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Bee-honey, propolis and *Eucalyptus globulus* extract: Pre-clinical toxicity study in Rodents

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ABSTRACT

This study was designated to evaluate the preclinical toxicity of the phytomedicine - bee honey, propolis (1.8%) and extract of *Eucalyptus globulus* (3,7%), commonly used in Brazil in treatment of breathing diseases. In order to evaluate the acute toxicity, groups of Swiss mice (n=10/group) received a single dose of phytomedicine (7.5, 15, 25 or 35mL/kg; p.o.) or saline 0.9% (5mL/kg; p.o.). This essay registered 20% and 40% of mortality rate with doses of 25 and 35 ml/kg, respectively. Animals presented lethargy and deaths were preceded by convulsion. The absence or presence of phytomedicine chronic toxicity was evaluated through biochemical and hematological analysis on rats (n=10/group) blood samples using daily oral doses of phytomedicine (7.5 or 15 ml/Kg) or saline 0.9% (5ml/kg; p.o.), during 90 days. The chronic toxicity essay did not show any treatment-related abnormalities in hematological parameters (red blood cell, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration; leucocytes and platelets). Concerning to biochemical parameters (glucose, urea, cholesterol, HDL-cholesterol, triglycerides; alanine aminotranferase, aspartate aminotranferase, alkaline phosphatase and total proteins), only the group treated with phytomedicine (7.5 mg/ml), revealed significant difference ($p < 0.01$) in the concentration of triglycerides (102.0 ± 8.8 in males and 100.0 ± 8.8 in females) when compared to control group (86.8±8.8 in males and 84.6±8.8 in females). No significant differences were found between the treated and control group in regard to body weight gain mean, neither organs (heart, spleen, liver, kidney, suprarenal gland, stomach, lungs, testicles, ovaries and womb) weight. The macroscopic analysis of many visceras did not show any significant differences between treated and control group. Thus, through this preclinical assay, it appears that no toxicological hazard (acute and chronic) is related to the use of tested phytomedicine.

KEYWORDS – Acute toxicity, chronic toxicity, *Eucalyptus globulus*

INTRODUCTION

The medicinal plants therapeutic use, or phytotherapy, is a modality in expansion around the world, constituting a quite promising market. This kind of therapeutic approach comes turning more and more popular on industrialized world. Today, only 5% of world flora was studied in their medicinal properties, and only 1% is used as raw material in pharmaceutical industry (13).

The growing phytomedicine market and the renewed interest by the general phytotherapy become necessary to review the sale situation of phytomedicine products, which can be acquired, surprisingly, in fairs, supermarkets, natural products stores, drugstores or industries, and in varied forms, since product in rude state (drought plant) until even tablets, liquid mixtures or capsules. Many phytomedicines are sold based in popular tradition and justify its recommendation in puerile suppositions. Rare products were submitted to rigorous scientific tests and, in spite of exhibiting recognized therapeutic properties, many products didn't have the risk potential or side effects evaluated. This lack of medical and sanitary control, absence of accurate botanical identification or purity certification of diverse phytomedicines may represent great danger to population health (2, 8).

The toxicity investigation is an essential procedure of medicinal plant characterization, once that offers all

demanded information by authorities involved in its regulation, moreover, it contributes to scientific knowledge of plant toxicity, and, more important, supplies useful data to health protection in long or short term (6, 22).

In order to standardize studies on the toxicity and effectiveness of herbal medicines, some countries have established guidelines to ensure verification of their safety and therapeutic efficacy. These guidelines should be followed for validation and obtaining registration and marketing of those products (4, 5).

Pre-clinical toxicity essays should indicate the trust degree that a phytomedicine must have, for its human species administration. Its includes analysis of a substance or chemical composition with the objective of classifying it toxically and, at the same time, to supply information regarding the correct use form, as well as the preventive and healing steps when of its inadequate use (16, 17).

