

# Effect of Sacha Inchi Oil (*Plukenetia volubilis* L.) on Genotoxicity in Mice (*Mus musculus*) and Subchronic Toxicity in Goldfish (*Carassius auratus*)

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## ABSTRACT

**Introduction:** *Plukenetia volubilis* is known as Sacha Inchi (SI) is originary from the peruvian amazone and it has been cultivated and consumed since the pre and Inca's time. **Objective:** Sacha inchi oil was assessed for genotoxicity in Balb C albino mice and subchronic toxicity in goldfish (*Carassius auratus*). **Material and Methods:** The genotoxicity was assessed in *Mus musculus* Balb C (n = 25) separated into five groups randomly selected of twenty-five each one. Groups were 10 mL / kg normal saline (NS), 40 mg / kg cyclophosphamide group (CP) and the three other groups received cyclophosphamide and sachá inchi oil of concentrations 10, 100 and 1000 mg / kg respectively. The substances were administered three times during 24 hours. The genotoxicity in mice was evaluated determining micronucleus levels in blood and bone marrow. The subchronic toxicity was assessed in goldfish (*Carassius auratus*) (n = 48) separated into four groups randomly selected of six each: normal saline group (control) and three groups that received doses of 10, 100 and 1000 µg sachá inchi oil per litre of water respectively for 45 days. **Results:** The values of weight, length, growth rate, condition factor (K) and number of survivors were recorded. CP group showed higher micronuclei levels in blood and bone marrow compared with sachá inchi oil 10, 100 and 1000 mg / kg groups (ANOVA Test  $p < 0.01$  Scheffe's Post Hoc  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively). The subchronic toxicity assessment in goldfish showed isometric growth, a decline in "K" and a similar specific percentage growth rate per day in all groups (ANOVA test  $p > 0.05$ ). **Conclusion:** Sacha inchi oil was not toxic under experimental conditions.

**Key words:** Sacha Inchi Oil, *Plukenetia Volubilis*, Genotoxicity, Subchronic Toxicity.

## INTRODUCTION

Medicinal plants have important uses in the traditional medicine in worldwide due to its content of phytochemicals and source of drug discovery. Their medicinal value is based on their pharmacological action on the human and animal organism.<sup>1</sup> However, these substances can also have toxic effects. It is known that 80% of the world population relies on using plants to alleviate the pain of some illnesses. Therefore, the WHO (World Health Organization) insists that the use of medicinal plants can be of great use to primary health care systems, but based on scientific evidence that shows the safety, effectiveness and quality required for human consumption.<sup>2,3</sup>

Sacha inchi (*Plukenetia volubilis* L.; Family: Euphorbiaceae) is distributed from Central America to Bolivia. In Peru, it is found mainly in the high and low forest, at heights ranging from 100 to 2000 meters in height, areas with average annual rainfall of 1084 mm<sup>3</sup> and temperatures ranging between 30 and 36.6 °C.<sup>4</sup> Studies carried out in this plant reveals high content of omega 3 and 6 with respect to soybean, corn, peanut, sunflower, cotton,

palm and olive seeds.<sup>5</sup> Sacha inchi oil is extracted from seeds using cold pressing (commercial oil press), Soxhlet extraction, and supercritical CO<sub>2</sub> methods. Furthermore, it is known that present considerable amounts of polyunsaturated fatty acids (PUFA), which are essential for the human organism.<sup>6</sup> Otherwise, it has been described numerous effects as cardioprotective, antiarrhythmic, antiinflammatory, hypotensive, neuroprotective, hypotriglyceridemic and hypocholesterolemic.<sup>7</sup>

The Guidelines of the Scientific Committee on Food for safety assessment of substances used in food contact materials recommend genotoxicity and subchronic studies in the core set of tests. In Peru and other countries, sachá inchi oil is commercialized and consumed based on its potential beneficial effects, there are no evidences reported of genotoxicity assays of sachá inchi oil. It is outstanding one whose results show that oral administration for 60 days in rats is safety, and the lethal dose 50 (LD<sub>50</sub>) above 37 g / kg of body weight in mice was determined.<sup>8</sup> Based on that, the present study aimed to evaluate the genotoxicity and subchronic toxicity of Sacha inchi oil (*Plukenetia volubilis* L.) when it is administered in mice and fish, respectively.

