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Preclinical toxicological assessment of a phytotherapeutic product – CPV (based on dry extracts of *Crataegus oxyacantha* L., *Passiflora incarnata* L., and *Valeriana officinalis* L.)

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Associations of plants have been widely used, for centuries, in Ayurveda and in Chinese medicine and have been increasingly acknowledged in Western medicine. The objective of this study is to assess the level of toxicity of an association of three plants: *Crataegus oxyacantha*, *Passiflora incarnata*, and *Valeriana officinalis* (CPV extract). This association was administered to rats, mice, and dogs, both acute and chronically for 180 days. The tests used in the acute experiments were: observational pharmacological screening, LD_{50}, motor coordination and motor activity. Chronic tests carried out were: weight gain/loss and behavioral parameters in rats and in mice; estrus cycle, effects on fertility, and teratogenic studies in rats and of mutagenic features in mice, in addition to the Ames test. The following parameters were assessed in dogs: weight gain/loss, general physical conditions, water/food consumption and anatomopathological examination of the organs subsequent to the 180 days of treatment. All of the results were negative, showing that CPV administered in high doses and over a long period of time presents no toxicity, suggestive of the fact that this is an association devoid of risk for human beings. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: preclinical toxicity; *Passiflora*; *Crataegus*; *Valeriana*; association of plants.

INTRODUCTION

Among phytotherapeutic products, special attention must be given to associations of plants with a view to better therapeutic effect. The associations are widely used in some oriental types of medicine such as Ayurveda (Wu *et al.*, 1998; Williamson, 2002) and Chinese medicine (Guo *et al.*, 2004; Kuribara *et al.*, 2004) and have been increasingly acknowledged in Western medicine (Williamson, 2001; Gilbert and Alves, 2003). In Brazil, we find phytomedications with a composition involving three or more plants – amongst these *Passiflora incarnata* L., *Passiflora edulis* Sims, *Valeriana officinalis* L., *Melissa officinalis* L., *Crataegus oxycantha* L., *Salix alba* L. – used therapeutically as a sedative for nervous excitation, as an anxiolytic, as a hypnotic, and in treating neurovegetative disorders.

Valeriana officinalis L. (Valerianaceae family) is a perennial plant, native to Europe and Western Asia (Grieve, 1994). The subterraneous parts of the plant have been in use from antiquity; Dioscórides described the use of this plant as a light sedative in the first century AD (Morazzoni and Bombardelli, 1995). Its sedative and anxiolytic effects have been confirmed in various recent studies (Andreatini *et al.*, 2002; Fernandez *et al.*, 2004).

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Passiflora incarnata L. (Passifloraceae family) is a creeper native to tropical America. The leaves of this plant are used for the treatment of anxiety, nervousness and neuralgia, and the plant is classified as an official species in Chilean, French, and Spanish pharmacopoiea (Soulimani *et al.*, 1997). Pharmacological studies over the last few decades confirm its anxiolytic and sedative effects (Dhawan *et al.*, 2001a, b; Krenn, 2002).

Crataegus oxyacantha L. (Rosaceae family) is a shrub native to Europe. Its fruit has been used over the course of time as a diuretic, for dyspnea, and renal calculi. There are also studies that show its sedative and anxiolytic effects (Hanus *et al.*, 2004). The majority of studies on this species, however, produce evidence of its cardiotonic properties (Degenring *et al.*, 2003). Other studies also describe its activities: hypolipidemic (Shanthi *et al.*, 1996) hypocholesterolemic, and anti-arterosclerotic (Rajendran *et al.*, 1996).

Although the three plants cited above are used therapeutically, associated one to the other in various pharmaceutical products, there are no toxicological studies of these associations. The existence of the few preclinical toxicological studies carried out with these plants in isolation showed no toxicity worthy of note (Fehri *et al.*, 1991; Fisher *et al.*, 2000).

The purpose of this study is to assess the acute and chronic toxicity of an association containing *Valeriana officinalis*, *Passiflora incarnata*, and *Crataegus oxyacantha*, an association commonly used by people in Brazil and for which no toxicological studies have previously been performed. The pharmacological action of this association is the subject of a separate publication (Tabach *et al.*, submitted).

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EXPERIMENTAL

Animals

Swiss albino mice (male and female) aged 3 to 4 months, weighing 30–40 g and Wistar rats (male and female), 3 months old, weighing between 300 and 400 g, kept in rooms with temperature control (23 ± 1 °C), light-dark cycle (12/12 h) with water and food *ad libitum*. Dogs (male and female), *Beagle* breed, average age 7 months at the start of the experiment.

Botanical material

The dry extracts of *Crataegus oxyacantha* (Batch No. 04804/M1), *Passiflora incarnata* (Batch No. 04675/M1) *and Valeriana officinalis* (Batch No. 26961/M2) that are part of this association were purchased by Biolab Sanus Farmacêutica Ltd from Laboratório Indena® having been identified through spectrophotometry or high efficiency liquid chromatography and subsequently assessed as to their physicochemical characteristics, residual contamination by organic solvents, and microbiological contamination following standards of reference, in accordance with the official reports supplied by Laboratório Indena® (analysis certificates numbers 11580- *C. oxyacantha*, 11636- *P. incarnata* and 62706 – *V. officinalis*). These extracts were mixed in the following proportion: *Crataegus oxyacantha*: 0.8 kg (26.7%), *Passiflora incarnata*: 1.0 kg (33.3%) and *Valeriana officinalis*: 1.2 kg (40%), the association having been given the name of CPV. This proportion was chosen based on the composition of a popular marketed product in Brazil that possesses the same amounts of the three extracts.