The acute toxicity study objectives to characterize the dose/effect relation that leads to DL50 value. This parameter, that represents the statistical probability of a dose to cause death in 50% of an animal population, is useful to identify the substance relative toxicity. Chronic toxicity study establishes, or not, the existence of adverse effects and, subsequently, identifies and characterizes possible affected organs due to cumulative effects of the administered substance (9, 14).

The phytomedicine bee honey, propolis and extract of *Eucalyptus globulus* is largely used by Brazilian population in breathing diseases. The bee honey is constituted of different carbohydrates, especially fructose and glucose (maltose, sucrose and others polysaccharides could be present), amino acids, enzymes, organic acids, minerals, pollen, and a limited number of fungi, algae and yeasts (1, 10). Bee honey is used as valuable energy source, also presenting laxative effect, also being indicated as preventive and curative on physical and mental fatigues. As topical agent, bee honey presents antimicrobial activity and several studies had demonstrated **its** effectiveness against acute and chronic gastric lesions and antioxidant effects (7, 21, 31). However, there is an advice against the utilization of bee honey in patients who already presented allergic reactions to pricked of bees, in view of allergic manifestations risk (10).

Propolis is a resin that bees collect of plants sprouts and barks, increased of saliva secretions. Several pharmacological activities have been attributed to propolis: antimicrobial (15, 24), healing (3), antiviral and fungicide (15), anti-ulcerating and anti-inflammatory (32), immune-stimulation, hypo tensor and antioxidants (25). According to HAUSEN (1987), propolis use is also contraindicated in individuals that develop allergic reactions when pricked by bees (11).

Eucalyptus globulus Labill of the family Myrtaceae is an Australian origin tree. The eucalyptus leaves contains essential oil, tannins, phenolic acids, flavonoids and waxes. The oil is constituted, predominantly of eucalyptol (1,8-cineol) besides monoterpenic hydrocarbons, sesquiterpenes, aldehydes and ketones. (27). The *E. globules* pharmacological actions are due, fundamentally, to essential oil (cineol), that presents spasmolytic, antiseptic, balsamic expectorant, antibiotic, antifungal and anti-inflammatory properties (33). Eucalyptus leaves are suitable for breathing ways diseases (bronchitis, influenzas, pharynx inflammations, sinusitis, irritant coughs, and asthma); urinary infections; diabetes; feverish diseases; rheumatism; ulcers and wounds. In agreement with Matos (1998), high doses of essential oil can provoke gastric irritations, hematury, proteinury, nauseas, palpitation, convulsions and delirium. Administration during gestation or nursing period, according to that author, is not recommended (18).

The traditionalism of medicinal plants use is not enough to validate them scientifically as effective and safe medicines. As strange body in organism, the products of phytomedicines metabolism are potentially poisonous until be proven its effectiveness without damages to human organism. Phytotherapy used incorrectly, without phyto-sanitary control, can cause serious damages to human species.

The aim of the present study was to evaluate preclinical toxicology data of the phytomedicine- bee honey, propolis and *E. globolus* extract in rats and mice, in order to increase the confidence in extrapolating safety to humans, particularly with reference to its use as a herbal medicine.

MATERIALS AND METHODS

The Phytomedicine Product

The phytomedicine - bee honey, propolis and extract of *E. globulus*) was kindly ceded by PRONATU Laboratories (São Paulo – Brazil).

Composition of the phytomedicine

Alcoholic Solution of propolis.....	*1.80% (p/v)*
Eucalyptus globules L. Extract.....	*3.70% (p/v)*
Bee Honey q.s.p.....	*100 ml*

The phytomedicine comes as a dense liquid with shade varying of clear beige to brown, bottled in amber glass containing 100 ml, in syrup form. The doses indicated for this product are 03 tablespoons (15 ml) of syrup, three times a day.

Animals

The animals used were albino Swiss mice and Wistar rats (male and female) from the biothery of the Physiological and Pharmacological Department of the Biological Sciences Center of the Federal University of Pernambuco (UFPE). The animals were being maintained in controlled temperature (23±2 ºC) in 12-hour dark/light cycles with water and rations balanced *ad libitum*. All experiments were in accordance with the guidelines for Care and Use of Laboratory Animals.

