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Acute oral toxicity of a novel functional drink based on *Ilex guayusa*, *Vernonanthura patens,* and cocoa husk

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ABSTRACT

A novel functional drink with nutraceutical properties was formulated from the aqueous extracts of *Ilex guayusa*, and *Vernonanthura patens* leaves, and cocoa husks. This juice contains various bioactive compounds, such as phenolic compounds and methylxanthines, with antioxidant and stimulant properties of pharmacological interest. However, it is known whether herbal extracts' interaction may have adverse toxic effects on human health. To evaluate this functional drink's innocuity, we estimated the acute oral toxicity (AOT) in experimental mice. This paper presents the AOT evaluation of two formulations of a functional drink (pre-formulation and microencapsulation) at a single dose of 2000 mg/kg of body weight (b.w.). No signs of adverse toxicity and mortality were observed after a single oral dose of 2000 mg/kg b.w. Likewise, no significant body and organ weight changes, food and water consumption behavior, and no histopathological changes were observed in the main organs evaluated. In conclusion, this functional drink can be categorized as low toxicity " according to the Globally Harmonized Classification System (GHS), making it a potential beverage with high nutritional and pharmacological value.

1. Introduction

Nowadays, there is a growing demand for food, which supplies sufficient nutritional needs and could help prevent or treat human health diseases [1]. The necessity of functional food has promoted the development of natural products from medicinal plants and other plant extracts with therapeutic bioactivities [2]. Herbal medicine consumption to treat various health ailments has been utilized for centuries up to the present time [3]. Although natural medicine is commonly thought to be safe due to its long-term use, it can also be detrimental to human health at the wrong doses [4]. Since the number of natural and medicinal plant-based preparations has grown exponentially worldwide,

Abbreviations: AOT, Acute oral toxicity; b.w., body weight; GHS, Globally Harmonized Classification System; MCCH, Maquita Cushunchic Fundation; LD50Median, lethal dose; INSPI, National Institute of Public Health Research of Ecuador; OECD, Organization for Economic Cooperation and Development; ANOVA, One-way analysis of variance; ROW, Relative organ weight; SD, Standard deviation.

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toxicological studies are an absolute requirement to prevent adverse toxicity and determine a safe dose range for human consumption [4].

Most native Ecuadorian populations use medicinal plants as the first resources to treat their health-related issues since traditional medicine has a meaningful value for them, and in many cases, their access to conventional health care is somewhat limited [5,6]. The public WHO global report on traditional and complementary medicine indicates that in developing countries, 80 % of the population relies on plant-derived medicines for health care [7]. Guayusa (*Ilex guayusa*) is usually consumed as an infusion by Amazonians, who consider it an excellent source of energy and healing benefits [8]. Many phenolic compounds, such as chlorogenic acids and quercetin, and alkaloids, such as caffeine and theobromine, have been identified in Guayusa's leaves. Together, these metabolites are known to provide adequate amounts of antioxidant, anti-inflammatory, and stimulant activity [9,10]. Laritaco (*Vernonanthura patens*) is a bush that grows wild in Ecuador's several provinces [11]. Its leaf's decoction is traditionally consumed to alleviate fever, inflammation symptoms or used for treatment against cancer or Leishmania [12]. It contains phenolic compounds, such as caffeic acid and pentacyclic triterpenoids, such as lupeol and epilupeol, which confers Laritaco good antioxidant, anti-inflammatory, and antimicrobial properties [11,13]. Cocoa husk (*Theobroma cacao*) is an agro-industrial by-product with high nutritional value. It is rich in phenolic compounds and alkaloids, such as theobromine and caffeine, which provide antioxidant and stimulating properties [14]. Thus, the consumption of these plant products (Guayusa and Laritaco leaves and cocoa husk) as an infusion may harness their therapeutic benefits. In a previous study, our research group developed the formula of a drink based on the aqueous extract of *I. guayusa* and *V. patens leaves* and cocoa husks (commercial registration: 11591SECRETOCIBE). This functional drink contains high amounts of phenolic compounds, such as chlorogenic acids, with high antioxidant capacity, granting potential benefits for human health, for instance, the prevention of diseases associated with metabolic syndromes [15]. Besides, combining these three species has been reported to promote synergistic anti-inflammatory activities [16]. To further rule out potential adverse toxicity of this functional drink, the present study aims to evaluate this plant extract formulation's acute oral toxicity based on *I. guayusa*, *V. patens*, and cocoa husk, using an *in vivo* test in experimental mice.