Administering the extract

The CPV extract, diluted in water as needed, was administered either orally or intraperitoneally in constant volume of 0.1 ml/10 g of weight in mice and 0.1 ml/100 g of weight in rats. Different concentrations of the extracts were used in order to obtain the desired posology (mg/kg) for the animals; in all cases, a very fine stable suspension was obtained.

The doses employed in the rats were based on the human daily dosage of the marketed phytomedicine available in Brazil (a 300 mg pill corresponding to 4.3 mg/kg for an 70 kg adult; doses 10, 100 and 200 times larger were also employed).

In the study with dogs, the original extract (without dilution) was administered at a dose of 430 mh/kg as bait, mixed to a damp ration.

Acute toxicity

Observational pharmacological screening. Seven groups of three mice were given the following doses of the CPV extract (mg/kg) intraperitoneal (i.p.) route: 160, 320, 640, 1250, 2500, 5000; the control group was treated with water. The animals were subsequently observed for 24 h with annotation of the presence or absence of 17 signs such as: urination, defecation, writhing (contraction of the abdomen with distention of posterior paws), bristly fur, palpebral ptosis, motor activity, muscular tonus, tremors or convulsions, ataxia, sensitivity to pain, signs of stereotype behavior, yawning, sleep, grooming, lacrimation, exophthalmia, and salivation (Carlini, 1972; Mendes *et al.*, 2002).

LD_{50}. Eight groups of ten mice were given water (control) or the following doses (mg/kg), via i.p. of CPV extract: 3000, 3250, 3500, 3750, 4000, 6000, and 8000 mg/kg and were subsequently observed for one week for index of mortality. In the same way, six groups of ten mice received, respectively, water (control) or the following doses (mg/kg) of CPV extract orally: 640, 1250, 2500, 5000, and 8000, and were also observed for one week for index of mortality. LD_{50}, via i.p., was calculated using a method of linear regression (Graphpad Prism® – GraphPad Software, Inc).

Motor coordination (the rotarod apparatus). Motor coordination in mice was assessed by means of the rotarod apparatus consisting of a rotatory bar 3 cm in diameter by 60 cm in length, divided by disks, 15 cm in radius, into five equal compartments. The system is kept raised 40 cm above the counter, rotating at a steady speed of 12 rpm. Seven groups of ten mice (previously selected for their capacity to stay on the bar for 30 seconds), were treated, respectively, with water (control) or with CPV extract in doses of 160, 320, 640, 1280, 2500, and 5000 mg/kg via i.p. The mice were then tested on the rotarod apparatus 30, 60, and 120 minutes following drug administration, with the amount of time assessed that each animal remained on the rotarod over a span of 30 seconds (Carlini and Burgos, 1979). This procedure was also carried out with oral administration of 5000 mg/kg of CPV extract.

Motor activity. Four groups of ten mice were treated, via i.p., with water (control) or with 640, 1250, and 5000 mg/kg of CPV extract. The animals were then placed in boxes equipped with photoelectric cells for recording motor activity after 30 and 60 minutes (Mendes *et al.*, 2002).

Chronic toxicity

Weight gain/loss and behavioral parameters in mice. Eight groups of 12 animals each (6 males and 6 females) were treated orally for 180 days, respectively, with water (controls) or with 160, 320, and 640 mg/kg of CPV extract. They were assessed weekly for weight gain/loss, general aspect, and food consumption and, every 30 days, for motor activity and coordination on the rotarod.

Weight gain/loss and behavioral parameters in rats (open field apparatus). Four groups of ten male rats, treated orally with water (control) or with 43, 430, and 860 mg/kg of CPV extract for 180 days, were assessed weekly for weight gain/loss, general aspect, water consumption and, at 45, 90, and 180 days, by the open field test (Sousa *et al.*, 2004). At the end of the treatment, the animals were decapitated, the blood collected for biochemical and hematological analysis and the viscera forwarded for anatomopathological examination.

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Effects on estrus cycle in rats. Three groups of ten female mice were treated orally with water (control) or with 430 and 860 mg/kg of CPV extract over the course of 60 days. From days 1 to 15 (the first 15 days) and from days 45 to 60 (the last 15 days) vaginal material was collected daily in order to determine the different phases of the cycle (proestrus, estrus, metestrus, and diestrus), in addition to assessing the number of oestrus, the interval between estrus, and total duration of each cycle (Formigoni *et al.*, 1986; Shivalingappa *et al.*, 2002).

Effect on fertility in rats and possible teratogenic effect on the offspring. Three groups of ten female rats were treated orally with water (control) or with 430 and 860 mg/kg of CPV extract for 10 days. On the eleventh day, without interrupting the treatment, the females were placed in contact with the males (three females to one male) with three shifts among the males every three days. After the males were removed, the females continued to undergo treatment until the day immediately preceding the date of delivery. The litters were then assessed as to possible teratogenic effect. The main parameters assessed were: number of pregnancies to term, duration of pregnancies, number of offspring, external signs of malformation of the litter, weight gain/loss, posture reflex, day eyes opened, locomotor activity, and open field test of the litter (Oliveira *et al.*, 1991).

