxyl groups are beneficial for flavonoid antioxidant activity, specially the presence of 3',4'-dihydroxy system in the B ring and the 3-OH on the C ring, that act as a radical target.

Robles et al. (2003) reported that the amounts of total phenols in *Pinus halepensis* growing at five different polluted areas can be indicative of the presence of NO. On the other hand, flavonoids are good indicators of O_3 pollution, as observed by a significant positive correlation between these compounds and ozone concentrations (Rezende and Furlan, 2009). Loponen et al. (1998) found that strongly polluted areas appear to affect accumulation and variability of some phenolics in *Betula pubescens* leaves; these changes are probably related to the effect of environmental contamination on shikimate and phenylpro-panoid pathways (Loponen et al., 2001).

Psidium guajava L. (Myrtaceae), the guava tree, is a native fruit tree in the Tropical Rain Forest. Its fruits are widely consumed either fresh or processed (beverages, syrup, ice cream, and jams) and it is also used in ethno-medicine for several purposes (Qian and Nihorimbere, 2004). Guava plants were shown to be effective accumulators of sulfur and fluoride in bio-monitoring studies, and sensible to ozone in semi-controlled experiments (Moraes et al.,

2002, Moraes et al., 2004; Furlan et al., 2007a; Furlan et al., 2007b; Resende and Furlan, 2009).

Since pollutants such as NO_2 and SO_2 may generate oxidative stress in plant cells, and flavonoids are potential protectors toward attacks by ROS, the present work aimed first to evaluate the effect of pollutants on the contents of leaf flavonoids of guava tree saplings exposed to a highly polluted and a non polluted areas around the industrial complex of Cubatão, São Paulo.

Secondly, we consider that the levels of air pollutants greatly vary among seasons at that industrial region, mainly due to the marked seasonality in climatic factors that governs their dispersion. Air pollutants, like NO_X, SO_2 and PM_{10}, are generally more concentrated in the winter than in the summer time (CETESB, 2007). We assume that such seasonality may affect the plant metabolism depending on the period of the year in which they are actively growing in their natural environment. Therefore, this work is also aimed to investigate if the profile of guava flavonoids changes when the saplings grow under more or less intensely polluted conditions. Two partially superimposed field experiments, each one lasting 12 months, were proposed in order to verify if such expected changes during plant growth would temporally vary in response to a gradient of environmental conditions.

2. Experimental

2.1. Experimental area

The study areas are located near the industrial complex of Cubatão in the state of São Paulo, Brazil (23°45' - 23°55' S, 46°15' - 46°30' W). The topographic location of the industrial complex and the unfavorable meteorological conditions hinder the dispersion of pollutants. The climate is tropical with annual mean temperatures of 23°C, high humidity (generally above 80% throughout the year) and annual precipitation of 2 100 mm on the coastal plain.

Cubatão is the largest industrial complex of South America, housing 10 petrochemical and chemical companies, seven fertilizer industries, one steel foundry, one paper industry and one cement factory, totaling 260 emission sources of air pollutants (CETESB, 2007). Part of the local pollution stems from the 7.5 million vehicles and trucks annually traversing the local highways.

The particular location of the industrial complex near the slopes of Serra do Mar, combined with the geographic distribution of industries, characteristics of air circulation and mass transport, delimit areas with distinct influences of air pollution. A highly polluted and a non-polluted site, i.e., Mogi River Valley (MV) and Pilões River Valley (PV), respectively, were selected to set up the exposure system. The choice was based on previous studies which demonstrated differences between the two sites regarding only the local air contamination (Furlan et al., 1999; Klumpp et al., 2000; Furlan et al., 2007a). Similar elevations (around 50 m) and meteorological conditions prevail at both exposure sites (Moraes et al., 2002).

MV is at the entrance of the valley, close to the core of the industrial complex, subject to winds blowing from the pollutant sources and showing severe vegetation damage. High contamination of particulate matter, fluorides, sulfur and nitrogen compounds is assumed to predominate at MV (Jaeschke, 1997). The local monitoring station in 2000 reported annual mean concentrations of 184 μg m^{-3} of total particulate matter, 35 μg m^{-3} of nitrogen oxides and 29 μg m^{-3} of sulfur dioxide (CETESB, 2003). PV site is located southwest of the industrial complex and is affected by low concentrations of air pollutants. PV is only slightly contaminated by industrial pollutants due to geographic barriers and a location unaffected by the land-to-sea breeze (Jaeschke, 1997).