For accomplishment of the toxicity essay, a pool of phytomedicine product marketed was used, belonging to different selling lots.

Pre-Clinical Toxicity

ACUTE TOXICITY (DL 50)

05 groups of mice (both sexes; 20-30g; n=10 animals/group), submitted to 12 hours fasting were used. These received growing doses of orally phytomedicine (7.5, 15, 25 or 35 ml/kg). Control group received saline solution 0.9% (5ml/kg; p.o.). The administered volume varied of 0.25ml (7.5ml/kg) to 1ml (35ml/kg), once dilutions for uniformize the volume would provoke alterations in product original composition. Larger doses were, thus, impracticable, once it would surpass the maximum volume (1ml) that may be orally administered in that animal species. The groups in study were meticulously observed during the 30, 60, 120, 240 and 360 minutes and later, every 24 hours, for 14 days. At the end of experiment, surviving animals were sacrificed through deep ethereal anesthesia and were accomplished macroscopic exams of following organs: heart, lungs kidneys, liver, stomach, ovaries, uteruses and testicles. The mortality percentage was observed by 72 hours and DL50 was made calculations, according to method of probito (19).

Chronic Toxicity

Three Wistar rats groups of either sex (150-281g; n=10 animals/group) were used. These received orally the phytomedicine (7.5 or 15 ml/kg; p.o.) or saline solution 0.9% (5ml/kg;v.o) during 90 consecutive days. All the animals were food deprived two hours before and two hours after the treatment. Body weights were registered weekly for dose adjustment. At the end of treatment and after 12 hours fasting, samples of blood were draw, by ocular puncture, for accomplishment of hematological and biochemical exams (Central Laboratory of Clinical Hospital of Federal University of Pernambuco). Subsequently, the animals were sacrificed through deep ethereal anesthesia, being proceeded organs (heart, spleen, liver, kidneys, suprarenal gland, stomach, lung, testicles, ovary and uteruses) examination (macroscopic analysis and weight).

The blood was processed for determination of the blood count (Coulter TKS): hemoglobin (Hb), hematocrit (Ht), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), leucocytes and platelets. The sanguine smears were colored by May-Grunwald-Giemsa method, and were used for differential counting of leucocytes (segmented, eosinophils, lymphocytes and monocytes). Biochemical parameters were accessed in serum samples, according to manufacturer (LABTEST). Concentration of each constituent was determined by spectrophotometer: glucose (Ortho-toluidine); urea (Urease); cholesterol (Huang mod.); HDL-cholesterol (enzymatic method); triglycerides (Soloni mod.); alanine amino-transferase-ALT (Frankel-Reitman); aspartate amino-transferase-AST (Frankel-Reitman); alkaline phosfatase (Bessey, Lowry mod.); total proteins (Biureto mod.).

Statistical Analysis

The values were expressed as averages ± standard error mean. Only results that presented probability of occurrence of nullity hypothesis in 5% ($p < 0.05$) or 1% ($p < 0.01$) were considered statistically different. The variance analyses and respective tests of average comparison were accomplished through the GLM procedure (SAS Institute, 1989).

RESULTS

Acute toxicity

It was not observed any toxicity signals, nor any mortality registration in the groups treated with the phytomedicine in 7.5 and 15 ml/kg doses, when compared to the group controls (table 1).

The mortality percentile registered in 25 ml/kg treated group was 20%. Sixty minutes after the administration of that dose, the animals presented high sedation degree and one of the deaths was preceded by convulsion.

In the 35 ml/kg treated group, a mortality percentile of 40% was registered and the animals presented the same alterations described previously for the 25 ml/kg treated group, however in a more pronounced manner. Two deaths were preceded by convulsion.