Chronic experiments on dogs. The tests below were carried out by UNITOX – Laboratório Universitário de Análises Toxicológicas da Universidade de Santo Amaro (UNISA). Two groups of six Beagle dogs (three males and three females) were treated; one group with water (control) and the other with 430 mg/kg of CPV extract (orally) for 180 days. The animals were assessed weekly for weight gain/loss, general physical condition, consumption of water and food. Blood and urine were collected before the start of the treatment (day zero) and on days 30, 90, and 180 of the treatment and the animals were then forwarded for an anatomopathological examination.

Genotoxicity and mutagenicity

The tests below were carried out by Genotox – Laboratório de Genotoxicidade da Universidade Federal do Rio Grande do Sul.

The Ames test. CPV extract was analyzed to induce reverse mutation in four lineages of *Salmonella tiphymurium* in the absence of and in the presence of metabolic activation of a post-mitochondrial fraction of rat liver induced by Aroclor 1254 (S-9), according to that described by Ames *et al.* (1975).

The Micronucleus test. CPV extract was tested for its capacity to induce chromosomal mutations in the bone marrow in Swiss mice treated in accordance with the method described by Schmid (1976).

Statistical analysis. The data were analyzed utilizing the Analysis of Variance (ANOVA) followed, when necessary, by the Duncan Multiple Comparison Test.

RESULTS

Acute toxicity

Pharmacological screening. The administering of CPV extract produced a reduction in ambulation as from the dose of 320 mg/kg, via i.p., with a tendency for the animals to keep to one corner of the cage. The fur bristled as from 640 mg/kg and there was a reduction in muscular tonus, in addition to an increase in abdominal contortions in the first 4 h following i.p. administration of 2500 and 5000 mg/kg. No alterations worthy of note were observed in the following parameters: urination, defecation, tremor, hind limb alterations, ataxia, salivation, palpebral ptosis, lacrimation or exophthalmia.

These initial data show that the extract does not present toxicity that is worthy of note; the signs observed are not severe by nature and are only in evidence with very high doses. The abdominal contortions showed that the extract must have produced some peritoneal irritation which might have caused the reduced ambulation and onset of bristly fur. The variation of pH found in several doses of CPV extract utilized in this test was small, oscillating between 4.42 (in a dose of 160 mg/kg) and 4.92 (in a dose of 5000 mg/kg).

LD_{50}. It was not possible to calculate the LD_{50} orally, for even a dose of 8000 mg/kg did not produce deaths up to seven days following administering of the extract. Through i.p. (3000 to 8000 mg/kg), the extract induced death in the animals, which allowed the 50% lethal dose to be calculated at 4570 mg/kg (Fig. 1), suggesting a very low toxicity of this product (Hodgson, 2000).

Motor coordination (rotarod apparatus). CPV extract up to a dose of 5000 mg/kg via i.p. or orally did not significantly alter the motor coordination of mice compared to the respective controls (results not shown).

Motor activity. Table 1 shows that the CPV extract, i.p. route, in the three doses utilized, significantly reduced the motor activity of mice at 30 and 60 minutes in relation to the control group. The reduction

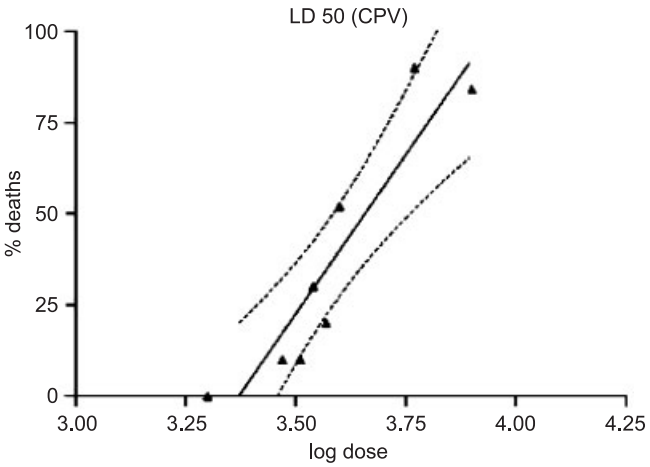

Figure 1. Determining the lethal dose 50% in mice treated acutely with CPV (ip) and observed over the course of seven days. LD_{50}, 4570 mg/kg was calculated using a method of linear regression (GraphPad Prism* – GraphPad Software, Inc).

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Table 1. Motor activity in mice (n = 10 per group) subsequent to acute administration, via ip, of different doses of the CPV extract. The values represent the average (X) followed by the standard deviation (sd)

Treatment	Dose (mg/kg)	Ambulation (X ± dp)		% of reduction in relation to controls	
		30 min	60 min	30 min	60 min
Control	–	1174.3 ± 358.8	1939.2 ± 792.4	–	–
CPV	640	668.0 ± 581.0*	1038.7 ± 902.6*	43.1	46.5
CPV	1250	506.1 ± 226.2*	740.8 ± 359.4*	56.9	61.8
CPV	5000	606.0 ± 415.0*	821.6 ± 507.2*	48.4	57.6

* $p < 0.05$ differs in relation to control (ANOVA, followed by the Duncan test).

