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Evaluation of Acute and Subacute Toxicities of a Recipe Based on Three Leafy Vegetables in Laboratory Rodents

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Abstract

The consumption of a mixture of leafy vegetables has always been a practice anchored in the dietary habits of the people of Brazzaville. The objective of this study was to evaluate the acute and subacute toxicities of the aqueous extract of the recipe based on three leafy vegetables (*Amaranthus hybridus* L., *Spinacia oleracea*, *Brassica campestris* L.) in laboratory rodents. The aqueous extract of the food recipe was administered orally using a cannula. Regarding acute toxicity, the administration of single doses of 5000 and 10,000 mg/kg to mice had no significant effects on behavior. On the other hand, weight loss was significantly (**p < 0.01) compared to control mice (1 mL/100g). The results on subacute toxicity showed that daily administration of the aqueous extract of the food recipe at a dose of 1000 mg/kg for 21 days in rats resulted in a non-significant weight gain, and caused changes in some biochemical parameters including HDL-cholesterol levels, blood sugar levels and ALAT activity.

Keywords

Leafy Vegetable, Food Recipe, Acute Toxicity, Subacute Toxicity, Biochemical Parameters

1. Introduction

In many countries in the world, the food and the lifestyle of the inhabitants are of major importance for public health. In Africa as elsewhere, leafy vegetables have always had a place of choice in the diet of many people [1]. Due to its immeasurable composition in microphone and macronutrients, in particular vitamins, mineral salts, fibers as well as carbohydrates, the consumption of leafy vegetables would contribute to the well-being of the organism [2]. According to doctors-nutritionists, the food and nutrition factors considerably influence the different physiological functions of the organism, including heart rate, intestinal transit regulation, blood pressure and memory [3]. This is why scientists have shown that the quantity of certain food molecules, in particular the flavanols contained in certain foods can influence the blood pressure of sensitive people [4]. In the Republic of Congo, certain uses of leafy vegetables differ from one region to another, obviously according to manners, and culinary practices, for example certain vegetables used as food in such a region are not consumed in another [5]. The mixture of leafy vegetables is also part of the Congolese eating habits actively. As a result, several studies highlight today that certain diseases are closely linked to the body's nutritional state [6]. Unfortunately in the experimental framework, the mixture of certain fire vegetables has not yet been the subject of an in-depth study in order to note the metabolic impacts within the human organism. It is in this context that this study is part of the evaluating the acute and subabiger toxicities of a food recipe based on three leafy vegetables (*Amaranthus hybridus* L., *Spinacia oleracea* L., *Brassica campestris* L.) in the Laboratory rodents.

2. Material and Methods

2.1. Material

2.1.1. Plant Material

The plant material used consisted of leafy vegetables. These are: *Amaranthus hybridus* L., *Spinacia oleracea* and *Brassica campestris* L. The whole plants were purchased from a state market in October 2020. The leaves were dried on a bench for three weeks at room temperature (26˚C ± 1˚C). Then, they were ground using a wooden mortar and sieved. The powders obtained were used as plant material for the toxicity assessment.

2.1.2. Animal Material

Male albino mice and Wistar rats, with body weights between 20 and 30 g, aged 14 ± 2 weeks for mice, 140 and 190 g, aged 18 weeks for rats, were used. All these animals were provided by the animal facility of the Faculty of Science and Technology of Marien NGOUABI University in Brazzaville, Republic of Congo. These animals were kept under standard conditions (12 hours of light and 12 hours of darkness) at room temperature of 26˚C ± 1˚C. All animals had free access to standard food and tap water.

2.2. Methods

2.2.1. Preparation of the Aqueous Extract of the Food Recipe

The aqueous extract of the food recipe was prepared by decoction. 50 g of leaf powder mixture (16.7 g for each vegetable) was placed in a glass flask containing

500 mL of previously boiled distilled water. The flask was hermetically sealed for thirty (30) minutes after which the solution obtained was filtered with cotton wool. The filtrate thus collected was then evaporated under reduced pressure at 50˚C - 60˚C. The dry extract obtained was used for toxicity tests.

