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Petiveria alliacea L.: Plant Drug Quality Control, Hydroalcoholic Extract Standardization and Pharmacological Assay of Lyophilized Extract.

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SUMMARY. Preparations such as fluid extract decoction and infusion of roots and leaves from *Petiveria alliacea* L. (Phytolacaceae) known popularly as *guiné* have been used by the population for the most different symptoms and even as sedative. Though experiments have been carried out and verified the analgesic and anticonvulsive action of roots aqueous and infused extract in mice, there are no studies on standardization for both plant drugs and their extract solutions. The aim of the present study was to evaluate the aerial parts of *guiné* plant drug and its turbo-extract in respect to its quality control, anxiolytic and stress gastric lesions protective activities, and the standardized turbo-extract. The results suggest anxiolytic and stress gastric lesion protective effect for aerial parts extract of *P. alliacea* in animals. A 0.630 mm granulometry and a 20% (m/V) plant: solvent proportion were the factors that determined the highest yield of total flavonoid content in turbo-extract in the present experiment.

RESUMEN. "*Petiveria alliacea* L.: Control de Calidad de la Droga Vegetal, Estandarización del Extracto Hidroalcohólico y Ensayo Farmacológico del Extracto Liofilizado". La población nativa del Brasil utiliza extractos fluidos, decocción e infusión de las raíces y hojas de *Petiveria alliaceae* L. (Phytolacaceae, *guiné*) ante a los más diferentes tipos de síntomas y también como sedativo. Se realizaron experimentos en ratas para verificar la actividad analgésica y anticonvulsiva de extractos acuosos e infusión de las raíces. Por otro lado, no hay estudiós de estandarización de los extractos y tampoco de la droga vegetal. El objetivo de este trabajo fue el de evaluar las partes aéreas del "guiné" y su turbo-extracto desde el punto de vista del control de calidad, actividad ansiolítica y protección de lesiones gástricas por estrés, así como el turbo-extracto estandarizado. Los resultados en animales sugieren que las partes aéreas de "guiné" contienen componentes con efectos ansiolíticos y de protección de las lesiones gástricas por estrés. La granulometría de 0,630 mm y la proporción planta:solvente a 20% (m/V) fueron los factores que determinaron la más alta concentración de flavonoides en el extracto turbolizado.

INTRODUCTION

Petiveria alliacea L. (Phytolacaceae), popularly known as *guiné, pipi, erva-de-tipi, erva-de-alho, embiaiendo, emboiaembo, ocoembo, mucara-caá, amansa-senhor,* presents a wide distribution in South America [1]. Fluid extract, decoction and infusion of roots and leaves have been used *p.o.* and topically by the native population as sedative, diuretic, diaphoretic, antirheumatic, antithermic, anesthetic, antihelminthic, sudorific and purgative [1,2].

Lima *et al.* [3] demonstrated that the aqueous extract from the roots of *P. alliacea* presented analgesic and depressing effects on the central nervous system (CNS) of mice. Its infusion also proved to be partially protective against convulsions induced by pentylenotetrazole and electroshock [4].

The toxicity of fractionated root powder used in high doses was described by Souza [5] with indications of behavioral alterations as stimulation, insomnia and hallucinations followed by depressing symptoms, and as abortive.

KEYWORDS: Antiulcerogenic effect; Anxiolytic effect; *Petiveria alliacea;* Phytolacaceae; Quality control.
PALABRAS CLAVE: Actividad ansiolítica; Control de calidad; Efecto antiulcerogénico; *Petiveria alliacea;* Phytolacaceae.

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Although there have been experiments carried out with *P. alliacea*, there are no studies on the standardization of this plant drug or its extract solutions.

Extract standardization can be obtained through factorial design using statistical methods to assure reliable significance of the results thus decreasing the probability the occurrence of random errors [7,8].

The aim of the present experiment was to evaluate the vegetal drug of *P. alliacea* as well as the quality control of it and to produce a standardized extract. Furthermore the anxiolytic action and protection against stress gastric lesion have been carried out.

MATERIAL AND METHODS

Plant Material and Extract Preparation

The samples were collected at the "Horto de Plantas Medicinais - Universidade Estadual de Maringá (UEM)" (24°S, 32°W), March, 1998, and a voucher specimen was deposited at HUM-Herbarium under the number 4704. The material was dried at room temperature (25 °C) and milled (Tecnal mod. TE-048). The milled material was used in the preparation of hydroalcoholic extract (20 min/40 °C; Skymsen mod. LSV-04), followed by the elimination of organic solvent and then lyophilized (EBG, Christ-Alpha 1-2).

