Original article

Acute Oral Toxicity of Soybean Lecithin by Acute Toxic Class Method with Limit Test

Toxicidad aguda oral de la lecitina de soya por método de clases tóxicas aguda con prueba límite

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ABSTRACT

Introduction: It is known that the Cuban population has increased the consumption of soy lecithin due to the properties attributed to it, but research is still required to corroborate its effects and safety in its consumption for therapeutic purposes.

Objective: To determine the acute oral toxicity of soy lecithin in Wistar rats.

Methods: An experimental study of preclinical pharmacology was carried out in the Laboratory of Antibodies and Experimental Biomodels of the Center of Molecular Immunology (LABEX-CIM) and the Center of Toxicology and Biomedicine of the University of Medical Sciences of Santiago de Cuba, between February and November

2020, in which five female rats of the Wistar line, normopoietic of 200 ± 70g, nulliparous of ten weeks of age were used, with which a limit test was carried out, with observation for 14 days, estimating clinical variables and signs of toxicity defined in the standard as established in the guide of acute oral toxicity, by the method of classes of acute toxicity.

Results: In the rats there was no modification of body weight and no apparent signs of affections were observed except in their gastrointestinal, hemlymphopoietic and integumentary apparatus. During the development of the investigation no animal showed signs of evident toxicity leading to death, according to the criteria described, nor modifications in their macroscopic anatomopathological studies indicating toxicity.

Conclusions: Soy lecithin showed no evidence of toxicity in the acute oral toxicity test. There is reliable evidence that it does not represent a concern for human health, however, further evidence is needed to corroborate that the abuse and chronic dosage of this supplement does not pose a risk, and it is recommended that the toxicological profile of the product be completed.

Keywords: soy lecithin; preclinical experimentation; acute oral toxicity.

RESUMEN

Introducción: Se conoce que la población cubana ha incrementado el consumo de lecitina de soya por las propiedades que se le atribuyen, pero aún se requieren investigaciones que corroboren sus efectos y seguridad en su consumo con fines terapéuticos.

Objetivo: Determinar la toxicidad aguda oral de la lecitina de soya en ratas Wistar.

Métodos: Se realizó un estudio experimental de farmacología preclínica en el Laboratorio de Anticuerpos y Biomodelos Experimentales del Centro de Inmunología Molecular (LABEX-CIM) y el Centro de Toxicología y Biomedicina de la Universidad de Ciencias Médicas de Santiago de Cuba, entre febrero y noviembre del año 2020, en el que se utilizaron cinco ratas hembras de la línea Wistar, normopeso de 200 ± 70g, nulíparas de diez semanas de edad, con las que se realizó una prueba límite, con

observación durante 14 días, estimando variables clínicas y signos de toxicidad definidos en la norma según establece la guía de toxicidad aguda oral, por el método de clases de toxicidad aguda.

Resultados: En las ratas no existió modificación del peso corporal y no se observaron señales aparentes de afecciones excepto en sus aparatos gastrointestinales, hemolinfopoyéticos y tegumentarios. Durante el desarrollo de la investigación ningún animal mostró signos de toxicidad evidente que condujera a la muerte, según los criterios descritos, ni modificaciones en sus estudios macroscópicos anatomopatológicos que indicaran toxicidad.

Conclusiones: La lecitina de soya no mostró evidencias de toxicidad en el ensayo de toxicidad aguda oral. Se dispone de evidencia confiable de que no representa una preocupación para la salud humana, no obstante, se necesitan otras pruebas que corroboren que el abuso y dosificación crónica de este suplemento no supone riesgo, por lo que se recomienda completar el perfil toxicológico del producto.

Palabras clave: lecitina de soya; experimentación preclínica; toxicidad aguda oral.