2.2.2. Assessment of Acute Toxicity

The OCDE Test Guideline [7], slightly modified was used, a sequential process using three animals (mice) of the same sex, was used to study acute toxicity. This method allows substances to be ranked in order of toxicity in a similar way. The experiment was carried out over a period of fourteen (14) days. For this purpose, three (3) batches of three (3) mice each, of the same sex, were constituted as follows:

- ✓ Lot 1 (control): mice received distilled water at a dose of 1 mL/100 g;
- ✓ Lot 2 (treated): mice received the aqueous extract of the food recipe at a dose of 5000 mg/kg;
- ✓ Lot 3 (treated): mice received the aqueous extract of the food recipe at a dose of 10,000 mg/kg.

After a single administration of the products (aqueous extract and distilled water), the mice were placed in individual cages for observation. The macroscopic symptoms observed included ptosis, piloerection, urinary excretion, reaction to external stimuli, stool condition and general behavior of the animals (aggressiveness, mobility, vocalization, convulsions, etc.). They were observed at 1/2, 1, 2, 3 and 4 hours after administration of each product. Mortality was assessed within 48 hours following administration. The mice were kept under observation for 14 days to detect the late onset of signs of toxicity. Body weight as well as food and water consumption were recorded every other day for 14 days.

2.2.3. Assessment of Subacute Toxicity

The method used is that described by the OCDE [7] slightly modified.

Two batches of 5 rats (males) were divided as follows:

- ✓ Batch 1 (control): the rats received distilled water at a dose of 0.5 mL/100g;
- ✓ Batch 2 (treated): the rats were treated with the aqueous extract of the food recipe at a dose of 1000 mg/kg.

These rats were treated with aqueous extract and distilled water orally and weighed daily for a period of 21 days. During this period, the body weights of the animals were measured at the end of each week. On the 22nd day, all rats were anesthetized and then sacrificed, arteriovenous blood was collected using dry tubes for biochemical analyses (creatinine, transaminases including ASAT and ALAT, alkaline phosphatases, blood sugar, total cholesterol, triglycerides, uric acid, urea, HDL-cholesterol and LDL-cholesterol). Then the organs (heart, liver, and spleen, left and right kidneys) were removed and observed macroscopically to identify possible lesions. The relative weights of the organs were calculated using the relationship below:

$$P_r = \frac{P_o}{P_a} \times 100$$

with P_r the relative weight of the organ in grams per 100 g, P_o the weight of the organ in grams and P_a the body weight of the rat in grams.

2.2.4. Determination of the Phytochemical Profile or Screening of the Food Recipe

To identify the different chemical groups or secondary metabolites (alkaloids, flavonoids, tannins, free anthraquinones, saponins, steroids, holoside sugars and mucilages), a phytochemical screening of the dry leaves of vegetables was carried out. For this, we used the classical phytochemical tests based on the colouring and precipitation reactions [8] [9].

2.2.5. Statistical Analysis

Statistical analysis was performed using Excel software (Office 2016) and the results were expressed as mean ± SEM. Analysis of variance was performed for the comparison of treated batches with the control batch by applying the Student t test ($p < 0.05$; $p < 0.01$; $p < 0.001$). The significance threshold was set at $p < 0.05$.

3. Results

3.1. Study of the Acute Toxicity of the Aqueous Extract of the Food Recipe

3.1.1. Effect of Aqueous Extract of Food Recipe on General Behavior in Mice

Table 1 below shows the effect of the aqueous extract of the food recipe on general behavior in mice. At doses of 5000 and 10,000 mg/kg the aqueous extract of the food recipe did not modify the general behavior of mice compared to those that received distilled water (1 mL/100g).

Table 1. General condition of animals after administration of products.

Settings	Treatments		
	E.D (1 mL/100 g)	E.A (5000 mg/kg)	E.A (10,000 mg/kg)
Number of animals	3	3	3
Mobility	N	N	N
Aggressiveness	N	N	N
Stool condition	N	N	N
Trembling	A	A	A
Sleep	A	A	A
Sensitivity to pain	N	N	N
Vomiting	A	A	A
Vocalization	A	A	A

Continued

Pilo-erection	A	A	A
Ptosis	A	A	A
Vigilance	N	N	N
Number of deaths	0	0	0

ED: Distilled water; EA: Aqueous extract; A: Absent; N: Normal.

3.1.2. Effect of Aqueous Extract of Food Recipe on Body Weight in Mice

Figure 1 below shows the weight changes of the mice over the 14 days of the test. Mice treated with the different doses (5000 and 10,000 mg/kg) lost weight significantly (*p < 0.05; **p < 0.01) compared to mice in the control group (1 mL/100g). This weight loss is more remarkable in mice treated at the dose of 10,000 mg/kg.