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## MATERIAL AND METHODS

### Chemicals

The materials applied in this research are as follows: Cyclophosphamide monohydrate ( $C_7H_{15}C_{12}N_2O_2P \cdot H_2O$ ) and fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA); Giemsa stain; EDTA; Triton X-100 (Merck, Lima, Peru); and all chemicals used in our study were of analytical or reagent grade.

### Sacha inchi oil analysis

Sacha inchi oil was elaborated in our laboratory by cold pressing. The determination of the fatty acids was carried out using the method of Ichihara and Fukubayashi (2010), which consisted of adding 0.2 mL of toluene, 1.5 mL of methanol and 0.3 mL of the mixture (HCl: methanol 1: 4.3) to 1 mg of sachá inchi oil and 0.1 mg of methyl tricosanoate (internal standard). Next, the mixture is incubated at 45°C for 14 hours for the derivation, the methylesters were extracted with 1.5 mL of n-hexane and 1 mL of water to be analyzed by gas chromatography in a Varian equipment CP-3800, under the following conditions: The gas chromatograph was equipped with a column Restek, FAMEWAX® WCOT fused silica, of 30 m x 0.25 mm x 0.25 µm film thickness. The oven temperature was held at 120 °C for 1 min, 30 °C/min until 160 °C, 160 °C for 1 min, 4 °C/min until 240 °C, 240 °C for 7 min, and maintained at 250 °C for 30.33 min. Helium was used as carrier gas at 22 psi and the injection volume was 1 µL.<sup>9</sup>

### Genotoxicity oral assessment in normal mice

Oral genotoxicity of the Sacha inchi oil (SI) was assessed in normal mice. According to OECD 474<sup>10</sup> and Schmid.<sup>11</sup> Twenty Balb/C albino mice with average weights of  $25 \pm 5$  g from the National Institute of Health of Peru (INS) were kept in the bioterium of the Faculty of Medicine (UNMSM). The experiment began with a 48-hour pre conditioning period. Mice were given food and water *ad libitum* access, 12 hours' light / dark cycle, temperatures between 22-26 °C and  $60 \pm 10\%$  of relative humidity.

During the entire experimental process, international ethical principles for research using laboratory animals were respected.<sup>12</sup> The evaluation separated the research specimens into 5 groups of 5 mice each one: the control group received 10 mL/ kg of normal saline (NS); cyclophosphamide group (CP) received cyclophosphamide 40 mg / kg and the three other groups (SI-10, SI-100, SI-1000) received cyclophosphamide and sachá inchi oil of concentrations at 10, 100 and 1000 mg / kg respectively. Animals were orally exposed to the substances, twice a day during 14 days. At the end of the experiment, mice were placed into a glass chamber where they became anesthetized with ether. Peripheral blood was obtained by cardiac puncture to prepare blood film which allows evaluate the micronuclei frequency in blood.

Then, mice were euthanized by intraperitoneal injection of sodium pentobarbital 100 mg / kg. Next, the femoral bone marrow was extracted with 3 ml of fetal bovine serum (FBS) and centrifuged at 1000 rpm for 10 minutes. Once the supernatant was removed, the cell button was dispersed over slides. The slides (minimum 2 per animal) were left to dry at room temperature for 24 hours. They were fixed with methanol for 5 minutes and stained with 5% Giemsa stain for 12-15 minutes. The slides were coded and examined by two independent observers (blind) under Olympus BH-2 microscope (100x oil immersion lens). The number of micronuclei per field was recorded.

### Subchronic toxicity assessment in gold fish

The subchronic toxicity assessment of SI in normal goldfish was carried out according to OECD 305<sup>12</sup> and Gutierrez *et al.*<sup>13</sup> Forty-eight goldfishes (*Carassius auratus*) of 15-days of age were kept into tanks of

20 L of capacity, which contained 1 L of dechlorinated water per gram of body weight for each fish. Fishes were acclimatized to experimental conditions 15 days prior to the start of the experiment. They were exposed to a 12-hour light/dark cycle. The fish were fed with a standard diet, which it contained marine algae in amounts ranging from 1 to 2% of body weight for each fish. Water parameters were measured and controlled such as: temperature: 22-26 °C; pH: 6.0-8.5; and more than 60% of available oxygen. The study was carried out using four experimental groups randomly selected of six goldfish each one: normal saline group (NS) and three groups (SI-10, SI-100, SI-1000) that received doses of 10, 100 and 1000 µg of sachá inchi oil per litre of water respectively.

