

***Protium neglectum*, *podocalyx loranthoides*, and *brosimum utile* source of flavonoids and other phenolic compounds with antioxidant activity**

***Protium neglectum*, *Podocalyx loranthoides*, y *Brosimum utile* Fuente de flavonoides y otros compuestos fenólicos con actividad antioxidante**

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Resumen

En los últimos años se ha enfocado el interés en el uso de los antioxidantes que protegen el organismo del daño oxidativo producido por los radicales libres los cuales juegan un papel importante en ciertas enfermedades. Existe información sobre la presencia de antioxidantes naturales en algunas plantas medicinales. En este trabajo se evaluó el contenido de polifenoles totales, flavonoides y taninos así como su actividad antioxidante en tres plantas medicinales venezolanas *Brosimum utile*, *Protium neglectum* y *Podocalyx loranthoides*. Los compuestos fenólicos fueron determinados por métodos espectrofotométricos específicos y las propiedades antioxidantes con los métodos del radical 1, 1-difenil-2-picrilhidracil, el poder reductor del ferricianuro-cloruro férrico y la decoloración del β -caroteno. Los resultados muestran que las plantas estudiadas son mas ricas en compuestos fenólicos que las frutas y vegetales, presentando un contenido de polifenoles totales en el rango de 61.83-125.31 mg-equivalentes de ácido gálico/g muestra en base seca, y los flavonoides y taninos en los rangos de 8.92-30.72 y 3.91- 44.83 mg-equivalentes de rutina y catequina/g de muestra en base seca, respectivamente. Todas las plantas presentaron actividad antioxidante que puede estar relacionada con sus propiedades medicinales como resultado de la presencia de compuestos fenólicos, de ciertas estructuras características de algunos de ellos y la concentración de taninos. Estas plantas se pueden considerar como buenas fuentes de antioxidantes para uso medicinal y comercial en la industrial farmacéutica, cosmética y alimentaria.

Palabras clave: *Protium neglectum*, *Podocalyx loranthoides*, *Brosimum utile*, flavonoides, actividad antioxidante

Abstract

Great attention has been focused on the use of antioxidants, to protect the human body from the oxidative damage by free radicals, which play an important role in human diseases. It has been reported that some medicinal plants contain a wide variety of natural antioxidants. Total polyphenols, flavonoids and tannins content, and the antioxidant properties of three Venezuelan medicinal plants *Brosimum utile*, *Protium neglectum* and *Podocalyx loranthoides* were evaluated. General methods were used for assessment of the phenolic compounds, while the antioxidant properties are investigated using the 1,1-diphenyl-2-picrylhydrazyl the ferricyanide and β -carotene bleaching methods. Results show that these plants are richer than fruits and vegetables in polyphenolic compounds, presenting a total phenolics range of 61.83–125.31 mg gallic acid equivalents/g dry weight, flavonoids and tannins range were 8.92–30.72 and 3.91–44.83 mg rutin and catechin equivalents/g dry weight, respectively. All plants demonstrated significant antioxidant activity which might be related to the medicinal properties as a result of polyphenolic compounds, the structure characteristic of some active phenolics and the concentration of tannins. These plants can be considered as good sources of antioxidants for medicinal and commercial uses in the pharmaceutical, cosmetic and food industry.

Keywords: *Protium neglectum*, *Podocalyx loranthoides*, *Brosimum utile*, flavonoids, antioxidant activity.

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Introduction

The antioxidant activity of fruits and vegetables is correlated mainly with their contents of polyphenols, carotenoids and vitamins C and E (Cadenas & Packer, 2002). Thus much attention has been focused on the use of antioxidants, specially natural antioxidants, to inhibit lipid peroxidation or to protect the human body from the oxidative damage by free radicals. Free radicals can be generated by metabolic pathways within body tissues; they can be introduced by external sources, with food, or drugs, can be caused by environmental pollution, etc. Oxidative damage caused by free radicals plays a significantly pathological role in human diseases. Cancer, emphysema, cirrhosis, atherosclerosis, and arthritis have been correlated with oxidative damage (Halliwell and Gutteridge, 1984; Anderson et al., 2001).

Among antioxidants, polyphenols constitute a large and complex category of compounds. They include flavonoids, the largest and most-studied group of polyphenols. It has been reported that some medicinal plants contain a wide variety of natural antioxidants, such as phenolic acids, flavonoids and tannins, which possess more potent antioxidant activity than dietary plants (Wong et al., 2006), and can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.

Nevertheless, scientific information on antioxidant properties of various plants, particularly those that are less widely used as foodstuffs and medicine, is still rather scarce.