2.2. Plant exposure

In order to verify differences of sapling responses to synergic action of air pollution and meteorological conditions, two partially superimposed field experiments, each lasting 12 months, were carried out. The first period (period I), covered July/2000 - June/2001 and started in early winter, a period with the highest concentration of air pollutants. In Cubatão, winter is the dry season, with 300 mm of mean accumulated rainfall and mean temperature of 19 °C. Over the first eight months of period I, values of rainfall and PM_{10} concentrations increased, while those of SO_2 and NO_2 decreased. The second period (period II), covered December/2000 - November/2001 and started in early summer, a season with low concentrations of air pollutants, a mean temperature of 24 °C, and mean accumulated rainfall reaching to 1 000 mm; all these factors converge to favor plant growth.

Higher numbers of unfavorable days for pollution dispersion were observed during the initial months of period I and the last eight months of period II. It is important to point out that CETESB makes no record of fluoride concentration at MV. As PV is regarded by CETESB as pollution-free, pollution parameters for the area are not determined.

For each period, 50 saplings about 30 cm high were obtained from a nursery and planted in pots of 50 L, containing a uniform substrate (Eucatex Plantmax: vermiculite, 3:1 by v/v). For acclimation, before submission to treatment conditions, they were kept for 30 days in a greenhouse to recover from transplant disturbances. Nylon wicks guaranteed water supply during cultivation and later field exposure. Plastic boxes served as water reservoirs. Hoagland's solution was added to the pots once a month. Plants were grown in the field protected from sunlight in such a way to avoid 50% of the incident radiation. Analyses were carried out with ten saplings after 4, 8, and 12 months of exposure. Exposed plants were monitored throughout the experiment in order to ensure protection against insects and pathogens. Spraying to protect the plants was not necessary.

2.3. Flavonoid analysis

Leaves corresponding to 4, 8 and 12 months of pollution exposure were collected, air dried (at 60 °C) and grinded. Two hundred and fifty mg of samples were extracted 3 times with 50 mL methanol (80%) at 150 °C. The extracts were combined and then concentrated in rotary evaporator under reduced pressure at 40 °C for total solvent removal (Markham, 1982). To eliminate low polarity contaminants, the residue was dissolved in 1 mL of toluene and centrifuged at 10 000 g for 5 min. The supernatant solution was reserved and the process repeated twice, each time with 1 mL of toluene. The combined supernatants of the three treatments were evaporated to dryness, constituting TF (toluene fraction). The residue was dissolved in 1 mL of methanol. A centrifugation at 10 000 g for 5 min was carried out, the supernatant was reserved and the process was repeated twice, each time with 1 mL of methanol. The combined supernatant solutions were dried for further analysis, constituting MF (methanol fraction).

Residues TF and MF were dissolved in 1 mL of methanol and the solution analyzed by HPLC-DAD (HP series II 1090). Constituent compounds were identified by comparison of retention times and UV spectra with a data of a standards library. Elution was performed at a flow rate of 0.5 mL min^{-1}, using 0.1% acetic acid and acetonitrile as the mobile phase with a gradient starting with 12% of the latter and increasing it to 20% (5-8 min), for 20 min and increasing to 50% (28-38 min), 65% (38-48 min) and 100% (48-50 min), isocratic for 5 min and decreasing to 12% (55-60 min). The column used was a RP-18 Zorbax (250 mm, 4.6 mm, 5 μm), at 40 °C (modified from Schieber et al., 2001). The injection volume for all samples was 25 μL and detection was achieved at 352 nm.

Compound concentrations were determined using standard solutions as reference.

For compounds not matching library data, MF was analyzed by preparative paper chromatography (Whatman 3MM) with acetic acid (15%) and then by cellulose TLC (Merck) for evaluation of purity of isolated compounds. The latter were submitted to UV-VIS absorption spectroscopy (240 – 500 nm) for identification, using methanol solutions and ionization (KOH, NaOAc) and complexing ($AlCl_3$, HCl, H_3BO_3) shift reagents. Flavonoid glycosides were hydrolyzed with 1N HCl and the resulting sugars and aglycones identified by cellulose TLC (Markham, 1982). The identified compounds were analyzed by HPLC-DAD and the corresponding data added to the library. As complementary analysis, MF fraction was analyzed by HPLC-ESI-MS (Shimadzu M10AVP–Esquire 3000 Plus–Bruker Daltonics). HPLC conditions were the same as described above, with flow reduction to 0.1 mL min^{-1}. MS conditions were: HV capillary 4 000 V, skimmer 40 V, dry temperature 325 ^{o}C, dry gas 7 L min^{-1}, nebulizer 27 psi.