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Introduction

The use of natural medicine versus its chemical alternative dates back many centuries before science appeared.[1] Man has used plants for medicinal purposes since prehistoric times. It is estimated that around 10 000 plant species are used for medicinal purposes in the world, most of them in traditional medicine systems. These still constitute an important field of pharmacological research, finding that several of their components are found in the form of herbal medicine or raw material in the pharmaceutical industry.[2] Although ancestral knowledge of plants for therapeutic purposes has been of vital importance throughout history, we cannot limit ourselves

to popular wisdom, since scientific validation of certain properties of medicinal plants is a necessity.[1]

Toxicology is defined as the study of the adverse effects of chemical, physical or biological agents on living organisms and ecosystems and is based on the principle of Paracelsus, initiator of pharmacological chemistry in the 16th century, which states that any substance can be toxic if it is consumed in sufficient quantity.[3] The acute toxicity study evaluates the toxicity induced by the substance under study as a result of the administration of high doses, either by single or repeated administration in an interval not exceeding 24 h, providing information on the maximum toxicity of the product and the possible risk associated with its acute exposure. In addition, it provides valuable information for the selection of dosage levels to be used in studies with repeated doses.[4]

Methods that use as few animals as possible and reduce their suffering are advocated internationally, such as fixed dose and acute toxic class methods. The acute toxic effects and the organic or systemic toxicity of a substance can be evaluated by various toxicity tests from which, after a single dose, a preliminary indication of toxicity can be obtained. Depending on the toxicity of the substance, a limit test may be considered which is primarily used in situations where the experimenter has information indicating that the test material is likely to be non-toxic, or toxic only above the regulatory dose limits, or the complete determination of the LD50.[4,5]

At present, industries and scientific institutions around the world are making efforts to obtain natural drugs that may be more than alternatives, complementary therapies to solve the health problems facing humanity. Researchers from the Technological Innovation Group of the Orient Pharmaceutical Laboratory, developed a product registered as a nutritional supplement with the LECISAN® trademark, from the use of a by-product from the soybean oil refining process in the processing factory of that legume, located in the Santiago de Cuba province.[6,7]

This pharmaceutical product has been well received by the population and is being prescribed by doctors from various medical specialties as a therapeutic regimen,

despite only being registered as a nutritional supplement, due to the properties attributed to it as an antioxidant, lipid-lowering agent and weight reducer, among others. Generally, the chemicals used in the preparation of medicines cure a condition in a short period of time, but they can harm other sensitive organs of the body, so their constant use is not always recommended.[2]

In the last five years, some preclinical investigations have been carried out using different biomodels, with the purpose of studying the effects of soy lecithin as dietary supplementation.[6,8] The effects observed *in vitro* and in experimental models are the basis of its use, and some studies show the need for multiple clinical trials in humans, to confirm the beneficial effects for human health.[9]

It is known that the Cuban population has increased the consumption of soy lecithin due to the properties attributed to it as an antioxidant, lipid-lowering agent and in reducing body weight. However, there is not enough evidence, through endorsed studies, to determine the degree of damage that its components may cause after excessive or prolonged administration, once the product is dispensed in our networks of natural product pharmacies and under free sale. Therefore, research is required to corroborate its effects and safety in its consumption for therapeutic purposes, for the benefit of human health.

Given its usefulness in the food industry, it has been considered slightly toxic. Endorsing the use of varied sources of lecithin's, reference can be made to the final report on the evaluation of the safety lecithin and hydrogenated lecithin published in 2001 by Fiume[10] and the scientific opinion article on its safety and efficacy for all animal species published in 2016 by the EFSA Panel on additives and products used in food,[11] in addition to the Re-evaluation lecithin's (E 322) as a food additive carried out by the panel itself in 2017.[12]

However, current legislation requires that, prior to registration and marketing, the safety of all types of products be evaluated, so it is essential to use toxicity tests in experimental biomodels in order to predict their behavior in humans.

Taking these aspects into consideration, this research is carried out with the objective of determining the acute oral toxicity of soy lecithin in Wistar rats, which could contribute to the knowledge of the pharmacology of this product registered in the country and lay the foundations for clinical investigations that justify its therapeutic use.

Methods

General aspects of the study

An experimental study of preclinical pharmacology was carried out in the Laboratory of Antibodies and Experimental Biomodels of the Center for Molecular Immunology (LABEX-CIM) of Santiago de Cuba, the Center of Toxicology and Biomedicine of the University of Medical Sciences of Santiago de Cuba, between February 2019 and November 2020, to evaluate single-dose oral toxicity of oral administration of soy lecithin.

