Antioxidant effects of *Phyllanthus niruri* tea on healthy subjects

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**ABSTRACT**

**Objective:** To investigate the potential antioxidant effects of *Phyllanthus niruri* (*P. niruri*, Euphorbiaceae) tea on healthy subjects. **Methods:** Five non-smoking, male healthy volunteers, 20 to 31 years old, were enrolled. Each subject was treated twice, following a randomized crossover fashion regarding the ingestion of *P. niruri* infusion (5 g/750 mL) (tea group) or 750 mL of water (control group). Fasting venous blood samples were collected prior to and at 1, 2 and 4 h after infusion drinking. Samples were tested for plasmatic gallic acid and ascorbic acid levels, erythrocytic catalase and superoxide dismutase activities, and intracellular DCFH fluorescence in granulocytes, monocytes and lymphocytes. **Results:** Catalase and superoxide dismutase activities were not altered by tea ingestion. Plasma levels of gallic acid were significantly increased at 1, 2 and 4 h after *P. niruri* ingestion and plasma ascorbic acid at 1 h after *P. niruri* ingestion. **Conclusions:** Ingestion of *P. niruri* tea is associated with a slight increase in antioxidant markers in human blood (ascorbic acid and gallic acid), which may contribute to its pharmacological effects.

**Keywords:** Antioxidants, Polyphenols, *Phyllanthus niruri*, Human, Ascorbic acid, Gallic acid

1. Introduction

Oxidative stress has been implicated in various pathological conditions including cardiovascular diseases, cancer and aging[1]. Positive association between the consumption of polyphenol-rich foods and beverages and the prevention of these conditions has been shown by several epidemiological studies[2]. Tea is one of the most popular beverages consumed by humans and many different types have been shown to contain polyphenols[3–5], which are known for their antioxidant properties. Moreover, the reducing power of polyphenols associated with other dietary antioxidants can protect the body tissues against oxidative stress[6–8].

*Phyllanthus niruri* (*P. niruri*, Euphorbiaceae) is a plant widely found in Brazil, locally known as quebra-pedra (stone crusher) and is popularly used for the treatment of renal pathologies, particularly urolithiasis[9,10]. The medicinal properties of this plant have been associated with some of its active components such as lignans, glycosides, alkaloids, ellagitannins, terpenes and phenylpropanoids, besides flavonoids and polyphenols, such as quercetin,
rutin and gallic acid (GA)[11]. Although many in vitro
and in vivo antioxidant effects of P. niruri extracts have
been shown[11–13], which seem to be determined by its
polyphenolic components[11,14,15], the bioavailability of those
components and their impact on human health need further
investigation.

Recently, we have shown that P. niruri exhibits antioxidant
effects in in vitro and in vivo models of liver toxicity in
mice[11]. That raises the possibility that this plant can
have a broader antioxidant effect in mammals. Therefore,
considering the presence of GA, one of the most readily-
absorbed polyphenolic compounds from vegetables[15], and
the lack of studies investigating the effects of P. niruri tea
ingestion, we focused the current study on determining
the plasma levels of GA following the ingestion of the
teas. Additionally, we have also investigated the potential
antioxidant effects of P. niruri tea ingestion on healthy
subjects.

2. Materials and methods

2.1. Preparation of P. niruri infusion

To prepare the infusion, a commercially available brand
of P. niruri (Prenda®) tea was used. The phytochemical
composition of the commercial preparation was identical
to that of a known P. niruri voucher specimen (number
SMDB 13142 at the UFSM herbarium). The infusion was
prepared with five packets (5 g) in 750 mL of boiling water.
The infusion was allowed to cool for 15 min before being
given to the subjects. The volunteers were then instructed to
comsume the tea within 10 min.

2.2. Participants

The study protocol n.0034.0.243.000–10 was approved by
the Human Ethics Committee of the Universidade Federal
de Santa Maria and informed consent was obtained from all
of the participants. Five non-smoker, male subjects with no
history of previous chronic diseases and not under treatment
for any current chronic or acute diseases (as self declared
by the volunteers), average age of (27.2 ± 2.3) range
22.8–27.4 kg/m², were enrolled. Exclusion criteria included
smoking, high alcohol intake (above 20 g of alcohol/d),
consumption of vitamins, mineral supplements or the acute
or chronic use of any other licit or illicit drugs. Each subject
was tested twice following a randomized crossover design
regarding the ingestion of the P. niruri tea or the same
amount of lukewarm water. Fasting venous blood samples
were collected into 10 mL heparinized syringes at 0 (before
drinking), 1, 2 and 4 h after either tea or water ingestion.
Blood samples were always drawn between 8:00 am and 1:00
pm. The volunteers were instructed by a nutritionist to avoid
the consumption of flavonoid-rich (apples, grapes and their
derivatives such as wine and juices), phytate- or tannin–
containing (coffee, tea, chocolate, among others) alimentson
the day before and during the blood sampling period.

