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*Full Length Research Paper*

# Evaluation of antioxidant capacity, phenolic content, antimicrobial and cytotoxic properties of *Guettarda uruguensis* flowers and fruits

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**Antioxidant capacity, phenolic content, antimicrobial and cytotoxic properties of extracts and fractions from flowers and fruits of *G. uruguensis* were evaluated. Total phenolic content ranged between 406.8 ± 20.47 and 57.98 ± 1.734 gallic acid equivalent in mg $g^{-1}$ (mg GAE $g^{-1}$) samples. Ethyl acetate and butanol fractions from flowers had the greatest rates in phenolic content and antioxidant activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS) assays. Pearson´s coefficient showed a strong association between total phenolic compounds in DPPH ($r = -0.83$, $p < 0.01$) and TBARS ($r = 0.85$, $p < 0.01$). Eleven out of the 12 samples tested were toxic in the brine shrimp assay (LC50 < 100 µg $mL^{-1}$). Antimicrobial susceptibility profile revealed flower remaining fraction and fruit extract with moderate activity against *Enterococcus faecalis*. Results indicate that *G. uruguensis* is a good source of compounds with antioxidant and cytotoxic activities. Further studies to identify compounds causing these activities are recommended.**

**Key words:** *Guettarda uruguensis*, total phenolics, antioxidant activity, antimicrobial, brine shrimp lethality.

## INTRODUCTION

The Rubiaceae family has approximately 650 genera and 13,000 species, predominantly distributed throughout the tropical regions (Delprete and Jardim, 2012). Rubiaceae plants demonstrate several secondary metabolites and important biological activities, such as caffeine in *Coffea arabica* L., quinine in *Cinchona pubescens* Vahl. and emetin in *Psychotria ipecuanha* (Brot.) Stokes. The three compounds mentioned above are among the 30 substances isolated from the most important plants for medicinal use (Gerlach et al., 2010). In Brazil, the plants of the Rubiaceae family are popularly used in the manufacture of phytotherapics, such as herbal medicine prepared from *Uncaria tomentosa* (Wild.) DC., popularly known as cat's claw (WHO, 1997; BRASIL, 2013).

Members of the *Guettarda* genus are distributed between East Africa and the islands of the Indian and Pacific Oceans to Neotropical regions (Achille et al., 2006). In Brazil, twenty species are distributed in the

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Amazon, Caatinga, Savannah, Atlantic Rainforest and Pampa regions (Barbosa, 2015). In fact, several species from this genus have been traditionally used as medicine, particularly against inflammatory diseases (Albuquerque et al., 2007; Capasso et al., 1998; Agra et al., 2007; Agra et al., 2008; Matos, 1997; Brandão, 1985).

Phytochemical investigations on the genus revealed several compounds, including alkaloids (Kan-Fan and Husson, 1979; Capasso et al., 1998), iridoids (Inouye et al., 1988; Ferrari et al., 1986; Naressi et al., 2015), triterpenes (Aquino et al., 1988; Aquino et al., 1989; Bhattcharyya and Almeida, 1985) and other classes of substances, such as phenolic esters (Oliveira et al., 2008; Testa et al., 2012; Naressi et al., 2015).

Several kinds of *Guettarda* extracts have been reported for their biological activities, including anti-inflammatory, antioxidant, antiviral, antimicrobial and anti-convulsive properties (Pina et al., 2012; Duarte et al., 2014; Barros et al., 2012; Saravana et al., 2009).

*Guettarda uruguensis* Cham. & Schltdl. (Rubiaceae), a species commonly known as velvetseed, has eatable fruits (Corrêa and Penna, 1984; Kunkel, 1984) and sweet-scented flowers (Kinupp, 2007). Since species of the genus have been employed from time immemorial for therapeutic purposes, with acknowledged pharmacological activities, and since studies on the biological activities of its fruits and flowers are scarce, current study determines the antioxidant capacity, phenolic content, antimicrobial and cytotoxic proprieties of extracts and organic fractions of the flowers and fruits of *G. uruguensis*.

## MATERIALS AND METHODS

### Plant material

Flowers and fruits of *G. uruguensis* were collected in Curitiba, Brazil, between November 2012 and February 2013. Plant material was identified by botanist José Tadeu Weidlich Motta of the Municipal Botanic Museum of Curitiba. A voucher specimen was deposited and registered under MBM 386376.

### Chemicals

Ascorbic acid, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, caffeic acid, rutin, tris, thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), ammonium molybdate, sodium phosphate and carbonate sodium were purchased from Sigma Co. (St. Louis,MO, USA). All other chemicals used were of analytical grade.

### Extraction and liquid/liquid partition

Dried flowers (306 g) were extracted by ethanol (5 L) in a Soxhlet extractor. Ethanol (FLE, 58.2 g) was extracted under reduced pressure in a rotatory evaporator by removing solvents. Partition was performed in a modified Soxhlet extractor (Carvalho et al., 2009), employing solvents within an increasing polarity scale. Hexane (FLH, 2.2 g), chloroform (FLC, 0.5 g), ethyl acetate (FLA, 3.7 g), butanol (FLB, 14.5 g) and remaining fractions (FLR, 27.3 g) were extracted. Fractions of dried fruits (200 g) were also extracted in modified Soxhlet (Carvalho et al., 2009) with solvents in an increasingly polarity scale. Hexane (FRH, 2.7 g), chloroform (FRC, 1.5 g), ethyl acetate (FRA, 0.8 g) and remaining fraction (FRR, 24.3 g) were extracted. Dried flowers (145 g) were extracted with ethanol (1.5 L) at room temperature (three times a week). The extract was concentrated in a vacuum at 40°C, and the crude alkaloid fraction (FLALC, 0.3 g) was obtained by classical methods (Batista et al., 1996). Dried flowers (200 g) were infused in hot water (1 L) for 60 min. The procedure was repeated three times. The combined extract (FLAQ, 32.6 g) was collected by filtration, concentrated under reduced pressure, and lyophilized.

### Total phenolic content

Total phenolic contents of flowers and fruits were determined by modified Folin-Ciocalteu method (Singleton and Rossi, 1965). Aliquots of ethanol extracts and its fractions (200 µL), dissolved in methanol, were added to Folin-Ciocalteu reagent (200 µL), sodium carbonate saturated solution (400 µL) and distilled water (3.2 mL). Absorbance was measured at 760 nm after 30 min. A standard curve was prepared with gallic acid at a concentration range between 2.5 and 20 µg $mL^{-1}$ ($y = 0.0392x - 0.0583$, $r = 0.9964$). Total phenolic content was expressed as gallic acid equivalent (GAE) in mg $g^{-1}$ samples.

### HPLC fingerprint of flower and fruit ethyl acetate fractions

A fingerprint HPLC analysis of ethyl acetate fractions was performed with high performance liquid chromatography (Merck-Hitachi LaChrom Elite®HPLC System) equipped with a pump (L-2130), UV–VIS detector (DAD L-2450), rheodyne manual injector (loop 20 µL). The fractions were dissolved in methanol and filtered with Milli pore membrane (0.45 mM pore diameter). The samples diluted in methanol (10 $mg.mL^{-1}$) were eluted using column Waters Xterra® reverse phase column C18 5 µm (4.6 x 250 mm). Total run time was 43 min with mobile phase A: methanol, B: acid phase, gradient elution: 0 to 40 min: 20 to 100% A, 40 to 43 min 100% A. Methanol used was HPLC grade (TEDIA) and acid phase was composed of 1% acetic acid. Flow rate at 1 mL $min^{-1}$ and an injection volume of 20 µL were employed and peak was detected at 329 nm.

### Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of flowers and fruits was evaluated according to Prieto et al. (1999). An aliquot mixture of 0.3 mL of extract sample solution (200 µg $mL^{-1}$) was mixed with 3 mL of mixture reagent solution (0.6 M sulfuric acid, 30 mM sodium phosphate and 4 mM ammonium molybdate). Sample tubes were sealed and incubated in a water bath at 95°C for 90 min. When reactant samples cooled to room temperature, sample absorbance was measured at 695 nm. Antioxidant activity of samples was expressed in relative antioxidant activity (AAR%), as compared to ascorbic acid and rutin standards (Equation 1).

$$AAR\% = [(Asample - Ablank)/(Astandard - Ablank)]\, x\, 100 \qquad (1)$$

Where, $A_{sample}$: absorbance of sample; $A_{blank}$: absorbance of sample blank; $A_{standard}$: absorbance of standard.

**Radical scavenging assay**

DPPH radical scavenging activity was determined following Mensor et al. (2001), with modifications. Samples (71 µL) at various dilutions were added to 29 µL of 0.3 mmol $mL^{-1}$ DPPH solution in a 96-well microplate. The solution was incubated in the dark at room temperature and absorbance was measured at 540 nm employing a microplate reader. The antioxidant capacity was calculated by Equation 2.

$$Inhibition\ (\%) = 100 - [(Asample - Ablank/Acontrol)]\ x\ 100 \quad (2)$$

where, $A_{control}$: absorbance of control; $A_{sample}$: absorbance of sample; $A_{blank}$: absorbance of sample blank. Antioxidant activity was expressed as IC50 (µg $mL^{-1}$), or rather, the concentration of the sample which was required to cause a 50% decrease in absorbance at 540 nm. IC50 was calculated by linear regression and the linear range was established by equation y = ax + b.

**Determination of thiobarbituric acid reactive substances (TBARS)**

Reactive species to thiobarbituric acid was determined according to methodology by Morais et al. (2006), with adaptations. Filtered egg yolk solution in SDS was employed as lipid source and butylhydroxidetoluene (BHT) as standard. Extracts and its fractions (100 µL of 1000 mg $L^{-1}$ solution) were added to filtered water (400 µL), yolk solution 5% (500 µL) and TBA solution 0.4% (1500 µL) and placed in a water bath (95°C) for one hour. After cooling, 1500 µL n-butanol were added to enhance lipid extraction. Supernatants were collected after tube centrifugation (3000 rpm, 3 min) and their absorbance was determined at 532 nm, with results as antioxidant content (Equation 3).

$$IA\% = [(Asample/Acontrol)]\ x\ 100 \quad (3)$$

where, Asample: sample absorbance, Acontrol: control absorbance.

**Antimicrobial assay**

Extracts and fractions were evaluated against six microorganisms, including two Gram-positive [*Staphylococcus aureus* ATCC 25923 (*S. aureus*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*)], three Gram-negative [*Escherichia coli* ATCC 25922 (*E. coli*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Klebsiella pneumonia* ATCC 700603 (*K. pneumonia*)] and one yeast [*Candida albicans* ATCC 10231 (*C. albicans*)]. Minimum inhibitory concentration (MIC) rates were determined by the broth microdilution method (CLSI, 2008a). The analysis for antifungal activity comprised serial dilutions of extracts and fractions within a concentration range between 1000 and 7.81 µg $mL^{-1}$. Dilutions were prepared with liquid medium RPMI 1640 in 96-well U-shaped bottom sterile microplates (CLSI, 2008b).

**Brine shrimp lethality bioassay**

The brine shrimp toxicity assay was adapted from method described by Meyer et al. (1982). Cysts of *A. salina* (0.5 g $ml^{-1}$) were added to aired saline water and incubated in an environmental chamber at 27 ± 2°C and relative humidity 80 ± 5%, for 48 h. Nauplii were collected and immediately used. Samples were tested in 10, 100 and 1000 µg $mL^{-1}$ concentrations (0.5% of dimethyl sulfoxide (DMSO) in saline water). The test was performed in triplicate, with 10 organisms per replicate. Saline water and dodecyl sulfate (SDS) were respectively employed as negative and positive controls.

**Statistical analysis**

Statistical analyses were performed by one-way ANOVA, followed by Dunnett's and Tukey´s post-hoc tests. Differences were statistically significant when $p < 0.05$. Total phenolic content data and antioxidant activities were analyzed by Pearson´s correlation and analysis of main components (PCA). Microsoft Office Excel 2010, GrahPad Prism 5.Ink and Matlab R2015a softwares were used for calculations.

## RESULTS AND DISCUSSION

### Total phenolic content of samples

Analysis results for total phenolic content demonstrated that *G. uruguensis* is a good source of phenolic compounds (Table 1). Total phenolic contents (expressed in mg GAE $g^{-1}$) for flowers ranged between 109.8 ± 3.44 and 406.8 ± 20.47, and were higher than those from fruits (between 57.98 ± 1.34 mg and 86.72 ± 2.961). Among the samples analyzed, the lowest phenolics concentrations (<100 mg $L^{-1}$) revealed FRR < FRC fractions. FLA (406.8 ± 20.47 mg GAE $g^{-1}$) and FLB (339.3 ± 20.31 mg GAE $g^{-1}$) showed the highest phenol contents. Phenolic content rates in FLA were approximately two times higher than FRA (184.1 ± 11.27 mg GAE $g^{-1}$).

Phenolic compounds are the secondary products of plant metabolism that constitute a large and complex group. These molecules are essential for plants' growth and reproduction, and their synthesis is induced under biotic and abiotic stress conditions, such as infections, injury, UV radiation, ozone, salinity, water stress and heat. They are partially responsible for color, astringency, aroma, and oxidative stability in food (Manach et al., 2004).

According to Singleton and Rossi (1965), phenolic compounds have different responses to Folin-Ciocaulteu assay, depending on their chemical structure. Phenolic compounds contribute to multiple biological effects, including antioxidant activity. This activity is believed to be mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994).