Although it remains unclear which of the compounds of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities and total antioxidant potential (Djeridane et al., 2006; Katalinic et al., 2006; Wong et al., 2006). Therefore, the assessment of such properties is interesting and useful, to find new sources of natural antioxidants, development of functional foods and nutraceuticals (Miliauskas et al., 2004).

The Amazon Region of Venezuela has a great variety of exotic food and medicinal plants with antioxidant activity, whose characterization could present some potential for sustainable non destructive forest-product development in Amazonia.

Protium neglectum, known in Venezuela as «curucay» or «taca-majaca», is a plant belonging to the genus *Protium* (Burseraceae) which is known for its ability to produce oleoresin exudates, which occur as a result of injuries to the bark (Siqueira et al.,

1995). The essential oil from leaves and resin of some *Protium* species as well as the leaves of *Podocalyx loranthoides* (Euphorbiaceae, *sensu lato*) known also as «palo de agua» or «reventillo», have been reported to have medicinal uses for the management of certain inflammatory diseases (Dowiejua et al., 1993; Siani et al., 1999).

The resins and leaves of *Protium* species are also commonly used in Brazilian folk medicine for the healing of ulcers and for the treatment of inflammatory complaints (Correa, 1984). The leaves of *Podocalyx loranthoides* are used in traditional medicine, in the Venezuelan Amazonas State, for some digestive problems and as a wound healer.

Brosimum utile (Moraceae), called «palo de vaca», «lechero», «vaca», «vacuno» is another oleoresin producing tree, found from Costa Rica, Brazil, Colombia, Venezuela, Ecuador, to Peru. As with the other two plants, it has been used in folk medicine in Amazonia for treatment of asthma and other respiratory diseases.

These genera (*Brosimum*, *Protium* and *Podocalyx*) contain flavonoids which have beneficial health effects in the prevention of cardiovascular diseases, certain diseases associated with oxidative stress, some cancer types, as an anti-inflammatory, and, during women's menopause, prevention of osteoporosis (Torres et al., 1997; Suárez et al., 2003).

Although, a multitude of natural antioxidants have been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Ramarathnam et al., 1995). However, there is a lack of information with regard to polyphenolic content and antioxidant properties of extracts of *Protium neglectum*, *Podocalyx loranthoides* and *Brosimum utile*. The aim of the study of these Venezuelan plants used in folk medicine was to evaluate the *in vitro* antioxidant properties, including antioxidant activity, reducing power, scavenging abilities on radicals, and to link these properties with their content of phenolic compounds.

Experimental

SAMPLE PREPARATION

All samples were collected in Venezuela. *Brosimum utile* bark in Cerro de Valle Seco, Puerto Cabello, Carabobo State; *Podocalyx*'s leaves close to the Sipapo River, Puerto Ayacucho, Amazonas State, and *P. neglectum* leaves in Maturín, Monagas State. Samples were cleaned, identified botanically and deposited at the Herbarium V.M. Ovalles (MYF), Facultad de Farmacia, Universidad Central de Venezuela (UCV).

They were cleaned, dried in a convection oven at 60°C for 48h, milled, sieved through 60 mesh sieves, and stored in plastic jars at ambient temperature.

EXTRACTION

One (1) g of each sample was extracted at room temperature for 60 min with constant agitation, first with 40 ml of acidified methanol-water (50:50 v/v) and after with 40 ml of acetone-water (70:30 v/v). After centrifugation (15 min, 3000 rpm) supernatants were combined and diluted with a 50:50 (v/v) mixture of the two previous extracting solutions to 100 ml. Sample extracts were used to assess the different types of phenolics and the antioxidant properties.

All reagents were analytical grade and extraction and measurements were done in triplicates. Absorbance readings were done in a Shimadzu UV/VIS spectrophotometer model UV-1700, Kyoto, Japan.

TOTAL PHENOLIC CONTENT

The total phenolic content was determined using the Folin-Ciocalteu method (Singleton et al., 1999), and slightly modified by Dewanto et al. (2002). To 125 µL of the suitably diluted sample extract 0.5 mL of deionised water and 125µL of the Folin-Ciocalteu reagent were added. The mixture was allowed to stand for 6 min and then 1.25 mL of a 7% aqueous Na₂CO₃ solution was added. The final volume was adjusted to 3 mL. The mixture was allowed to stand 90 min and the absorption was measured at 760 nm against water as a blank. The amount of total phenolics was expressed as gallic acid equivalents (GAE mg /g dried sample). The calibration curve range was 20–500 µg/mL.