The macroscopic exam of vital organs (heart, liver,

kidneys, lungs, stomach) of the surviving animals, 14 days after the phytomedicine administration (7.5; 15; 25 and 35 ml/kg; p.o.) did not presented alterations, when compared to control group.

Chronic Toxicity

Hematological parameters (table 2) not showed differences statistically significant among treated (7.5 or 15ml/kg; p.o.) and control groups.

Except for triglycerides values for male (102.0±8.8) and females (100.0±8.8) mice in treated group with 7.5 ml/kg of the phytomedicine, no statistically significant differences were observed for any other biochemical parameter, when compared to the control group (table 3).

Table I. Percentage of mortality in mice (males and females) in groups treated with phytomedicine

	Phytomedicine				Control group Saline 0.9%
Dose	7,5 ml/kg	15 ml/kg	25 ml/kg	35 ml/kg	5 ml/kg
Deaths/Total animals	0/10	0/10	02/10	04/10	0/10
% Mortality	0	0	20	40	0

Table II. Hematological parameters in Wistar rats treated with the phytomedicine for 90 days (n = 10/group).

Parameters	Gender	Treatment		
		Phytomedicine 15ml/kg	Phytomedicine 7.5ml/kg	Saline 0.9% 5ml/kg
Erythrocyte (mm^3)	M	6.8 ± 0.16	6.7 ± 0.16	6.9 ± 0.16
	F	6.3 ± 0.16	6.7 ± 0.16	6.1 ± 0.16
Hemoglobin (g/ml)	M	21.4 ± 0.51	21.0 ± 0.51	20.6 ± 0.51
	F	19.6 ± 0.51	21.0 ± 0.51	20.2 ± 0.51
Hematocrit (%)	M	64 ± 1.46	64 ± 1.46	63 ± 1.46
	F	59 ± 1.47	63 ± 1.46	60 ± 1.46
MCH (mm^3)	M	94.1 ± 0.51	94.8 ± 0.51	94.0 ± 0.51
	F	94.6 ± 0.51	94.6 ± 0.51	93.8 ± 0.51
MCV (mm^3)	M	31.2 ± 0.33	31.6 ± 0.33	31.3 ± 0.33
	F	31.4 ± 0.33	31.4 ± 0.33	31.5 ± 0.33
MCHC (%)	M	33.2 ± 0.27	33.2 ± 0.27	32.9 ± 0.27
	F	33.2 ± 0.27	33.2 ± 0.27	32.5 ± 0.27
Leucocytes (mm^3)	M	5400 ± 0.34	6080 ± 0.34	5640 ± 0.34
	F	5660 ± 0.34	5920 ± 0.34	5460 ± 0.34
Segmented (%)	M	39 ± 1.01	41 ± 1.01	39 ± 1.01
	F	41 ± 1.01	41 ± 1.01	40 ± 1.01
Eosinophils (%)	M	2.4 ± 0.56	4.0 ± 0.55	2.4 ± 0.55
	F	1.6 ± 0.56	2.2 ± 0.55	1.8 ± 0.55

Lymphocytes (%)	M	55 ± 1.18	53 ± 1.18	54 ± 1.18
	F	53 ± 0.18	53 ± 0.18	56 ± 0.18
Monocytes (%)	M	3,2 ± 0.30	2,2 ± 0.30	3.2 ± 0.30
	F	2.8 ± 0.30	3.0 ± 0.30	2.6 ± 0.30
Platelets (mm^3)	M	318 ± 19.0	335 ± 19.0	316 ± 19.0
	F	260 ± 19.0	299 ± 19.0	284 ± 19.0

Erythrocytes values must be multiplicated by 10^6. Platelets values must be multiplicated by 10^3. Males and females are represented by M and F respectively.

Table 3. Biochemical parameters in Wistar rats treated with the phytomedicine for 90 days. (n = 10/group).