2.3. Sample processing and quantification of GA by HPLC

The blood samples were stored at −20 °C for up to 2 months.
The samples were then thawed on ice and centrifuged for
1 min at 10 000 r/min at 4 °C. The plasma fractions (1.5 mL)
were transferred to 15 mL plastic tubes containing 13.5 mL
of nitrogen–saturated methanol (HPLC grade) and stored
at −20 °C for up to 2 months. The tubes were vigorously
vortexed and then centrifuged at 1 200 r/min for 10 min, at
4 °C. The supernatants were decanted into glass tubes under
a stream of nitrogen and the tubes were screw–capped right
away[16]. The chromatographic analyses were carried out in
isocratic conditions using a RP–C18 column (4.6 mm × 250.0
mm) packed with 5 μm diameter particles. The mobile
phase consisted of methanol:acetonitrile:water (40:15:45,
v/v) containing 1% (v/v) acetic acid. Aliquots (20 μL) of
the clear methanolic plasma extract were injected and run at
0.8 mL/min with detection at 254 nm. The mobile phase
was filtered through a 0.45 μm membrane filter and then
degassed by ultrasound immediately before use. Reference
GA solutions were prepared in the HPLC mobile phase at
centration ranging from 0.006 to 0.250 mg/mL[17–18]. The
retention times of the peaks from the plasma samples were
compared to those obtained for the reference GA solutions
and quantitated by peak integration using the external
standard method. The calibration curve for GA was
\[ y = 53.985x + 0.206 \quad \text{(r=0.985 9)} \]
All chromatographic procedures
were performed at room temperature and in triplicate.

2.4. Determination of plasmatic ascorbic acid (AA)

Plasma AA levels were measured as described by Jacques–
Silva et al.[19]. Plasma was precipitated with one volume of
100 g/L cold trichloroacetic acid solution and centrifuged
at 3 000 r/min for 5 min at 4 °C. Aliquots (300 μL) of
the supernatants were mixed with 2,4-dinitrophenylhydrazine
(4.5 mg/mL), CuSO₄ (0.075 mg/mL) and 133 g/L trichloroacetic
acid (final volume 1 mL), and incubated for 3 h at 37 °C.
Then, 1 mL of 65% H₂SO₄ (v/v) was added to the medium. The
AA content was calculated using a standard curve (1.5–4.5 μmol/L
AA freshly prepared in sulfuric acid) and expressed as μ
mol of AA/mL of plasma.
2.5. Erythrocyte catalase (CAT) activity

CAT activity was measured by the method of Aebi\textsuperscript{[20]}. Packed erythrocytes (10 μL) were hemolyzed by adding 100 volumes of distilled water (990 μL). Then, 20 μL of the hemolysed red blood cells (RBC) were transferred to a 1 mL quartz cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mmol/L H\textsubscript{2}O\textsubscript{2} in 50 mmol/L phosphate buffer (pH 7.0) to a final volume of 1 mL. The rate of H\textsubscript{2}O\textsubscript{2} decomposition was measured spectrophotometrically at 240 nm for 120 s. CAT activity was expressed as μmol H\textsubscript{2}O\textsubscript{2}/mL packed RBC per minute.

2.6. Erythrocyte superoxide dismutase (SOD)

SOD activity was assayed spectrophotometrically as described by Boveris and Cadenas\textsuperscript{[21]}. This method is based on the capacity of SOD to inhibit the autoxidation of adrenaline to adrenochrome. Adrenaline autoxidation was monitored at 480 nm. One unit of enzyme activity is defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C.

2.7. Determination of ROS by flow cytometry

Intracellular H\textsubscript{2}O\textsubscript{2} was determined using DCF–DA (Sigma Chemical Co.) as described by Hasui \textit{et al.}\textsuperscript{[22]} with modifications. Intracellular peripheral blood mononuclear cells DCF–DA fluorescence was determined by flow cytometry (FACScalibur analyzer, BD Biosciences). The leukocytes were then isolated by mixing total blood with lysing solution (BD FACS\textsuperscript{TM}) as indicated by the manufacturer. The cells (1×10\textsuperscript{6}/mL) were washed twice with PBS (pH 7.4), centrifuged at 1 800 r/min for 5 min, and resuspended in ice-cold PBS. Cells were then incubated with DCF–DA (2 μmol/L final) for 30 min at 37 °C. Excess DCF–DA was removed by washing the cells once with PBS and centrifuging at 1 800 r/min for 5 min. At least 50 000 events were counted for each blood sample.

2.8. Statistical analysis

Data are expressed as mean±standard deviation (SD). Statistical analyses were performed using analysis of variance (ANOVA), followed by post hoc Duncan’s multiple range test. When appropriate, repeated measures (ANOVA) were used, followed by post hoc Duncan’s multiple range test. Results were considered significant when \(P<0.05\).