Animal model

The biomodels were supplied by the National Center for the Production of Laboratory Animals (CENPALAB). Five female rats of the Wistar line, normal weight of 200 ± 70g, nulliparous and 10 weeks old, were used, applying the principle of reduction in animal experimentation and adhering to the norm, which establishes this number of animals per dose at research.[13]

Preparation and administration of doses

The soy lecithin by-product, supplied as raw material by the soy processing plant in Santiago de Cuba, to the Oriente Pharmaceutical Laboratory (LBF) was used for the production of the nutritional supplement registered as LECISAN®.[6,7] The test substance was administered in a single dose, using a gastric cannula. It was prepared

shortly before the administration to guarantee its stability. The animals were fasted prior to dosing (food was withheld, but not water overnight) and after this, food was withheld for a further 3-4 h.

Procedures for keeping and handling experimental animals

The animals were handled according to the recommendations oriented in this regard, complying with the quarantine procedures.[14] They were randomly selected and housed in translucent plastic boxes (T2 boxes), with stainless steel mesh lids, replaceable and previously sterilized. Commercial food for the species, supplied by CENPALAB (pellet), and water were supplied *ad libitum*. The room had a lighting regime of 12 h of light and 12 h of darkness, ambient temperature of 22 ± 2ºC and relative humidity of 60 ± 5%.

Experimental design

As established by the Organization for Economic Cooperation and Development (OECD) in the Guidelines for the Testing of Chemicals (OECD TG), Test No. 423: Acute oral toxicity - Acute toxic class method,[4] the study limit test was applied observation at 2000 mg/kg to one animal (followed by dosing to four other animals) on the basis that it was expected to produce some signs of toxicity without causing serious toxic effects or mortality with a 24 h period between doses administered to the first animal and the dosage to the others. Individual records were kept for each animal. The animals were observed individually during the first 30 min, every 30 min during the first 4 h, and then every 1 h until completing the first 24 h. They were then observed daily at least once for a total of 14 days, paying attention to the appearance of toxic reactions, emphasizing the time of appearance and the duration of the recovery period.

The individual weights of the animals were determined before the administration of the test substance and thereafter at least once a week and at the time of death or sacrifice. All test animals underwent gross necropsy for recording of gross pathological

changes, with microscopic examination of organs showing evidence of changes considered to provide useful information.

The clinical variables observed included general affectations in the digestive systems (changes in stool consistency or diarrhea and changes in eating pattern), respiratory (presence of dyspnea), cardiovascular, hemlymphopoietic, and integumentary systems (changes in the skin and coat), eyes and mucous membranes) and nervous system (changes attributed to damage to the autonomic and central nervous systems, emphasizing somatomotor activity and behavior pattern and the appearance of tremors, convulsions, salivation, lethargy, sleep and coma). The evident toxicity was determined from the description of visible signs of toxicity after the administration of the test substance, considering the variables imminent death, moribund state, predictable death and late death, as established by the standard.[4]

Method of obtaining, processing and interpreting the data

Observation, systemic method, experimentation and documentary review were applied as empirical methods in this research. Numerical and statistical analyses were used as mathematical methods. The data collected was organized and coded in a database and the statistical-mathematical processing of the results was carried out with the IBM SPSS Statistics v23.0 system (SPSS, Inc., Philadelphia) for WINDOWS©® (Microsoft, Redmond, Virginia). Descriptive statistics, with the chi-square test, through hypothesis contrast (goodness-of-fit tests) were used as a method to verify if the frequencies observed in each category were compatible with the independence between variables. The variables of interest were presented in tables and figures.

Ethical aspects

The experiments were carried out in accordance with the ethical principles recommended in the International Guidelines and in the Republic of Cuba[14] for research with laboratory animals and with the approval of the Ethics Committee of the Laboratory of Antibodies and Experimental Biomodels (LABEX-CIM) from Santiago de

Cuba. All study protocols, including the method of euthanasia, by cervical dislocation, were submitted for consideration, analysis and approval by the ethics committee in the institutional setting, also observing the provisions of biological safety regulations.