		Treatment		
Parameters	**Gender**	**15 ml/kg Phytomedicine**	**7.5 ml/kg Phytomedicine**	**15 ml/kg Saline 0.9%**
Glucose (mg/dl)	M	77.5 ± 9.7	72.4 ± 9.7	66.7 ± 9.7
	F	60.4 ± 9.7	79.3 ± 9.7	68.5 ± 9.7
Urea (mg/dl)	M	50.1 ± 2.2	43.3 ± 2.2	41.8 ± 2.2
	F	36.1 ± 2.2	39.8 ± 2.2	41.2 ± 2.2
Cholesterol (mg/dl)	M	94.0 ± 7.3	71.2 ± 7.3	89.7 ± 7.3
	F	109.2 ± 7.3	102.8 ± 7.3	96.6 ± 7.3
HDL (mg/dl)	M	36.5 ± 5.4	33.2 ± 5.4	39.1 ± 5.4
	F	56.6 ± 5.4	41.9 ± 5.4	42.7 ± 5.4
Triglycerides (mg/dl)	M	96.0 ± 8.8	102.0 ± 8.8 **	86.8 ± 8.8
	F	74.0 ± 8.8	100.0 ± 8.8 **	84.6 ± 8.8
AST (U/L)	M	27.6 ± 4.2	40.4 ± 4.2	34.2 ± 4.2
	F	34.6 ± 4.2	36.6 ± 4.2	31.2 ± 4.2
ALT (U/L)	M	30.4 ± 4.6	43.0 ± 4.6	37.2 ± 4.6
	F	39.0 ± 4.6	41.4 ± 4.6	39.4 ± 4.6
Alkaline Fosfatase (U/L)	M	77.0 ± 0.49	82.0 ± 0.49	78.0 ± 0.48
	F	80.0 ± 0.49	78.0 ± 0.49	81.0 ± 0.49
Proteins (g/100ml)	M	8.8 ± 0.37	8.8 ± 0.37	8.3 ± 0.37
	F	7.9 ± 0.37	8.6 ± 0.37	8.2 ± 0.37

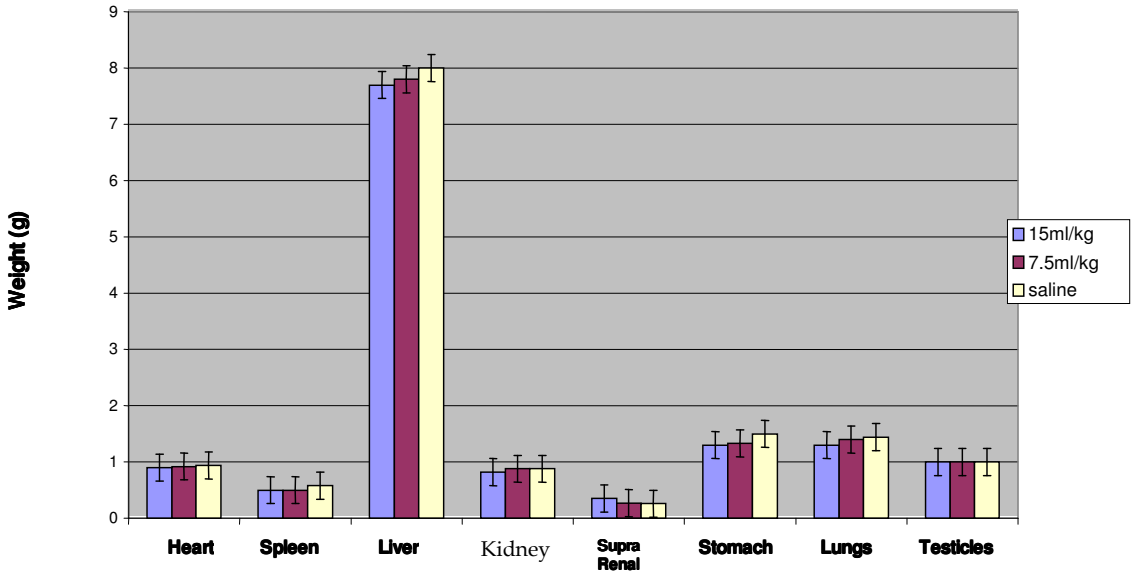

Figure 1a. Weight (g) average of phytomedicine (bee honey, propolis and E. Globulus extract) treated male Wistar rats during 90 days (n = 10 animals/group)