3. Results

GA is a compound found in plants both in a free and in a bound form. It is found in large amounts in \textit{P. niruri} leaves, from which it can be extracted by hot water infusions. HPLC analysis of the commercial \textit{P. niruri} tea indicated the presence of GA and an unknown related compound as the major components. The quantity of GA found in the different filtrates (tea of \textit{P. niruri} with hot water varied from 55 to 70 mg/mL (Figure 1). Plasma levels of GA were significantly increased at 1, 2 and 4 h after \textit{P. niruri} ingestion (paired comparisons of sample times 1, 2 and 4 h with baseline [before ingestion]) showing that GA was rapidly absorbed (Figure 2). No significant variation in GA levels, compared to baseline levels, was observed among control subjects [(1.32±0.15), (1.36±0.11), (1.40±0.11) and (1.36±0.15) μmol/L at 0, 1, 2 and 4 h, respectively]. The identity of GA in plasma was confirmed by the UV–VIS spectra of the corresponding standard peak of GA (Figure 3). In order to investigate whether these increases in plasma GA are associated with antioxidant effects, we determined the plasma AA levels, RBC CAT and SOD activities and peripheral blood mononuclear cell reactive oxygen species (ROS) formation by flow cytometry, before and after the ingestion of \textit{P. niruri} tea.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Chromatograms of standard gallic acid (A) and \textit{P. niruri} (B). The peak 1 corresponds to gallic acid and the peak 2 is from an undetermined compound. Chromatographic conditions are described in the experimental section.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Gallic acid levels in plasma after \textit{P. niruri} ingestion. Data are expressed as the differences between each time point minus the baseline values for each subject. *\(P<0.05\) compared with that before treatment (Wilcoxon test).}
\end{figure}
SOD (Table 1) activities were altered after *P. niruri* intake. On the other hand, a decrease in DCF–DA fluorescence was observed at 2 h after the ingestion of *P. niruri* tea in peripheral blood mononuclear cells (Figure 5).

![Figure 3](image-url)

**Figure 3.** Peak marked refers to the increase in the area after ingestion of *P. niruri* tea.

Peak marked (chromatograms after 1, 2 and 4 h post *P. niruri* ingestion) refers to the increase in the area of gallic acid after ingestion of *P. niruri* tea, when compared to the chromatograms after 1, 2 and 4 h post water ingestion. Peaks are indicated by arrows.

![Figure 4](image-url)

**Figure 4.** Plasmatic ascorbic acid after *P. niruri* ingestion.

Data are expressed as the differences between each time point minus the baseline values for each subject. *P*<0.05 compared with that before treatment (Wilcoxon test).

### 4. Discussion

*P. niruri* is commonly used in folk medicine to treat renal disease, particularly urolithiasis, and there is a clinical evidence supporting that *P. niruri* extracts can be an effective adjuvant in the treatment of renal calculus[11,23]. Of particular importance, urolithiasis has an inflammatory component, which is associated with oxidative stress[24]. Here, we detected only a modest antioxidant effect of *P. niruri* tea ingestion in healthy subjects, as indicated by a transitory increase in AA and by a sustained increase in plasma GA up to 4 h after tea ingestion. This increase might be related to the presence of AA in *P. niruri* extract[25]. Accordingly, experimental studies using rodents have shown that chronic ingestion of aqueous extracts of *P. niruri* enhances the cellular antioxidant defense system, including the levels of vitamin C, glutathione, as well as SOD, CAT, and glutathione peroxidase activities[26–27]. In contrast to vitamin C, in the present study, the activities of antioxidant enzymes (SOD and CAT) were not altered by the

<table>
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<th>Treatment time (h)</th>
<th>Catalase (μ mol H₂O₂/mL erythrocytes per minute) Control</th>
<th><em>P. niruri</em></th>
<th>SOD activity (IU) Control</th>
<th><em>P. niruri</em></th>
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Results are expressed as mean±SD.
ingestion of *P. niruri* tea. The absence of alteration in those enzymes activities might be related to the fact that *P. niruri* was given as a single dose. Recently, Ferk et al reported that supplementation of drinking water with GA in humans for 3 d caused a reduction of DNA damage in lymphocytes of healthy individuals[28]. That effect was paralleled by an increase in the activities of antioxidant enzymes, whereas the total antioxidant capacity and malondialdehyde levels in serum were not modified. Other studies have shown the modest transient increases in plasma antioxidant capacity after tea ingestion in humans[29]. Thus, although the plasma level of antioxidants can be expected to influence the intracellular antioxidant capacity of different cell types[30], this direct correlation does not always occur. Taken together, these results indicate that an overlap between extra- and intra-cellular antioxidant capacity is not always the case.

Here, we have observed a significant, though modest, decrease in DFC–DA fluorescence in monocytes, but no increase in the activities of antioxidant enzymes, whereas significant alteration was detected in granulocytes or modest transient increases in plasma antioxidant capacity for 3 d caused a reduction of DNA damage in lymphocytes of healthy individuals[28]. That effect was paralleled by an increase in circulating GA. Therefore, *P. niruri* tea ingestion seems to be associated with a modest increase in antioxidant markers in human plasma. However, further studies are required to determine the effects of chronic ingestion of *P. niruri* and its potential as an antioxidant modulator of peripheral blood oxidative stress in healthy human subjects and whether or not this can be involved in the therapeutic effects of this plant.

**Conflict of interest statement**

The authors declare no conflicts of interest.

**Acknowledgments**

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**References**