There were two series for each experimental group. The solution of sachá inchi oil was prepared using 50 mg of polysorbate 80 per litre of dechlorinated water. It was added to the tanks every 48 hours for 45 days (7 weeks), immediately after the water change. The fish were weighted and measured longitudinally from head to tail weekly. Behavioural signs as motility, colour, motion complexity, appearance of fungi *respect to the control group* were observed daily. The number of surviving fish was recorded weekly.

### Statistical analysis

#### Genotoxicity in mice

Numerical variables were described with measures of central tendency and dispersion, mean and standard deviation, respectively. The normality and homogeneity of variance were evaluated using the Shapiro-Wilk's and Bartlett's tests, respectively. Subsequently, an analysis of variance (ANOVA) with post hoc Tukey's test was used with variables that showed significant difference ( $p < 0.05$ ) between the groups. All statistical analysis was performed using SPSS V 21.0 software.

#### Subchronic toxicity to fish

The length-weight relationships were established using linear regression. The LWR was estimated by using the equation  $W = aL^b$  ( $W$  = total weight expressed in grams,  $L$  = length expressed in centimeters).<sup>13,14</sup> Since the length is a linear scale and the weight is equal to the cube of it; if the shape of the fish is consistent throughout development, the growth is isometric ( $b = 3$ ). When  $b > 3$ , the fish grows faster in weight than in length. The fish growth is positive allometric. In contrast; when  $b < 3$ , the fish grows faster in length than in weight. The fish growth is negative allometric. In the study, if  $b$  ranged between 2.5 and 3.5, the fish growth was considered isometric.<sup>15</sup>

The condition per individual was estimated using the Fulton index ( $K$ ),  $K = (W/L^3) \times 100$ , where  $W$  is the whole body wet weight expressed in grams and  $L$  is the length expressed in centimeters. The annual variation of  $K$  was estimated using a one-way ANOVA and analyzing the data. In order to estimate the growth rate in weight and length, the equation of specific percentage growth rate per day regarding the length and weight [ $SGR (\% \text{ X day})$ ] =  $[(\ln \text{ final } X) - \ln (\text{initial } X)] / \text{treatment time}] \times 1000$  was used. According to Gill,  $X$  can be weight or length.<sup>16</sup>

## RESULTS

In the analysis by gas chromatography was determined: Palmitic acid (C16: 0)  $10.67 \pm 0.06\%$ , Stearic acid (C18: 0):  $4.36 \pm 0.01\%$ , Oleic acid (C18: 1):  $21.10 \pm 0.02\%$ , Linoleic acid (C18: 2):  $50.36 \pm 0.01\%$ ,  $\alpha$ -linolenic acid (C18: 3):  $12.20 \pm 0.02\%$  and Vaccenic acid (C18: 7):  $1.31 \pm 0.004\%$  (Figure 1 and Table 1).

The genotoxicity in mice was evaluated by determining micronucleus levels in blood. The CP group showed  $13.20 \pm 0.22$  micronuclei. The level of micronucleus was higher in CP group compared with the groups that received 10, 100 and 1000 mg / kg of sachá inchi oil (ANOVA Test

$p < 0.01$  Scheffé's Post Hoc  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively). The inhibition of micronuclei was higher than 50% for both the group of 100 and 1000 mg / kg of sachu inchi oil (Table 2).

The evaluation of genotoxicity in mice by determining micronucleus levels in bone marrow showed  $1.80 \pm 0.14$  in the CP group. The level of micronucleus was higher in CP group compared with the groups that received 10, 100 and 1000 mg / kg of sachu inchi oil (ANOVA Test  $p < 0.01$  Scheffé's Post Hoc  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively). The inhibition of micronuclei was higher than 60% for both the 10 and 100 mg / kg of sachu inchi oil (Table 3).

