Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **29** (5): 783-8 (2010)

Original Article

Received: June 17, 2009
Accepted: December 24, 2009

Antioxidant Activity and Acute oral Toxicity of *Phrygilanthus acutifolius* Flowers

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SUMMARY. **Antioxidant activity of ethanol and water extracts and infusion of the flowers of *Phrygilanthus acutifolius* were evaluated. Total phenolic contents were determined using Folin- Ciocalteau reagent. The ethanol extract was more effective in scavenging radical 1,1 biphenyl -2-picrylhydrazyl (DPPH), with lower IC50 values. The water and ethanol extracts were the most effective antioxidants based on the inhibition of lipid peroxidation (β carotene bleaching method). The acute toxicity test in rats indicated that oral administration of 3.200 mg/Kg of flowers produced neither mortality, nor changes in behaviour or any other physiological activities. There were no significant differences in the body and organ weight between controls and treated animals. In blood chemistry analysis, aspartate transaminase, alanine transaminase, and alkaline phosphatase activities, no significant changes occurred. In view of the doses consumed empirically in traditional medicine of Amaicha del Valle, Tucumán, Argentina, there is a wide margin of safety for the therapeutic use of *Phrygilanthus acutifolius* flowers.**

INTRODUCTION

Phrygilanthus acutifolius, currently named Thrypodanthus acutifolius, is a member of the Loranthaceae family. The flowers are commonly used in folk medicine for throat pain, respiratory infections and hypertensive diseases. Recently, our research group demonstrated antibacterial [1], antiinflammatory, antinociceptive, antipyretic [2] and diuretic activities [3] in the flower extracts.

Many herbal medicines are believed to have preventive effects on chronic diseases due to their scavenging on antioxidant properties [4]. In herbal products, phenolic compounds have been shown to be effective antioxidant constituents. Many phenolic compounds exert more powerful in vitro activity than vitamin E and inhibit lipid peroxidation by chain-breaking peroxil-radical scavenging.

The reactive oxygen species (ROS) and free radicals are known to play a causative role in the tissue inflammation. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA. The oxidative damage is a crucial etiological factor implicated in several chronic diseases such as hypertension [5].

Despite the wide use of *Phrygilanthus acutifolius* flowers in folk medicine of the northwest of Argentine, no study has been published in the scientific literature about its toxicological profile. Considering the ethnopharmacological applications of the plant, the goal of this work was to investigate the possible toxic effects on rats.

MATERIAL AND METHODS

Plant material

The plant materials used in this study consisted of the flowers of *Phrygilanthus acutifolius* (Ruiz & Pav.) Eichler (Corpus) (Loranthaceae) collected by Mag. Adriana Daud during the flowering season (May–June) in Amaicha del Valle, in the province of Tucumán, Argentina.

KEY WORDS: Acute toxicity, Antioxidant activity, *Phrygilanthus acutifolius*.

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The voucher herbarium (N° 511125) specimen was identified by using morphological, anatomical and histochemical techniques, and by comparison with an identified specimen at the Miguel Lillo herbarium, Tucumán, Argentina.

Preparation of extracts

Ethanol and aqueous extracts and infusions were prepared from the flowers of the plant under study. The dried materials were milled into particles of about 5–10 mm in diameter. To prepare the ethanol extracts, 20 g of flowers were soaked for 5 days in 100 ml of 70 % ethanol. For the aqueous extracts, the flowers were soaked for 2 days in 100 ml of water. The infusion was prepared by extracting 20 g of dried flowers with boiling water (200 ml) for 15 min. All extracts were filtered through Whatman Paper No. 1 and evaporated by a lyophilizer to dryness. All extracts were dry-stored in sterile conditions at 4 °C until used.

Determination of total phenol content

Total phenolic content was estimated by the Folin–Ciocalteu method [6] with some modifications. Two hundred microlitres of diluted sample were added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. 4 minutes later, 800 µl of saturated sodium carbonate (7.5 %) was added and after 30 min of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0-10 mg/l) was used for the standard calibration curve. The results were expressed as mg gallic acid equivalent (GAE)/g dry weight, and calculated as mean value ± SD (n = 3).