Table 2. Assessment of the behavior in open field of male rats (n = 10 per group) treated by mouth for 180 days with different doses of CPV extract, with assessment of locomotion, rearing, time (in seconds) spent grooming, freezing and the number of fecal boluses. The values represent the average (X), followed by the standard deviation (sd)

Treatment	Dose (mg/kg)	Locomotion (X ± sd)	Rearing (X ± sd)	Grooming (X ± sd)	Freezing (X ± sd)	Defecation (X ± dp)
Control	–	59.6 ± 42.5	26.9 ± 13.7	26.5 ± 24.2	2.9 ± 3.1	2.2 ± 1.9
CPV	43	41.7 ± 16	14.3 ± 10*	19.3 ± 20	5.7 ± 8.7	2.9 ± 2.1
CPV	430	51.5 ± 31.2	11.9 ± 7*	22.3 ± 9.9	3.3 ± 3.9	3.9 ± 2.1
CPV	860	38.1 ± 22.5	13.6 ± 6*	15.7 ± 13.3	2.1 ± 2.1	4.9 ± 1.8*

* $p < 0.05$ differs in relation to control (ANOVA, followed by the Duncan test).

was dose-dependent for 640 and 1250 mg/kg; however, a ceiling effect seems to have occurred with a dose of 5000 mg/kg.

Chronic toxicity

Weight gain/loss and behavioral parameters in mice. Oral administration for 180 days of 160 mg/kg to 640 mg/kg of the CPV extract did not modify food consumption, the general aspect, and weight gain in mice over the course of time, neither in males nor in females, as compared to the control group. The animals treated also presented no differences in relation to the controls as to performance on the rotarod apparatus, (data not shown), even after 180 days of treatment, indicative of the absence of the effects of CPV extract on motor coordination and on muscular tonus.

Weight gain/loss and behavioral parameters in rats (open field). After 180 days of treatment, all of the animals presented weight gain over the course of time, there being no statistical difference between the groups. Table 2 shows a reduction in locomotion, in rearing and grooming in all three doses utilized, although only that of rearing was significant. On the other hand, there was an increase in defecation, but only in the animals treated with 860 mg/kg.

Biochemical, hematological analysis and anatomopathological examination of the organs (rats). Chronic administration for 180 days of different doses of the CPV extract did not alter the serum biochemical parameters (glucose, cholesterol, amylase, creatinine, uric acid, urea, albumine, total proteins, GGT, TGP, alkaline phosphatase, and serum glutamic oxalacetic transaminase) and hematological (red series: erythrocytes, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobine; white series: leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils) and platelets, when compared to the control group (Table 3). No significant result was found in the anatomopathological examination of the organs that might be attributed to chronic treatment with CPV extract, evidencing absence of toxic effects (data not shown).

Estrus cycle in rats. The treatment with different doses of CPV extract over the course of 60 days did not produce significant alterations in the various parameters of the estrus cycle in rats (number of estrus, interval between estrus, and total duration of the cycle both on the first 15 days, and on the last 15 days of treatment). At the termination of the chronic treatment, the females were killed, and their organs subjected to an anatomopathological examination. No alteration worthy of note was observed in the macroscopic and microscopic examination of the organs.

Possible effect on fertility in rats and teratogenic effect on offspring. In Table 4, it can be observed that all of the parameters analyzed were similar to those observed in the control group. Parameters related to: weight gain/loss, day eyes opened, day auditive pavillion opened, day for adult walking, postural reflex, as also behavior of male offspring in open field at 70 days of life were not altered in relation to the controls (data not shown). Effects of treatment on ambulation on the eighth and thirteenth day of life of the offspring (male and female) born from treated rats, are presented in Table 5. It is seems that no statistical differences were found between control and treated groups.

An analysis of results of the open field test (Table 6) shows that female offspring presented an increase in ambulation that was statistically significant only with the greater dose in relation to the control group, a fact that was not observed in male offspring.

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Table 3. Biochemical and hematological parameters in rats after chronic administration for 180 days of different doses of the CPV extract. The values represent the average (X), followed by the standard deviation (sd)