Quality Control of Plant Material

The following pharmacopoeia methods were used to control the quality of the plant drug: a) loss on drying [9]; b) loss on desiccation [10]; c) dry residues for aqueous and hydroalcoholic extracts at 50 °GL and 70 °GL [10]; d) extract content [11]; e) total flavonoid content [12] and f) granulometric analysis [13].

Animals

Male Wistar rats weighing 200-250 g were housed in polyethylene-walled cages in groups of five with food and water *ad libitum*. Lights were on from 6 a.m. to 6 p.m. and temperature was kept at 23 ± 10 °C.

Acute Stress Lesion Induction

Acute gastric lesions by stress were induced in groups of ten rats according to the model of Nagura, modified by Bacchi and Sertié [14]. After oral administration of 0.9% NaCl, cimetidine and extracts of *Petiveria alliacea* (EBG), each rat was immobilized in a cylindrical cage and immersed vertically to the level of the xiphoid for 17 h at 23 - 25 °C. The rats were previously deprived of food for 24 h, but water was allowed.

The EBG extract was administered orally 30 min before the procedures at doses of 200, 400, and 600 mg/kg. Cimetidine (32 mg/kg) and 0.9% NaCl were used as reference drugs. After each treatment the animals were killed, the stomachs were removed and their inner surface examined. The gastric lesions were counted and the mean ulcerative index (UI) was calculated as follows: I = presence of edema, hyperemia, and single submucosal punctiform hemorrhages (petechiae); II = presence of submucosal hemorrhagic lesions with small erosions; III = presence of deep ulcer with erosions and invasive lesions.

$$UI = \frac{(\text{n lesion I}) + (\text{n lesion II})^2 + (\text{n lesion III})\,3}{\text{N animals}} \quad [1]$$

Anxiolytic Effect Test

The elevated plus-maze was made of wood, according to the specifications of Pellow *et al.* [15]. The apparatus consisted of two opposed open arms measuring 50 x 10 cm, crossed at right angle with two opposed arms of the same size enclosed by 40 cm-high walls, except for the entrance. The four arms delimited a central area of 10 cm^2. The whole apparatus was elevated 70 cm above the floor. To avoid rats falling down, a rim of 1 cm-high Plexiglas was made to surround the open arms. Illumination was provided by a 80 W light suspended 160 cm above the maze. The experimental sessions were recorded by a vertically-mounted video camera, linked to a TV monitor and a VCR in an adjacent room. Videotapes were later analyzed by an observer unaware of treatment conditions.

The experimental session was conducted 30 min after EBG oral injection. The rat was placed at the central square facing and enclosed arm, and allowed to freely explore the elevated plus-maze for 5 min. Before the next rat was introduced, the maze was cleaned with a solution of 70% ethanol and dried. The number of entries with the four paws onto open an enclosed arms, and the time spent on open and enclosed arms were recorded. From these data, the percentage of entries onto and of time spent on open arms was calculated [100 x open/(open + enclosed)]. This parameter was considered to reflect anxiety while the number of entries into enclosed arms was used as an index of locomotion [16].

Acute Toxicity (DL_{50})

Acute toxicity studies of *P. alliacea* by lyophilized extract were performed on mice and estimated by the method described by Miller and Tainter [17]. Increasing doses of extracts were administered to groups of 10 animals for each dose level after a 24 h interval.

Data analysis

The pharmacological analysis results were expressed as the mean ± S.E.M. for each group, and the single-factor ANOVA followed by the multiple range Tuckey test were used. A p value of 0.05 or less was required for significance.

Factorial Design

A 2^3 factorial design was used to evaluate the influence of three factors on the properties of *P. alliacea* hydroalcoholic extract obtained by turbo-extract (Table 1). The factors were selected according to the experimental conditions of the present pharmacological studies, randomly designed to minimize systematic errors [18] and carried out without reposition (Table 2).

The effects of one or more factors for the factorial design according to the equations in Table 4 resulting from Table 3 was made on the total flavonoid content (TFC) of the extract solution.