Antioxidant test

DPPH scavenging activity

The antioxidant activity was assessed by the measurement of the scavenging ability of extracts towards the stable free radical 1,1 biphenyl -2-picrylhydrazyl (DPPH). The radical DPPH is reduced to the corresponding colourless hydrazine upon reaction with hydrogen donors [7]. After dissolving the samples in ethanol, aliquots of 0.75 ml (1.56-200 µg/ml) were added to 0.25 ml of an ethanol solution of DPPH (300 µM). The mixtures were vigorously shaken and left to stand at room temperature for 20 minutes in the dark [8,9]. Absorbance at 517 nm was measured versus ethanol as a blank. Quercetin (1.56-200 µg/ml), a natural antioxidant and butylated hydroxytoluene (BHT) (1.56-200 µg/ml), a synthetic antioxidant one, were used as reference solutions. All experiments were carried out in triplicate.

The degradation of DPPH was evaluated against a control (0.25 ml of DPPH solution and 0.75 ml ethanol 96 %). Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined by the equation [**1**] [10]:

$$AA\ \% = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad [\mathbf{1}]$$

β-carotene bleaching method

The antioxidant activity of extracts from *Phrygilanthus acutifolius* flowers was evaluated using β–carotene–linoleate model system, as described by Sun & Ho [11]. Two milligrams of β–carotene were dissolved in 10 ml chloroform and 1 ml of β–carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween 20 as emulsifier. Chloroform was then evaporated at room temperature under vacuum at reduced pressure using a rotary evaporator and the resulting mixture was immediately diluted with 50 ml of distilled water. 4.8 ml aliquots of this emulsion were transferred into different test tubes containing 0.2 ml of plant extracts (200 µg/ml) or the reference antioxidants (Quercetin and BHT) and mixed well. The absorbance at 470 nm, which was regarded as to, was measured immediately against a blank consisting of the emulsion without β–carotene. The caped tubes were placed in a water bath at 50 °C and the absorbance was measured every 20 min up to 120 min. Quercetin or BHT were used as positive control and distilled water or solvent were the negative control. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β–carotene by using the equation [**2**]:

$$AA = [1 - (A_0 - At\ /\ A_0^0 - A_t^0)] \times 100 \quad [\mathbf{2}]$$

where A_0 and A_0^0 were the absorbance values before incubation for test sample and control, respectively. A_t and A_t^0 were the respective absorbances of the test sample and the control after incubation for 120 min. The results were expressed as % of the prevention of bleaching of β–carotene [12].

Animals

Wistar male rats weighing 190 to 240 g were used for this study and were obtained from the Bioterio of the Facultad de Bioquímica, Química y Farmacia, Instituto de Biología (INSIBIO). All

animals were kept under normal laboratory conditions of humidity, temperature (25 ± 1 °C) and light (12 h day: 12 h night), and allowed free access to food and water ad libitum for at least 5 days. The studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

Acute toxicity

Healthy rats male that had fasted overnight, but which were allowed access to water *ad libitum* were randomly divided into eight groups (n = 5). Groups 1-2 (negative controls group) received orally water and 70 % ethanol, respectively. Groups 3 to 5 were orally treated with *Phrygilanthus acutifolius* ethanol extract at the doses of 128, 1600 and 3200 mg/kg body weight, respectively. Groups 6 to 8 were orally treated with *Phrygilanthus acutifolius* aqueous extract at the doses of 128, 1600 and 3200 mg/kg body weight, respectively.

Observations were made and recorded systematically 1, 2, 4, 6, 24 and 48 h after the administration of the test substance. Visual observations included changes in the skin and fur, eyes and mucous membranes, and also respiration.

Animals were observed for general behavioural and body weight changes, hazardous symptoms and mortality for a period of 48 h after treatment. All animals were euthanized with diethyl ether at 48 h and various tissues (kidneys, liver, heart, lungs, stomach, spleen) were isolated, weighed and visually inspected for any histopathological changes.

Hematological and biochemical analysis

Blood samples were assayed for glucose, creatinine, urea, total protein, albumin, total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

Haematological analyses were performed using an automatic haematological analyzer (ALCYON analyzer ISE y AXSYM System-ABOTT). The parameters determined included: red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb) and platelets count.

Statistical analyses

Values reported were expressed as mean ± S.E.M. A Student test p value higher than 0.05 was considered insignificant.

RESULTS AND DISCUSSION

Total phenol content

Total phenol content (TPC) was determined in comparison with standard gallic acid and the result was expressed in terms of mg GAE/g dry weight. The phenolic content of the ethanolic extract was 10.0 ± 0.3 mg/g, the water extract was 8.23 ± 0.16 mg/g and the infusion was 3.25 ± 0.65 mg/g, all as mg GAE/g dry weight.