Results

Acute oral toxicity was evaluated using the acute toxic class method with limit test, based on information indicating that the test substance is likely to be non-toxic, i.e., that it has toxicity only above the recommended doses, regulatory limits. No tests were performed in the ranges of category 5 of the GHS (2000 -5000 mg/kg) as results with direct relevance to the protection of humans or animals were considered unlikely.[4]

Table 1 shows the evolution of weight as a variable indicative of acute oral toxicity. The difference in weight between the animals did not exceed 20% of the average value of the group so that there was no difference between them and the mean values between the initial weight and the 7 days showed a difference of 0.46 and 1.60 between day 7 and day 14, with a general average gain of 2.06, in the normal range. To quantify the goodness of fit, the X^2 (chi-square) test was used, where it can be seen that the variation in weight in the animals is not visually significant. Many are the studies carried out on substances with pharmacological properties before moving on to the clinical phase, among these are the toxicological studies.

Table 1 - Weight evaluation in rats during acute oral toxicity study.

Biomodel estimates						Statisticians			
Weight (g)	R1	R2	R3	R4	R5	Mean values	Typical deviation	Variance	*p* value*
Initial	237.00	248.00	250.00	249.00	257.00	248.20	7.190	51.70	0.220
7 days	237.00	248.50	250.00	250.00	257.80	248.66	7.468	55.77	0.241
14 days	239.50	250.30	251.00	252.00	258.50	250.26	6.842	46.81	0.220

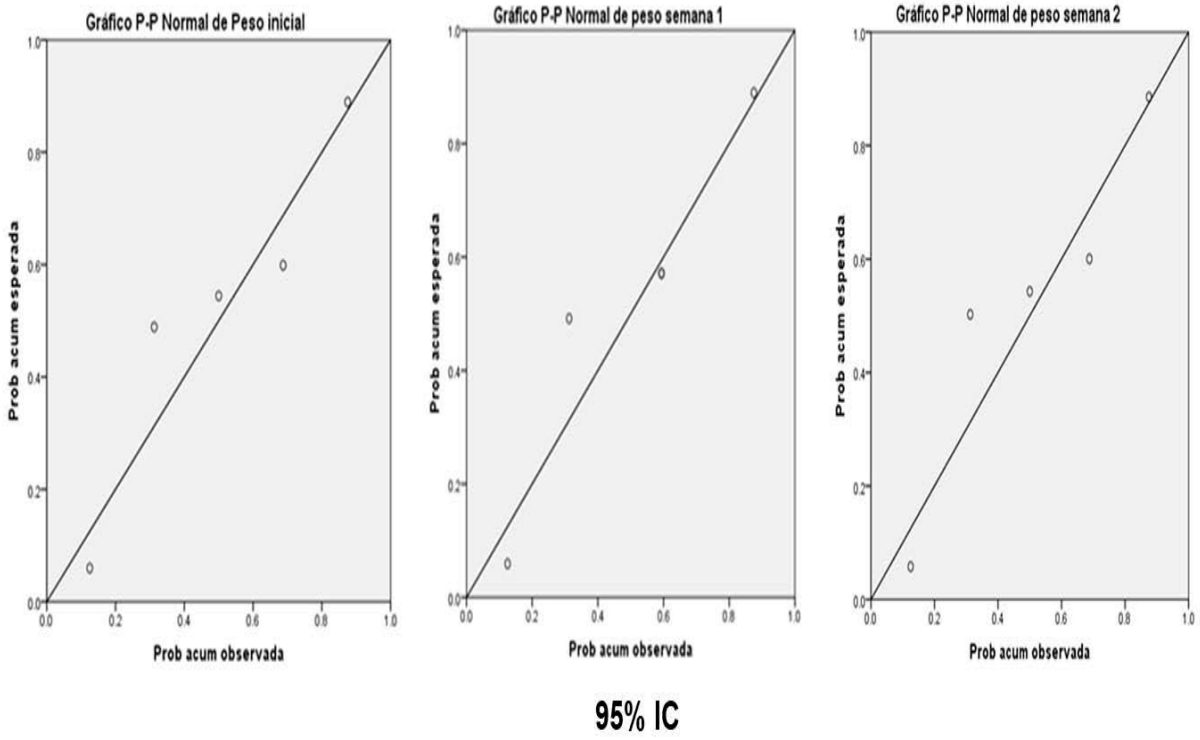
Legend: R1-rat 1; R2-rat 2; R3-rat 3; R4-rat 4; R5-rat 5.