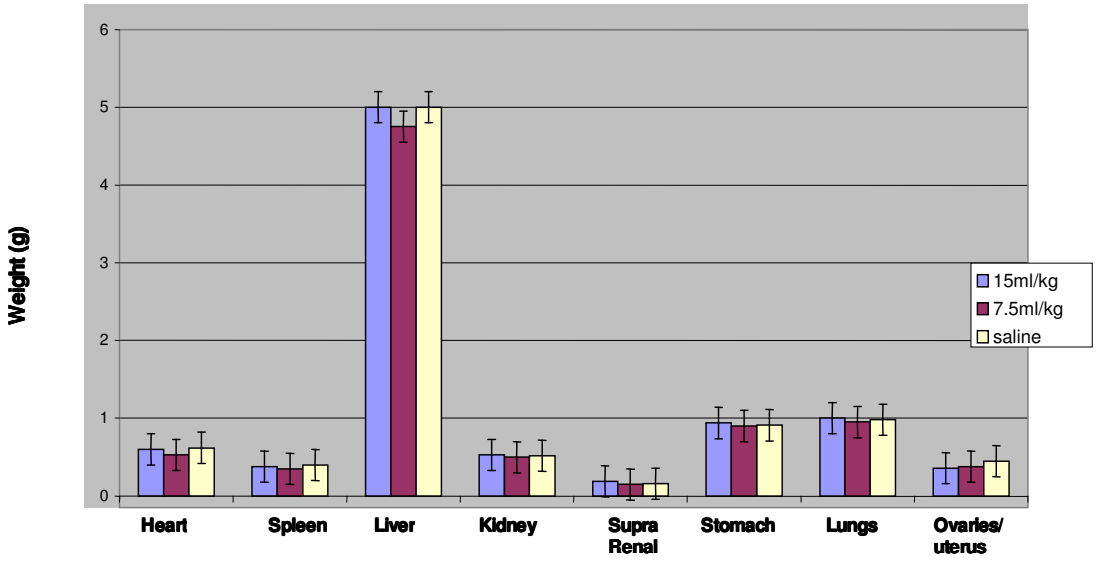

Figure 1b : Organs weight average of phytomedicine (bee honey, propolis and E. globulus extract) treated female Wistar rats during 90 days (n = 10 animals/group)

Figures 1a,b and 2a,b, show the organs weight and body weight evolution of treated and control groups. No significant differences were found between treated and control group in regard to body weight gain, neither organs weight (heart, spleen, liver, kidney, suprarenal gland, stomach, lungs, testicles, ovaries and womb). In the same way, at autopsy, the macroscopic analysis of some internal organs (e.g. heart, liver, lungs, kidneys) did not show any significant differences between treated and control group.