2. Materials and methods

2.1. Plant materials and preparation of the functional drink samples

Leaves of *I. guayusa Loes*. (*Aquifoliaceae*), (national herbarium of Ecuador QCNE-Quito code: CIBE020), obtained from the Taisha town, Morona Santiago; leaves of *V. patens Kunth* H. Rob. (*Asteraceae*), (CIBE037), obtained from the Marcabelí canton, El Oro; and cocoa husks provided by Maquita Cushunchic (MCCH), Guayaquil, Guayas, were used as plant materials [15]. The samples were collected with the environmental ministry's authorization (No. 013-2017-ICFLO-DNB/-MAE). The leaves of *I. guayusa* and *V. patens* and cocoa husks were individually dried, ground, and sieved; afterward, they were mixed according to the proportions established in the formula (11591SECRETOCIBE) [15]. Subsequently, two formulations of the functional drink were elaborated, a pre-formulation, and microencapsulation, following the methodology of Quijano-Avilés [15]. 10 g of the formula were weighed to prepare the pre-formulation and 10 g for the microencapsulation. These were infused individually in 1 L of distilled water for 5 min, and then the extracts obtained were filtered through Whatman #1 paper. The corresponding extract was brought to lyophilization (Freeze Dryer 4.5, Labconco™, USA) at -40 °C for 72 h. To obtain the microencapsulation, maltodextrin (10 %) was added to the extract, and then it was homogenized at 300 rpm. Then it was dried by spray dryer (Spray dryer SD Basic, Armfield ®, Blashford, United Kingdom) with a feed flow of 3.5 mL.min-1, a supply of compressed air of 50 mL. min-1 at 4 bars, and income air temperature at 120 °C [15].

2.2. Animals

Biologically healthy and nulliparous female albino mice strain CF1, approximately 8 weeks old, weighing 25 g, were used. These were obtained from the bioterium of the National Institute of Public Health Research of Ecuador (INSPI). The animals were housed inside polypropylene plastic cages and were provided with a standard diet and water ad libitum [17]. Controlled laboratory conditions were maintained throughout the experimentation period (22 ± 3 °C, relative humidity 30–70%, and light / dark cycles of 12 h), and all the animals went through an acclimatization period of one week before the start of the trial [18]. All procedures with animals were carried out in accordance to the 3 Rs principles [40] and the statements of the European Union regarding the handling of experimental animals (2010/63/EU) [19]. The experimental protocol was approved by the ethics committee on the use of research animals and teaching at the University of San Francisco de Quito (No. 2019-017-a).

2.3. Acute oral toxicity study

2.3.1. Experimental design

The acute oral toxicity test was carried out according to the standardized fixed-dose guideline No. 420 of the Organization for Economic Cooperation and Development [20]. The mice were randomly distributed in three groups of five animals each, and before dosing the treatments, they fasted for four h with free access to water [4]. Subsequently, body weights were recorded (UW8200S scale, Shimadzu®, Kioto, Japan), and then the treatments were dosed orally with the help of an oral-esophageal cannula. The mice of the first and the second group received the pre-formulation and the microencapsulation, respectively, dissolved in potable water (vehicle) at a single dose of 2000 mg/kg of body weight (b.w.); while the third group served as a negative control, which only received potable water (Supplementary material 1). The administration volume was 300 μL per animal, considering the maximum volume of liquid per administration in mice (1 mL/ 100 g of b. w.) [20].

2.3.2. Toxicological observations

Following different treatments, the animals were observed within the first 4 h on the first day and then once a day during the 14 days of the study [21] to observe clinical signs of pain, stress, or suffering associated with toxicity in animals [22]. Accordingly, changes in behavior patterns and physical appearance, bodyweight variations, and changes in diet and water consumption were monitored by evaluating drowsiness, skin changes (jaundice), fur (piloerection or rough coat), posture (hunch-backed spine), nasal or ocular secretions, dyspnea (gasping or nasal noise), tremors, seizures, coma, and death [20,23]. Body weights were recorded on the 1st day, the 7th day, and the 14th day of the trial [24]. Food and water consumption were recorded daily by measuring the quantities of food and water supplied and each group's remaining [21].