2.4. Statistical analysis

Average concentrations of total flavonoids were compared by three way analysis of variance, taking sites, periods and months of exposure as factors. The data were log10 transformed to reach equal variances. Normality and equal variance were tested. Interactions among factors were tested and the Tuckey test was applied to look for significant differences between pairs of average values ($p < 0.05$).

3. Results and Discussion

Predominant flavonoid constituents of *P. guajava* leaves were quercetin glycosides, mainly guaijaverin (quercetin 3-O-arabinoside) and two quercetin derivatives. High amounts of quercetin glycosides have been repeatedly reported, characterizing this species as an outstanding quercetin producer. Methanolic leaf extracts of *P. guajava* have yielded 5 quercetin glycosides: quercetin 3-O-alpha-L-arabinoside (guaijaverin), quercetin 3-O-beta-D-glucoside (isoquercetin), quercetin 3-O-beta-D-galactoside (hyperin), quercetin 3-O-beta-L-ramnoside (quercitrin) and quercetin 3-O-gentiobioside (Lozoya et al., 1994). Arima and Danno (2002) detected the same five flavonols and described two new ones, morin 3-O-alfa-L-xylopiranoside and morin 3-O-alfa-L-arabinopiranoside. More recently, Liang et al. (2005) have reported the same flavonols found by Lozoya et al. (1994), in addition to kaempferol and three non-identified compounds. Medicinal and pharmacological properties of guava extracts have been assigned to quercetin derivatives (Qian and Nihorimbere, 2004; Gutierrez et al., 2008).

HPLC analyses of the present work detected 20 peaks corresponding to flavonoids, as judged by UV/VIS spectra (Figure 1). Among these peaks, six matches with library data. All six flavonoids reported by Lozoya et al. (1994) were detected in the present study, in addition to kaempferol. Table 1 contains the HPLC-ESI-MS data for *P. guajava* extract (MF). The final identification of flavonoid constituents was obtained with the combination of HPLC, HPLC-MS and spectrophotometric analyses.

Period I comprised four initial months (including winter) with low rainfall. However, pollutant concentrations were not substantially higher than those observed during period II (Table 2). Comparing the atmospheric conditions along the four initial months of both periods, rainfall and particulate matter were higher in period II.

MV saplings showed a tendency of higher amounts of total flavonoids at the first four months for both periods (Table 3). After eight months of exposure, saplings maintained the trend of accumulating higher total flavonoid amounts at the polluted site (MV), although the difference with the non-polluted location (PV) was not significant. However, an inversion of this trend occurs after twelve months and MV saplings showed lower total flavonoid contents (Table 3). Significant differences were verified when comparing time of exposure. At both sites, saplings showed less flavonoid amounts at the end of exposure periods, suggesting physiological changes on flavonoid contents during plant growth.

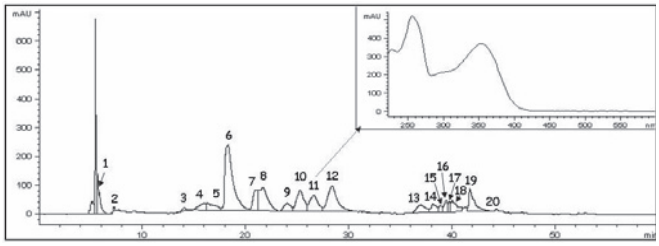

Figure 1. *HPLC chromatogram of a* P. guajava *methanol extract. Detail: UV/Vis spectrum of guaijaverin. Constituents: 1. possible triglycoside; 2. not identified; 3. not identified; 4. possible diglycoside; 5. possible quercetin diglycoside; 6. quercetin 3-O-dilgycoside; 7. quercetin 3-O-diglycoside; 8. quercetin 3-O-monoglycoside; 9. quercetin 3-O-monoglycoside; 10. quercetin 3-O-xyloside; 11. guaijaverin; 12. quercitrin; 13. possible kaempferol derivative; 14. possible isorhamnetin derivative; 15. quercetin; 16. not identified; 17. kaempferol; 18-20. not identified.*