Pearson's chi-square * P value <0.05 statistically significant

Source: Registry of parameters in biomodels.

There were no changes in the weight of the animals from the administration to the end of the observation period, the animals showing a normal weight gain throughout the weeks of experimentation. It was concluded that weight was not an element to be considered to evaluate possible toxic effects.

Through the analysis of pp graphs (probability to probability) the comparison of the cumulative distribution function of the two distributions (empirical and theoretical) was represented with each other and taking into consideration that the values are approximately aligned in a straight line we can state that the series data conforms to Gauss's law. The graphs for initial weight, at day 7 and at day 14, include the intermediate line and the expected percentile of the distribution based on estimates of the maximum probability parameter as observed (fig.1).



Note: Without applying standardization and considering a normal distribution, it can be seen that there were no variations when applying the estimation method of the average ranges of the values for the weight of the animals.

Source: Table1.

Fig. 1- Probability plots for estimated weight during the study.

Taking into consideration representative clinical elements of each apparatus or system, possible toxic effects were evaluated, as evidenced (table 2).

No apparent signs of affections in the respiratory and cardiovascular systems were observed. In the gastrointestinal tract, soft stools and decreased appetite appeared in all the animals (100%) after administration, being persistent in the first 48 h and then recovering between the third and fourth days.

In addition, during the administration period, no alterations of the nervous system, skin lesions, eye damage, or piloerection were observed, but the skin and integuments turned pale during the same period in which the gastrointestinal manifestations appeared.

Similarly, in all the biomodels, the mucous membranes were hypo colorized, being more evident at 72 h, persisting until the end of the observation.

Table 2 - Clinical elements indicative of acute toxicity from the exploration of variables in the different devices or systems.

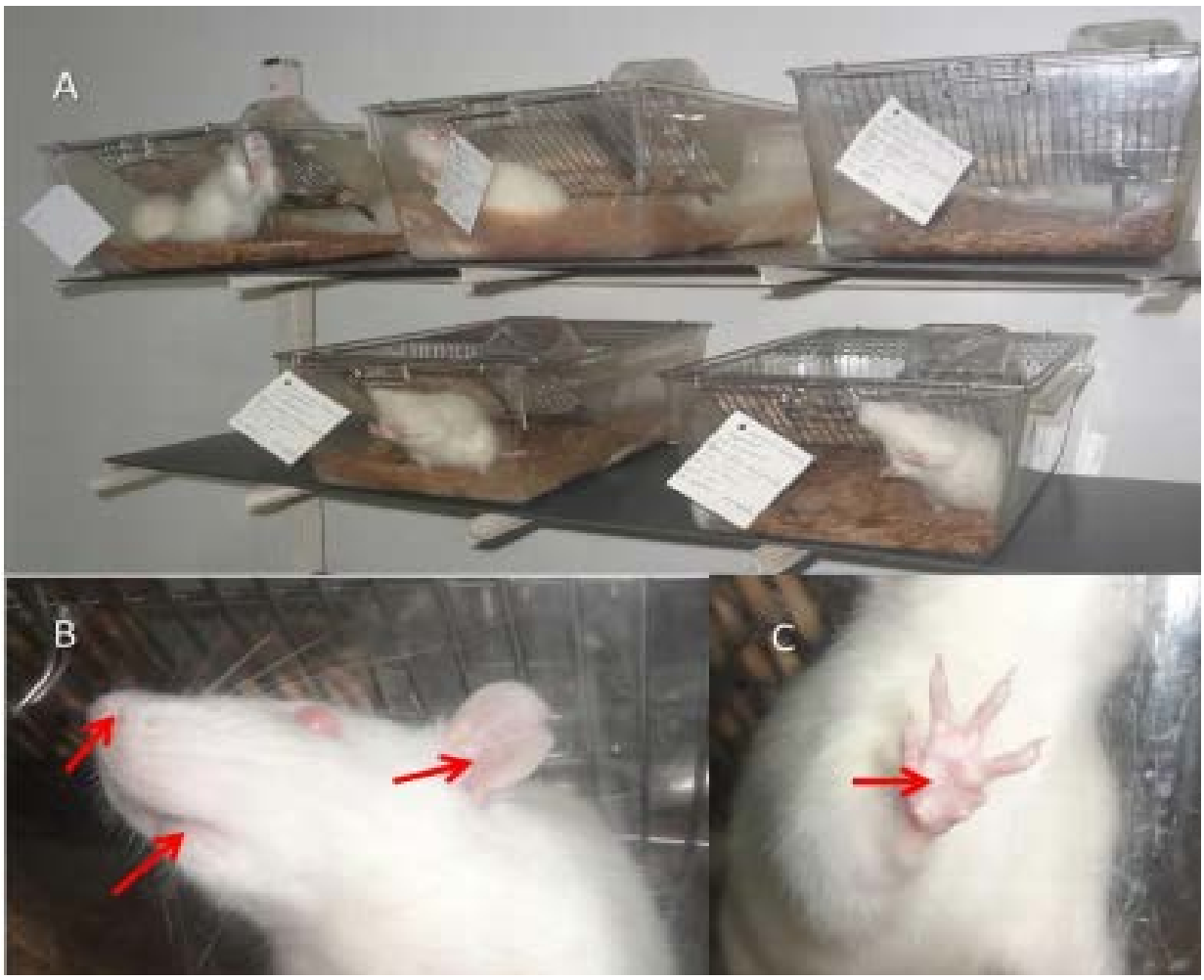
Devices or Systems	Clinical Elements of Acute Toxicity
Gastrointestinal	Loose stools/decreased appetite
Respiratory	Apparently, it hasn't happened
Cardiovascular	Apparently, it hasn't happened
Hem lymphopoietic	Hypocolored mucous membranes
Integuments	Pale coloration of skin and integuments
Nervous system	Apparently, it hasn't happened