		Treatment and Doses (mg/kg)			
Functions	Parameters[a]	Controls (N = 10)	CPV 43 (N = 10)	CPV 430 (N = 10)	CPV 860 (N = 10)
General	*glucose*	83.8 ± 11.8	81.5 ± 5.4	82.9 ± 12.0	81.2 ± 4.3
	Cholesterol	108.8 ± 27.7	113.5 ± 21.8	106.4 ± 11.6	116.4 ± 23.9
	Amylase	1764.2 ± 341.4	1649.1 ± 242.4	1606.8 ± 245.6	2091.5 ± 570.6
Renal	*Creatinine*	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
	Uric acid	1.7 ± 0.4	1.6 ± 0.3	1.4 ± 0.3	1.6 ± 0.3
	Urea	54.2 ± 6.6	55.1 ± 7.6	56.0 ± 5.4	56.4 ± 7.0
Hepátic	*Albumine*	4.0 ± 0.2	4.0 ± 0.3	4.0 ± 0.2	3.9 ± 0.2
	Total Protein	7.6 ± 0.3	7.4 ± 0.4	7.3 ± 0.6	7.5 ± 0.4
	GGT	10.3 ± 4.1	9.0 ± 0	9.0 ± 0	9.8 ± 2.8
	TGP	92.8 ± 16.6	92.6 ± 20.1	87.9 ± 16.6	101.9 ± 16.9
	*Alk P**	113.3 ± 53.8	112.2 ± 14.2	110.2 ± 24.6	115.5 ± 33.8
	TGO	251.8 ± 69.6	233.0 ± 63.5	239.9 ± 83.5	253.5 ± 51.3
	Erythrocytes	6.9 ± 0.9	6.3 ± 0.5	6.3 ± 0.5	16.6 ± 29.7
	Hemoglobin	12.0 ± 1.6	11.0 ± 0.7	10.9 ± 0.7	12.2 ± 1.6
Red	Hematocrit %	33.2 ± 4.0	31.7 ± 1.9	31.6 ± 2.0	35.8 ± 5.4
series	MCV**	48.5 ± 2.1	50.2 ± 2.4	50.0 ± 1.9	50.2 ± 2.4
	MCH***	17.5 ± 1.0	17.5 ± 0.8	17.2 ± 0.8	17.1 ± 0.8
	Leucocytes	6.2 ± 2.9	5.7 ± 0.9	5.4 ± 0.9	6.1 ± 1.4
White	Neutrophils (%)	13.1 ± 5.7	13.1 ± 5.2	10.7 ± 2.9	12.6 ± 7.2
series	Lymphocytes (%)	53.5 ± 10.1	53 ± 12.3	56.1 ± 6.9	51.9 ± 16.2
	Monocytes (%)	30.1 ± 6	30.8 ± 6.2	30.9 ± 4.3	32.5 ± 9.3
	Eosinophils (%)	2.7 ± 0.8	2.7 ± 1.2	1.8 ± 0.4	2.6 ± 1.3
	Basophils (%)	0.6 ± 0.2	0.7 ± 0.8	0.5 ± 0.2	0.5 ± 0.3
	Platelets	731.0 ± 144.1	662.0 ± 114.1	632.4 ± 111.1	609.6 ± 84.9

NS

* Alkaline phosphatase; *** mean cell volume; ** mean cell hemoglobin.

[a] *Units are as follow*: Hemoglobin: g/dl; Erythrocytes: $\times 10^6/mm^3$; Mean cell volume: μm^3; Mean cell hemoglobin: pg; Platelets and Leucocytes: $\times 10^3/mm^3$.

Table 4. Effects of administration of the CPV extract on females rats treated by mouth for 10 days prior to and throughout all of the pregnancy. The values represent the average (X), followed by standard deviation (sd)

Treatment	Doses (mg/kg)	Females crossed	Females with delivery	Days of pregnancy (X ± sd)	Number of offspring/delivery (X ± sd)	Nesting (X ± sd)
Control	–	12	10	27.7 ± 3.2	13.0 ± 2.3	14.9 ± 2.0
CPV	430	12	9	27.0 ± 2.3	10.4 ± 6.2	10.8 ± 5.7
CPV	860	12	10	26.0 ± 1.4	9.4 ± 4.8	10.2 ± 4.2

NS

Table 5. Effect from administering CPV extract by mouth on ambulation of offspring of females rats treated 10 days prior to and throughout all of the pregnancy. The values represent the average (X), followed by the standard deviation (sd)

Treatment	N	Doses (mg/kg)	Ambulation (8th day) (X ± sd)	Ambulation (13th day) (X ± sd)
Control	60	–	5.6 ± 2.3	13.0 ± 6.8
CPV	54	430	4.8 ± 2.5	16.4 ± 6.5
CPV	60	860	5.9 ± 3.5	15.7 ± 7.0

NS

Chronic experiments in dogs. Clinical-laboratory examinations obtained after 180 days of treatment did not present significant alterations in treated dogs, when compared with the control group (Table 7). In two of the six experimental animals and in one of the control group animals, there was an increase in thyroid (with associated parathyroid), although without statistical significance. Furthermore, isolated cases of gliose and medular hyperemia were also observed in the experimental group; in both cases, however, (increase in

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Table 6. Assessment of the behavior in open field of offspring (female and male) at the 70th day of life (n = 10 per group) of rats treated for 10 days before and throughout all of the pregnancy with different doses of CPV extract (oral), with assessment of locomotion, rearing, time spent grooming and the number of fecal boluses. The values represent the average (X) followed by the standard deviation (sd)

Treatment	Gender	N	Dose (mg/kg)	Locomotion	Rearing (X ± sd)	Grooming (X ± sd)	Defecation (X ± sd)
Control	F	10	–	107.8 ± 35.7	38.5 ± 13.2	27.8 ± 16.6	1.7 ± 2.4
CPV	F	10	430	137.2 ± 31.1	38.0 ± 10.0	21.8 ± 16.3	1.7 ± 2.6
CPV	F	10	860	181.6 ± 30.7*	44.7 ± 19.2	28.8 ± 21.0	2.4 ± 2.0
Control	M	10	–	94.7 ± 43.7	33.8 ± 17.6	15.3 ± 12.6	3.7 ± 1.5
CPV	M	10	430	109.5 ± 18.5	29.6 ± 9.2	19.9 ± 14	2.8 ± 1
CPV	M	10	860	107.2 ± 19	31.6 ± 9.7	35.6 ± 27.8	3.4 ± 2.5

* $p < 0.05$ differs in relation to control (ANOVA, followed by the multiple comparison test).