Table 1. *HPLC-ESI-MS data of a* P. guajava *extract. Compound numbers refer to peak numbers in Figure 1*

Compound	$[M+H]^+$	Fragments *m/z*
1. Possible triglycoside	707.3	203.1; 365.2; 527.2; 689.3; 707.3
2. NI	750.2	136.1; 276.2; 500.2
3. NI	804.2	149.0; 227.1; 525.1; 677.1
4. Possible diglycoside	617.2	149.0; 617.2
5. Possilbe quercetin diglycoside	617.2	149.0; 303.1; 617.2
6. Quercetin 3-*O*-diglycoside (hexoses)	629.2	149.0; 303.1; 383.1; 465.1; 629.2
7. Quercetin 3-*O*-diglycoside (hexoses)	629.1	149.0; 303.1; 465.1; 629.1
8. Quercetin monoglycoside (hexose)	465.1	149.0; 303.1; 465.1
9. Quercetin monoglycoside (pentose)	465.1	149.0; 303.1; 435.1
10. Quercetin 3-*O*-xyloside	435.1	149.0; 197.1; 303.1; 435.1
11. Guaijaverin	435.1	149.0; 303.1; 435.1
12. Quercitrin	449.1	149.0; 303.1; 449.1
13. Possible Kaempferol diglycoside	587.1	149.0; 287.1; 433.1; 587.1
14. Possible Isorhamnetin diglycoside	573.2	259.1; 315.1; 573.2
15. Quercetin	303.1	303.1
16. NI	641.2	149.0; 391.3; 611.2; 641.2
17. Kaempferol	287.1	287.1
18. NI	520.4	149.1; 284.4; 467.4; 520.4
19. NI	522.4	149.1; 284.4; 469.4; 522.4
20. NI	555.3	149.0; 284.4; 555.3

NI = not identified

Although rainfall is low in winter months (June to September), there is no real dry period in Cubatão. Heavy rainfall is characteristic of summer months (December to March). Rainfall is an important factor concerning air pollutant concentrations. During rainy season, pollutant dispersion and removal from the atmosphere is favored at polluted areas. Conversely, availability of pollutants and hence plant absorption is larger during dryer periods (CETESB, 2007).

Accumulated rainfall was not different between the two periods, but the rainfall was more evenly distributed across the period II compared to period I (Table 2). Comparing with the first eight exposure months, concentrations of NO_2 were higher during the last four months in both periods. Saplings growing during period II were exposed to eight months (April-November) comprising a higher number of days with unfavorable conditions for pollutant dispersion and higher NO_2 and particulate matter concentrations.

Table 2. *Accumulated precipitation (PPT- mm), number of unfavorable days for pollutant dispersion (UDD), mean concentrations (μg m^{-3}) of nitrogen dioxide (NO_2), sulfur dioxide (SO_2) and particulate matter (PM_{10}) at Cubatão (Mogi Valley) during the indicated periods of sapling exposure*

Period I	PPT[a]	UDD[b]	NO_2[b]	SO_2[b]	PM_{10}[b]
Jul/2000 - Oct/2000	537.5	19	54.0	32.3	159.9
Nov/2000 – Feb/2001	1093.9	-	49.0	21.3	213.2
Mar/2001 – Jun/2001	490.0	13	60.3	28.5	196.9
Accumulated	2121.4	-	-	-	-
Max[c]	-	-	113.0	114.0	557.0
Annual Mean	163.2	-	54.4	27.4	190.0
Period II	**PPT[a]**	**UDD[b]**	**NO_2[b]**	**SO_2[b]**	**PM_{10}[b]**
Dec/2000 – Mar/2001	874.2	-	54.0	22.8	219.2
Apr/2001 – Jul/2001	502.3	26	48.0	30.5	201.7
Aug/2001 – Nov/2001	625.5	11	62.3	27.5	243.7
Accumulated	2002.0	-	-	-	-
Max[c]	-	-	117.0	112.0	587.0
Annual Mean	151.7	-	54.8	26.9	221.5

[a] Data from EMAE (Empresa Metropolitana de Águas e Energia S.A. www.emae.com.br)

[b] Data from CETESB (Companhia de Tecnologia e Saneamento Ambiental www.cetesb.sp.gov.br; data from automated air monitoring station)

[c] NO_2 and SO_2, maximum concentration for 1 hour; PM_{10}, maximum concentration for 24 hours

The response of the saplings regarding flavonoid accumulation was different when comparing the first eight and the last four months of exposure (Table 3). In both periods, the eight initial months were similar as to pollution concentrations and a trend toward increase of total flavonoid contents. However, flavonoid amounts decreased in the following four months, a result possibly accounted for the increase of the amounts of NO_2 along the four last months of both periods.