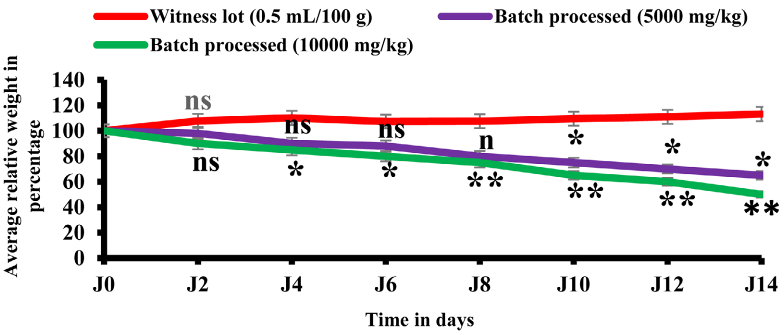

Figure 1. Effects of aqueous extract of the food recipe on the evolution of relative weight in albino mice. Results are expressed as mean ± standard error, n = 3 mice per group. **p < 0.01 significant difference compared to control mice Distilled water.

3.1.3. Effect of Aqueous Extract of the Recipe on Food Intake by Batch of Mice

Figure 2 below shows the effect of the aqueous extract of the recipe on food intake by batch of mice. In general, animals treated with the aqueous extract at the different doses (5000 and 10,000 mg/kg) consumed less food compared to animals in the control group (1 mL/100g). This decrease in food consumption is more remarkable in animals treated with the aqueous extract at the dose of 5000 mg/kg.

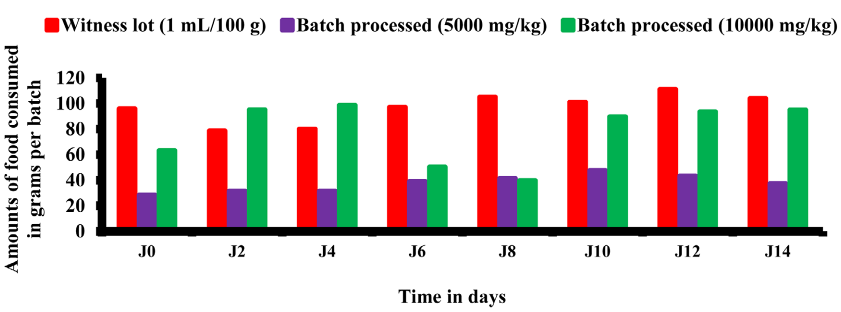

Figure 2. Effects of aqueous extract of the food recipe on food consumption in albino mice. Results are expressed as mean ± standard error, n = 3 mice per group.

3.1.4. Effect of Aqueous Extract of the Food Recipe on Water Intake by Batch of Mice

Figure 3 below shows the effect of the aqueous extract of the food recipe on water intake by batch of mice. There was a decrease in water intake in animals treated with the aqueous extract at a dose of 5000 mg/kg compared to treated animals (10,000 mg/kg) and animals in the control group (1 mL/kg).

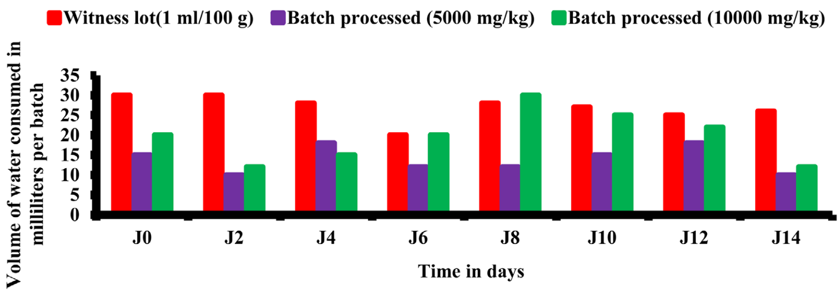

Figure 3. Effects of aqueous extract of the food recipe on water intake in albino mice. Results are expressed asmean ± standard error, n = 3 mice per group.