Plant phenols are widely distributed in the plant kingdom and they are sometimes present in surprisingly high concentrations (Harborne, 1993). The high content of phenolic compounds reported in current study has also been registered for different *Guettarda* species. Lima et al. (2009) also observed a higher content of phenolic compounds in *G. grazielae* with rates ranging from 347.75 ± 0.02 GAE $g^{-1}$ in MeOH-$H_2O$ fraction of stems and 298.03 ± 0.002 mg GAE $g^{-1}$ in EtOAc fraction of

**Table 1.** Total phenolic contents in extracts and organic fractions of flower and fruit of *G. uruguensis.*

| Samples | Total phenolic content (mg GAE $g^{-1}$) |
|---|---|
| FLE | 192.4 ± 15.94[a] |
| FLC | 109.8 ± 3.442[b] |
| FLA | 406.8 ± 20.47[f] |
| FLB | 339.3 ± 20.31[e] |
| FLR | 192.7 ± 8.123[ac] |
| FRC | 86.72 ± 2.961[bd] |
| FRA | 184.1 ± 11.27[ac] |
| FRR | 57.98 ± 1.734[d] |

Rates are given as mean ± S.E.M. (n= 3); rates with the same letter are not significantly different according to Tukey´s test ($p < 0.05$).

leaves. Revathi and Rajeswari (2015) reported a lower content for *G. speciosa* (115.81 ± 0.67 mg TAE $g^{-1}$). Total phenolic content for *G. uruguensis* was higher than that registered by other authors for different *Guettarda* species.

## HPLC fingerprint of flower and fruit ethyl acetate fractions

Flower and fruit ethyl acetate fractions fingerprint revealed chromatographic profiles (Figures 1 and 2, respectively). The two fractions showed chromatographic peaks with retention time 06.09 and 15.75 min, although peaks in the flower fraction had a higher concentration. Peaks that differentiated fractions were: peak at 13.75 min in flower and peak at 14:33 min in the fruit, both presenting different intensities and different ultraviolet profiles. Profile of the fruit´s ethyl acetate fraction is characteristic of flavonoids. Caffeic acid standard at 6:09 min revealed the same UV profile. However, flower fraction had higher concentrations as compared to those from fruit fractions.

## Antioxidant capacity

Several techniques have been used to determine *in vitro* antioxidant activity for a rapid screening of medicinal plants. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants combat free radicals and protect people from various diseases either by scavenging the reactive oxygen species or by protecting the antioxidant defense mechanisms.

The phosphomolybdenum assay has been routinely used to evaluate the antioxidant capacity of extracts (Prieto et al., 1999). Although, some extracts and fractions from flower and fruit demonstrated a higher relative antioxidant activity as compared to rutin standard, all samples showed a lower antioxidant activity than ascorbic acid (Table 2 and Figure 3). In the ranking of antioxidant capacity obtained by this method, the remaining fraction of *G. uruguensis* flower showed higher phosphomolybdenum reduction (26.01 ± 0.01% of ascorbic acid and 121.59 ± 0.01% of rutin), followed by remaining fraction of *G. uruguensis* fruit (27.1 ± 0.03% of ascorbic acid and 128.14 ± 0.03% of rutin).

Assessment of antioxidant activity by the DPPH method revealed a large variation among extracts and fractions from flower and fruit (Table 2 and Figure 3). The lowest $IC_{50}$ rate, or rather, the highest scavenging activity of DPPH radicals, was obtained from ethyl acetate and butanol fractions of flowers. FLA ($IC_{50}$ = 13.21 µg $mL^{-1}$) showed a rate ($p < 0.05$) which was statistically similar to that of ascorbic acid ($IC_{50}$ = 4.78 µg $mL^{-1}$) and rutin ($IC_{50}$ = 6.19 µg $mL^{-1}$). FLB also demonstrated a strong scavenging activity with IC50= 22.73 ± 0.29 µg $mL^{-1}$. In the case of fruit samples, the extracts' scavenger capacity was comparatively lower than extract and fractions of flowers. FRA ($IC_{50}$ = 68.8 ± 0.06 µg $mL^{-1}$) showed the highest scavenger capacity among other fruit extracts (Table 2 and Figure 3).

Specialized literature has scanty information on the antioxidant activity of the species *Guettarda*. DPPH assay was performed on *G. viburnoides* leaves and on the *G. uruguensis* stem bark. Naressi et al. (2015) showed higher antioxidant activity in *G. viburnoides*, with $IC_{50}$ rates for crude extract (24.69 µg $mL^{-1}$), ethyl acetate (18.92 µg $mL^{-1}$), aqueous-methanol (26.47 µg $mL^{-1}$) fractions from leaves, and also for grandifloroside (20.52 µg $mL^{-1}$), a compound isolated from leaves. In a previous paper, Duarte et al. (2014) reported antioxidant activity of crude extract and fractions from the stem bark of *G. uruguensis* and proved that ethyl acetate fractions (IC50 = 10.91 µg $mL^{-1}$) were greatly capable of quenching the DPPH radical.

Lipid peroxides are likely involved in many pathological events, including inflammation, metabolic disorders, oxidative stress and cellular aging. Table 2 and Figure 3 summarize the effects of the flower and fruit extracts and fractions of *G. uruguensis*. FLB (IA = 53.42 ± 4.29%) and FLA (IA = 52.08 ± 2.21%) obtained the highest antioxidant index from other samples analyzed, although both are not significantly different ($p > 0.05$) from BHT (IA = 54.6%).

The antioxidant activity of phenolic compounds was correlated to their chemical structures. The relationship of the structure activity of several phenolic compounds has been studied (Rice-Evans et al., 1996; Lien et al., 1999; Son and Lewis, 2002). Free radical scavenging and antioxidant activity of phenolics mainly depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules. However, it is also affected by other factors, such as glycosylation of aglycones, H-donating groups (-NH, -SH)