Few studies are available concerning air pollution effects on plant phenolic compounds. Pasqualini et al. (2003) reported higher amounts of phenolic compounds in *Pinus halepensis* exposed to SO_2 and ozone, but a decrease in the amounts of phenolics occurred after exposure to nitrogen oxides. A similar result was observed in the present work, since a trend was noted between higher NO_2 concentrations in the last four months of exposure and decreased foliar amounts of flavonoids.

Table 3. *Amounts (mg 100 g^{-1}, dry weight basis, mean ± standard deviation) of total leaf flavonoids of saplings of* P. guajava *(n=10) exposed to atmospheric pollution Cubatão: PV, non-polluted area; MV, polluted area. Period I: 4 (Jul/2000 - Oct/2000), 8 (Nov/2000 – Feb/2001), 12 (Mar/2001 – Jun/2001); Period II: 4 (Dec/2000 –Mar/2001), 8 (Apr/2001 – Jul/2001), 12 (Aug/2001 – Nov/2001). Average values followed by distinct letters in each column are significantly different by means of Tuckey test ($p < 0.05$). No interactions among factors were found*

Months of exposure	Period I		Period II	
	PV	MV	PV	MV
4	58.1±2.9 A	66.8±5.2 A	47.2±5.4 A	53.5±10.7 A
8	37.2±5.9 B	38.6±7.2 B	40.6±8.7 B	43.0±4.6 B
12	40.0±7.6 B	37.2±1.0 B	46.0±2.3 AB	35.3±3.3 B

When studying fluorine-treated leaves of *Hypericum Perforatum*, Fornasiero (2001) observed an increase of anthocyains amounts, and suggested that these compounds could be acting in association with other protective molecules in the plant cell. The author hypothesized that *H. perforatum* produces high amounts of proanthocyanidins (condensed tannins) which is related to the red-brown pigmentation of F-affected areas. Rezende and Furlan (2009) verified the same situation on *P. guajava* leaves after ozone fumigation, which presented higher amounts of tannins and anthocyanins associated with injured leaf area.

Furlan et al. (2007a) exposed saplings of *Tibouchina pulchra* and *P. guajava* at the same conditions described in this paper, and both species showed alterations in growth and biomass allocation, as well as increased leaf concentrations of nitrogen and fluoride at MV site. These authors observed higher fluoride accumulation during months with less rainfall. It is important to point out that fluoride concentrations, although higher at MV, were not monitored by CETESB. In the present study, anthocyanins were not analyzed although these compounds might be more important as an alternative antioxidant source for *P. guajava* than other flavonoids.

In *Betula pubescens* ssp. *czerepanovii* plants growing near a steel factory, Loponen et al. (2001) observed no effects of pollutants on the amounts of total phenolics, but when considering individual compounds they found a decrease in the amounts of gallic acid derivatives and no effect in the amounts of phenylpropanoids. Bialonska et al. (2007) observed higher contents of compounds absorbing at 280 and 325 nm from leaves of bilberry growing near a Zn-Pb smelter; these authors correlated the results to scavenging of free radicals produced by influence of metal ions.

According to the individual compounds analyzed in the present work, the MV saplings showed higher amounts of quercitrin and quercetin after four and eight months (Table 4). Saplings showed different response as regarding to amounts of guaijaverin. After four months of period I and twelve months of exposure during period II, the saplings showed less amounts of guaijaverin. On the other hand, a tendency of enhanced guaijaverin amounts was observed during eight and 12 months of period I as well as four and eight months of period II. As well as, quercetrin and quercetin amounts were reduced after 12 months of period II (Table 4).

The pollution impact on secondary metabolism is dependent on pollutant chemistry and on the species studied, since production of compounds is different among different species. Submitted to the same stimulus, some species activate some biosynthetic pathways, while others are affected in different ways.