Source: Registry of parameters in biomodels.

During the development of the investigation, no animal showed signs of evident toxicity that would lead to death, taking into consideration the criteria described and it is to be assumed that the LD 50 exceeds 2000 mg/kg based on what was observed and the previous information from which peer-reviewed research was available.[10,11,12,15,16,17]

Figure 2. Shows the external appearance of the animals where only slight paleness is observed, which becomes more evident at the mucosal level and through the skin of

the plantar pads and ears. When performing the observation of clinical variables, these elements correspond to the observation of changes in the hematopoietic and integumentary system.

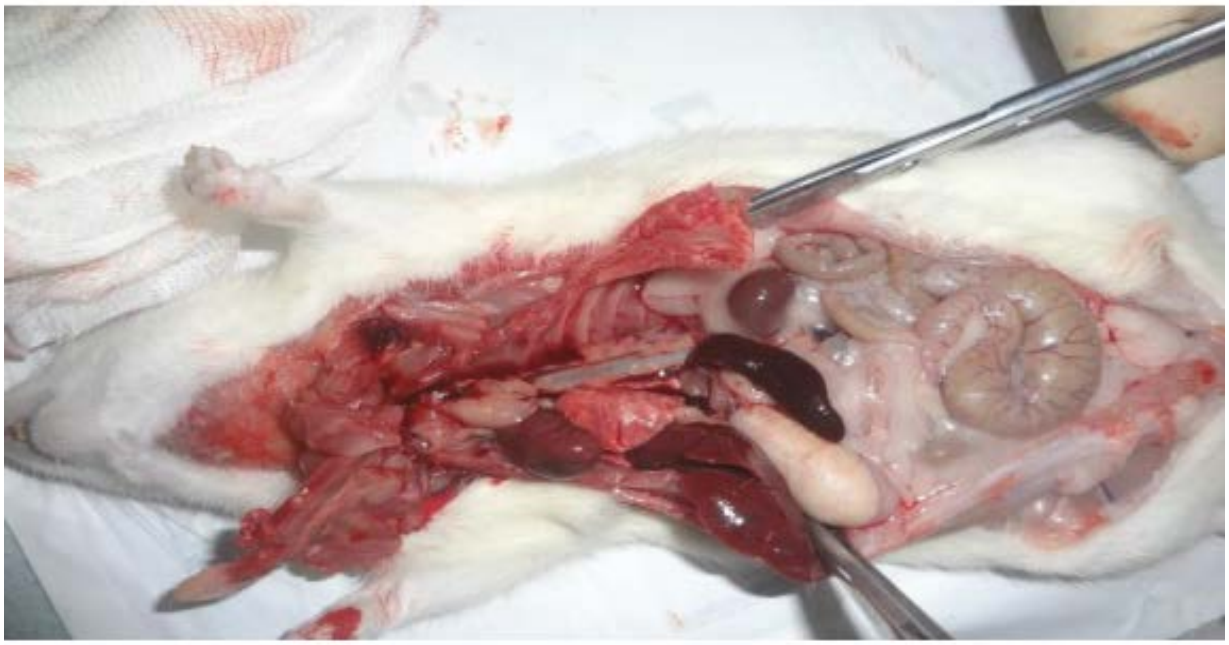


A) Animals under observation. B) Hypocoloration of the mucous membranes and integuments corresponding to the openings of the mouth, nose and skin of the ears marked with the red arrow. C) Hypocoloration of the plantar pads in the anterior train

Source: Photographs taken by the author during the study

Fig 2 - External appearance of the animals during the observation of clinical variables indicative of toxicity.

After 14 days of observation, the animals were sacrificed. No macroscopic changes were evident in the necropsies performed on the animals (fig. 3), so it was not necessary to carry out a microscopic study, taking into account the characteristics of the study.



Note: Photograph taken during the investigation where no morphological alterations indicative of toxicity in the organs evaluated are revealed.

Source: Photograph taken by the author during the study

Fig 3 - Macroscopic appearance of the organs and tissues in the animals after necropsy.

Discussion

The Organization for Economic Cooperation and Development (OECD) Guidelines for the testing of chemicals are regularly updated in light of scientific progress or changing assessment practices.[4,13] In 1984, the British Society of Toxicology suggested a new approach to acute toxicity testing based on administration at a series of fixed dose levels, avoiding the use of animal death as an endpoint and it was based on changes in the observation of clear signs of toxicity. Following UK *in vivo* validation studies, the procedure was adopted as a Test Guideline in 1992. Based on the recommendations of several expert meetings it was considered timely because international agreement had been reached on LD_{50} limit values Median Lethal Dose) for chemical classification and currently, testing on one sex (usually females) is considered sufficient.[4]