The extracts and infusion of the *Phrygilanthus acutifolius* flowers showed a range of variation with respect to total phenolic content, compared to the proposed scale by Hua-bin *et al.* [6] and Vasco *et al.* [13], with a medium content (5-10 mg GAE/g) for ethanol and water extracts and a low value (<5 mg GAE/g) for the infusion.

Phenolics compounds are widely distributed in plants and have gained much attention due to their antioxidant activities and free radical scavenging abilities, which potentially had beneficial implications for human health [14].

DPPH radical scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples 15. Antioxidants, when interacting with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The color changes from purple to yellow and its absorbance at wavelength 517 nm decreases.

All the extracts were capable of scavenging DPPH radicals in a concentration-dependent manner. Figure 1 shows the dose response curves of DPPH radical scavenging activities of the extracts from *Phrygilanthus acutifolius* flowers. The scavenging effect of the ethanol, water and infusion extracts on DPPH radicals increased with increasing concentrations from 1.56 to 200 µg/ml and was 89.72 ± 1.25 %, 86.35

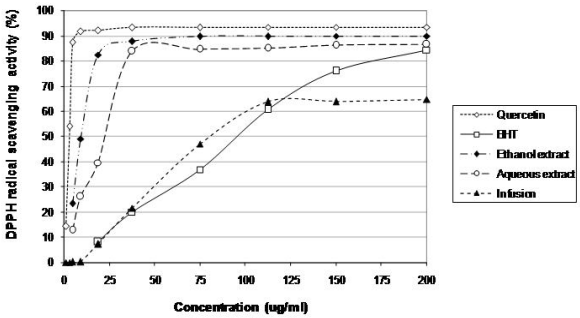

Figure 1. DPPH radical scavenging activity of the extracts of *Phrygilanthus acutifolius*'s flowers. Quercetin and BHT were used as reference antioxidants. Values are means ± SD (n = 3).

± 0.30 %, 64.15 ± 2.35 % at a concentration of 200 µg/ml, respectively. These results indicate that the ethanol and water extracts showed similar DPPH radical scavenging activity, while the infusion extract was a considerably less effective DPPH as radical scavenger. The scavenging effect of the standards, BHT and Quercetin were 84.50 ± 1.55 % and 93.43 ± 0.25 % at a concentration of 200 µg/ml, respectively.

The effective concentration (IC 50), defined as the concentration at which the DPPH radicals were scavenged by 50 %, was lowest for the ethanol extract measured at 9.6 ± 0.1 µg/ml, followed by the water extract at 24.5 ± 1.5 µg/ml and the infusion at 77.0 ± 2.5 µg/ml. IC 50 of the antioxidant controls were 2.10 ± 0.25 µg/ml for Quercetin and 80.00 ± 2.50 µg/ml for BHT. It was seen that ethanol extract had the highest DPPH radical scavenging activity, as shown by the lowest value of IC 50, while the infusion extract had the least activity. The IC 50 values for our materials were found to be Quercetin < Ethanol < water < infusion < BHT.

The present experiment show that the *Phrygilanthus acutifolius* extracts are a good scavenger of DPPH *in vitro,* demonstrating that their compounds may contribute to neutralize the oxidant agents produced during inflammatory states.

Antioxidant activity determined by β-*carotene bleaching method*

We have also evaluated the antioxidant potential of the extracts from flowers of *Phrygilanthus acutifolius* by the β-carotene bleaching method. Figure 2 shows the decrease in absorbance of β-carotene emulsion in the presence of either 200 µg/ml of the extracts or the reference antioxidants (BHT and Quercetin).

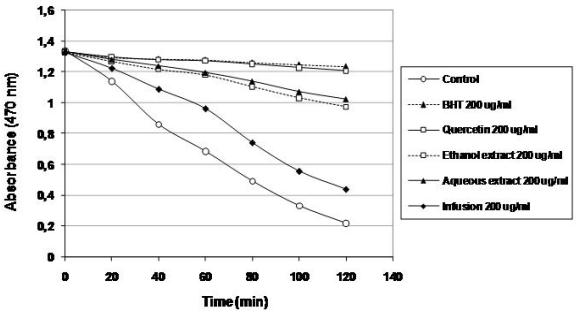

Figure 2. Antioxidant activities of ethanol, water and infusion extracts (200 µg/ml) from *Phrygilanthus acutifolius*'s flowers measured by β-carotene bleaching method. Quercetin and BHT were used as reference antioxidants. Values are means ± SD (n = 3).