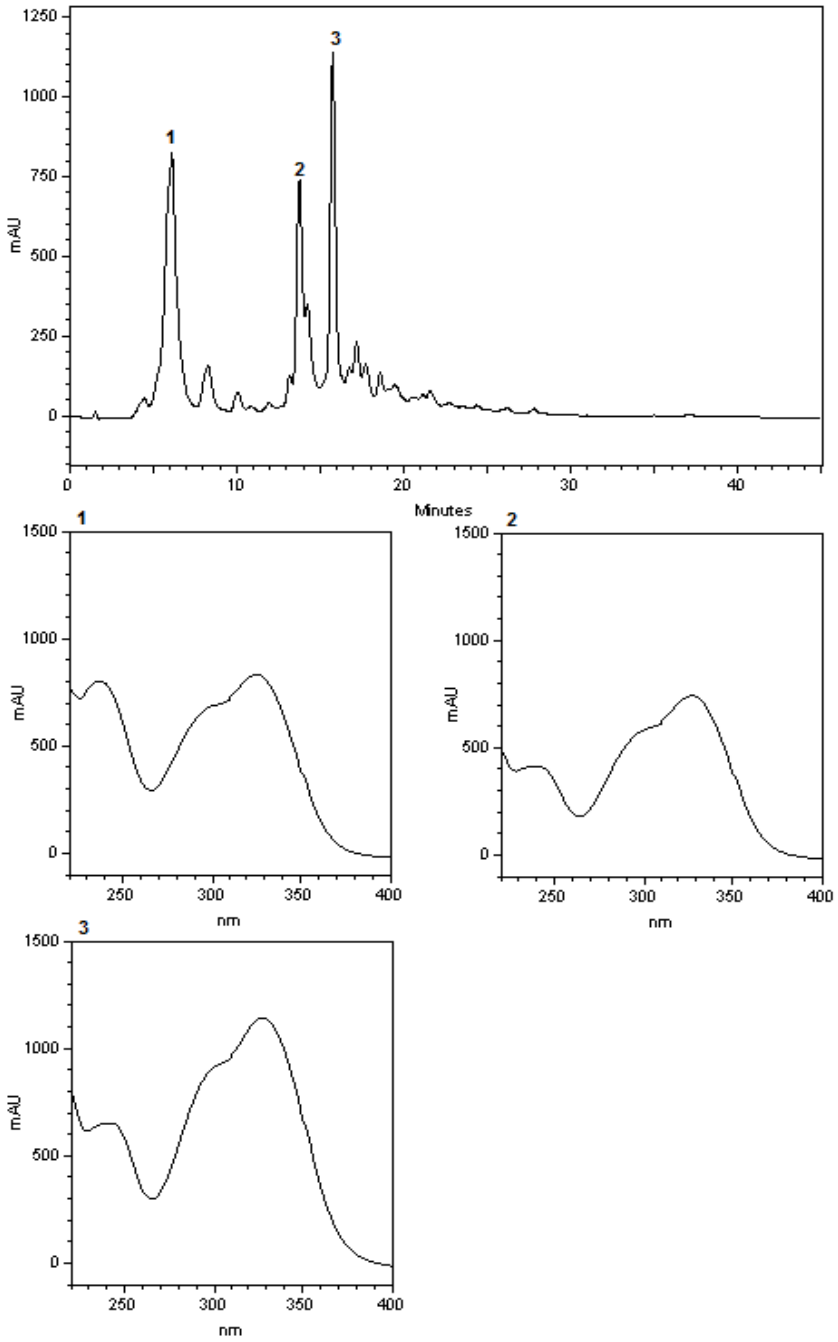


**Figure 1.** Fingerprint of ethyl acetate fraction of flower. Peak 1 – 6.09 min. λmax: 326 nm; Peak 2 – 13.75 nm. λmax: 326 nm; Peak 3 – 15.75 nm. λmax: 329 nm.

and others. There are currently several reports on antioxidant components, generally focusing on flavonoids and phenolic acids (Rice-Evans et al., 1996; Nakatani, 2000; Zheng and Wang, 2001).

The flavonoids, namely, quercetin-3-O-B-D-galactopyranoside, quercetin-3-O-B-D-glucopyranoside and grandifloroside (Naressi et al., 2015) and the phenolic acids, namely, 5-caffeoylquinic acid, 4,5-dicaffeoylquinic acid (Capasso et al., 1998, Oliveira et al., 2008, Testa et al., 2012) and shickimic acid (Capasso et al., 1998) were identified in the genus Guettarda. The literature also shows that 3,5- and 4,5-O-dicaffeoylquinic acids and 3-O-glucosilates derived from quercetin have significant scavenging abilities of free radicals, with IC50 rates close to those for FLA in the DPPH assay.

Antioxidant capacity and total phenolic rates may also

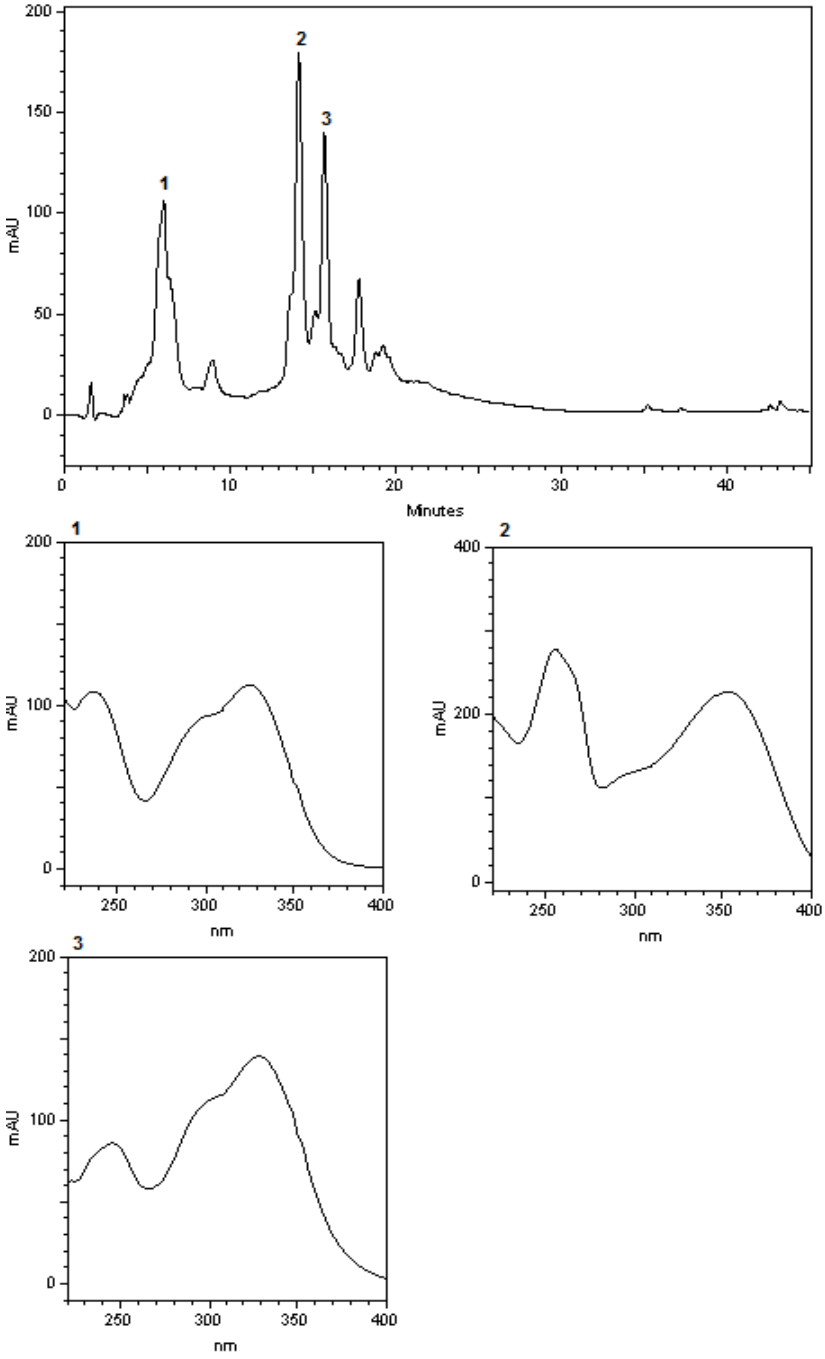


**Figure 2.** Fingerprint of ethyl acetate fraction of fruit. Peak 1 – 6.09 min. λmax: 326 nm; Peak 2 – 14.33 min. λmax: 353 nm; Peak 3 – 15.75 min. λmax: 329 nm.

enhance other investigations and co-relate such activity with other important ones, such as the anti-inflammatory activity which is directly related to the popular use of several species of the genus Guettarda. Specialized literature suggests the co-relationship between antioxidant and anti-inflammatory activities. In other words, several vegetal extracts decrease inflammation by eliminating superoxides known to participate in the recruitment of polymorphonuclear cells (PMN) occurring in inflamed tissues (Thambi et al., 2009; Ródenas et al., 2000).