2.3.3. Anatomopathological analysis

At the end of the trial, all animals were sacrificed by cervical dislocation, and different vital organs were collected (heart, liver, kidneys, and stomach) for further investigation [25]. The possible occurrence of alterations in the color, size, and consistency of the treated groups' organs was analyzed macroscopically (Stereomicroscope, Carl Zeiss™ Stemi™ DV4 series, Oberkochen, Germany) by the comparison with the control group [18]. Besides, the relative organ weight (ROW) of each animal was calculated and recorded using the formula: (ROW: the absolute weight of the organ (g)/ bodyweight of the animal at the time of sacrifice (g) x 100 %) [26]. Samples of liver, kidneys, and stomach were taken from each group and preserved in 10 % formalin for histopathological analysis [27].

2.4. Statistical analysis

Data were expressed as the mean ± standard deviation (SD), and the normality of the data was checked using the Shapiro-Wilks test. The significant differences of the quantitative parameters (corporal weight (g), food and water consumption (ml), and organ weight (%)) between the treated groups and the control group were calculated by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$ [3]. R software version 3.6.3 was used for the analysis.

3. Results and discussion

The use of herbal medicine has increased over the years worldwide. Since herbal preparations are composed of multiple bioactive phytochemicals with various therapeutic activities, their combination can be beneficial to treat or prevent several diseases such as cancer, chronic diseases, and metabolic syndrome diseases. Identification and scientific validation of plants with pharmacological activity is the main objective of medicinal plant research [4]. *Ilex guayusa* is a native Amazonian plant which is mainly consumed for its stimulant, diuretic, anti-inflammatory, and gastroprotective properties [10]. The main bioactive constituents identified are caffeine, theobromine, quercetin-3-O-hexose, and chlorogenic acid derivatives (Arteaga-Crespo et al., 2019; [9]. Chianese et al. [28] reported its ability to treat diabetes and metabolic syndrome associated with the ursolic acid component, regulating the lipids and glucose metabolism. Gamboa et al. [29] reported antimicrobial activity against periodontal disease. These findings allow to promote guayusa preparations for functional food, cosmetic or cosmeceutical formulations (Arteaga-Crespo et al., 2019). *V. patens* leaves are used in folk medicine against malaria, stomach pain, and antihelmintic. The main compounds are phenolic compounds and triterpenoids [30]. Some reports about *V. patens* leaves demonstrate antifungal activity associated with the pentacyclic triterpenoid lupeol [13,31]. The cocoa husk is the principal by-product of the cocoa agroindustry which still contains a high nutritional value [32]. Major bioactive compounds identified in cocoa husk are phenols, theobromine, caffeine, procyanidins, and catechins [33]. The cocoa shells' soluble dietary fiber promotes health beneficial effects on the regulation of cholesterol levels and reducing food intake and body weight gain [34]. Other studies have reported health protective effects on lipid and glucose metabolism and oxidative stress [32]. In a study with experimental animals, the cocoa soluble fiber from cocoa shells favorable changes of weight, insulinemia, lipids, glycemia, and blood pressure for the treatment of metabolic syndrome diseases [34].

Valverde-Zumba [16] evaluated in vivo anti-inflammatory activity of a mixed extract of this amazonian plants and observed maximal anti-inflammatory effects at a dose of 300 mg/kg, which maybe attributed to synergistic effects of multiple bioactive compounds. Quijano-Avilés [15] developed an optimized formulation with the same plant species to evaluate its antioxidant activity and observed that adding cocoa husk to *I. guayusa* and *V. patens* infusions increased the variety of phenolic compounds and the antioxidant power and further support its health beneficial effects against the metabolic syndrome. In the present study, we evaluated the acute oral toxicity of two formulations of a functional drink (pre-formulation and microencapsulation) based on the aqueous extract of dried leaves of *I. guayusa, V. patens*, and cocoa husk. For both formulations, a single oral dose of 2000 mg/kg b.w. was administered to individual mice, following the evaluation guidelines indicated in the OECD guide No. 420 [20]. Overall, we did not observe adverse toxicity signs or mortality at the end of the 14-day observational period in any of the treated animals with the pre-formulation and the microencapsulation. The evaluated treatments did not induce adverse changes in the behavior patterns, and all animals presented a normal physical appearance.