3.2. Study of the Subacute Toxicity of the Aqueous Extract of the Food Recipe

3.2.1. Effect of Aqueous Extract of the Recipe on Body Weight Evolution in Rats

Figure 4 below shows the weight gain of rats over three weeks. During this study, animals treated with the aqueous extract of the food recipe (1000 mg/kg) gained weight in a non-significant manner compared to animals in the control group (0.5 mL/100 g). This weight gain appears to be confounded with animals in the control group.

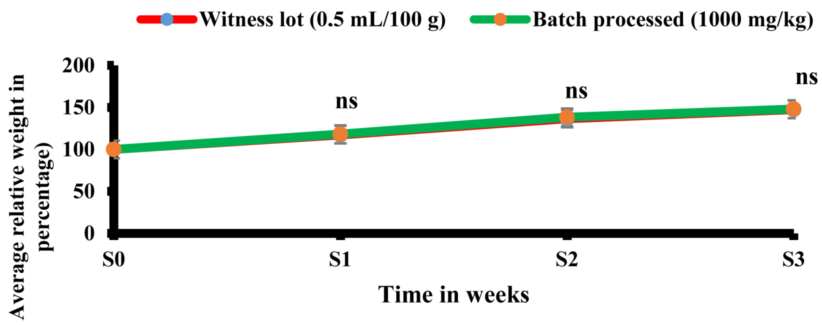

Figure 4. Effect of aqueous extract of the food recipe on the evolution of body weight in the wistar rat. (Results are expressed as mean ± standard error, n = 5 rats per group. No significant difference compared to animals in the control group).

3.2.2. Effect of Aqueous Extract of Food Recipe on Food Intake by Batch of Rats

Figure 5 below shows the effect of the aqueous extract of the recipe on food intake by batch of rats. This figure shows a slight variation in food intake between animals treated with the aqueous extract at a dose of 1000 mg/kg and those in the control group (0.5 mL/100g).

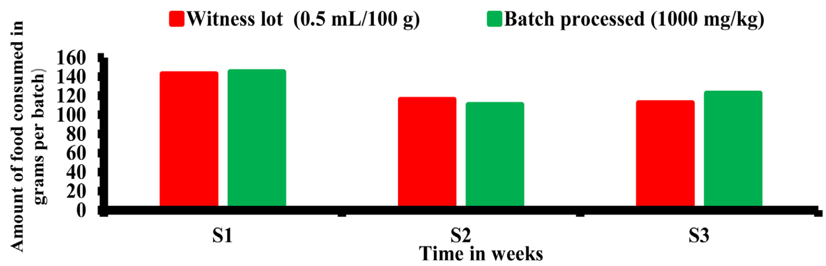

Figure 5. Effects of aqueous extract of the food recipe on food consumption of rats. Results are expressed as mean ± standard error, n = 5 rats per group; S: week.

3.2.3. Effect of the Aqueous Extract of the Food Recipe on the Water Intake by Batch of Rats

Figure 6 below shows the effect of the aqueous extract of the food recipe on the water intake by batch of rats. We note, a non-significant increase in the water intake of animals treated with aqueous extract at a dose of 1000 mg/kg compared to the animals of the control group (0.5 ml/100g).

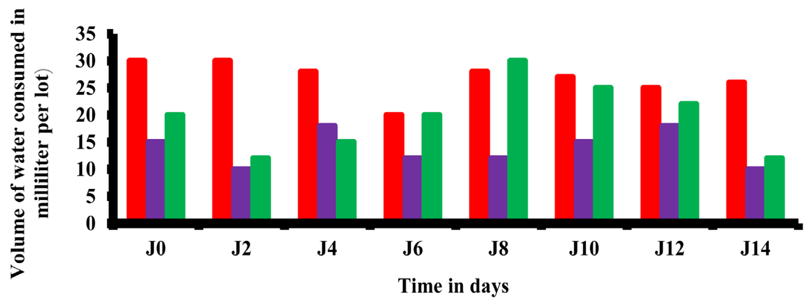

Figure 6. Effects of aqueous extract of the food recipe on water intake of rats. Results are expressed as mean ± standard error, n = 5 rats per group; S: week.

3.2.4. Effect of the Aqueous Extract of the Food Recipe on the Organs

Table 2 below shows the effect of the aqueous extract of the food recipe on the weight of the organs taken from witness rats at a dose of 0.5 ml/100g and treated daily for 21 days at the dose of 1000 mg/kg. After macroscopic observation, it emerges from this study that the aqueous extract of the recipe at a dose of 1000 mg/kg does not influence the mass of vital organs (liver, kidneys, spleen and heart).