Current analysis registered that flowers and fruits of *G. uruguensis* proved to have high antioxidant activity and elevated levels of phenolic compounds. It is a widely

**Table 2.** *G. uruguensis* flower and fruit extract and fractions antioxidant activity obtained by phosphomolybdenum, DPPH and TBARS methods.

| Samples | Assay | | | |
|---|---|---|---|---|
| | Total antioxidant activity | | DPPH | TBARS (IA%) |
| | Ascorbic acid | Rutin | $IC_{50}$ (µg $mL^{-1}$) | |
| FLE | 19.65 ± 0.01 | 91.66 ± 0.01 | 58.65 ± 0,98 | 22.41 ± 1.10 |
| FLH | 24.92 ± 0.01 | 116.51 ± 0.01 | 206.8 ± 16.99 | 30.53 ± 0.21 |
| FLC | 25.55 ± 0.00 | 119.43 ± 0.00 | 108.8 ± 3.58 | 32.36 ± 2.23 |
| FLA | 23.09 ± 0.01 | 107.95 ± 0.01 | 13.21 ± 0.66[a] | 52.08 ± 2.20[a] |
| FLB | 20.66 ± 0.02 | 96.59 ± 0.02 | 22.73 ± 0.29 | 53.42 ± 4.29[a] |
| FLR | 26.01 ± 0.01 | 121.59 ± 0.01 | 60.42 ± 0.82 | 28.94 ± 2.53 |
| FRH | 9.94 ± 0.03 | 46.43 ± 0.03 | >1000 | 22.71 ± 3.25 |
| FRC | 22.14 ± 0.02 | 103.4 ± 0.02 | 73.21 ± 1.13 | 24.18 ± 1.45 |
| FRA | 19.85 ± 0.03 | 92.8 ± 0.03 | 68.8 ± 0.06 | 20.82 ± 1.731 |
| FRR | 27.41 ± 0.03 | 128.14 ± 0.03 | 61.22 ± 2.79 | 22.65 ± 6.13 |
| AA | 100b | NA | 4.78 ± 0.04[a] | NA |
| RUTIN | NA | 100 | 6.19 ± 0.06[a] | NA |
| BHT | NA | NA | NA | 54.6[a] |

Rates are given as mean ± S.E.M. (n=3); rates with the same letter in each column are not significantly different by Dunnett´s test ($p < 0.05$). NA: not analyzed; AA: ascorbic acid; BHT: butylhydroxytoluene.

grown plant, possessing fruits used as food and aromatic flowers. Since there is an inverse relationship between dietary intake of antioxidant-rich foods and the occurrence of several human diseases, above results are interesting and research on the determination of antioxidant-rich foods is highly relevant and rewarding.

## Correlations between total phenolic contents and antioxidant activities

Antioxidant activities of medicinal plant extracts are often associated with redox proprieties when they function as reducing agents. Phenolic compounds constitute one of the major groups of secondary metabolites acting as free radical scavengers and antioxidants. The antioxidant activities of flower and fruit extracts and fractions of *G. uruguensis* were measured by the phosphomolybdenum, DPPH and TBARS assays. FLA and FLB registered a high level of antioxidant activity in different assays (Table 3). FLA (13.21 ± 0.66 µg $mL^{-1}$) provided a statistical result ($p < 0.05$) with DPPH similar to ascorbic acid (4.78 ± 0.04 µg $mL^{-1}$) and rutin (6.19 ± 0.06 µg $mL^{-1}$) controls. In the case of the TBARS method, FLA (IA = 52.08 ± 2.20%) and FLB (IA = 53.42 ± 4.29%) presented statistical antioxidant indexes similar ($p < 0.05$) to BHT control (IA = 54.6%).

Results of antioxidant assays were consistent and correlated with the polyphenolic contents assessed by linear regression analysis. Table 3 shows Pearson's correlation coefficients. An important correlation of flower and fruit extracts and respective fractions may be observed between the phosphomolybdenum: ascorbic-acid and rutin tests ($r = 1\ p < 0.01$). Pearson´s coefficient indicated a strong association ($r = 0.85\ p < 0.01$) of phenolic compounds and antioxidant activity TBARS, whereas negative correlation ($r = -0.83\ p < 0.01$) revealed an inverse relationship between phenolic compounds and IC50. In fact, IC50 is the necessary concentration to inhibit 50% of free radicals, in other words, lower IC50 rates point to better antioxidant activity.

Figure 4 shows two main principal components (PCs) characterizing total phenolic content and antioxidant capacity (phosphomolybdenum, DPPH and TBARS) of flowers and fruit fractions. The first principal component (PC1) accounted for 60.03% of variability in the data set, whilst the second PC (PC2) accounted for 32.78% of variance in the data.

PC1 distinguishes two groups: (*i*) ethyl acetate fractions (FLA and FRA), FLE and FLB from (*ii*) remaining fractions (FRR and FLR), FRC and FLC. The fractions obtained from the chloroform solvent (FLC and FRC) constitute a distinct group from the remaining fractions (FRR and FLR). The proximity of FLC to the FRC quadrant may be explained by the different extraction mode between flower and fruit. Consequently, chloroform and remaining fractions could be discriminated by the polarity scale in PC2. Figure 4 shows the five assays represented by vectors. Since they are inversely proportional, the coefficients for total phenol, TBARS and DPPH, demonstrate that the higher rates for the two tests were FLA and FLB. The best rates with the greatest contribution in the phosphomolybdenum tests were from