3.1. Body weight and food and water consumption

There were no significant differences observed in the animal body weights and water consumption between the treated and control groups. Animals of treated and control groups presented a progressive increase in body weight throughout the study period. The body weights on days 1, 7, and 14 and the treated groups' water intake and the control group are shown in Table 1. In food intake, we observed significant differences between the treated group with microencapsulation vs. the control group. Nevertheless, there were no differences between pre-formulation and microencapsulation (Table 1). It was observed that the group treated with microencapsulation consumed more food than the control group. This behavior could be associated with the maltodextrin component present in the microencapsulation formula. Maltodextrin is a carbohydrate with a high glycemic index, which increases blood glucose and accelerates glucose entry into cells, reducing blood sugar. This triggers a brain signal to increase blood sugar levels, producing a hunger sensation, stimulating greater food intake [35,36]. Contrary, in a toxicological context, it is instead expected that adverse toxicity or illness state caused by a harmful effect of a substance generates a decrease or absence of food and water consumption, which goes hand in hand with a significant loss of body weight [22,23]. It is relevant to mention that reducing more than 20 % in the bodyweight of dosed animals compared to the control is considered a critical state and is defined as one of the human endpoint criteria in many international guidelines [18,22]. Overall, in the present analysis, the treatments did not alter food and water consumption and body weights associated with toxicity in the experimental animals at the administered single oral dose of 2000 mg/kg b.w.

3.2. Macroscopic observations, organ weight, and histopathological analysis

No macroscopic alterations were observed following necropsy in the heart, liver, stomach, and kidneys of all animals. The animal's groups' organs were treated with the pre-formulation and the microencapsulation at a single oral dose of 2000 mg/kg b.w. presented a standard color, consistency, and size according to the control group. All organs presented a standard size and morphology, i.e., the heart of red color and hard consistency, the liver of wine red color and smooth consistency, the stomach of beige color and smooth and firm consistency, and the kidneys with a greyish-red coloration with a smooth and firm consistency (Fig. 1) (Supplementary material 2). No significant difference could be observed in the relative weights of the treated groups' organs concerning the control group (Table 2). In the histopathological analysis, no significant changes were detected in the microscopic analysis of the liver, kidneys, and stomach of the treated and control groups. The groups' organs were treated with the pre-formulation and the microencapsulation at a single oral dose of 2000 mg/kg b.w. presented a

Table 1
Body weight, food and water consumption of the treated animals with the functional drink and the control group.

Variable	Pre-formulation	Microencapsulation	Control	P-value
Body weight (g)				
1st day	27.90 ± 1.32	28.56 ± 1.84	27.08 ± 1.41	0.35
7th day	29.72 ± 2.61	30.86 ± 1.71	28.76 ± 1.34	0.28
14th day	31.58 ± 3.14	31.78 ± 2.26	30.18 ± 0.95	0.51
Food (g) and water (ml) intake				
Water	25.71 ± 2.81	26.86 ± 6.09	24.43 ± 4.52	0.40
Food	21.17 ± 1.56	22.55 ± 2.62	20.24 ± 2.30	0.03*

Values are expressed as mean ± SD; treated groups with a pre-formulation and a microencapsulation of the functional drink at a single oral dose of 2000 mg/kg b.w., and a control group using potable water; * $p < 0.05$. Body weight, n = 5; food and water consumption n = 14 days.