Table 7. Biochemical and hematological parameters in dogs after chronic administration with 430 mg/kg of the CPV extract for 180 days. The values represent the average (X), followed by the standard deviation (sd)

		Treatment (mg/kg)			
Functions	Parameters[a]	Male Controls (N = 3)	Male CPV (N = 3)	Female Controls (N = 3)	Female CPV (N = 3)
General	*Potassium*	4.3 ± 0.1	4.4 ± 0.3	4.6 ± 0.4	4.4 ± 0.6
	Glucose	85.5 ± 2.1	87.3 ± 6.8	82.0 ± 8.5	86.3 ± 7.6
	Cholesterol	128.5 ± 10.6	137.0 ± 8.7	175.7 ± 38.8	160.3 ± 38.1
	Sodium	146.5 ± 0.7	145.3 ± 0.6	145.0 ± 2.0	146.3 ± 0.6
	Amylase	927.5 ± 85.6	1017.3 ± 300.8	836.3 ± 174.0	649.3 ± 214
Renal	*Uric acid*	0.2 ± 0	0.2 ± 0	0.4 ± 0.3	0.2 ± 0
	Creatinine	0.9 ± 0.07	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.1
	Urea	35.4 ± 11.3	37.6 ± 12.6	35.4 ± 3.6	33.2 ± 6.0
Hepatic	*Albumine*	3.6 ± 0.1	3.4 ± 0.2	3.5 ± 0.1	3.7 ± 0.3
	Total protein	6.1 ± 0.1	6.1 ± 0.4	6.2 ± 0.2	6.4 ± 0.4
	GGT	13.0 ± 0	13.0 ± 2.7	10.3 ± 1.2	10.6 ± 1.5
	TGO	47.0 ± 1.4	40.3 ± 7.1	39.0 ± 8.5	46.3 ± 0.6
	TGP	67.5 ± 0.7	64.7 ± 8.1	43.6 ± 3.8	51.6 ± 5.0
	*Alk P**	60.0 ± 21.2	81.3 ± 33.3	78.0 ± 20.2	74.0 ± 20.1
Red series	Erythrocytes	7.1 ± 0.4	6.8 ± 0.1	6.7 ± 0.9	7.3 ± 0.3
	Hemoglobin	16.5 ± 1.0	15.7 ± 0.6	15.6 ± 2.0	17.0 ± 0.5
	MCH**	23.3 ± 0.03	23.2 ± 0.4	23.1 ± 0.5	23.4 ± 0.3
	Hematocrit %	49.7 ± 3.5	47.8 ± 0.9	47.1 ± 5.4	51.7 ± 1.4
	MCV***	70.0 ± 0.8	70.6 ± 0.6	70.0 ± 1.0	71.2 ± 1.2
White series	Leukocytes	12.7 ± 0.1	15.4 ± 2.3	12.6 ± 2.0	14.0 ± 2.9
	Neutrophils (%)	30.1 ± 3.2	35.6 ± 8.7	37.5 ± 10.6	33.4 ± 4.7
	Eosinophils (%)	2.6 ± 1.7	2.5 ± 0.9	1.3 ± 1	1.8 ± 0.2
	Basophils (%)	0.5 ± 0	0.4 ± 0.05	0.3 ± 0	0.6 ± 0.05
	Lymphocytes (%)	26.8 ± 0.9	26.4 ± 10.6	31.5 ± 10.5	29.2 ± 7.3
	Monocytes (%)	39.9 ± 5.9	35.1 ± 3.3	29.5 ± 20.7	34.8 ± 3.5
	Platelets	277 ± 56.6	321.7 ± 11.4	350.7 ± 170	311.7 ± 81.1

NS

* Alkaline phosphatase; ** mean cell hemoglobin; *** mean cell volume.

[a] *Units are as follow*: Hemoglobin: g/dl; Erythrocytes: $\times 10^6/mm^3$; Mean cell volume: μm^3; Mean cell hemoglobin: pg; Plateles and Leucocytes: $\times 10^3/mm^3$.

thyroid and in gliose) no evidence was detected of cell proliferation. All of the other alterations observed presented regular distribution between animals of the control and experimental groups, and could not be attributed to the treatment.

Tests for genotoxicity and mutagenicity

The Ames test. The results indicate that the CPV extract could not induce mutations in the four lineages of *Salmonella typhimurium*, according to a report supplied by the UFRGS Laboratory of Genotoxicity.

The Micronucleus test. Doses of up to 2000 m/kg of CPV did not produce signs of toxicity, or significant reduction in the production of erythrocytes, and were considered negative for chromosomic mutagenicity, according to the report supplied by the UFRGS Laboratory of Genotoxicity.

DISCUSSION

In former studies carried out in our laboratories (Tabach *et al.*, submitted), it was demonstrated that the CPV

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extract, based on plants *Crataegus oxyacantha*, *Passiflora incarnata*, and *Valeriana officinalis* possesses, in mice, effects similar to those of the benzodiazepinic anxiolytics. In fact, both extract and diazepam increased the number of entries and the time of permanence in the open arms of the elevated plus maze and enhanced the sleeping time of pentobarbital.

The present study also shows that CPV extract diminishes motor activity in mice in the same way as diazepam and other benzodiazepines (Siemiatkowski *et al.*, 2000); the data obtained in the open field also support the suggested anxiolytic effect of the CPV extract (Prut and Belzung, 2003).