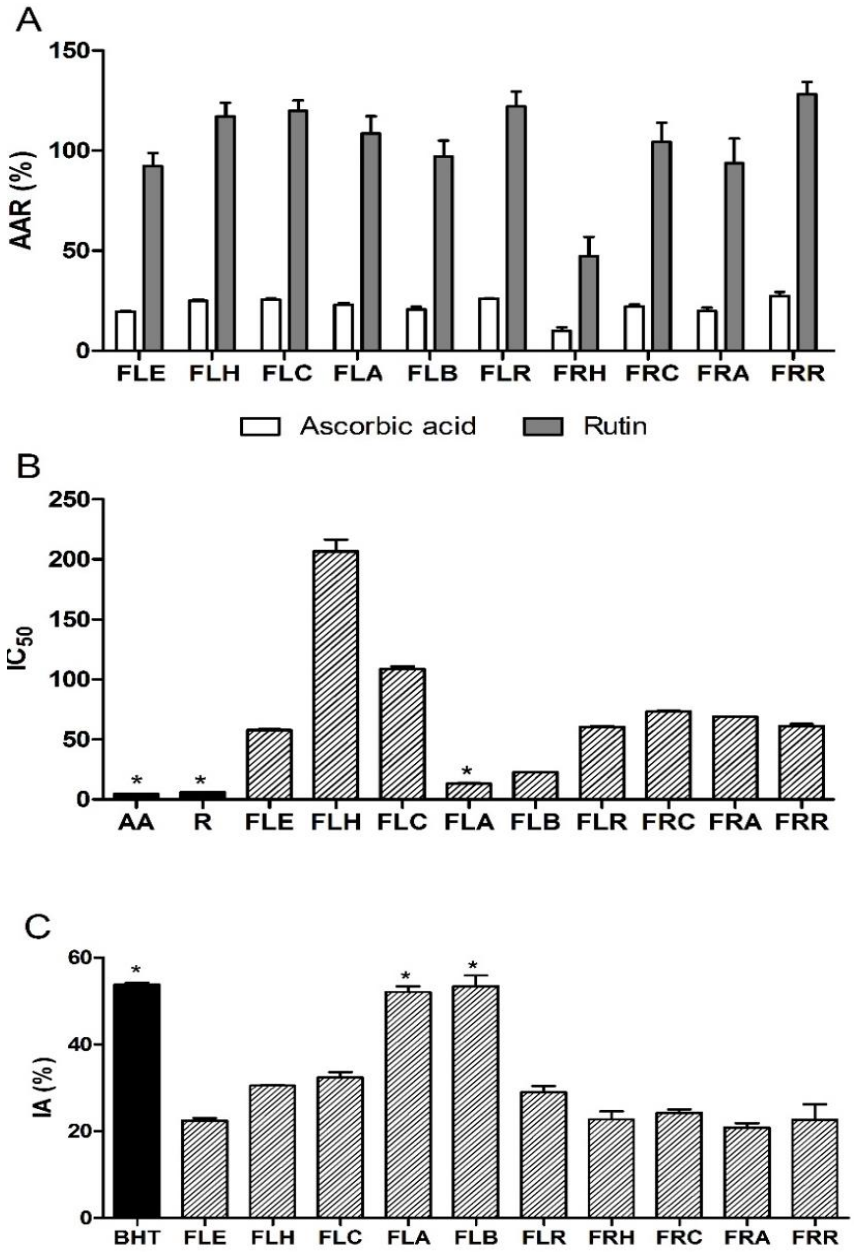


**Figure 3.** Antioxidant activity from flower and fruit extract and fractions of *G. uruguensis* obtained by the phosphomolybdenum method (panel A), DPPH (panel B) and TBARS method (panel C). Results are given as mean ± S.E.M. Statistical comparison was performed with analysis of variance (ANOVA) followed by Dunnett´s test; *p < 0.05 as compared to the control group.

**Table 3.** Pearson's correlation coefficients between antioxidant activities and the polyphenolic content of flower and fruit extracts and fractions of *G. uruguensis* Cham. & Scthdl. (Rubiaceae).

| Phenolics | | Ascorbic acid | Rutin | TBARS | DPPH |
|---|---|---|---|---|---|
| Phenolics | 1 | | | | |
| Ascorbic acid | Ns | 1 | | | |
| Rutin | Ns | 1* | 1 | | |
| TBARS | 0.85* | ns | ns | 1 | |
| DPPH | -0.83* | ns | ns | -0.69** | 1 |

*Significant correlation 0.01; ** Significant correlation 0.05.

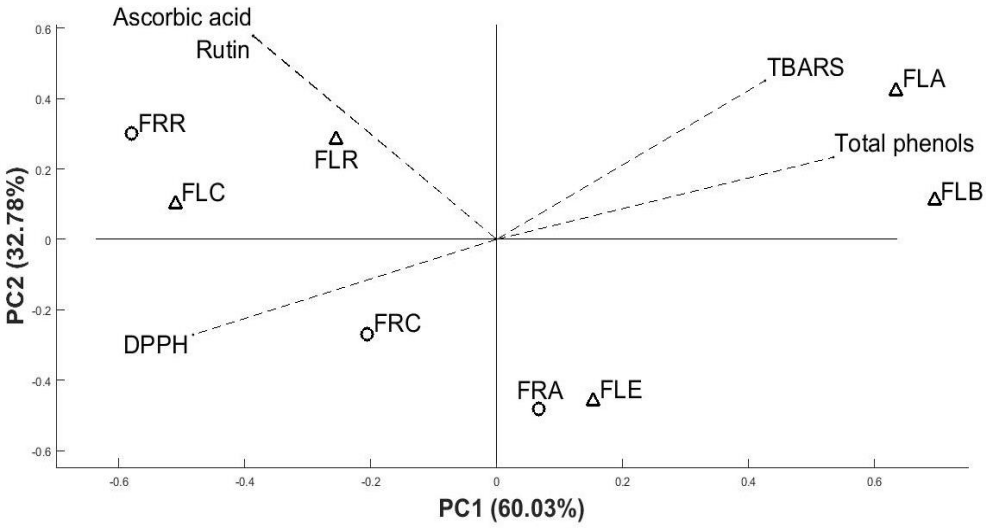


**Figure 4.** Data set of scores and loadings PCA plot.

FRR and FLR.

## Antimicrobial activity

There is no consensus at an acceptable inhibition level for the use of natural antimicrobial products when compared with known antibiotics (Aligianis et al., 2001). According to Ayres et al. (2008), MIC rates obtained were classified as having good inhibitory potential (<100 µg $mL^{-1}$); moderate activity (500-1000 µg $mL^{-1}$) and absence of inhibitory activity (>1000 µg $mL^{-1}$).

Assessment of antimicrobial susceptibility profile indicated that samples presented moderate activity, or rather, the flower´s remaining fraction (500 µg $mL^{-1}$) and fruit extract (500 µg $mL^{-1}$), against *Enterococcus faecalis*. Extracts and fractions were classified as having low activity (CIM=1000) or as inactive (CIM > 1000) in the remaining microorganisms.

In previous studies on *G. uruguensis*, Kelmer et al. (2011) demonstrated the stem bark´s activity (CIM=500 µg $mL^{-1}$) against *S. aureus* and that of the chloroform fraction (CIM=125 µg $mL^{-1}$) against *S. aureus*. The best performance of chloroform fraction was attributed to ursolic acid. Duarte et al. (2014) showed that crude extract and stem bark fractions of *G. uruguensis* had antimicrobial activity against *S. epidermidis* and *C. albicans*. Divergences among studies may be due to the fact that the biosynthesis of secondary metabolites was affected by environmental factors, such as seasonality, circadian rhythm and development. The collection period may be highlighted since the quantity and even the nature of chemical constituents are not constant throughout the year. Another explanation may comprise the difference between plant organs and the characteristics of extraction process (Gobbo-Neto and Lopes, 2007).

## Brine shrimp lethality bioassay

The lethal concentration ($LC_{50}$) obtained from regression and probit analysis (Bliss, 1934) in 24 h are presented in Table 4. Except FRR ($LC_{50}$>1000 µg $mL^{-1}$), other samples were also considered toxic. In fact, highest toxicity was presented by FLC ($LC_{50}$ < 10 µg $mL^{-1}$), significantly ($p$ < 0.05) similar to SDS (16.27 µg $mL^{-1}$).

In previously studies, current research team reported toxicity for chloroform fraction ($LC_{50}$ = 80.31 µg $mL^{-}1$) obtained from the stem bark of *G. uruguensis*. Toxicity may have been related to alkaloids in the chloroform fraction (Duarte, 2012). Alkaloids have also been described in other *Guettarda* species (Husson et al., 1977; Kan-Fan and Husson, 1979; Brillanceau et al., 1984; Kan-Fan et al., 1985; Ferrari et al., 1986; Montagnac et al., 1997; Capasso et al., 1998). In the wake of such results, an alkaloid extract was assessed to verify whether toxicity could be reproduced. The alkaloid extract actually provided a second high toxicity score ($LC_{50}$ < 21.54 µg $mL^{-1}$) and suggested that high toxicity by FLC may have been due to alkaloids.