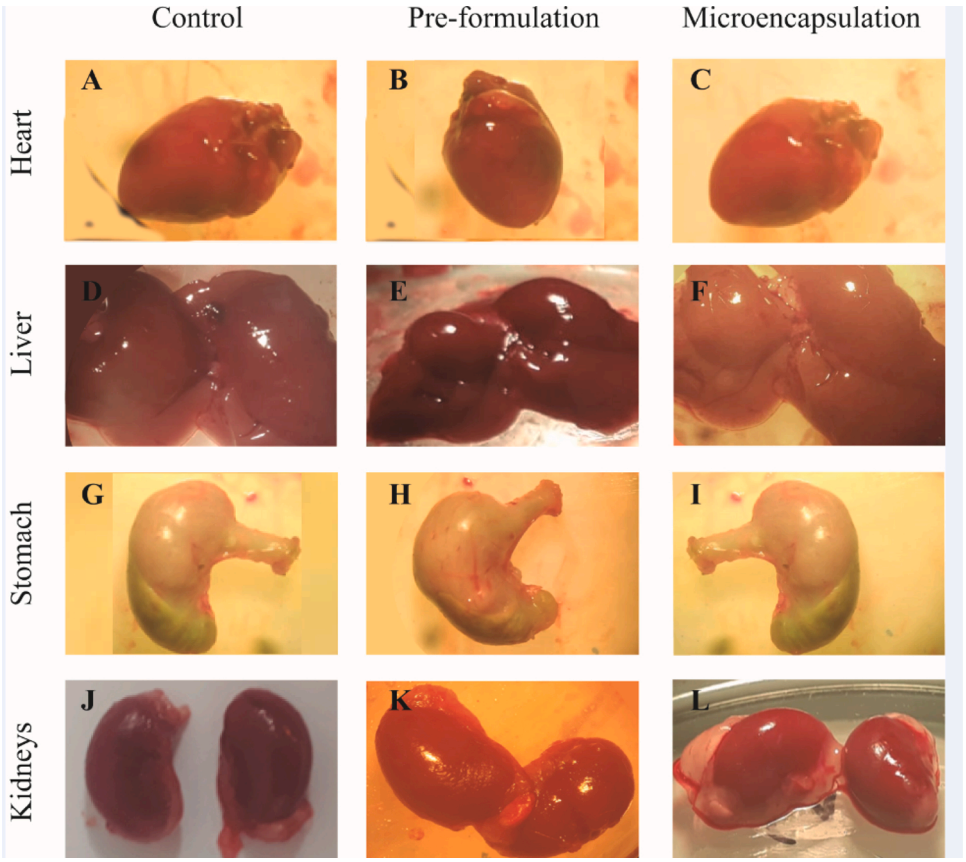

Fig. 1. Macroscopic visualization of the heart (A, B, and C), the liver (D, E, and F), the stomach (G, H, and I), and the kidneys (J, K, and L) for the comparison between the pre-formulation group (2000 mg/kg b.w.) with the control group, and the microencapsulation group (2000 mg/kg b.w.) with the control group.

Table 2
Relative organ weight of the treated animals with the functional drink and the control group (%).

Organs	Pre-formulation	Microencapsulation	Control	P-value
Heart	0.50 ± 0.05	0.48 ± 0.04	0.45 ± 0.06	0.334
Liver	4.77 ± 0.24	5.07 ± 0.52	4.59 ± 0.59	0.313
Stomach	1.31 ± 0.13	1.54 ± 0.16	1.64 ± 0.52	0.300
Kidneys	1.31 ± 0.06	1.35 ± 0.06	1.38 ± 0.15	0.582

Values are expressed as mean ± SD, n = 5; treated groups with a pre-formulation and a microencapsulation of the functional drink at a single oral dose of 2000 mg/kg b.w., and a control group using potable water.

standard architecture structure similar to the control group (Fig. 2). The absence of macro-and microscopic alterations in the morphology, size, and weight of the analyzed organs allowed us to conclude that the treatments did not cause direct organ damage at the single oral dose of 2000 mg/kg b.w. Some vital organs such as the liver, kidneys, heart, and stomach are often affected by substances with toxic effects.

For this reason, their evaluation to identify possible toxic signs is of utmost importance [37]. Overall, the evaluated parameters in this study were effective in assessing the beverage's acute oral toxicity. However, our study's limitation is the lack of biochemical parameters analyzed in blood, which may further support our conclusions at the molecular and cellular level.

In conclusion, according to the Globally Harmonized Classification System (GHS) [38], our results allow us to classify the functional drink (pre-formulation and microencapsulation) within the category 5″low toxicity," which means that the median lethal dose of this beverage is above 2000 mg/kg b.w. (LD50 > 2000 mg / kg b.w.). This study is the first scientific evidence for the lack of acute pathophysiological in vivo toxicity of a functional drink based on *Ilex guayusa*, *Vernonanthura patens*, and cocoa husk. However, additional toxicological studies, such as sub-acute and chronic toxicity studies, are necessary to strengthen its long-term use as a safe, functional nutraceutical beverage. An important dimension is that this functional beverage based on Ecuadorian plants has a wide variety of phenolic compounds such as chlorogenic acid, caffeic acid, and quercetin that provide high antioxidant activity. These compounds have excellent nutraceutical properties, which together can help with the prevention or treatment of metabolic syndrome diseases [15]. It is known that the chlorogenic acid in combination with caffeic acid can help glucose absorption and attenuate insulin resistance. Quercetin has beneficial properties for treating metabolic syndrome since it helps regulate blood pressure and cholesterol metabolisms. Also, it helps to reduce body weight and regulate insulin and glucose [39]. To further support its health-beneficial antioxidant properties for the treatment of syndrome metabolic diseases, further pharmacological follow-up studies such as hepatoprotective, antidiabetic of this beverage are recommended.

