In vitro antioxidant and cell viability of Pyrostegia venusta (Ker Gawl.) Miers


Abstract: Many diseases are associated with oxidative stress and inflammatory processes. The current research is directed toward evaluating the antioxidant potential and phytochemistry composition of P. venusta leaves. In this study, P. venusta leaves were dried and macerated, and the crude extract was partitioned. Phytochemical analysis was performed using standard methodologies, and the total flavonoid content was measured using a calibration curve with rutin. We evaluated the antioxidant potential of P. venusta leaves using 1,1-Diphenyl-2-picolylhydrazyl (DPPH), 2, 2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and a Trolox-like standard. Cell viability (CV) assays were done using macrophage RAW 264.7 cell lines and compared to four commercial anti-inflammatorines (acetylsalicylic acid, Indometacina, Betametasone, and Piroxicam).

Phytochemical analysis revealed the presence of steroids, coumarins, and flavone. The flavonoid content was 148.5 ± 7.65 µg as a rutin equivalent/mg of crude extract. The ethyl acetate fraction showed the best antioxidant activity in the methodologies of DPPH inhibition (IC50 = 38.62 µg/mL) and ABTS radical (IC50 = 28.58 µg/mL). Samples of P. venusta had CV values that were better than the commercial anti-inflammatory, which showed CV values below the negative control. The crude extract and the ethyl acetate fraction, showed CV values below the negative control and the hexane fraction obtained values above the negative control, these being best results.

Keywords: Pyrostegia venusta; leaves; antioxidant; DPPH; ABTS; cell viability

1. INTRODUCTION

Oxygen is essential for many biological processes, including the production of energy in heterotrophic organisms (Kreb’s Cycle) and others involved in electron transfer [1-2]. This process results in free radicals and other compounds like reactive oxygen species (ROS). The body naturally neutralizes this species, but the imbalance between neutralization and formation can induce oxidative damage to biomolecules, known as oxidative stress [1, 3-5].

Many diseases such as cancer, diabetes mellitus, cardiovascular diseases, degenerative disorders, arthrosclerosis, and inflammatory diseases are reported to involve this imbalance of free radicals [1, 6-8]. During an inflammatory process, an organism causes an oxidative stress for defense, known as a respiratory burst [9, 10].

The phagocytic leukocytes (macrophages, neutrophils and eosinophils), when activated by a pro-inflammatory mediator, are the most likely source of ROS. Initially, an NADPH oxidase catalyzes a large amount of superoxide radical (O2•-) production; this reactive species is used in enzymatic reactions to produce other ROS like HClO (hypochlorous acid), H2O2 (hydrogen peroxide), and OH• (hydroxyl radical), and these ROS kill pathogens as well as
adjacent cells [10-12]. However, inflammation can result in serious tissue damage; to prevent this, it is necessary use anti-inflammatory and antioxidant supplements [9, 12].

Many of these synthetic supplements are very effective, but they possess certain side effects [1, 13, 14]. Antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), for example, are carcinogenic [1, 13-15].

Recently, there has been increasing interest in plants used in alternative medicine for their potential antioxidant and anti-inflammatory effects [1, 3].

Pyrotagia venusta (Ker Gawl.) Miers is a member of the family Bignoniaceae, popularly known as “cipó de São João” and widely distributed in the Brazilian ecosystems of Amazonia, the Atlantic forest, and Cerrado [16, 17]. This plant is commonly used in folk medicine for the treatment of vitiligo, diarrhea, cough, flu, jaundice, infections, and inflammatory diseases of the respiratory system [18-20]. Previous investigations have shown that flower and root extracts of *P. venusta* have antioxidant, antimicrobial, and anti-inflammatory activity proprieties (4,7-O-β-D-rutinosil-3’,5-dihydroxy-4’-methoxyiflavanona) [21, 22], and the methanolic extract of roots contain allantoin (anti-oxidative and anti-inflammatory activities) [23], steroids, hesperidin (4,7-O- β-D-rutinosil-3’,5-dihydroxy-4’-methoxyiflavanona) (anti-oxidative), and 3-β-β-D-glucopyranosyl-sitosterol [1].

There are many studies on the roots and flowers of *P. Venusta* due to their extensive use in folk medicine; however, little has been studied regarding the leaves.