Body weight and head-tail length were monitored for 7 weeks. All the fish were observed thoroughly for the onset of any signs of subchronic toxicity. All the groups showed homogeneous growth rate (ANOVA test  $p > 0.05$ ). However, the body weight of the SI-10 group at the end of the study was greater than the NS group (Student's T test  $p > 0.05$ ) and the head-tail length of the SI-1000 group at the end of the study was greater than the NS group (Student's T test  $p > 0.05$ ) (Figure 2). There was a decrease in the condition factor ranging from  $17.76 \pm 0.97$  (Week

0) to  $10.48 \pm 0.87$  (Week 7) in all treatment groups (Figure 3). The specific percentage growth rate per day regarding the length and weight was similar in all treatment groups (ANOVA Test  $p > 0.05$ ) (Figure 4). Isometric growth was found with values obtained from b ranging from 2.6 and 3.4.

## DISCUSSION

Genetic toxicity or genotoxicity is the process made by an agent that induces damage to DNA and other cellular targets that control the integrity of genetic material.<sup>17</sup> The assessment of genotoxicity using micronuclei in blood and bone marrow of mammals is used to detect lesions in chromosomes or mitotic apparatus of erythroblasts. Erythrocytes taken from the bone marrow and / or peripheral blood of animals (usually rodents) are analyzed to determine chromosomal lesions in a living system. Genotoxic substances cause cytogenetic damage leading to the formation of micronuclei containing *chromosomal fragments* and whole *lagging chromosomes*<sup>24</sup>. When the erythroblasts of the bone marrow became polychromatic erythrocyte, the main nucleus is expelled and the micronucleus remains in the

**Table 1: Percentage of fatty acids determined in Sacha inchi oil.**

Fatty acids	(x ± SD) mg/g	%
Palmitic acid (16:0)	104.76 ± 2.66	10.67
Stearic acid (18:0)	42.83 ± 1.23	4.36
Oleic acid (18:1n-7)	12.85 ± 0.34	1.31
Linoleic acid (18:1n-9)	207.28 ± 5.45	21.10
α-linolenic acid (18:2n-6)	494.61 ± 13.02	50.36
Vaccenic acid (18:3n-3)	119.87 ± 3.09	12.20
U3/ U6	0.24	-

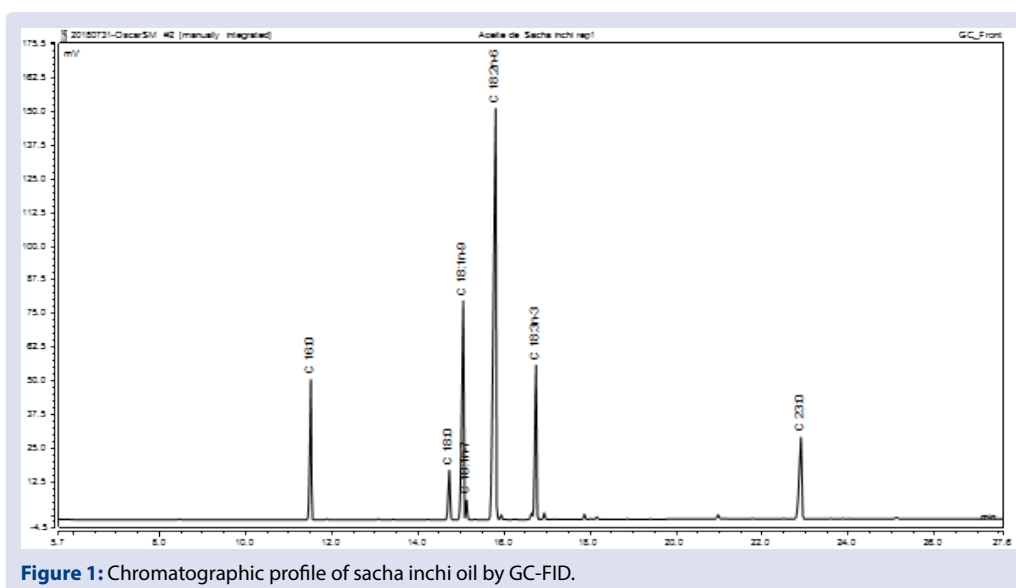
**Table 2: Number of micronuclei obtained to evaluate the genotoxicity of sachu inchi oil in erythrocytes of peripheral blood of *Mus musculus* (OECD 474).**