The selection of dose levels in toxicological studies has undergone changes with the introduction of these new methods. The long-accepted maximum dose level of 5000 mg/kg has since December 2002 been reduced to 2000 mg/kg in the Classes, Limit Test and Up and Down Methods, although it remains the maximum dose of choice in the Fixed Dose Method.[4,5] Before carrying out any type of toxicological evaluation, its main and secondary pharmacological effect must be known, derived from the interrelationships in its mechanism of action within the organism; as well as the dose at which these effects occur. For this reason, the first dose to be evaluated in toxicology studies is the pharmacological dose, knowing in advance the pharmacokinetics and pharmacodynamics of the active ingredient, all in order to determine the degree of safety of its use in humans.[1,3]

One of the objectives of preclinical tests is to demonstrate any type of toxic effect that morphologically or functionally affects the different organs or systems of the individual, or to classify it according to the effect to be investigated, either in the organ, system or by the type of damage. This requires a series of acute and repeat dose studies designed to determine the toxic effects of the compound on organ systems in animals.[15]

The statistical properties of the Fixed Dose Procedure are evaluated using mathematical models in a series of studies. *In vivo* and modeling studies have shown that the procedure is reproducible, uses fewer animals, and causes less suffering than traditional methods. Furthermore, they are able to classify substances in a similar way to the other acute toxicity test methods.[4,5] *Honda et al.*[16] conducted acute and sub chronic studies with purified soy lecithin phosphatidylinositol by administering up to 2000 mg/kg orally to male and female rats, with no deaths or clinical signs of toxicity in either group during the observation period, similarly occurring in our study. *Fiume*[10] considers lecithin to be practically non-toxic in acute oral studies, short-term oral studies and sub-chronic dermal studies in animals after its investigation with murine. *Heywood et al.*[17] in an investigation with Sprague-Dawley rats concluded that oral administration of a purified preparation of phospholipids obtained from bovine cerebral cortex indicated an LD50 greater than 5 g/kg of body weight.