This paper describes the investigation of crude extract of leaves of *P. venusta*, hexane and ethyl partitions. Phytochemical analysis was used to determine the biocompound classes present, and the total flavonoid content was measured for the crude extract. The tree extract was evaluated as a source of natural antioxidants (DPPH and ABTS tests) and using a RAW 246-7 (macrophages murines cell) in vitro to verify the safety of its use (immunotoxicology).

2. MATERIAL AND METHODS

Plant material

The leaves of *Pyrostegia venusta* were collected from Altinópolis-SP (Brazil), August 2012, and authenticated through the herbarium VIES of Federal University of Espirito Santo (UFES), Vitória, Brazil.

**Extraction and fractionation**

The *P. venusta* leaves were dried at a temperature below 40 °C for 7 days and powdered. The *P. venusta* crude extract was obtained by maceration in ethanol (96% (v/v)) for 24 h, and this procedure was repeated twice with ethanol and three times with ethyl acetate. The extracts (ethyl acetate and ethanol) were mixed and concentrated on a rotary evaporator generating the crude extract (yield 6.9% (w/w)). Part of the crude extract was reserved.

The concentrated extract was mixed with methanol: water (3:1) (v/v) and partitioned three times with hexane, the residual methanol: water layer was partitioned three times with ethyl acetate. The hexane and ethyl acetate layers were evaporated on a rotary evaporator under reduced pressure to produce the hexane and ethyl acetate extracts.

**Phytochemical analysis**

The presence of phytochemical compounds like alkaloids, steroids, terpenoids, tannins, saponins, coumarins, flavonoids, and naphthoquinones was qualitatively evaluated. The extracts were subjected to pharmacognosics classical tests to detect the classes of metabolites present [36, 37].

We prepared EtOH and CHCl₃ solutions of the extracts for the tests.

**Alkaloids**

1 mL of EtOH solution of the extracts was mixed with 1 mL of HCl (p.a.) and treated with a few drops of Dragendorff’s reagent. Orange precipitation indicated the presence of alkaloids.

**Steroids and triterpenes**

1 mL of chloroform solution of the extracts was mixed with 2 mL of Acetic anhydride and treated with a 3 drops of H₂SO₄ (p.a.). The change in color from blue or green indicates the presence of steroids, and the change from reddish-brown indicates the presence of triterpenes.

**Tannins**

The gelatine test was used to check the presence of tannins. 2 mL of the EtOH solution of the extracts was mixed with 5 mL of gelatine (2.5% (w/v)) and sodium chloride (10% (w/v)) solution. A precipitate...
indicated the presence of tannins.

**Saponins**

The presence of saponins was evaluated by the frothing test. 20 mL of EtOH solution of the extracts was mixed with 15 mL of distilled water and one mL of saturated solution of sodium carbonated and was boiled. 2 mL of the boiled mixture was filtrate taken aside a graduated cylinder; 98 mL of distilled water was added and shaken vigorously. The formation of stable and persistent froth indicated the presence of saponins.

**Coumarins**

In a filter paper, 1 drop of each EtOH solution of extract and after dried, the blots was observed in a UV chamber, in sequence, was added upon the blots, KOH (10% (w/v)) and was observed in the UV chamber again. The fluorescence indicates the presence of coumarins.

**Naphthoquinones**

1 mL of CHCl₃ solution of the extracts was mixed with 2 mL of NH₄OH (10% (v/v)) and shaken vigorously. The presence of a red halo in the water layer indicates the presence of naphthoquinones.

**Flavonoids**

Cyaniding reaction (Shinoda test): 1 mL of EtOH solution of the extracts was mixed with 1 mL of HCl (p.a.) and added to Mg powder. A change in color varying from brown until red indicates presence of flavonoids.

**Estimation of the total flavonoid content in the crude extract**

The flavonoid quantitative analysis of *P. venusta* was done using the rutin equivalent. The method was based on a calibration curve using rutin and the formation of a flavonoid-aluminium complex [34].

Calibration curve: a 0.5 mg/mL of methanolic solution of rutin was placed in 5 aliquots (0.25, 0.5, 1.0, 1.5, and 2.0 mL), methanol was added to each one until a volume of 2 mL was reached; then, 0.6 mL of acetic acid (p.a.), 10 mL of pyridine: water (2:8) (v/v) solution, and 2.5 mL of AlCl₃ methanolic solution (6.5% (w/v)) were added sequentially. Water was then added until reaching a volume of 25 mL. The negative control was made using 2 mL and the other reagents. After incubation at room temperature for 30 min, the samples’ absorbance was measured at 430 nm.