Treatment	Average	Estandar error	95% Confidence intervals		Value		%IM
			Lower limit	Upper limit	Minimum	Maximum	
Normal saline (NS)	0.20	0.09	0.01	0.39	0	1	----
Cyclophosphamide (CP) 40 mg/Kg.	13.20 <sup>a,b,c</sup>	0.22	12.73	13.67	12	14	0.00
CP + Sacha inchi 10 mg/Kg	11.95 <sup>a</sup>	0.33	11.26	12.64	10	13	9.47
CP + Sacha inchi 100 mg/Kg	6.30 <sup>b</sup>	0.16	5.96	6.64	5	7	52.27
CP + Sacha inchi 1000 mg/Kg	4.80 <sup>c</sup>	0.42	3.92	5.68	2	6	63.64

% IM = Inhibition of Micronuclei =  $(((CP-NS)/CP)*100)$

CP: Cyclophosphamide, SI: Sachu inchi oil

ANOVA Test  $p < 0.01$  Scheffé's Post Hoc  $a = p < 0.05$ ,  $b = p < 0.01$  y  $c = p < 0.001$



**Figure 1: Chromatographic profile of sachu inchi oil by GC-FID.**

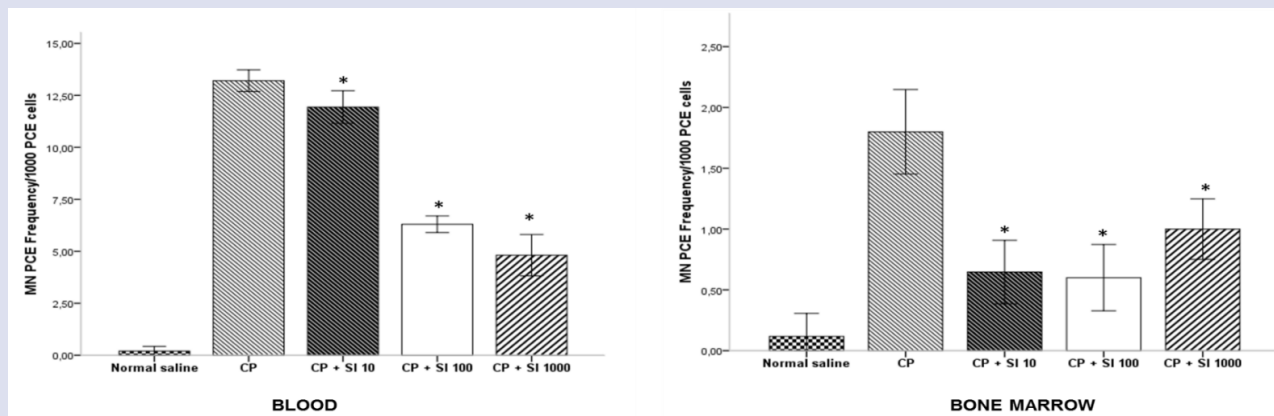
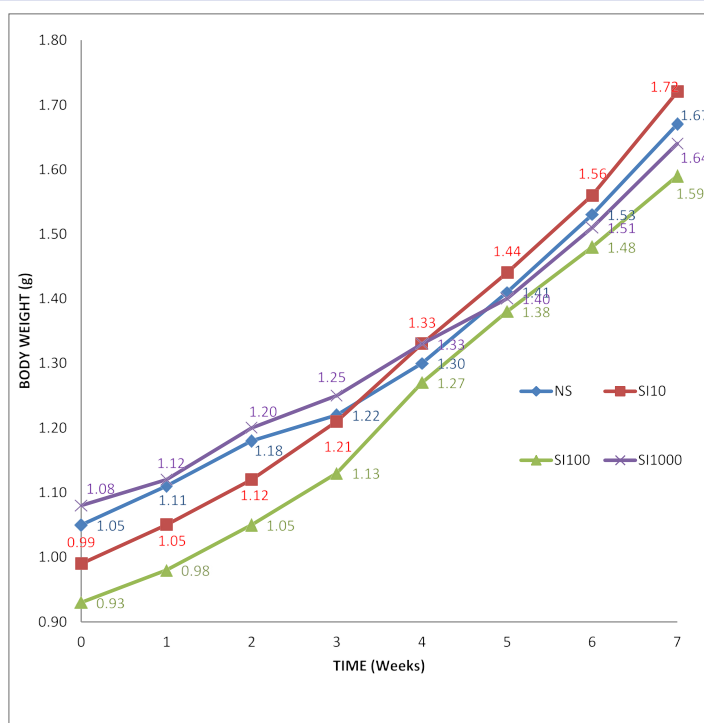
**Table 3: Number of micronuclei obtained to evaluate the genotoxicity of sacha inchi oil in erythrocytes of bone marrow of *Mus musculus* (OECD 474).**