Factors	Levels	
	-	+
A Alcoholic content (°GL)	50	70
B Granulometry (mm)	0.210	0.630
C Plant: Solvent proportion (%; m/V)	10	20

Table 1. Factors and levels for factorial design.

Experiment	Combinations	Factor and Level		
		A	B	C
1	(1)	-	-	-
2	A	+	-	-
3	B	-	+	-
4	AB	+	+	-
5	C	-	-	+
6	AC	+	-	+
7	BC	-	+	+
8	ABC	+	+	+

Table 2. Experimental factors combinations.

Treatments	Effects			Interactions				Results
	A	B	C	AB	AC	BC	ABC	
(1)	-	-	-	+	+	+	-	Y_1
A	+	-	-	-	-	+	+	Y_2
B	-	+	-	-	+	-	+	Y_3
AB	+	+	-	+	-	-	-	Y_4
C	-	-	+	+	-	-	+	Y_5
AC	+	-	+	-	+	-	-	Y_6
BC	-	+	+	-	-	+	-	Y_7
ABC	+	+	+	+	+	+	+	Y_8

Table 3. Standard evaluation of the main effects and interactions of a 23 factorial design.

Effects/ Interactions (E/I)	Equations
E_a	$1/4\ [(y_2 = y_4 + y_6 + y_8) - (y_1 + y_3 + y_5 + y_7)$
E_b	$1/4\ [(y_3 + y_4 + y_7 + y_8) - (y_1 + y_2 + y_5 + y_6)$
E_c	$1/4\ [(y_5 + y_6 + y_7 + y_8) - (y_1 + y_2 + y_3 + y_5)$
I_{ab}	$1/8\ [(y_1 + y_4 + y_5 + y_8) - (y_2 + y_3 + y_6 + y_7)$
I_{ac}	$1/8\ [(y_1 + y_3 + y_6 + y_8) - (y_2 + y_4 + y_5 + y_7)$
I_{bc}	$1/8\ [(y_1 + y_2 + y_7 + y_8) - (y_3 + y_4 + y_5 + y_6)$
I_{abc}	$1/8\ [(y_2 + y_3 + y_5 + y_8) - (y_1 + y_4 + y_6 + y_7)$

Table 4. Equations of evaluation of the experiments for a 2^3 factorial analysis.

Total Flavonoid Content of the Extract Solution (TFC)

A 4 mL of the extract solution was taken, corresponding to 0.4 g of the plant for the extract at 10% (m/V), and submitted to TFC determination as described by German Pharmacopoeia [12].

Factorial Design Data Analysis

The factorial design was statistically evaluated by one-entry variance analysis, followed by the methods of Yates [19] and Daniel [20] for non-replicate experiments.

RESULTS AND DISCUSSION

Quality control methods developed for *Petiveria alliacea* represent sufficient data for the plant drug characterization to support the galenic development [21]. However, the safety of the use of the drug or even its extracts will be provided by clinical and pre-clinical pharmacological and toxicological assays [22].

The values obtained through loss on drying and desiccation techniques were respectively 65.88 ± 1.06% and 65.21 ± 0.96%. From the technological point of view, loss on drying and desiccation may indicate the efficiency of the drying operation. They demonstrated to be statistically significant correlated to the fresh plant. For the plant drug dried and milled the value obtained was 91.47 ± 0.31% for the loss of water and volatile substances.

Extractive content was determined according to World Health Organization with the aim to know the plant drug properties after drying. This method can be related to the plant drug when the active constituents were not determined. The obtained value (21.83%) by the use of water as extractor liquid is similar to the values obtained for the hydroalcoholic extracts at 50 °GL and 70 °GL (21.25% and 22.48%, respectively).

One of the control methods of this plant drug was carried out using the determination of TFC which resulted in 0.49 ± 0.08%. Although flavonoids is not the only class of substances that could be responsible for the activity of *P. alliacea*, but its presence was confirmed before[23-25].

The granulometric analysis was considered as an important technological parameter for establishing the peculiar conditions of the milled drug. Granulometry may alter positively or negatively the extraction of active or other substances. The passage and retention curves were used to determine the mean diameter of particles (d_{50}=0.340 mm) and a slope of the ascendant curve suggests that the granulometric classes are homogeneously distributed. On the other hand, the histogram present a granulometric classes are above d_{50}, where the most part of the particles (37.7%) were placed between 0.630-0.840. The possible direct influence of this factor on TFC was evaluated through factorial analysis.