Sample: a 0.5 mg/mL methanolic solution of the extracts was placed in an aliquot of 1 mL and was prepared and measured in the same manner described for the calibration curve procedure.

**Antioxidant activity of the extracts**

**DPPH radical scavenging activity**

The free radical scavenging effects of the extracts on DPPH (2,2-diphenyl-1-picrylhydrazyl) (SIGMA cod D9132) radicals was determined using method of Rufino *et al.* [38]. Each extract was dissolved in EtOH, and serial dilution (300, 150, 75, 37.5, 18.75, and 9.375 µg/mL, 4 mL for each one) was prepared. Then, 1 mL of each concentration was mixed with 2 mL of 0.004% (w/v) DPPH EtOH solution (final concentration 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL), and the negative control was made by mixing 1 mL of each concentration with 2 mL of EtOH. The control was prepared using 1 mL of EtOH and 2 mL of 0.004% (w/v) DPPH EtOH solution. Trolox (SIGMA cod 238813) was used as an antioxidant standard. The samples were allowed to incubate for 30 min in the dark at room temperature, and the absorbance (Abs) was measured in 517 nm. The percentage inhibition is calculated by the following equation:

\[
\% \text{inhibition} = \left( \frac{\text{Abs}_{\text{Cont}} - \text{Abs}_{\text{Samp}}}{\text{Abs}_{\text{Cont}} - \text{Abs}_{\text{Neg}}} \right) \times 100
\]

IC₅₀ values were calculated from the linear or logarithmic regression of inhibition data.

**ABTS radical scavenging activity**

The free radical scavenging effects of the extracts on ABTS (2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (SIGMA cod A1888) radicals was determined using the method of Rufino *et al.* [39]. Each extract was dissolved in EtOH and serial dilution (300, 150, 75, 37.5, 18.75, and 9.375 µg/mL) was prepared. The ABTS solution radical cation was pregenerated by mixing 7 mM of ABTS stock solution with 140 mM of potassium persulfate and incubated for 16 h in the dark at room temperature until the reaction was complete. The absorbance of the ABTS solution radicals was equilibrated to 0.70 (70%) by dilution in EtOH.

Then, 30 µL of each concentration of extract solution was mixed with 1 mL of ABTS radical solution (final concentration 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL) and the negative control was made
by mixing 30 µL of each concentration with 1 mL of 
EtOH. The control was prepared using 30 µL mL of 
EtOH and 1 mL of ABTS radical solution. Trolox was 
used as an antioxidant standard. The absorbance (Abs) 
was measured in 734 nm. The percentage inhibition is 
calculated by the equation:
\[
\%\text{inhibition} = \left( \frac{\text{Abs}_{\text{Cont}} - \text{Abs}_{\text{Samp}}}{\text{Abs}_{\text{Cont}} - \text{Abs}_{\text{Neg}}} \right) \times 100
\]

IC\text{50} values were calculated from the linear or 
logarithmic regression of inhibition data.

**Immunotoxicology**

**Cell culture**

The RAW 264.7 murine macrophage cell line 
was obtained from Cell Bank of Rio de Janeiro (Rio de 
Janeiro, Brazil). These cells were grown at 37 ºC in 
DMEM medium supplemented with 10% FBS, 
penicillin (100 µg/mL) and streptomycin sulfate (100 
µg/mL) in a humidified atmosphere of 5% CO\text{2} [40].

**MTT assay for cell viability**

RAW 264.7 cells were mechanically scraped 
and plated at 2x105 cells/well. The 
well plates containing 100 µL of DMEM were supplemented and 
incubated for 2 h [41]. The extracts were dissolved in 
DMSO (10000, 7500, 5000, and 2500 µg/mL) [40]. After 2 h of incubation, the medium was discarded, 90 
µL of DMEM supplemented was added to all well 
plates, the test material was added (10 µL of each 
concentration), the same volume of DMSO was added 
to the negative control, the same volume of commercial 
anti-inflammatory solutions was added (positive 
controls), and the plates were incubated for 24 h; for 
the control, just DMEM supplemented was added (100 
µL). After being stored overnight, the medium was 
discarded and 90 µL of phosphate buffered saline (PBS) solution was added and incubated for 4 h at 37 ºC. Then, the medium was discarded and the formazan 
blue that formed in the cells was dissolved in 100 µL 
of DMSO. The optical density was measured at 540 nm [41].