This anxiolytic effect in mice seems to be specific, so much so that the CPV extract did not prove to have any analgesic, neuroleptic, antidepressive effect, or on the dopaminergic system (Tabach *et al.*, submitted). Furthermore, to reinforce the similarity in effect to the benzodiazepines, former experiments carried out in our laboratories showed that the CPV extract could also exert an amnesic effect in mice as, does, for instance, diazepam (Tabach *et al.*, submitted; Dhingra *et al.*, 2004).

On the other hand, the results of the present study show that the association of the plants *Crataegus oxyacantha* L., *Passiflora incarnata* L., and *Valeriana officinalis*, even when administered acutely or over a long period of time, presented little toxicity in the three species of animals studied. The absence of significant effects relating to acute or chronic toxicity (180 days) is a contrast to data in literature concerning benzodiazepines. Thus, LD_{50} of diazepam by mouth is 720 mg/kg in mice and 1240 mg/kg in rats (Coleman and Johnston, 1979), whereas 4570 mg/kg was the value found for the CPV extract. It must be emphasized that a drug possessing an LD_{50} of 5 g/kg or more may be considered atoxic (Hodgson, 2000). In the same way, there is a marked difference in potency between diazepam and CPV extract in the motor coordination of mice. Doses as low as 0.5–3.0 mg/kg of diazepam (Savic *et al.*, 2003; Jardim and Guimarães, 2004) markedly impaired to performance in mice, including on the rotarod apparatus; with CPV extract, even a high dose of 5000 mg/kg by mouth did not impair the performance of these animals.

Also, CPV extract practically did not alter the estrus cycle, did not interfere with the fertility of rats, and did not induce teratogenesis in the offspring born from females treated during the entire pregnancy; these facts are a contrast to the known effects of diazepam and other benzodiazepines on the parameters of pregnancy and the health of the offspring exposed *in vitro* (Ryan and Pappas, 1986).

Another important aspect relates to the fact that this extract does not possess any genotoxic or mutagenicity effect: both the Ames test and the micronuclei test presented negative results, as opposed to the data observed with the benzodiazepines (Giri and Banerjee, 1996).

In conclusion, the results obtained with this study indicate that the effects of CPV extract formed by the association of three different plants did not potentialize toxic effects that might be attributed to each one of the components of this extract, even when administered in high doses and for a long period of time, suggesting that this is an association devoid of risk for human beings.

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REFERENCES

Ames BN, McCann J, Yamasaky E. 1975. Methods for detecting carcinogens and mutagens with samonella/mammalian-microsome mutegenicity test. *Mutat Res* **11**: 347–364.

Andreatini R, Sartori VA, Seabra ML, Leite JR. 2002. Effect of valepotriates (valerian extract) in generalized anxiety disorder: a randomized placebo-controlled pilot study. *Phytother Res* **16**: 650–654.

Carlini EA. 1972. Screening farmacológico de plantas brasileiras. *Rev Bras Biol* **32**: 265–275.

Carlini EA, Burgos U. 1979. Screening farmacológico de ansiolíticos: metodologia laboratorial e comparação entre diazepam e clorobenzepam. *Rev Assoc Bras Psiquiat* **2**: 25–31.

Coleman JH, Johnston JA. 1979. *Handbook of psychoactive drug prescribing information*. Physicians Postgraduate Press: Memphis, USA.

Degenring FH, Suter A, Weber M, Saller R. 2003. A randomised double blind placebo controlled clinical trial of a standardised extract of fresh Crataegus berries (Crataegisan) in the treatment of patients with congestive heart failure NYHA II. *Phytomedicine* **10**: 363–9.

Dhawan K, Kumar S, Sharma A. 2001a. Anxiolitic activity of aerial and underground parts of *Passiflora incarnata*. *Fitoterapia* **72**: 922–926.

Dhawan K, Kumar S, Sharma A. 2001b. Comparative biological activity study on *Passiflora incarnata* and *P. edulis*. *Fitoterapia* **72**: 698–702.

Dhingra D, Parle M, Kulkarni SK. 2004. Memory enhancing activity of Glycirrhyza glabra in mice. *J Ethnopharmacol* **91**: 361–365.

Fehri B, Aiache JM, Boukef K, Memmi A, Hizaoui B. 1991. *Valeriana officinalis* and *Crataegus oxyacantha*: toxicity from repeated administration and pharmacologic investigations. *J Pharm Belg* **46**: 165–176.

Fernandez S, Wasowski C, Paladini AC, Marder M. 2004. Sedative and sleep-enhancing properties of linarin, a flavonoid-isolated from Valeriana officinalis. *Pharmacol Biochem Behav* **77**: 399–404.

Fisher AA, Purcell P, Couteur DGL. 2000. Toxicity of Passiflora incarnata L. *J Toxico Clin Toxicol* **38**: 63–66.

Formigoni MLO, Lodder HM, Gianotti-Filho O, Ferreira TMS, Carlini EA. 1998. Pharmacology of lemongrass (Cymbopogon citratus stapf). II Effects of daily two months administration in male and in female rats and in offspring exposed "in utero". *J. Ethnopharmacol* **17**: 65–74.

Gilbert B, Alves LF. 2003. Synergy in plant medicines. *Curr Med Chem* **10**: 13–20.

Giri AK, Banerjee S. 1996. Genetic toxicology of four commonly used benzodiazepines: a review. *Mutat Res* **340**: 93–108.

Grieve M. 1994. *A modern herbal: The medicinal, culinary, cosmetic and economic proprieties, cultivation and folklore of herbs, grasses, fungi, shrubs and trees with all their modern scientific uses*. Tiger: London.