For pharmacological assays, the lyophilized extract was prepared with hydroalcoholic mixture at 70 °GL (EBG). Besides the dry residue value obtained with water is similar to the one obtained with alcohol extraction. The alcohol presence can prevent the extract from the microbial contamination and can also allow the extraction of a great variety of substances.

The antiulcerogenic effects of the total EBG extract in the acute stress-induced gastric lesions in rats are summarized in Table 5. Gastric lesions induced by acute stress were significantly reduced by oral administration of the 600 mg/kg dose of extract. These reductions in gastric lesions were comparable to those produced by the reference drug, cimetidine (32 mg/kg).

Treatment	Dose (mg/kg)	N	Ulceractive Index (X ± SD)	Inhibition (%)
Control	-	7	25.3 ± 2.3	-
Cimetidine	32	8	11.8 ± 1.0 *	53.4
EBG	600	8	15.8+1.3 *	62.5

Table 5. Effect of oral doses of lyophilized EGB extract of *Petiveria alliacea* on the incidence of acute gastric lesions induced by 17-hour stress, with restraint plus water immersion. 0.9% NaCl was utilized as control. Cimetidine (32 mg/kg) was used as reference. ANOVA and Tukey's test were utilized for comparisons (*p<0.05).

Following oral administration of EBG at doses of 600 mg/kg, the animals submitted to elevated plus maze significantly increased the percentage of open arm entries [F(3,23)=7.500, p = 0.00206]. Reported results have shown that this measure is connected with anxiety [16]. The lower aversion expressed by the higher number of entries onto the open arms of the plus maze seems to be due to an anxiolytic effect of EBG extract.

The percentage of time spent on the open arms [F(3,23) = 3.198, p= 0.1259] and the total number of entries into the enclosed arms was not significantly changed [F(2,23)=1.749, p=0.5492]. Locomotion as indicated by the number of enclosed arm entries [16] was not increased by the extract, suggesting that anxiolytic effect of EBG is selective (Table 6).

According to the special literature, an infusion of roots of this compound produced a sig-

Treatment	% Open/ Total Arms Entries	% Open/ Total Arms Time	Enclosed Arms Entries
Control	25.3 ± 7.4	16.1 ± 6.9	5.9 ± 1.1
EBG200 mg/kg	28.5 ± 7.6	5.3 ± 2.3	7.6 ± 3.8
EBG400 mg/kg	15.3 ± 10.0	8.9 ± 5.9	3.5 ± 0.9
EBG600 mg/kg	57.3 ± 2.1 *	26.9 ± 4.9	3.9 ± 0.6

Table 6. EBG and saline (control) injected by p.o. in rats submitted to elevated plus maze. One factor ANOVA detected a significant effect ($F(3,23) = 7.50$, $p = 0.0011$) and post-hoc comparisons with Tuckey's multiple comparison test showed that the 600 mg/kg of the drug differed from control (*$p < 0.05$) in the percentage of open/total arm entries ($n = 6$-8).

nificant reduction in locomotion activity and an increase in threshold to electroshock-induced convulsions in mice [4], which suggests its central depressor action and justifies its popular use as sedative.

Since the active compounds present in the extract are not known, it is impossible to affirm which are precisely the compounds involved in *P. alliacea* central effects. The results suggest that the extract contains at least one anxiolytic active principle.

The results also demonstrated that oral administration of *P. alliacea* extract (up to 3.0 g/kg) did not produce any sign of toxicity in mice. The results also indicated that *P. alliacea* crude extract must be administered in doses five times higher than the above in order to produce anxiolytic and gastric ulcer reduction effects thus showing that the principle responsible for its effects has low acute toxicity. The low toxicity verified for *P. alliacea* confirms literature data obtained in experiments with rats and mice [4,26].

Its use makes possible to determine the occurrence of interactions between the factors on their different levels and thus detect which may influence the standardization process [7,8,7].

The results of the hydroalcoholic extract solutions were initially submitted to one-entry variance analysis (ANOVA) for the values of TFCx10^4 obtained (Table 7). The change of levels for the factors evaluated through variance analysis showed a highly significant difference ($F_{calc} = 345.20$; $F_{crit\ 0.01[7.16]} \geq 4.14$) for TFC.