From a pharmacological point of view, the brine shrimp lethality test was employed to detect general toxicity. It proved to be a good detector of compounds with antiviral, insecticidal, anti-parasitic and anti-tumoral activities (Siqueira et al, 1998; McLaughlin et al., 1998). With the exception of FRR, extracts and fractions obtained from the flower and fruit of *G. uruguensis* displayed a strong activity against brine shrimp, which is highly suggestive of bioactivity and its pharmacology potential. Since *G. platypoda* and *G. pohliana* revealed antitumoral activities in human cancer cell strains (Oliveira, 2013; Pina et al.,

**Table 4.** Brine shrimp lethality bioassay of flower and fruit extracts and fractions of *G. urugensis.*

| Sample | $LC_{50}$ (µg $mL^{-1}$) | Range of confidence limit |
|---|---|---|
| FLE | $142.51^{a}$ | 35.34-574.70 |
| FLH | $51.79^{bcd}$ | 18.35-146.17 |
| FLC | $<10^{ef}$ | - |
| FLA | $38.99^{bg}$ | 9.82-154.78 |
| FLB | $69.73^{chi}$ | 42.12-115.43 |
| FLR | $25.12^{ej}$ | 5.33-118.39 |
| FLAQ | $84.83^{hk}$ | 34.26-210.09 |
| FLALC | $21.54^{fgj}$ | 11.53-40.27 |
| FRH | $149.62^{l}$ | 74.20-301.70 |
| FRC | $69.78^{dik}$ | 12.55-388.13 |
| FRA | $153.99^{al}$ | 71.58-331.27 |
| FRR | >1000 | - |

Statistical comparison was performed with analysis of variance (ANOVA) followed by Tukey's test.

2012), the antitumoral capacity of *G. uruguensis* should be evaluated.

## Conclusion

Ethyl acetate and butanol fractions of *G. uruguensis* flowers showed high levels of total phenolic contents and a relevant antioxidant capacity. They were efficient as and sometimes better than standard antioxidants. The evaluation of antimicrobial susceptibility profile indicated that samples were active against *E. faecalis*. Strong activity against brine shrimp suggests bioactivity and pharmacology potential. However, further studies are recommended to reveal their chemical composition and toxicity and to determine the pharmaceutical potential of *G. uruguensis*.

## Conflict of Interests

The authors have not declared any conflict of interest.

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## REFERENCES

Achille F, Motley TJ, Lowry II PP, Jérémie J (2006). Polyphyly in *Guettarda* L. (Rubiaceae, Guettardeae) based on nrDNA ITS sequence data. Ann. Missouri Bot. Gard. 93:103-121.

Agra MF, França PF, Barbosa-Filho JM (2007). Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Rev. Bras. Farmacogn. 17:114-140.

Agra MF, Silva KN, Basílio JLD, Freitas PF, Barbosa-Filho JM (2008). Survey of medicinal plants used in the region Northeast of Brazil. Rev. Bras. Farmacogn. 18(3):472-508.

Albuquerque UP, Monteiro JM, Ramos MA, Amorim ELC (2007). Medicinal and magic plants from a public market in northeastern Brazil. J. Ethnopharmacol. 110(1):76-91.

Aligianis N, Kalpoutzakis E, Mitaku S, Chinou IB (2001). Composition and antimicrobial activity of the essential oil of two *Origanum* species. J. Agric. Food Chem. 49(9):4168-4170.

Aquino R, De Simone F, Pizza C, Cerri R, De Mello JF (1988). Quinovic acid glycosides from *Guettarda platypoda*. Phytochemistry. 27(9):2927-2930.

Ayres MC, Brandão MS, Vieira-junior GM, Menor JCAS, Silva HB, Soares MJS, Chaves MH (2008). Antibacterial activity of useful plants and chemical constituents of the roots of *Copernicia prunifera*. Br. J. Pharmacogn. 18:90-97.

Batista CVF, Schipsema J, Verpoorte R, Rech SB, Henriques AT (1996). Indole alkaloids from *Rauwolfia sellowii*. Phytochemistry 41:969-973.

Barbosa MR (2015). *Guettarda in* Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro.

Barros AV, Araújo LM, Oliveira FF, Conceição AO, Simoni IC, Fernandes MJB, Arns CW (2012). In vitro evaluation of the antiviral potential of *Guettarda angelica* against animal herpes viruses. Acta Sci. Vet. 40(4):1068.

Bhattacharyya J, De Almeida MZ (1985). Isolation of the constituents of the root-bark of *Guettarda platypoda.* J. Nat. Prod. 48(1):148-149.

Bliss CI (1934). The Method of Probits. Science 79:38-39.

Brandão MGL, Botelho MGA, Krettli AU (1985). Quimioterapia experimental antimalárica com produtos naturais: uma abordagem mais racional? Ciência e Cultura. 37(7):1152-1163.

Brillanceau MH, Kan-Fan C, Kan SK, Husson HP (1984). Guettardine, a possible biogenetic intermediate in the formation of Corynanthe-Cinchona alkaloids. Tetrahedron Lett. 25(26):2767-2770.

Capasso A, Balderrama L, Sivila SC, De Tommasi N, Sorrentino L, Pizza C (1998). Phytochemical and pharmacological studies of *Guettarda acreana.* Planta Medica 64(4):348-352.

Carvalho JLS, Cunico MM, Dias JFG, Miguel MD, Miguel OG (2009). Term-stability of extractive processes from *Nasturtium officinale* R. Br., brassicaceae for Soxhlet modified system. Quím. Nova. 32(4):1031-1035.

Clinical and Laboratory Standards Institute (2008a). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M07-A8, Wayne, PA, USA: CLSI.

Clinical and Laboratory Standards Institute (2008b). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard Document M38-A2. Wayne, P.A.: CLSI.

Corrêa MP, Penna LA (1984). Dicionário de plantas úteis do Brasil e das exóticas cultivadas. Rio de Janeiro: Instituto Brasileiro de Desenvolvimento Florestal.

Duarte AFS, Hirota BCK, Oliveira VB, Campos R, Murakami FS, Miguel MD, Miguel OG (2014). Assessment of the antioxidant and antimicrobial activity of the crude ethanolic extract and organic fractions obtained from the stem bark of the specie Guettarda uruguensis Cham. & Scthdl. (Rubiaceae). Rev. Ciênc. Farm. Básica Appl. 35(4):607-614.

Delprete PG, Jardim JG (2012). Systematics, taxonomy and floristics of Brazilian Rubiaceae: An overview about the current status and future challenges. Rodriguésia 63(1):101-128.

Duarte AFS (2012). Estudo fitoquímico, toxicidade e atividades biológicas (antioxidante, antimicrobiana e alelopatica) de cascas do caule de *G. uruguensis* Cham. & Schltdl. (Rubiaceae). Master´s Dissertation in Pharmaceutical Sciences – Departamento de Ciências Farmacêuticas. Universidade Federal do Paraná, Curitiba.

Ferrari F, Mesana I, Botta B, De Mello JF (1986). Constituents of *Guettarda platypoda.* J. Nat. Prod. 49:1150-1151.