P. guajava showed high diversity of quercetin derivatives. Although the hydroxyl group substitution occurs always at position 3, they differ on sugar moiety number and sugar type. During the last exposure months, under higher NO_2 concentration and probably higher intense oxidative stress, saplings showed reduced amounts of guaijaverin, quercitrin and quercetin, suggesting a relevance of those compounds as antioxidants, although differences regarding contents of individual flavonoids were relatively small.

Phenolic compounds are strongly active as antioxidants due to the high reactivity of the phenolic hydroxyl groups (Amic et al., 2003). Such reactivity may act as an antioxidant defense, inactivating reactive oxygen species (ROS) during an oxidative stress such as the presence of air pollutant (Pietta, 2000; Harborne and Williams, 2000). According to Pietta (2000), flavonoids have been described as inhibitors of some enzymes involved in physiological processes that generate ROS, such as xantin oxidase, lipooxygenase, glutathion S-tranferase and NADH-oxidase. Due to the low redox potential presented by flavonoids (0.23 – 0.75 V), these molecules are able to reduce highly oxidized free radicals (redox potential slightly above 2 V) such as superoxide, peroxyl and alcoxyl. In recent years, many papers have shown the correlation between the antioxidant capacity and chemical structure of flavonoids, as well as the in vitro antioxidant activity of flavonoids has been assessed. Pietta (2000) pointed out that the redox

Table 4. *Amounts (mg 100 g^{-1}, dry weight basis, mean ± standard deviation) of leaf flavonoids of saplings of* P. guajava *(n=10) exposed to atmospheric pollution Cubatão: PV, non-polluted area; MV, polluted area. Period I: 4 (Jul/2000 - Oct/2000), 8 (Nov/2000 – Feb/2001), 12 (Mar/2001 – Jun/2001); Period II: 4 (Dec/2000 –Mar/2001), 8 (Apr/2001 – Jul/2001), 12 (Aug/2001 – Nov/2001). Values followed by * correspond to means statistically different comparing PV and MV at the same exposure period (p <0.05)*