The effects described in our investigation for soy lecithin on the digestive tract are likely due to its fatty nature. Various studies have shown that due to the high content of linoleic acid (47%) and phospholipids (40%) the digestibility of the ethereal extract of commercial lecithin's is similar to that of the oil from which it comes and around 92-95%.[7]

In the last five years, preclinical investigations have been carried out using different biomodels, with the purpose of studying the effects of soy lecithin as dietary supplementation.[6] Gastrointestinal disorders, diarrhea and platelet alterations have been reported with its prolonged use and in high doses, and the effect on body weight is controversial, but it is not clear if they are due to toxic effects related to prolonged consumption.[18]

Bell et al.[19] in an investigation carried out concluded that animals exposed for life to soy lecithin in the diet were hypoactive and had poor postural reflexes, which indicates that lecithin during development leads to behavioral and neurochemical abnormalities in the offspring by finding early sensorimotor deficits in rats and relating it to elevated levels of choline acetyltransferase.

There are not many preclinical studies conducted to show toxic effects of soy lecithin due to years of consumption without reports of serious effects. Almost everything published deals with soy products in a general sense and refers to compounds such as phytoestrogens that they contain. Genistein, a typical soy isoflavone, is an important antioxidant, but the compound has some pro-oxidant potential.[6,9] The clinical effects observed in the integumentary and hem lymphopoietic system of the biomodels in this research may be related to the presence of genistein in the administered formulation.

McClain et al.[20] conducted safety studies with genistein present in the diet of Wistar rats, including two acute studies, two sub chronic studies and a chronic dietary mixture study, reporting low toxicity, but with a decrease in food consumption in the animals. They also observed a number of minor changes considered not toxicologically significant.

At the request of the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to issue a scientific opinion

on the safety and efficacy of lecithin for all animal species. The additive consisted predominantly of lecithin derived from grape, sunflower, and soybean seeds, with other plant extracts, and were safe in the study for all target species. Considering that in animal feed it does not pose any risk and that they are effective as emulsifiers at the recommended use levels.[11,12]

Although soy and its components, such as genistein, have been consumed at high levels in various Asian populations without apparent adverse effects, concern has been raised about possible adverse effects due to its estrogenic effects. A sub chronic study revealed no histopathological findings related to genistein treatment, but the chronic study revealed, in both sexes, reversible histological changes in the reproductive organs and in the bones, kidneys, heart, liver, and spleen.[18]

McClain et al.[20] observed relatively few macroscopic changes at necropsy; only in the chronic study changes in the weight of the organs in rats were evidenced. In addition, osteopetrosis (hyperostosis) was observed along with a compensatory increase in extramedullary hematopoiesis in the spleen, hepatocellular hypertrophy, and minimal proliferation of the bile ducts. Almost all the findings were attributed to the estrogenic properties of genistein considering the changes of a functional nature. Therefore, they were not interpreted as adverse effects, considering that the no observed effect level (NOEL) was 5 mg/kg/day based on functional changes induced by hormones at higher doses.

The study of medicinal plants has followed a route that consists of different steps in its preclinical stage: selection of the plants to be investigated, correct botanical identification, minimum phytochemical characteristics, pharmacological study and toxicological study, to decide whether to continue or abandon the study traditional use. Toxicological studies should not be limited only to carrying out toxicity tests (acute, sub chronic or chronic) in animals, but depending on their results, they should include genotoxicity, carcinogenicity and teratogenicity tests with a view to detecting possible risks for man, of genetic, oncological damage or on the reproduction and development of their offspring. The World Health Organization has insisted that the use of medicinal

plants can be of great application, but on scientific bases that support the safety, effectiveness and quality required for human administration.[1,2]

Conclusions

Soy lecithin showed no evidence of toxicity in the acute oral toxicity test. Reliable evidence is available indicating that its acute toxic effects, by extrapolation or estimation, do not represent a human health concern and assignment to a more hazardous category is not warranted. However, other tests are needed to corroborate that the abuse and chronic dosage of this supplement do not put human health at risk, so it is recommended to complete the toxicological profile of the product.

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Conflict of interests

The authors declare that does not exist an interest conflict.

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