TOTAL FLAVONOIDS

Flavonoid content in extracts was determined spectrophotometrically (Miliauskas et al., 2004) using rutin as a reference compound; this method is based on the formation of a complex with aluminum. One ml of plant extract was mixed with 1 ml AlCl₃ in ethanol (20 g/L) and diluted with methanol to 25 ml. The absorbance of the reaction mixture was measured at 415 nm after 40 min at 20°C. Blank of samples were prepared taking 1 ml plant extract and 1 drop acetic acid, and diluted to 25 ml. The absorbance of a rutin calibration curve in the range 20–200 µg/mL in methanol was measured under the same conditions. Flavonoids are expressed as rutin equivalents (RTE mg/g dried sample)

TOTAL CONDENSED TANNINS

The analysis of condensed tannins (procyanidins)

was carried out according to the vanillin modified method (Broadhurst and Jones, 1978; Heimler et al., 2005). To 50µL of the suitably diluted sample, 3 mL of a 4% vanillin methanol solution and 1.5 mL of HCl were added. The mixture was allowed to stand for 15 min and the absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as (+) catechin equivalents (mg CTE /g dried sample). The calibration curve ranged from 50-600 µg/mL.

Antioxidant Properties

REDUCING POWER:

This was determined by the method of Yen and Duh (1993). Briefly, 1 ml of extract in an appropriate solvent was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml (1 %) K₃Fe (CN)₆ solution. After 30 min at 50°C, 2.5 ml (10 %) trichloroacetic acid (TCA) was added and the mixture was centrifuged for 10 min (2000 rpm). Finally, a 2.5 ml aliquot was mixed with 2.5 ml ultra-pure water and 0.5 ml (0.1%) FeCl₃ and the absorbance was recorded at 700 nm, comparing absorbance reading versus an ascorbic acid standard curve in the range of 50-500 µg/ml, since ascorbic acid is a well characterized natural reducing agent. Results are expressed as AsAE (mg ascorbic acid equivalents/g dry weight (dw)).

DPPH RADICAL SCAVENGING ASSAY

Free radical scavenging activity of plant extracts against stable DPPH• (1,1-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich St. Louis, MO) was determined spectrophotometrically. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep-violet to light-yellow) were measured at 515 nm on a UV/visible light spectrophotometer. The antiradical capacity of the sample extracts was estimated taking 0,025mL of sample extracts suitably diluted that were added to 0.975 mL DPPH• solution (60 µM in MeOH), and the absorbance was read at *t* = 0 and at regular time intervals at 515 nm, until the reaction reach a plateau (time at the steady state) (Arnaous et al., 2002). The antioxidant dose required to cause a 50% inhibition (antioxidant activity) is expressed as EC₅₀ (g sample/g DPPH•); TEC₅₀, time needed to reach the steady state to the concentration corresponding at EC₅₀ was calculated, and AE was also calculated (Sanchez-Moreno, et al., 1998); also the radical scavenging activity was calculated by the following formula:

$$\% \text{ inhibition} = ((A_B - A_A)/A_B) \times 100$$

Where A_B = absorbance of blank sample (t=0min); A_A = absorbance of tested extract solution (t=30 min).

β-Carotene/Linoleate

The β-carotene bleaching method (Suja et al., 2005) was used; in brief, 0.2 mg β-carotene in 1mL chloroform, 20 mg of linoleic acid and 200mg of Tween 40 were transferred into a 50 mL volumetric flask. Once the chloroform had been removed by nitrogen flush, distilled H₂O was added and the resulting mixture was stirred vigorously. 4 mL aliquots of the emulsion were transferred to test tubes containing either a) 0.2 mL of sample extract, b) 0.2 mL of 100ppm butyl hydroxyanisol (BHA) methanol solution for comparison, c) 0.2 mL of methanol as a control, and d) 0.2mL methanol and 4mL of an emulsion prepared without the addition of β-carotene as a blank. After mixing, the absorbance (Abs t=0) at 470nm was recorded. All tubes were placed in a water bath at 50°C, taking readings at 15 min intervals until the β-carotene color in the control tube was bleached. Antioxidant activity was measured by the antioxidant activity coefficient (AAC), which is an estimation of the relative oxidation in the presence and absence of extracts:

$$AAC = \frac{(\text{Sample Abs}_{(t=100)} - \text{Control Abs}_{(t=100)}) / \text{Control Abs}_{(t=0)} - \text{Control Abs}_{(t=100)}}{1000}$$

The antioxidant activity (AA) is expressed as percent inhibition relative to the control after 100 min. incubation using the following equation: $AA = 100 (DR_C - DR_S) / DR_C$ where AA is the antioxidant activity, DR_C is the degradation rate of the control ($=\ln(a/b)/100$), DR_S is the degradation rate in the presence of the sample ($=\ln(a/b)/100$), a is the initial absorbance at time 0, and b is the absorbance at 100 min.