Compounds	Rt	Period I (months)						Period II (months)					
		4		8		12		4		8		12	
		PV	MV	PV	MV	PV	MV	PV	MV	PV	MV	PV	MV
1. Possible triglycoside	5.6±0.9	2.7±0.9	2.7±1.1	2.4±0.7	2.7±0.6*	2.6±0.8	2.7±0.7*	2.6±0.9	2.6±0.8	2.0±0.5	1.7±0.3	1.9±0.2	1.9±0.2
2. NI	7.2±0.2	2.5±0.7	2.6±0.9	1.6±0.4	2.0±0.7	2.5±0.8	1.5±0.7	2.9±0.7	2.7±0.7	2.3±0.6	1.9±0.3	2.3±0.3	2.0±0.4*
3. NI	14.0±0.4	6.0±1.5	5.1±2.6	4.9±1.7	4.2±1.1	5.9±2.1	5.1±1.3	6.7±2.1	6.3±2.0	6.5±2.3	6.9±2.2	6.4±0.8	4.8±1.8
4. Possible diglycoside	15.9±0.4	3.6±1.1	4.0±1.6*	2.9±1.5	3.2±1.5*	2.7±0.8	2.7±0.7	2.9±0.7	2.9±0.9	1.9±0.3	2.0±0.2	2.2±0.3	2.1±0.3
5. Possible Quercetin diglycoside	16.3±0.2	t	t	1.5±2.8	t	t	t	t	t	t	t	t	t
6. Quercetin 3-*O*-diglycoside	18.3±0.2	1.1±1.5	t	1.1±1.5	t	t	1.5±0.8	t	t	t	1.5±0.2	t	1.6±0.2
7. Quercetin 3-*O*-diglycoside	21.0±0.1	3.5±1.2	3.6±1.9	2.5±0.7	2.6±0.6	2.3±0.8	1.9±0.6	3.2±1.1	3.4±1.1	2.8±1.0	2.6±0.9	2.7±0.8	2.2±0.5
8. Quercetin monoglycoside	21.2±0.2	3.4±1.0	3.7±0.8*	2.4±0.6	2.6±0.6*	2.6±0.8	2.6±0.8	3.1±0.8	3.1±0.8	2.5±0.7	2.5±0.8	2.5±0.5	2.1±0.6
9. Quercetin monoglycoside	24.0±0.2	6.7±1.9	6.7±3.2	2.4±0.6	2.6±0.8	2.9±0.5	3.1±1.0	4.5±2.1	5.3±1.7	3.8±1.1	4.6±1.1	4.9±1.1	3.0±1.3*
10. Quercetin 3-*O*-xyloside	25.3±0.4	4.1±1.6	5.2±1.3*	2.5±0.7	2.7±0.9	2.7±0.6	3.1±1.0*	3.6±1.0	3.7±0.9	2.4±0.5	2.6±0.7	2.6±0.3	2.5±0.6
11. Guaijaverin	26.6±0.4	7.7±2.2	4.6±2.0*	3.2±0.8	3.5±1.0	3.4±0.7	3.6±1.1	4.8±1.6	5.3±1.8	4.5±1.6	4.8±1.7	5.2±0.6	3.9±1.4*
12. Quercetrin	28.3±0.5	5.8±2.5	6.4±3.8*	2.8±0.8	3.0±0.9*	3.4±0.9	3.4±0.8	4.1±1.4	4.3±1.6	3.1±0.8	3.1±0.7	3.7±0.7	2.9±0.8*
13. Possible Kaempferol derivative	36.9±0.6	t	T	1.2±0.6	t	t	t	t	t	t	t	t	t
14. Possible Isorhamnetin derivative	38.1±0.5	t	1.0±0.6	1.1±0.5	t	t	t	t	t	t	t	2.1±0.2	t
15. Quercetin	38.8±0.5	3.1±0.4	3.5±0.8*	1.0±0.5	1.4±1.1	t	t	1.5±1.1	2.5±0.3	1.4±0.3	1.8±0.4*	1.9±0.3	1.0±0.3*
16. NI	39.3±0.4	1.5±0.7	1.6±0.9	t	t	t	t	t	t	1.4±0.2	t	t	t
17. Kaempferol	39.7±0.4	2.4±1.0	3.5±1.7	2.7±0.9	2.7±0.9	3.1±0.6	3.1±0.6	3.4±1.5	4.1±1.6	2.6±0.9	2.9±0.8	2.9±0.6	2.1±0.3
18. NI	40.1±0.2	t	3.4±0.5	T	t	2.3±0.8	2.6±0.8	2.3±0.8	2.5±0.8	1.9±0.7	1.7±0.2	1.8±0.3	1.7±0.1
19. NI	41.7±0.3	t	2.9±0.5	t	t	2.8±0.8	2.4±0.8	2.2±0.6	2.5±0.8	t	1.7±0.2	1.8±0.4	1.6±0.1
20. NI	46.0±0.2	t	t	1.7±0.5	1.8±0.6	2.8±0.5	2.2±0.5	t	t	1.8±0.3	1.7±0.4	1.9±0.3	1.7±0.3
12. Quercetrin	28.3±0.5	5.8±2.5	6.4±3.8*	2.8±0.8	3.0±0.9*	3.4±0.9	3.4±0.8	4.1±1.4	4.3±1.6	3.1±0.8	3.1±0.7	3.7±0.7	2.9±0.8*
13. Possible Kaempferol derivative	36.9±0.6	t	T	1.2±0.6	t	t	t	t	t	t	t	t	t

Rt = retention time (min); NI = not identified; t = trace

potential presented by flavonoids is influence by aspects of their molecular structure, particularly the substituents in the B ring and the presence of a hydroxyl group at position third Hydroxyl and methoxyl groups at 5 and 7 positions are less important to define the redox potential of a flavonoid. It ends up that flavonols comprise the flavonoid class with higher redox potential (in special those with free 3-hydroxyl), followed by anthocyanins and flavones. So far, flavonoids identified in material from *P. guajava* have been exclusively flavonols.

It seems that different pollutants can affect phenolic responses on different manners. According to Grassmann et al. (2002), peroxide and superoxide are less reactive and can diffuse over large distances even crossing plasma membranes. It has been postulated also that most secondary metabolites, including flavonoids, are synthesized in the cell cytoplasm and then transported to the vacuole. Although the cytoplasmatic presence of flavonoids is considered to be transitory, a large proportion may remain in the cytoplasm in some tissues, for example contributing to flower color in some species (Markham et al., 2001).

When exposed to industrial air pollution of Cubatão, with high concentrations of fluoride, particulate matter and sulfur dioxide, saplings of *P. guajava* showed no significant differences on total foliar flavonoids when comparing to saplings at a non-polluted area. Studies using controlled conditions are crucial to elucidate flavonoid responses regarding different pollutants and their concentrations.

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