3. RESULTS AND DISCUSSION

**Phytochemical analysis**

The screening of *P. venusta* extracts revealed 
the presence of steroids, coumarins, and flavonoids. (Table 1).

Table 1. Phytochemical screening of *P. venusta* leaves.

<table>
<thead>
<tr>
<th>Test</th>
<th>Crude</th>
<th>Hexane</th>
<th>EtOAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkaloids</td>
<td>Drageroff's test</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>2. Steroids and triterpenes</td>
<td>(+) steroids(green color)</td>
<td>(-)</td>
<td>(+) steroids(green color)</td>
</tr>
<tr>
<td>3. Tannins</td>
<td>Gelatin test</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>4. Saponins</td>
<td>trothing test</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>5. Coumarins</td>
<td>(+) Blue</td>
<td>(-)</td>
<td>(+) yellow/greenish</td>
</tr>
<tr>
<td>6. Naphthoquinones</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>7. Flavonoids</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

(+) presence, (-) absence.

Table 1 shows that the phytochemical profile of 
the crude extract and the ethyl acetate fraction are very 
similar: both contain steroids, flavonoids, and 
coumarins, while the hexane fraction had no detectable 
amount of any of the classes of compounds discussed. 
A comparison of these results with the flowers and 
roots of *P. venusta* done by Roy et al. [1] in which the presence of terpenoids, alkaloids, tannins, steroids, and 
saponins was verified, showed several differences 
against the present work, like the presence of 
coumarins; these compounds have been reported in the 
literature as anticoagulant, hypotensive, antimicrobial, 
anti-inflammatory, and antitumor activities [1, 24]. The 
antimicrobial activities of *P. venusta* leaves describe 
by Fernandes et al. were consistent with the presence of 
coumarins [25].

Steroids are partly responsible for anti-diarrheal 
activity [26, 27], and their presence in the leaves, as
well as in the flowers and roots, may justify their use in folk medicine for the treatment of diarrhea [19].

The antioxidant activity of *P. venusta* described in the works of Roy et al. [1], Veloso et al. [22], and Roy et al. [21] can be correlated with the presence of flavone (a polyphenol) in the phytochemical analysis and the presence of rutin in the leaves described by Blatt et al. [28].

**Antioxidant activity of extracts**

A substance shows antioxidant activity when it significantly delays or inhibits the oxidation process. The rate of inhibition of an oxidative process can be measured by an oxidative process in the presence of an antioxidant. Antioxidants’ efficiency is associated with their ability to scavenge free radicals [29, 30].

**DPPH radical scavenging activity**

DPPH is a very stable organic nitrogen radical with ultraviolet-visible absorption (515–520 nm). If an active antioxidant compound is present, these radicals are inhibited and the absorbance of a sample decays.

The results at various concentrations of *P. venusta* extracts are shown in Figure 2.

![Figure 2. DPPH inhibition (%).](image)

**Figure 2.** DPPH inhibition (%) ± standard deviation, for *P. venusta* extracts.

IC$_{50}$ values (concentrations of samples required for 50% inhibition) were calculated by linear regression for the extracts: 38.62 µg/mL for the ethyl acetate fraction, 79.72 µg/mL for the hexane fraction, and 83.04 µg/mL for the crude extract. The IC$_{50}$ value of Trolox was calculated by logarithmic regression, to provide higher linearity than the linear regression, resulting in a value of 2.55 µg/mL.

When comparing the activity of extracts to each other, the striking predominance of antioxidant activity by the ethyl acetate, in the three highest concentrations, is clear over the crude and hexane extracts. The antioxidant activity is linked to the presence of phenolic compounds such as flavonoids and coumarins. The results of phytochemical tests showed that these compounds (flavonoids and coumarins) are only present in the crude extract and ethyl acetate fraction, possibly being concentrated in this fraction by the fractionation process, which explains the higher values of DPPH inhibition to this fraction (ethyl acetate).