Treatment	Average	Estandar error	95% Confidence intervals		Value		% IM
			Lower limit	Upper limit	Minimum	Maximum	
Normal saline	0.10	0.10	-0.11	0.31	0	2	----
Cyclophosphamide (CP) 40 mg/Kg.	1.80 <sup>a,b,c</sup>	0.14	1.51	2.09	0	2	0.00
CP + Sacha inchi 10 mg/Kg	0.65 <sup>a</sup>	0.11	0.42	0.88	0	1	63.89
CP + Sacha inchi 100 mg/Kg	0.60 <sup>b</sup>	0.11	0.36	0.84	0	1	66.67
CP + Sacha inchi 1000 mg/Kg	1.00 <sup>c</sup>	0.00	1.00	1.00	1	1	44.44

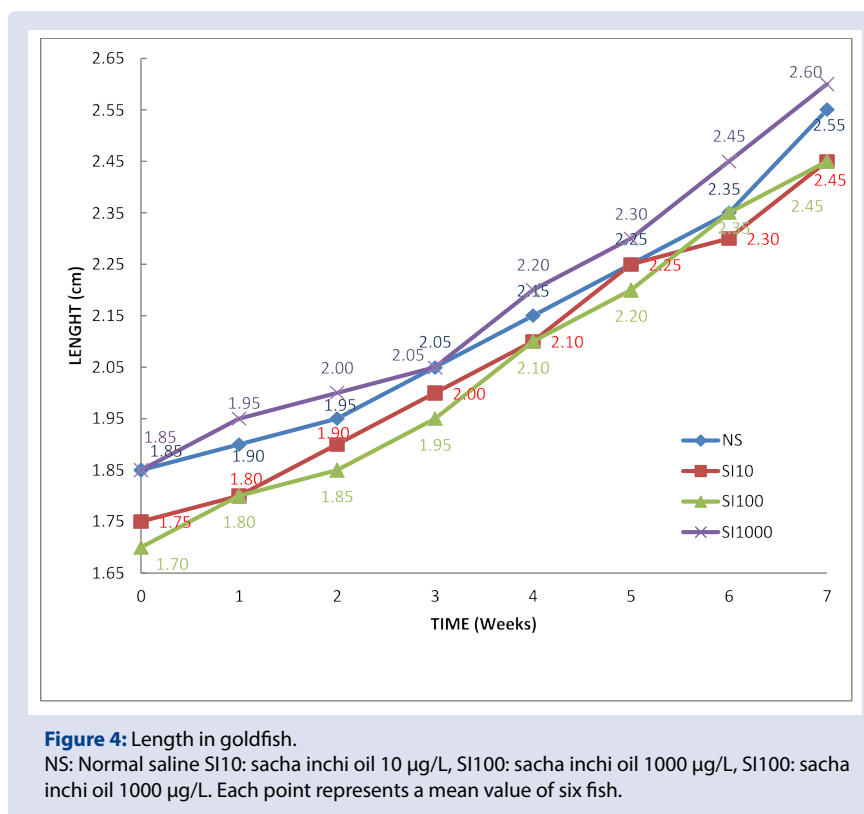
% IM = Inhibition of Micronuclei =  $(((CP-NS)/CP)*100)$

CF: Cyclophosphamide, SI: Sacha inchi oil

ANOVA Test  $p < 0.01$  Scheffé's Post Hoc  $a = p < 0.05$ ,  $b = p < 0.01$  y  $c = p < 0.001$