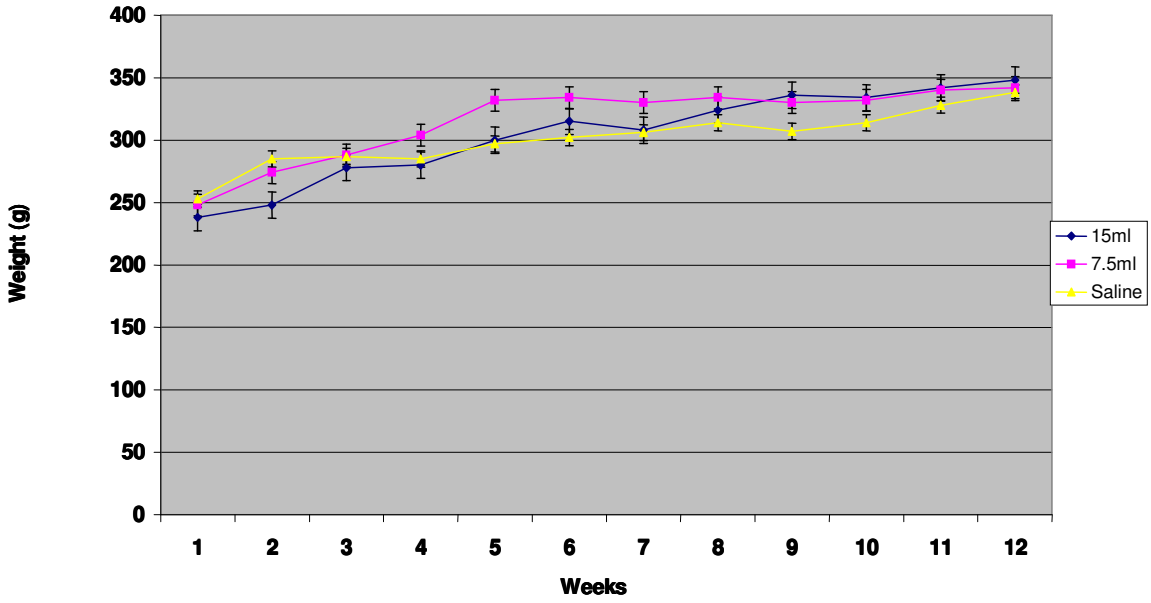

Figure 2a. Body weight evolution of Phytomedicine (bee honey, propolis and E. globulus extract) treated male Wistar rats during 90 days (n = 10 animals/group)

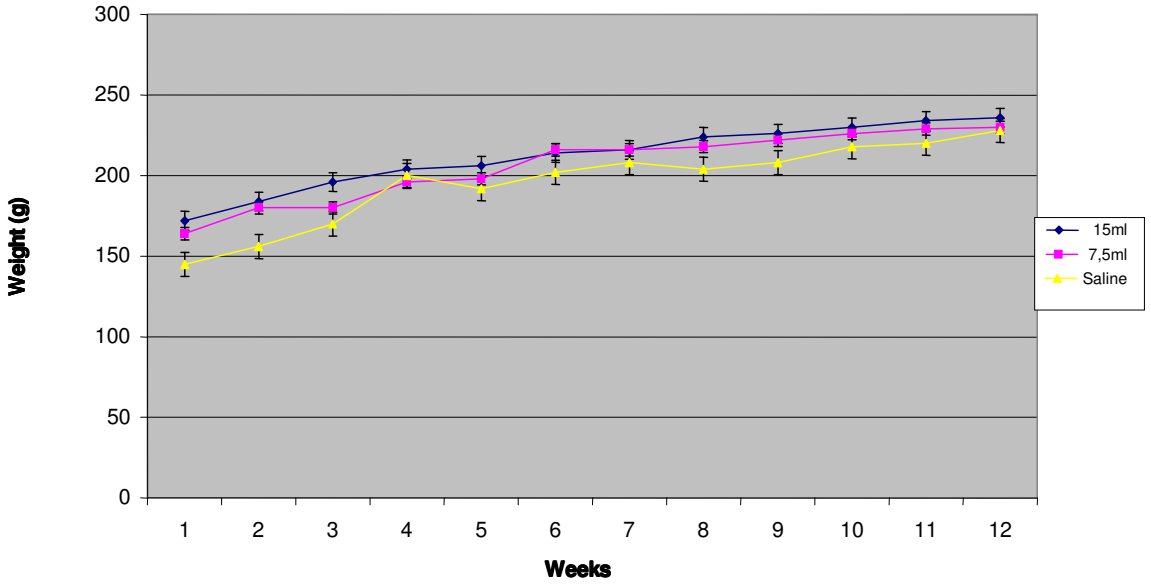

Figure 2b. Body weight evolution of phytomedicine (bee honey, propolis and E. globulus extract) treated female Wistar rats during 90 days (n = 10 animals/group).