The addition of *Phrygilanthus acutifolius* extracts, BHT and Quercetin at 200 µg/ml was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene, in comparison with the control ($p < 0.05$), which contained no antioxidant component. The water (75.85 ± 0.91 %) and ethanol (69.24 ± 2.75 %) extracts were most effective, whereas the effects of infusion (20.93 ± 1.90 %) were not as strong. Both extracts were less effective than BHT (91.40 ± 2.37 %) and Quercetin (91.30 ± 2.37 %), at the same concentration. The results indicated that the extracts were effective antioxidants in a β-carotene linoleic acid model system.

In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50 °C. The presence of antioxidants in the extracts minimizes the oxidation of β-carotene by hydroperoxides. The degradation rate of β-carotene-linoleate depends on the antioxidant activity of the samples being analyzed. There was a correlation between degradation rate and the bleaching of β-carotene, the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity.

All extracts had lower antioxidant activity than BHT and Quercetin, in agreement with the results from Ozsoy [12]. Notably, the extracts used have antioxidant activity at low concentrations (µg/ml).

In addition, the total phenolics compounds content of the *Phrygilanthus acutifolius* extracts correlated significantly with the DPPH radical scavenging activity ($r^2 = 0.99$, $p < 0.01$) and the bleaching of β-carotene ($r^2 = 0.93$, $p < 0.01$).

Acute toxicity

No toxic symptoms or death were observed in any of the animals after oral administration of the different doses of the aqueous or ethanol extracts. No changes were observed in body weight, food and water intake in either the control or treated group. When the emotionally parameters such as the number of grooming and faecal boluses were analyzed, no significant differences between the control and the experimental group was detected.

Relative organ weights of animals treated with a dose of 3200 mg/kg, equivalent to 25 times the human dose, are presented in Table 1. There were no remarkable differences in relative weights of kidneys, liver, heart, lungs, stomach and spleen between the control and treated

Treatment	Liver *	Kidney *	Lung *	Heart *	Spleen	Stomach *
Control	2.99 ± 0.31	0.74 ± 0.09	0.62 ± 0.04	0.28 ± 0.01	0.16 ± 0.01	0.57 ± 0.04
3200 mg/Kg aqueous extract	2.81 ± 0.28	0.83 ± 0.08	0.59 ± 0.05	0.29 ± 0.01	0.18 ± 0.02	0.55 ± 0.05
Control	2.88 ± 0.29	0.82 ± 0.08	0.60 ± 0.03	0.30 ± 0.02	0.17 ± 0.01	0.54 ± 0.03
3200 mg/Kg ethanol extract	2.77 ± 0.30	0.90 ± 0.07	0.54 ± 0.03	0.28 ± 0.02	0.19 ± 0.02	0.52 ± 0.04

Table 1. Effect of acute treatment with extracts of *Phrygilanthus acutifolius* flowers on the weight of the principal organs in rats. * Organ/bodyweight (%). Values are mean ± SEM (n = 5). No statistical difference between control and treated. (P > 0.05).

groups. Similar results were observed with doses of 128 and 1600 mg/kg of the extracts (data not shown). Pathological examinations of the tissues on a gross basis showed no detectable abnormalities.

Since the aqueous and ethanol 1-3 extracts have previously been shown to be pharmacologically active with a minimum active dose of 100 mg/kg, it is possible to conclude that the active compounds present in the extracts exhibit a rather low acute oral toxicity profile. Neither the aqueous nor the ethanol extracts produce any mortality or alter the behavioural pattern of rats as compared with the control group, in doses up to 3200 mg/kg.

Results of the haematological studies are presented in Table 2. The data shows that the haemoglobin concentration, the red blood cells and platelets had no statistically significant differences between the control and the extract treated group. However, white blood cells counts were significantly lower but still within normal limits throughout the treatment period (3-17 x 10^3). Similar results were also observed by Amida *et al.* [16]. These authors suggested that the defence mechanism may be altered at high dose.

No significant treatment-related changes in the levels of plasma concentrations of glucose, creatinine, urea, protein, albumin, cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL) were observed at the termination of the study (Table 2). The lack of significant alterations in the levels of cholesterol and creatinine, good indicators of liver and kidney functions respectively [17], suggests that the extracts ingestion did not alter the hepatocytes and kidneys of the rats, or the normal metabolism of the animals. Also, the oral extract administration did not induce any changes in the activity of the marker enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Values were not altered with respect to normal, indicating that there might not be any potential liver damage [18].