	Treatments							
	(1)	A	B	AB	C	AC	BC	ABC
1	3090	3390	2000	2810	6480	7200	3720	5490
2	3060	3330	1985	2830	6260	7560	3770	5480
3	3120	3360	1970	2820	6700	7380	3820	5500
Mean	3090	3360	1985	2820	6480	7380	3770	5490
± S	42,43	42,43	21,21	14,14	311,13	254,56	70,71	14,14
CV(%)	1.37	1,26	1,07	0,50	4,80	3,45	1,88	0,26

Table 7. Total flavonoid content (TFCx104) for hydroalcoholic extract solutions of *Petiveria alliacea* (n=3).

A preliminary evaluation of the (positive/negative) type and the intensity of the effects and their interactions with TFC may be observed in the results obtained through standardized evaluation of factorial projects (Table 8).

The estimated values (Table 8) demonstrated that among the main factors, the change from lower levels (-) to higher levels (+) provided a TFC increase for factors A and C and decrease for factor B. Secondary interactions seem to influence positively the extracts yielding, except for BC.

Effect/Interaction	Estimated Value
E_a	931,25
E_b	-1561,25
E_c	346,25
I_{ab}	2966,25
I_{ac}	378,75
I_{bc}	-738,75
I_{abc}	63,75

Table 8. Effects and interactions (E/I) resulting from standardization of factorial planning.

In order to estimate errors in this type of experiment, it must be supposed that certain higher interactions constitute no significant influence and combine their mean squares to estimate the errors. Daniel [20] provides a way to avoid this problem by representing the estimated effects in a net of normal probability. According to this method, the non significant effects are graphically distributed along a straight line tending to zero, while the important effects are not situated along this line.

The estimated effects ordered by intensity in data net of normal probability (Table 9) may be better observed through a graphic representation (Fig. 1). Thus the effects and interactions C, A, BC and B are important and must be interpreted.

One mean of corroboration of the data from Table 8 and Fig. 1 is the use of variance analysis according to Yates [19] for the main effects and interactions

The results of the variance analysis showed that factor C presented a highly significant influence on TFC (98.71 for $\alpha \leq 0.01$; 98.5). Factor B also presented a significant influence (27.35 for $\alpha \leq 0.05$; 18.5), but three times lower than factor C (Fig. 1 and Table 10). The factor A and the interactions were not significant.

Order (j)	E/I	Estimated Value	Pj
7	C	2966,25	0,9286
6	A	931,25	0,7857
5	AC	378,75	0,6429
4	AB	346,25	0,5000
3	ABC	63,75	0,3571
2	BC	-738,75	0,2143
1	B	-1561,25	0,0714

Table 9. Data for determining the effects and interactions of a net of normal probability. Pj = (j-0,5)/7.

Effects/Interactions	GL	MS	Fcalc
A	1	1734453	9,73
B	1	4875003	27,35*
AB	1	239778	NS
C	1	17597278	98,71**
AC	1	286903	NS
BC	1	1091503	6,12
ABC	1	8128	NS
Total	7	25833047	
Error		178269,76	

Table 10. ANOVA results for total flavonoid content. $F_{crit.(1,2)}$: $\alpha \leq 0,05 = 18,5^{*}$, $\alpha \leq 0,01 = 98,5^{**}$.

Granulometric increase (0.630 mm) influenced TFC negatively due to the surface decrease (lixiviation phase) and particle thickness increase (diffusion phase) thus making difficult the contact with the extractor liquid. But when plant: solvent proportion was increased (from 10% to 20%), a positive influence on TFC was verified.

To corroborate the model used in the experiment, an adjusting test consisting of a residue analysis was applied. The evaluation demonstrated that the only significant effects are due to B (granulometry) and C (plant: solvent proportion), with estimated values of 1,561.25 and 346.25 respectively. Considering these results