Linoleic acid in the presence of oxygen forms a hydroperoxide radical which produces the β-carotene oxidation and its bleaching with time. The antioxidant activity (AA) is evaluated in terms of β-carotene bleaching using BHA for comparative purposes.

Statistical Analysis

All data on all antioxidant activity tests are the average of triplicate analyses. The data were recorded as mean ± SD. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by student's-t test, p values < 0.05 were considered as significant.

Results and Discussion

Polyphenolic composition is presented in Table I. Phenolic compounds are known as powerful chain-

Table I
Polyphenolic composition of three Venezuelan medicinal plants

Plant	Polyphenols GAEmg/g d.w	Flavonoids RTE mg/g d.w	Tannins CTE mg/g d.w
<i>Brosimum utile</i>	61.83 ± 4.12 ^a	8.92 ± 0.32 ^a	3.91 ± 0.40 ^a
<i>Protium neglectum</i>	85.15 ± 2.49 ^b	30.72 ± 0.66 ^b	4.10 ± 0.51 ^a
<i>Podocalyx loranthoides</i>	125.31 ± 3.04 ^c	25.47 ± 1.70 ^{bc}	44.83 ± 2.79 ^b

GAE: Gallic acid equivalents, RTE: Rutin equivalents, CTE: Catechin equivalents. Means with different letters in the same column were significantly different at the level p<0.05.

breaking antioxidants (Shahidi and Wanasundara, 1992). Phenolic compounds are very important constituents of plants and their radical scavenging ability is due to their hydroxyl groups (Hatano et al., 1989). The phenolic compounds may contribute directly to antioxidant action (Duh et al., 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka et al., 1998).

Total phenolic content presented a range of 61.83–125.31 GAEmg/g (d.w), while the flavonoids and condensed tannins ranges were 8.92–30.72 and 3.91–44.83, RTE and CTE mg/g (d.w) respectively. *P. loranthoides* presented the highest content of all polyphenolic compounds tested. Data on polyphenolic composition of these plants was not found in the literature.

When these results are compared to total phenolic, flavonoid, and tannin content reported for *Brassicaceae* edible plants (white cabbage, broccoli, kale, cauliflower and Brussels sprouts) (Heimler et al., 2005), it was found that the plants studied showed much higher values for all compounds. These results show that these plants are rich in total polyphenols, flavonoids and tannins.

Antioxidant Properties

There are several methods for assessment of antioxidant activities. Plant extracts are a mixture of different phenolic compounds with different functional groups, polarity, and chemical behavior, which could lead to scattered results, depending on the test used. Therefore, the use of several assays for evaluating the antioxidant potential of extracts would be more informative and even necessary, to understand the biological activity of an antioxidant (Cao and Prior, 1998). In this study the ferric reducing power,

DPPH• radical scavenging activity, and the β -carotene bleaching methods were used. The results of the antioxidant properties are presented in Tables II and III.

Table II
Antioxidant properties of Venezuelan medicinal plants as assayed by the reducing power and β -carotene bleaching method

Sample	Antiox. Activ. Coef. (AAC)	Antiox. Activ. (Aa)	Red. Power AsAE mg/g
<i>Brosimum utile</i>	413.07	61.11	59.71±0.37 ^a
<i>Protium neglectum</i>	702.06	79.82	62.75±2.05 ^b
<i>Podocalyx loranthoides</i>	538.55	70.35	60.33±2.78 ^{ab}
BHA	673.19	85.61	

Means with different letters were significantly different at the level $p < 0.05$

AsAE: Ascorbic Acid Equivalents

Table III
Antioxidant properties *in vitro* as scavenging abilities on radicals

Sample	EC ₅₀ g/gDPPH	%TEC ₅₀ min	AE	% Inhibition
<i>Brosimum utile</i>	1,28	27,60	0,028	82,95
<i>Protium neglectum</i>	0,64	29,97	0,05	85,44
<i>Podocalyx loranthoides</i>	0,18	29,35	0,185	86,68
DL- <i>-tocopherol</i>	0,201 ^a	9,52 ^a	0,52 ^a	89,76 ^b
BHA	0,093 ^a	103,85 ^a	0,10 ^a	73,44 ^b

^aSanchez-Moreno et al., 1998; ^bÖnay-Uçar et al., 2006. EC₅₀: Amount of antioxidant (g of sample) necessary to decrease 50% the initial DPPH• concentration. TEC₅₀: Time needed to reach the steady state to the concentration corresponding at EC₅₀. AE: Anti-radical Efficiency.