**ABTS radical scavenging activity**

The ABTS radical is a blue chromophore (absorbance measured in 734 nm); if an active antioxidant compound is present, these radicals are inhibited and the absorbance of a sample decays. The results are shown in Figure 3.

Because of the complexity of plant extracts, the antioxidant activity should be evaluated by more than one method [31]. In this case, the mechanism used to evaluate antioxidant activity in the ABTS radical test was the same as that used for the DPPH test, but the ABTS method is better when applied to a variety of plant foods containing high-pigmented antioxidant compounds (hydrophilic or lipophilic). As extracts of...
P. venusta have marked pigmentation, this second method was used to confirm and determine the reliability of the results obtained in the DPPH test [3, 32, 33].

As expected, acetate extract had the best results, in all tested concentrations. IC₅₀ values were calculated by linear regression for the extracts: 27.58 µg/mL for the ethyl acetate fraction, 58.09 µg/mL for the hexane fraction and 42.86 µg/mL for the crude extract. The IC₅₀ value of Trolox was calculated by logarithmic regression, to provide higher linearity than the linear regression, resulting in a value of 0.59 µg/mL.

These results confirm that P. venusta is considered a natural source of antioxidants.

Estimation of the total flavonoid content in the crude extract

Phytochemical analysis confirmed the presence of flavonoids in the crude extract, which are known to be antioxidants; the amount of these could justify, or not, this biological activity. Thus, an experiment to quantify these compounds was carried out.

The flavonoid content was calculated using a calibration curve of rutin (Figure 4) (r² = 0.999) and found to be 148.5 ± 7.65 µg/mg of extract as the rutin equivalent [34].

In relation to the mass of dried leaves obtained in a proportion of 1.02 ± 0.05% (w/w) equivalent of rutin, a low value, confirming that described by Blatt, Salantino and Santos [28], and Santos and Blatt [35], who indicated that the leaves of P. venusta had low levels of flavonoids. However, the content of the rutin equivalent relative to the mass of extract 148.5 ± 7.65 mg/mg (14.85% (w/w)) showed that the steeping process was effective in extracting and concentrating these compounds, showing that flavonoids may be responsible for the observed antioxidant activity.

The effect of the extracts from P. venusta on RAW264.7 in cell viability (CV) was determined by the formation of formazan blue. As a preliminary study, the results were compared with four commercial anti-inflammatories (acectysalicylic acid, Indometacina, Betametasona, and Piroxicam). The results are shown in Figure 5.
The crude extract and the ethyl acetate fraction reached lower values of solvent viability (negative control, 0 mg / mL), demonstrating that these have a toxic effect on cells. However, the hexane fraction had positive values of cell viability over the respective negative controls.

When analyzing the results of the commercial anti-inflammatory, it is observed that all CV, in at least one of the tested concentrations, resulted in negative values of CV, below the negative control. As occurred for the crude extract and ethyl acetate fraction, this indicates that they also have a certain degree of toxicity to macrophages.

Although only the results of the hexane fraction can be considered good in terms of cell viability, a subsequent study of inflammation (such as model stimulated macrophages) should certainly include the crude extract and the ethyl acetate fraction, since the anti-inflammatory also presented immunotoxicity, which is not an exclusion criterion in further studies using these samples.

4. CONCLUSION

The phytochemical analysis revealed the presence of steroids, flavonoids, and coumarins in the P. venusta leaf extract. The total flavonoid content of crude extract was 148.5 ± 7.65 µg/mg (14.85 % (w/v)) in rutin equivalents.

When analyzing the results of the commercial anti-inflammatory, it is observed that all CV, in at least one of the tested concentrations, resulted in negative values of cell viability, below the negative control. As occurred for the crude extract and ethyl acetate fraction, this indicates that they also have a certain degree of toxicity to macrophages.

Although only the results of the hexane fraction can be considered good in terms of cell viability, a subsequent study of inflammation (such as model-stimulated macrophages) should certainly include the crude extract and the ethyl acetate fraction, as the anti-inflammatory also presented immunotoxicity, which is not an exclusion criterion in further studies using these samples.

The results of antioxidant activity tests confirm that P. venusta can be considered a natural source of antioxidants, and in both approaches the ethyl acetate fraction showed the highest antioxidant potential.

5. ACKNOWLEDGMENTS

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