Conflict of interest

The authors declare no conflict of interest.

Credit author statement

Geovanna Paladines-Santacruz: Term, Investigation, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Andrea Orellana-Manzano:** Term, Conceptualization, Investigation, Methodology, Formal analysis, Resources, Validation, Visualization, Writing - Review & Editing, Supervision, Project administration. **Glenda Sarmiento:** Investigation, Resources, Supervision. **Glenda**

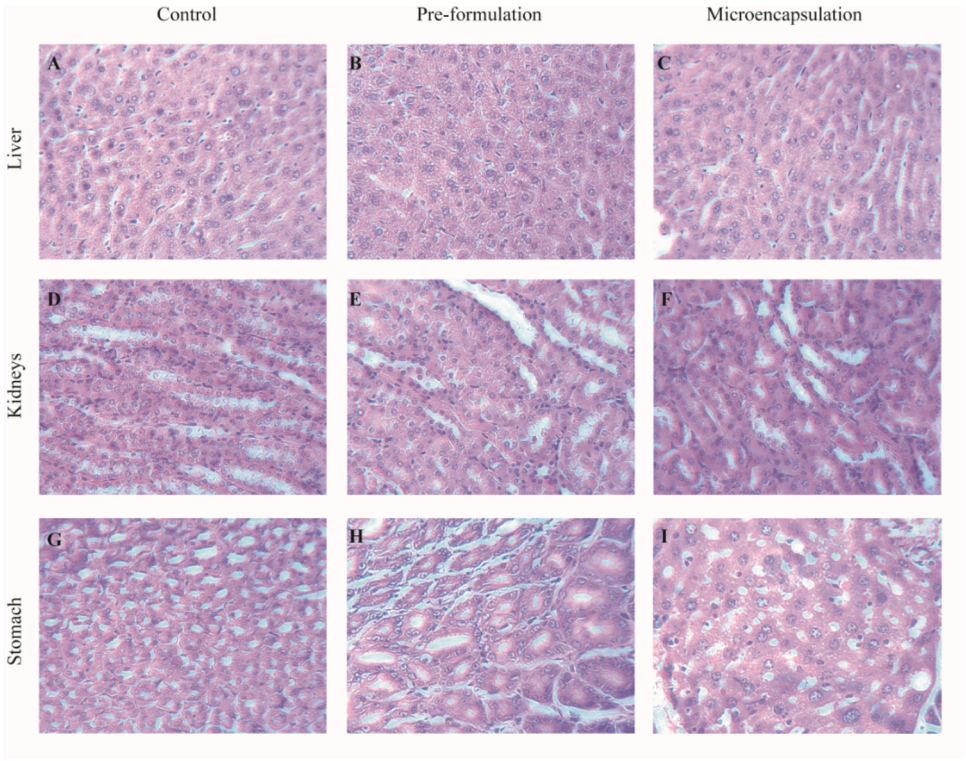

Fig. 2. Microphotographs of histopathological evaluation of the liver (A, B, and C), the kidneys (D, E, and F) and the stomach (G, H, and I) of the treated groups and the control group from histological sections of organs embedded in paraffin and stained with hematoxylin and eosin, for the comparison between the pre-formulation group (2000 mg/kg b.w.) with the control group, and the microencapsulation group (2000 mg/kg b.w.) with the control group.

Pilozo: Investigation, Formal analysis. **Elsy Iñiga:** Investigation, Formal analysis. **Fausto Zaruma Torres:** Resources, Writing - Review & Editing, Supervision. **Johana Ortíz Ulloa:** Formal analysis, Resources, Writing - Review & Editing, Supervision. **María Quijano Avilés:** Conceptualization, Resources. **Davide Di Grumo:** Formal analysis, Visualization, Writing - Review & Editing. **Silvia Orellana-Manzano:** Formal analysis, Validation, Resources, Writing - Review & Editing. **María del Carmen Villacrés:** Resources, Supervision, Project administration. **Patricia Manzano Santana:** Term, Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Win Vanden Berghe:** Writing - Review & Editing.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.03.026.

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