REDUCING POWER

The reducing properties are generally associated with the presence of reductones (Duh, 1998; Tanaka et al., 1998) as these species are known to be free radical chain terminators (Gordon, 1990). Therefore, it was considered important to determine the reductive capacity of these three medicinal plant extracts as this may indicate their potential as antioxidants. The extracts were capable of reducing Fe³⁺ ions, as can be seen in Table II, and all plants have similar effectiveness at reducing Fe³⁺, with ascorbic acid equivalents (AsAE) values in the range of 59.71-62.75 AsAE mg/g, even though there were significant differences between *Brosimum* and the other two plants. Accordingly, *Podocalyx* and *Protium* might contain higher

amounts of reductones, which could react with free radicals to stabilize and block chain reaction through donating a hydrogen atom. These results are higher than the ones reported, for methanol extracts of *Draconocephalum moldavica* L. (Lamiaceae) (41.74 mg AsAE/g) (Dastmalchi et al., 2007), that characterized their extracts as having mild reducing activity in comparison with values presented by reference compound such as gallic acid and quercetin (1,333.30 and 794.35 mg AsAE/g). These results suggest that these plants might be potential preservatives that could be used in foods or cosmetics.

DPPH RADICAL SCAVENGING ASSAY

A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades or disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via free-radical attack on the DPPH molecule) and convert them to a colorless/bleached product, resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases the more potent the antioxidant activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. When the antioxidant activity is expressed as TEC₅₀ (time which the antioxidant requires to cause a 50% inhibition of the DPPH•), the values found allow classifying the antioxidants in these plants as "intermediate" taking into account the kinetic classification proposed for some polyphenolic compounds (Sanchez-Moreno et al., 1998). This classification also considers butylhydroxyanisole (BHA), a synthetic antioxidant used in the oxidation prevention of fats and oils, as a «slow» and α -tocopherol as an intermediate antioxidant.

From the percentage scavenging values (% inhibition), it can be seen that all plant extracts presented similar scavenger activity. All of the extracts were more effective than quercetin (73.87%), much less effective DPPH• scavengers than ascorbic acid (AsCA) (103.57%) or gallic acid (GA) (102.27%) (Dastmalchi et al., 2007). Nevertheless, they presented higher effectiveness than BHA (73.76%) and were as effective as DL- α -tocopherol (89.76%) (Önay-Uçar et al., 2006). Moreover, taking in consideration values for the EC₅₀ and the anti radical efficiency (AE), the best of all plants as a radical scavenger was the *P. loranthoides*, which also presented the highest polyphenol and tannin content. This difference could be due to differences in structure conformation and hydroxyl group positions on the flavonoid molecules, which

determine antioxidant properties (Miliauskas et al., 2004; Choi et al., 2002), and also to the presence of two biflavonoids I 7, II 4-dimethylamentoflavone and II 4'-methylamentoflavone (Suárez et al., 2003). In general these properties depend on the ability to donate hydrogen or an electron to a free radical.

The DPPH• free radical scavenging activity suggests that components within the extracts are capable of scavenging free radicals via electron or hydrogen-donating mechanisms and, thus, should be able to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices, e.g. biological membranes.

β-CAROTENE/LINOLEATE

In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by antioxidants from the extracts. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts. There is an indirect correlation between degradation rate and the bleaching of β-carotene, where the extract with the lowest degradation rate exhibited the highest antioxidant activity. *Brosimum* extract presented the lowest antioxidant activity while the other two plant extracts showed antioxidant activity almost similar to BHA (Fig. 1). Nevertheless, *Podocalyx*, which presented the highest polyphenolic content, did not show the best antioxidant activity, this might be related to the high tannin content. Good correlation was shown with the reducing power assay.

Conclusions

The results presented in this study are the first information on the antioxidant activities of these Venezuelan plants.

These results revealed that the medicinal plants studied exhibit clearly a higher antioxidant activity and contain more phenolic compounds than common vegetables and fruits. Due to differences in the results of the antioxidant properties of the plants by the methods used it can also be concluded that the antioxidant activity of plant extracts is not only the result of polyphenol, and flavonoid content, but may also be related to the presence of some individual active phenolic compounds and concentration of tannins. These plants can be considered as good sources of natural antioxidants for medicinal and commercial uses, and the beneficial effects against

some diseases could be attributed to the antioxidant activity and polyphenol content.

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