Parameters	Control	3200 mg/kg aqueous extract	Control	3200 mg/kg ethanol extract
Glucose(g/l)	0.75 ± 0.05	0.96 ± 0.07	1.03 ± 0.09	1.03 ± 0.07
Creatinine (mg/l)	0.80 ± 0.06	0.79 ± 0.08	0.81 ± 0.07	0.76 ± 0.04
Urea (g/l)	0.40 ± 0.03	0.49 ± 0.04	0.53 ± 0.05	0.45 ± 0.03
Protein (g/l)	6.73 ± 0.57	7.65 ± 0.59	7.30 ± 0.61	8.00 ± 0.69
Albumin (g/dl)	3.66 ± 0.16	3.59 ± 0.19	3.41 ± 0.20	3.55 ± 0.18
Cholesterol (mg/dl)	96.00 ± 4.00	94.00 ± 5.00	89.00 ± 5.00	93.00 ± 3.00
Triglycerides (mg/dl)	79.00 ± 4.00	83.00 ± 5.00	73.00 ± 3.00	85.00 ± 6.00
HDL (mg/dl)	67.90 ± 3.50	66.80 ± 4.30	70.60 ± 5.20	63.00 ± 4.70
LDL (mg/dl)	15.00 ± 0.80	16.80 ± 1.00	17.30 ± 1.02	15.60 ± 0.90
AST Units/l	243.00 ± 11.00	239.00 ± 10.00	250.00 ± 12.00	235.00 ± 9.00
ALT Units/l	57.10 ± 3.70	61.50 ± 4.10	55.00 ± 2.10	63.50 ± 4.60
ALP Units/l	187.00 ± 9.00	185.00 ± 7.00	172.00 ± 7.00	178.00 ± 8.00
RBC (106/μl)	7.70 ± 0.90	8.15 ± 0.22	6.64 ± 0.75	8.17 ± 0.65
WBC (103/μl)	9.813 ± 0.973	5.021 ± 1.031*	6.903 ± 0.721	3.220 ± 0.085*
Hb (g/dl)	14.5 ± 1.3	14.7 ± 1.1	14.2 ± 0.9	14.4 ± 1.0
Plabelets (103/μl)	885 ± 65	915 ± 70	935 ± 76	926 ± 85

Table 2. Biochemical and hematological parameters at termination of treatment. *Data presented as mean ± SEM. N = 5. Significantly different from control* P<0.05. Reference values of WBC 3-17x10^3/μl.

The acute oral administration of *Phrygilanthus acutifolius* flowers aqueous and ethanol extracts did not induce significant alterations in almost any morphological, behavioural, biochemical and haematological parameters in Wistar rats. The results of the present study support the concept that the flowers extracts are safe for human use.

Based on the antioxidant activity, antibacterial, anti-inflammatory and diuretic properties before demonstrated, it can be concluded that these results support strongly the ethnomedical use of the flowers of *Phrygilanthus acutifolius*. Hence, the flowers of *Phrygilanthus acutifolius* merits further investigation to identify active constituents.

CONCLUSIONS

It was found that the ethanol and water extract and infusion of the flowers of *Phrygilanthus acutifolius* evidenced antioxidant activity with increasing concentration.The extracts showed similar DPPH radical scavenging activity, while the infusion was a considerably less effective DPPH as radical scavenger. Similar results were obtained based on the inhibition of lipid peroxidation (β-carotene bleaching method). It was demonstrated that extracts contains high levels of phenolic compounds.

The data on extraction procedures and antioxidant activity assessment obtained in these experiments single out ethanol and water as the most promising sources for the isolation of natural antioxidative compounds from the flowers of *Phrygilanthus acutifolius*.

At the oral doses tested the flowers extracts can be considered safe as it did not cause either any lethality or adverse changes in the general behaviour in the acute toxicity studies in rats. The extracts not induce significant alterations in almost all biochemical and hematological parameters. Hepatic function has been monitored by the evaluation of the serum level transaminases ALT, AST and ALP. These enzymes were not altered indicating that there may be no potential liver damage.

It can be conclude that ethanol and water extracts ensured the medicinal properties of the plant *in vivo* correlate with its antioxidant activity. The results of the present study support the concept that the flowers extracts are safe for human use.

Acknowledgments. This research was supported by Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT) grants to A. Sánchez Riera.

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