**Figure 2: Effect of Sacha inchi oil (*Plukenetia volubilis* L.) on the frequency of micro nucleated polychromatic erythrocytes (MNPCE).**

**Figure 3: Body weight in goldfish.**

NS: Normal saline SI-10: sachu inchi oil 10 µg/L, SI-100: sachu inchi oil 100 µg/L, SI-1000: sachu inchi oil 1000 µg/L. Each point represents a mean value of six fish.



cytoplasm, otherwise the cell would be anucleated. Because of the lack of a main nucleus, is easier to visualize the micronuclei in these cells. An increased micronuclei frequency in polychromatic erythrocytes is indicative of the existence of induced chromosome damage.<sup>18</sup>

Studies with cyclophosphamide demonstrate an intimate relationship between chromosomal damage and teratogenic effects caused by this substance in fetal liver, and during pregnancy in mice.<sup>19-21</sup> Likewise, Cancino<sup>22</sup> and Matuo<sup>23</sup> demonstrated the efficient use of cyclophosphamide as a positive control in the assays of teratogenicity in rats and mice for their clastogenic and aneugenic effects. Tables 2 and 3 show the sensitivity of cyclophosphamide to evaluate the genotoxicity of sachu inchi oil in erythrocytes of blood and bone marrow. The values were within normal limits, 13.20 and 1.80 micronuclei respectively. Cyclophosphamide is an alkylating agent which reparative mechanisms have ruptures effects formatting monoadducts and cross-links between chains. This delays the anaphasic onset in somatic cells.<sup>24</sup>

Vizoso<sup>25</sup> evaluated the genotoxicity of extracts of *Plantago lanceolata* L. and *Matricaria recutita* L. and found that their components did not have effect on hereditary processes and the observed effects may be due to the presence of antimutagenic substances. Lopez<sup>26</sup> evaluated the extract of *Aloysia citriodora* and concluded that it did not have cytotoxicity or genotoxicity effect due to the absence of active ingredients that cause any damage to single or double stranded DNA. The presence of polyphenols and monoterpenes, and their antioxidant activity would stimulate the elimination of free radicals suppressing the genotoxicity of chemical agents.<sup>27</sup>

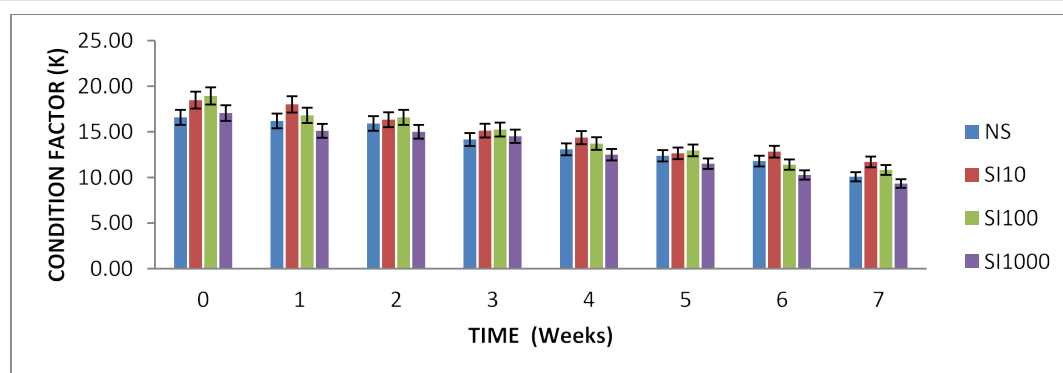
The genotoxicity assessment in blood micronucleus (Table 2) shows a greater decrease in the average number of micronuclei in the group that received cyclophosphamide 40 mg / kg and sachu inchi oil 1000 mg / kg. Its inhibition percentage was 63.64% of the one of the group that received only cyclophosphamide 40 mg / kg. The genotoxicity assessment of bone marrow shows greater decrease in the average

number of micronuclei in the group that received cyclophosphamide and sachu inchi oil 100 mg / kg, which showed an inhibition of 66.67% (Table 3). The presence of phytochemicals as monounsaturated ( $\omega$ -9), polyunsaturated ( $\omega$ -3 and  $\omega$ -6) and other fatty acids (malvalic, stercularic and abietic), terpenes, steroids,  $\beta$ -carotene, chlorophyll, and flavonoids can explain the results. These antioxidants interrupt radical chain oxidation by donating a hydrogen atom (H) to quench lipid peroxyl radicals and becoming another more stable radical. This new radical does not react with lipids but with other similar molecules; generating a non-radical product or quinones, which can be considered antioxidants, after undergoing further oxidation.<sup>28</sup>