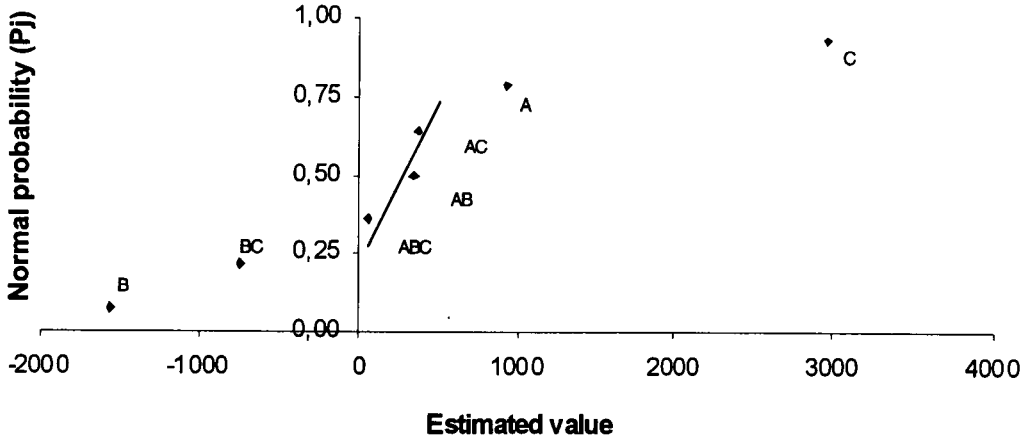

Figure 1. Graphic representation in a normal probability net of the effects and interactions estimated according to Daniel (1959) [20].

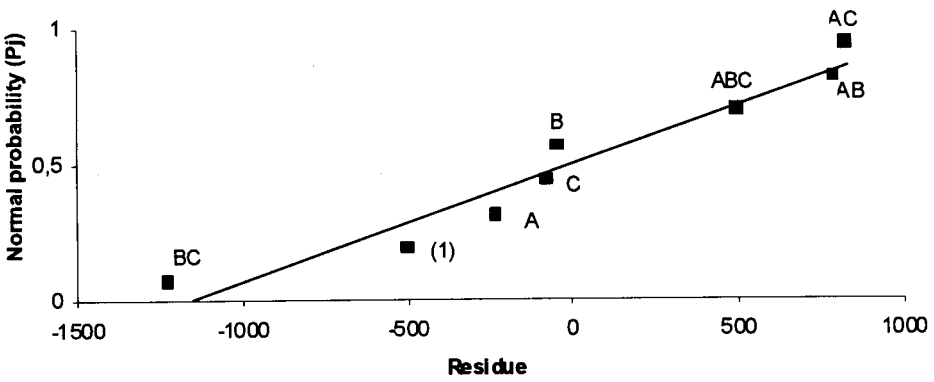

Figure 2. Graphic representation in normal probability net for factorial planning residue analyses.

accurate, the calculated TFC (TFC_{calc}) is represented by the formula [2]

$$TFC_{calc} = 4,297.5 + (-1,561.25/2)x2+(346.25/2)x3 \quad [2]$$

in which: 4,297.5 corresponds to the mean value of TFC; X2 and X3 assume values +1 or -1, according to the signal of the values estimated in Table 8.

The residues obtained by the difference between TFC_{exp} and TFC_{calc} (Table 11) can be represented graphically. In the normal probability paper a slight deviation of linearity was observed (Fig. 2), which suggests that the method was adequate to the analysis.

Treatments	TFC exp (10^4)	TFC calc (10^4)	Residue	Pj
(1)	3090	3595,00	-505,00	0,1875
A	3360	3595,00	-235,00	0,3125
B	1985	2033,75	-48,00	0,5625
AB	2820	2033,75	786,25	0,8125
C	6480	6561,25	81,25	0,4375
AC	7380	6561,25	818,75	0,9375
BC	3770	5000,00	-1230,00	0,0625
ABC	5490	5000,00	490,00	0,6875

Table 11. Residues for 2^3 factorial planning using *Petiveria alliacea* hydroalcoholic extract solutions.

The factorial design demonstrated that the use of 0.210 mm granulometry and 20% (m/V) plant: solvent proportion provided a significant yielding increase of TFC. For the alcoholic content, no difference between the two levels was observed. However, it may be observed in Table 10 that in all assays in which factor A (alcoholic content) was present at the higher level, the results were always non significant, which may be considered as an indication that 50 °GL alcoholic content provides a higher yielding than 70 °GL content.

In conclusion, the present results suggest that the extract of *P. alliacea* aerial parts present anxiolytic and antiulcerogenic effects in animals. This fact provides, at least in part, experimental support to the popular use of this plant as sedative. Additional tests should be carried out to identify the active substance or at least the class of compounds responsible for this effect.

The factors employing the factorial design of the type 2^3 used in the present study determined the highest yield of total flavonoid content in the extract obtained through turbo-extract were 0.630 mm granulometry and 20% (m/V) plant: solvent proportion.

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