Table 2. Variation of relative weights of the organs of witnesses and treated animals to the recipe for leafy vegetables.

Organs	Mass of organs (G)	
	Distilled water (0.5 mL/100 g)	Aqueous extract (1000 mg/kg)
heart	0.341 ± 0.021	0.359 ± 0.011^{ns}
Liver	3.407 ± 0.072	3.643 ± 0.159^{ns}
Spleen	0.161 ± 0.009	0.164 ± 0.005^{ns}

Continued

Left kidney	0.266 ± 0.006	0.315± 0.013^{ns}
Right -back	0.278 ± 0.006	0.317± 0.016^{ns}

ns = not significant.

3.2.5. Effect of the Aqueous Extract of the Recipe on the Biochemical Parameters of Rats

Table 3 below shows the effect of the aqueous extract of the food recipe on the biochemical parameters. The aqueous extract of this recipe in a dose of 1000 mg/kg, does not cause any significant effect on the levels of creatinemia, total cholesterol, triglyceride, uric acid and urea compared to the animals of the group of the group Witness (0.5 ml/100 g), however at this same dose (1000 mg/kg), the aqueous extract leads to a non-significant increase in the rate of LDL-cholesterol, ASAT and alkaline phosphatase activities. Only at this same dose (1000 mg/kg), the aqueous extract causes a significant increase (*p <0.05) of the ALAT activity, the level of blood sugar and a significant decrease (**p < 0.01) of the HDL-cholesterol rate compared to control rats (0.5 ml/100 g).

Table 3. Variation of the biochemical parameters of animals witness and treated with the recipe for leafy vegetables.

Biochemical parameters	Treatments	
	Distilled water (0.5 mL/100 g)	Aqueous extract (1000 mg/kg)
ALAT (UI/L)	26.134 ± 3.039	52.584 ± 4.398*
ASAT (UI/L)	69.062 ± 18.273	70.296 ± 10.138^{ns}
Créat (mg/l)	0.86 ± 0.04	0.66 ± 0.08^{ns}
CT (g/l)	82.88 ± 14.43	56.11 ± 6.07^{ns}
HDL (g/l)	103.54 ± 6.63	78.38 ±2.92**
LDL (g/l)	320.46 ± 71.60	328.72 ± 73.46^{ns}
PAL (UI/L)	37.2 ± 6.64	48.62 ± 7.39^{ns}
TG (g/l)	124.36 ± 17.09	87.158 ± 15.073^{ns}
Glycémie (g/l)	1.062 ± 0.061	2.292 ± 0.494*
Acide Urique (mg/l)	2.68 ± 0.49	2.30 ± 0.77^{ns}
Urée (g/l)	82.6 ± 8.75	62.5 ± 8.12^{ns}

ns = not significant; ** = significant.

3.3. Phytochemical Screening of the Aqueous Extract of the Food Recipe

The objective of this study was to highlight the secondary metabolites which could be responsible for all the effects observed during our experiment. The classic chemical analysis of the aqueous extract of the food recipe has shown the presence of eight (8) main chemical groups which are: flavonoids, saponosides, steroids, triterpenoids, tannins, mucilage, free anthraquinons, Holosid dares and alkaloids (**Table 4**).

Table 4. Phytochemical screening results.

Secondary metabolites	Aqueous extract from the food recipe
Flavonoids	+++
Saponosides	+++
Steroids and terpenoids	++
Tannins	+++
Mucilage	+++
Anthraquinones	++
OSE-Holosides	+++
Alkaloids	+

(+): presence; (+++): strong presence.