Guo Y, Kong L, Wang Y, Huang Z. 2004. Antidepressant evaluation of polysaccharides from a Chinese herbal medicine Banxia-houpu decoction. *Phytother Res* **18**: 204–207.

Hanus M, Lafon J, Mathieu M. 2004. Double-blind, randomised, placebo-controlled study to evaluate the efficacy and safety of a fixed combination containing two plant extracts

(*Crataegus oxyacantha* and *Eschscholtzia californica*) and magnesium in mild-to-moderate anxiety disorders. *Curr Med Res Opin* **20**: 63–71.

Hodgson E. 2000. Introduction to toxicology. In *Textbook of modern toxicology Second Edition*, Hodson E, Levi PE (eds). McGraw-Hill Companies: Singapore, 1–25.

Jardim MC, Guimarães FS. 2004. Role of glutamate ionotropic receptors in the dorsomedial hypothalamic nucleus on anxiety and locomotor behavior. *Pharmacol Biochem Behav* **79**: 541–546.

Krenn L. 2002. [Passion Flower (*Passiflora incarnata* L.) – A reliable herbal sedative]. *Wien Med Wochenschr* **152**: 404–406.

Kuribara H, Tomioka H, Takahashi R, Onozato K, Murohashi N, Numajiri T, Iwata H, Koya S. 2004. An antidepressant effect of sho-ju-sem, a Japanese herbal medicine, assessed by learned helplessness model in mice. *Phytother Res* **18**: 173–176.

Mendes FR, Mattei, R, Carlini, EA. 2002. Activity of *Hypericum brasiliense* and *Hypericum cordatum* on the central nervous system in rodents. *Fitoterapia* **73**: 462–471.

Morazzoni P, Bombardelli E. 1995. *Valeriana officinalis*: traditional use and recent evaluation of activity. *Fitoterapia* **66**: 99–112.

Oliveira MG, Monteiro MG, Macaúbas C, Barbosa VP, Carlini EA. 1991. Pharmacologic and toxicologic effects of two Maytenus species in laboratory animals. *J Ethnopharmacol* **34**: 29–41.

Prut L, Belzung C. 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* **463**: 3–33.

Rajendran S, Deepalakshimi PD, Parasakthy K, Devaraj H, Devaraj SN. 1996. Effect of tincture of Crataegus on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. *Atherosclerosis* **123**: 235–241.

Ryan C, Pappas, BA. 1986. Intrauterine diazepam exposure: eefects on physical and neurobehavioral development in rat. *Neurobehav Toxicol Teratol* **8**: 279–286.

Savic MM, Obradovic DI, Ugresic ND, Bokonjic DR. 2003. The influence of diazepam on atropine reversal of behavioural impairment in dichlorvos-treated rats. *Pharmacol Toxicol* **93**: 211–218.

Schmid W. 1976. The micronucleus test for cytogenetic analysis. In *Chemical mutagens principles and method for their detection.* Hollaender A (ed.). Plenum Press: New York, 31–53.

Shanthi R, Parasakthy K, Deepalakshmi PD, Niranjali DS. 1996. Protective effect of tincture of crataegus on oxidative stress in experimental atherosclerosis in rats. *J Clin Biochem Nutr* **20**: 211–223.

Shivalingappa H, Satyanarayan ND, Purohit MG, Sharanabasappa A, Patil SB. 2002. Effect of ethanol extract of *Rivea hypocrateriformis* on the estrous cycle of the rat. *J Ethnopharmacol* **82**: 11–17.

Siemiatkowski M, Sienkiewicz-Jarosz H, Czlonkowska AI, Bidznski A, Plaznik A. 2000. Effects of buspirone, diazepam and zolpidem on open field behavior, and brain [3H] muscimol binding after buspirone pretreatment. *Pharmacol Biochem Behav* **66**: 645–651.

Soulimani R, Younos C, Jarmouni S, Bousta D, Misslin R, Mortier F. 1997. Behavioural effects of *Passiflora incarnata* L. and its indole alkaloid and flavonoid derivatives and maltol in the mouse. *J Ethnopharmacol* **57**: 11–20.

Sousa FCF, Melo CTV, Monteiro AP, Lima VTM, Gutierrez SJC, Pereira BA, Barbosa-Filho JM, Vasconcelos SMM, Fonteles MF, Viana GSB. 2004. Antianxiety and antidepressant effects of riparin III from *Aniba riparia* (Nees) Mez (Lauraceae) in mice. *Pharmacol Biochem Behav* **78**: 27–33.

Tabach R, Mattei R, Carlini EA. 2008. Pharmacological evaluation of a phytotherapeutic product-CPV (dry extract *of Crataegus Oxyacantha* L*, Passiflora Incarnata* L*, and Valeriana Officinalis* L) in Laboratory Animals (**Submitted**).

Williamson EM. 2001. Synergy and other interactions in phytomedicines. *Phytomedicine* **8**: 401–409.

Williamson EM. 2002. *Major herbs of Ayurveda*. Churchill Livingstone: London.

Wu Y, Zhang Y, Na Wu J, Lowell T, Gu M, Yuan CS. 1998. Effects of Erkang, a modified formulation of Chinese folk medicine Shi-Qua-Da-Bu-Tang, on mice. *J Ethnopharmacol* **61**: 153–159.

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