Gerlach SL, Burman R, Bohlin L, Mondal D, Ransson UG (2010). Isolation, Characterization, and Bioactivity of Cyclotides from the Micronesian Plant *Psychotria leptothyrsa*. J. Nat. Prod. 73(7):

1207-1213.
Gobbo-Neto L, Lopes NP (2007). Medicinal Plants: Factors of influence on the content of secondary metabolites. Quím. Nova. 30: 374-381.
Harborne JB (1993). Introduction to Ecological Biochemistry (Fourth Edition). Academic Press, London. P 318.
Husson HP, Kan-Fan C, Sevenet T, Vidal JP (1977). Structure de la cathenamine intermediaire cle de la biosynthese des alcakloides indoliques. Tetrahedron Lett. 22:1889-1892.
Inouye H, Takeda Y, Nishimura H, Kanomi A, Okuda T, Puff C (1988). Chemotaxonomic studies of rubiaceous plants containing iridoid glycosides. Phytochemistry 27(8): 2591-2598.
Kan-Fan C, Brillanceau MH, Pusset J, Chauviere G, Husson HP (1985). Three quinicine derived alkaloids from *Guettarda trimera*. Phytochemistry 24(11): 2773-2775.
Kan-Fan C, Husson HP (1979). Isolation and biomimetic conversion of 4,21-dehydrogeissoschizine. J. Chem. Soc. Chem. Commun. 22:1015-1016.
Kelmer F, Lopes DS, Marrega TP, Camacho DP, Moura VM (2011). Perfil de susceptibilidade antimicrobiana do extrato bruto e frações da planta *Guettarda uruguensis*. In: XII Encontro Maringaense de Biologia/XXVI Semana de Biologia, Maringá.
Kinupp VF (2007). Plantas alimentícias não-convencionais da região metropolitana de Porto Alegre, RS. [Tese]. Porto Alegre: Programa de Pós-Graduação em Fitotecnia, Universidade Federal do Rio Grande do Sul.
Kunkel G (1984). Plants for human consumption: An Annotated Checklist of the Edible Phanerogams and Ferns. Koenigstein: Koeltz Scientific Books.
Lien EJ, Ren S, Bui H, Wang R (1999). Quantitative structure-activity relationship analysis of phenolics antioxidants. Free Radic. Biol. Med. 26:285-294.
Lima GS, Moura FS, Lemos RPL, Conserva LM (2009). Tritepenes from *Guettarda grazielae* MRV MArbosa (Rubiaceae). Rev. Bras. Farmacogn. 19:284-289.
Matos FJA (1997). O formulário fitoterápico do Professor Dias de Rocha. Fortaleza: UFC. P 258.
McLaughlin JL, Rogers LL, Anderson JE (1998). The Use of Biological Assays to Evaluate Botanicals. Drug Info. J. 32:513-524.
Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, Leitao SG (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15(2):127-30.
Manach C, Scalbert A, Morand C, Remesy C, Jimenez L (2004). Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79:727e47.
Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45:31-34.
Montagnac A, Litaudon M, Pais M (1997). Quinine and quinicine derived alkaloids from *Guettarda noumeana*. Phytochemistry 46(5):973-975.
Morais SM, Catunda-Jr EA, Silva ARA, Martins-Neto JS (2006). Atividade antioxidante de óleos essenciais de espécies de *Croton* do nordeste do Brasil. Quim Nova 29:907-910.
Nakatani N (2000). Phenolic antioxidants from herbs and spices. Biofactors 3:141-146.
Naressi MA, Manholer DD, Ames FQ, Bersani-Amado CA, Formagio ASN, Pereira ZV, Da Costa WF, Baldoqui DC, Sarragiotto MH (2015). Chemical constituents, anti-inflammatory, and free-radical scavenging activities of *Guettarda viburnoides* Cham. & Schltdl. (Rubiaceae). Quím. Nova 38(7):932-936.
Oliveira PRN, Testa G, Sena SB, Costa WF, Sarragioto MH, Santin MO (2008). Chemical constituents of the roots of *Guettarda pohliana* Müll. Arg. (Rubiaceae). Quím. Nova 31(4):755-758.
Oliveira PRN, Testa G, Medina RP, Oliveira MA, Kato L, da Silva CC, Carvalho JE, Santin MO (2013). Cytotoxicity activity of *Guettarda pohlinana* Müll. Arg. (Rubiaceae). Nat. Prod. Res. 27(18):1677-1681.
Osawa T (1994). Novel antioxidants for utilization in food and biological system. In: Uritani I, Garcia VV, Mendoza EM, editors.Postharvest Biochemistry of Plant Food Materials in the Tropics. Japan Scientist Societies Press; Tokyo, Japan. pp. 241-251.
Pina EML, Araújo FWC, Souza IA, Bastos IVGA, Silva TG, Nascimento SC, Militão GCG, Soares LAL, Xavier HS, Melo SJ (2012). Pharmacological screening and acute toxicity of bark roots of *Guettarda platypoda*. Braz. J. Pharmacogn. 22: 1315-1322.
Prieto P, Pinedo M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity though the formation of phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem. 269(2):337-341.
Revathi D, Rajeswari M (2015). Chemical Profiling of *Guettarda speciosa* Linn. by GC-MS. Inter. J. Emerging Technol. Adv. Eng. 5(9):114-118.
Rice-Evans CA, Miller NJ, Paganga G (1996). Structure antioxidant activity relationship of flavonoids and phenolic acids. Free Radic. Biol. Med. 20(7):933-956.
Saravana KA, Amutha P, Gandhimathi R, Dhanapal R (2009). Study on phytochemical profile and antiepileptic activity of inner bark of *Guettarda speciosa* (L.). Iran J. Pharm. Ther. 8(1):73-76.
Ródenas J, Carbonell T, Mitjavila MT (2000). Diferent roles for nitrogen monoxide and peroxynitrite in lipid peroxidation induced by activated neutrophils. Free Radic. Bio. Med. 28:374-380.
Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16(3):144-158.
Siqueira MJ, Bomm DM, Pereira NFG, Gareez WS, Boaventura MAD (1998). Estudo fitoquimico de Unonopsis lindmanii Annonaceae, biomonitorado pelo ensaio de toxicidade sobre *Artemia salina* Leach. Quim Nova 21:557-559.
Son S, Lewis BA (2002). Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: Structure activity relationship. J. Agric. Food Chem. 50:468-472.
Testa G, Oliveira PRN, Silva CC, Schuquel ITA, Santin SMO (2012). Constituintes químicos das folhas e avaliação anti-inflamatória de extratos das raízes e folhas de *Guettarda pohliana* Müll. Arg. (Rubiaceae). Quím. Nova 35(3):527-529.
Thambi PT, Kuzhivelil B, Sabu MC, Jolly CI (2006). Antioxidant and anti-inflammatory activities of the flowers of *Tabernaemontana coronaria* (L) R. Br. Indian J. Pharm. Sci. 68:352-355.
World Health Organization (1997). Medicinal Plants Monographs (red. Blumenthal M), Herbal Gram: Salerno-Paestum, Italy, 40:38.
Zheng W, Wang SY (2001). Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49:5165-5170.