4. Discussion

The results of the acute toxicity study have shown that, the aqueous extract of the recipe based on three leaf vegetables does not change the general behavior and does not cause the mice death to the doses of 5000 and 10,000 mg/kg. These results suggest that mice tolerate the aqueous extract from the food recipe to the dose of 10,000 mg/kg supposedly very high. These results make it possible to suggest that the lethal dose of the aqueous extract of this food recipe would be at doses greater than 10,000 mg/kg. Compared to other medicinal plants as food, which would cause mice death from the dose of 2500 mg/kg and present a DL_{50} at 3300 mg/kg [10]. Many studies have also shown similar results concerning other extracts from medicinal and food plants in high doses [11] [12]. Regarding weight evolution, it appears that the aqueous extract of the recipe has exerted significant effects on the loss of body weight in mice in doses (5000 and 1000 mg/kg), but it is more remarkable in the mouse treated at a dose of 10,000 mg/kg. This weight decrease on the one hand could be caused by low food consumption. On the other hand, this weight loss in animals treated with aqueous extract could be explained by the effect of a significant presence of thylakoids from the spinach membranes because a study carried out in 2014 in Lund, in Sweden, consisted of consuming an extract of spinach based on the membranes of the green leaves, called thylakoids, had decreased the desire to eat up to 95% and favored the weight loss of 43% [13]. Also, it has been demonstrated by scientists, that apart from water, no other food contains few calories than leaf vegetables. Foods that contribute to weight loss are generally hypocaloric foods (sorrel, cabbage, lemon, etc.) which help save calories, dietary fibers and protein foods that provide long-term satiety. Given the vegetables to generally contain minerals and vitamins which facilitate weight loss, in particular calcium, magnesium, potassium, iron and vitamin C. It is admitted that the association of These three leafy vegetables would increase the content of these nutrients. It is therefore clear that leafy vegetables are the ideal food loss group. The results of the subaigious toxicity made it possible to observe, at the maximum dose of 1000 mg/kg a non-significant increase in body weight. This increase in body weight of rats could be explained by significant food consumption caused by a presence of plant steroids in this recipe [14]. Numerous studies have shown that natural steroids containing in leaf vegetables have the role of modifying energy proteins. This process therefore allows human muscles to develop and work well during an effort. As a result, this chemical family has a direct impact on muscle mass gain [15]. Regarding biochemical tests, the aqueous extract of the dose of dose of 1000 mg/kg causes the significant increase in ALAT activity and the level of blood sugar ($p < 0.05$) and a significant decrease in the HDL-cholesterol rate ($p < 0.05$ and $p < 0.01$) compared to control rats (0.5 ml/100g). The increase in ALAT activity in animals treated with aqueous extract at a dose of 1000 mg/kg could be explained by hepatitis infection caused by a significant presence of steroids and tannins contained in this extract. However, on the one hand it has been shown that steroids have numerous and varied side effects, some of which are irreversible, including: hepatic and renal functions [16]. On the other hand, the tannins destroy the cells and will therefore deteriorate the intestinal mucosa, the liver and the kidneys [17]. In Ghana, studies carried out by Adinortey [18] who worked on extracts from the sheets of *Launaea taraxacifolia*, revealed a presence of tannin as is the case in this study. Also, these steroids would be the cause of a significant decrease in the rate of HDL-cholesterol because according to the researchers, in high doses, their harmful effects cause changes in cholesterol levels (increase in low density lipoproteins—LDL cholesterol and a decrease in high density-hdl cholesterol lipoproteins [19]. Effect of the maximum dose of 1000 mg/kg of this food recipe. Having a high glycemic index then promoting hyperglycemia [19]. In view of the results found, the aqueous extract studied at a dose of 1000 mg/kg has not caused significant variations on the other parameters, in particular the levels of creatinemia, total cholesterol, triglyceride, uric acid, of Urea, LDL-cholesterol, ASAT activities and alkaline phosphatase. This is all the more justified since it is beautiful and a mixture of three leafy vegetables consumed regularly in the Brazzavilloise diet without any fear prior to normal quantities.

5. Conclusion

The consumption of leafy greens mixture has always been part of the dietary habits of the Brazzaville population. According to acute and subacute toxicities, our studies revealed that the combination of these three leafy greens (*Amaranthus hybridus* L., *Spinacia olerace* L. and *Brassica campestris* L.) would significantly decrease body weight and HDL-cholesterol levels and significantly increase ALAT activity and animal blood sugar. Thus, we can say that the consumption of excessive leafy greens mixture (food recipe) could have harmful impacts on our health. Despite its good nutritional characteristics, it would be preferable to consume them reasonably in order to avoid all kinds of toxicities or inconveniences within our body. These results obtained following this preclinical study have never been the subject of a clinical analysis that is to say in humans. It would therefore be desirable to carry out this study in humans in order to compare the results obtained in laboratory rodents.

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Author Agreement

All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript. This is our original work and it has not been considered or reviewed by any other publication and has not been published elsewhere in the same or a similar form.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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