DISCUSSION

Regarding the phytomedicine (bee honey, propolis and extract of E. globules) acute toxicity study, no mortality registration was verified to 15 ml/kg dose. However, for 25 and 35 ml/kg doses, a percentile of mortality of 20% and 40%, respectively, was observed. These last two doses represent 39 and 55 times that used in humans. It was not possible to determine DL50 value, in function of the maximum volume (1ml)

already administered with the dose of 35 ml/kg. All the animals of those last two groups presented sedation, being the death of two of them preceded by convulsion. In the consulted literature we didn't find any registration of acute toxicity with extracts of that species. However, there is data on acute toxicity for essential oil (DL50 = 3480 mg/kg; p.o.) of *E. globulus* (23, 30). Considering that until 15 ml/kg dose, which is equivalent to approximately 23.5 times that those indicated for upper air ways diseases treatment, it did not provoke any death, we can suggest that the product presents a relative safety range in studied animal species.

Among the information obtained through the studies of acute toxicity, DL50 is just one of the parameters that can be obtained (22). The poisonous effects evaluation and necropsy also supply important information for integration of pharmacological/toxicological studies. The interpretation of those data is important reference on risks evaluation of man exposition to a chemical agent.

General observations showed that the acute treatment with the phytomedicine did not revealed any toxicity signs, when compared to control group, since the beginning until the 14th day after the treatment, for the surviving animals. Such observations indicate absence of long term toxicity for the studied product. Although it has not been possible to determine the phytomedicine DL50, our results reveal a low degree of toxicity, consequently, supplying indicative of relatively safety of its acute use on tested animal species.

Chronic toxicity is an important parameter in the evaluation of drug safety range on which bases the calculation of initial dose to be used in clinical tests. Chronic toxicity essay with the phytomedicine (bee honey, propolis and extracts of E. globules) orally administered at doses of 7.5 ml/kg (187 mg/kg of the extract of E. globulus) or 15 ml/kg (374mg/kg of extract of E. globulus) revealed absence of toxicity effects. Those doses are 11.2 and 23.5 times those used for human's chronic treatment (0.64 ml/kg or 24mg/kg; p.o. of extract). Hematological analyses did not show any statistically significant differences between experimental and control groups. The values range found in treated and control groups were similar and were maintained within the reference limits (20).

Regarding the biochemical parameters, only triglycerides concentration analysis revealed statistically significant difference ($p < 0.01$) for phytomedicine treated group, at dose of 7.5ml/kg, when compared to the control group. Variations in triglycerides concentration could be caused by alterations on pancreas (calculus, biliary cirrhosis, cholestasis), liver (hepatic lesions), in some types of renal diseases, coronary heart disease and malnutrition. Physiologically that alteration may occur after meals (12). However, the macroscopic analysis of those organs did not reveal any alteration when compared to control group. Therefore, although the average corresponding to triglycerides values was statistically different, this does not suggest physiologic alterations, considering that they are in a normality range, as described in specialized literature (20, 26, 28, 29) Besides, those alterations did not present a dose-dependent effect when we analyzed the same parameters in animals treated with higher doses (15ml/kg; p.o.). Additionally, the evolution of body weight of phytomedicine treated animals (7.5 or 15 ml/kg) did not differ of control.

Considering that the dose of 7.5 ml/kg is equal to 11.2 times that indicated for upper air ways diseases treatment in humans (according to information contained on product label), we can suggest that the product presents good safety range on animal species studied.

During the whole experimental period (90 days), the animals were observed and analysed by individual clinical evaluation of body weight, locomotor's apparatus, behavioral aspects and skin. None treated group with the phytomedicine presented differences in relation to control group. Finally, toxicity signs were not evidenced on hematological or biochemical parameters, organs weigh or body weight evolution by the groups treated with the phytomedicine.

CONCLUSION

Our results give strong support that, in equivalent doses in human terms, the phytomedicines (7.5 or 15 ml/kg) did not present important toxicity (acute and chronic) effects in Swiss mice and Wistar rats of both sex.

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