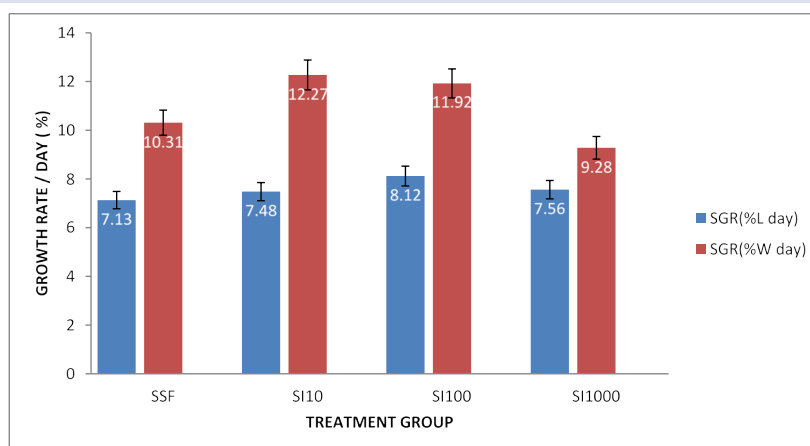
This study sought to evaluate the subchronic toxicity in *Carassius auratus*. The growth of all the goldfish was isometric. On the other hand, there were slight variations in the condition factor of all experimental groups throughout the study. There was no significant difference in the normal evolution of the species. Therefore, sachu inchi oil does not influence on the quality of life of *Carassius auratus*. The values of b ranging from b = 2.6 and b = 3.4 show a general trend to increase thickness proportional to the size during growth.<sup>29</sup>

The values of the condition factor (K) observed in goldfish showed a slight decrease throughout the treatment period (Figure 5). This is consistent with the findings of Visser *et al.*<sup>30</sup> The highest values of K were during the first three weeks and would be associated with the rapid growth of goldfish when they are young, the growth rate decreased throughout weeks.

Growth in weight and length, condition factor (K), specific percentage growth rate per day regarding the length [SGR (%L day)] and weight [SGR (%W day)] were evaluated weekly for 45 days (Figure 6). All experimental groups showed similar growth patterns. This homogeneity is due to factors such diet, temperature, photoperiod, growth stage and the absence of behavioral disorders and deaths in all groups.



**Figure 5:** Condition factor (k) of each week in the 45 days of treatment. NS: Normal saline SI-10: sachu inchi oil 10 µg/L, SI-100: sachu inchi oil 100 µg/L, SI-1000: sachu inchi oil 1000 µg/L. Where:  $K = (W/L^3) \times 100$ ; (n=6).



**Figure 6:** Specific percentage growth rate per day regarding the length [SGR (%L DAY)] and weight [SGR (%W DAY)] in groups in 45 days of treatment. NS: Normal saline SI10: sachu inchi oil 10 µg/L, SI100: sachu inchi oil 100 µg/L, SI1000: sachu inchi oil 1000 µg/L. Where:  $SGR (\%L \text{ day}) = \frac{(\ln(\text{Final length}) - \ln(\text{Initial length}))}{\text{treatment time}} \times 1000$ ;  $SGR (\%W \text{ día}) = \frac{(\ln(\text{Final weight}) - \ln(\text{Initial weight}))}{\text{treatment time}} \times 1000$ . (n=6).

## CONCLUSIONS

Based on the evidence described, the present study concludes that sachu inchi oil is safe. Observational studies are needed to quantify the actual consumption of sachu inchi oil among human population prior to experimental studies. The decrease in the level of micronuclei in the different experimental groups demonstrates that sachu inchi oil is not genotoxic.

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## CONFLICTS OF INTEREST

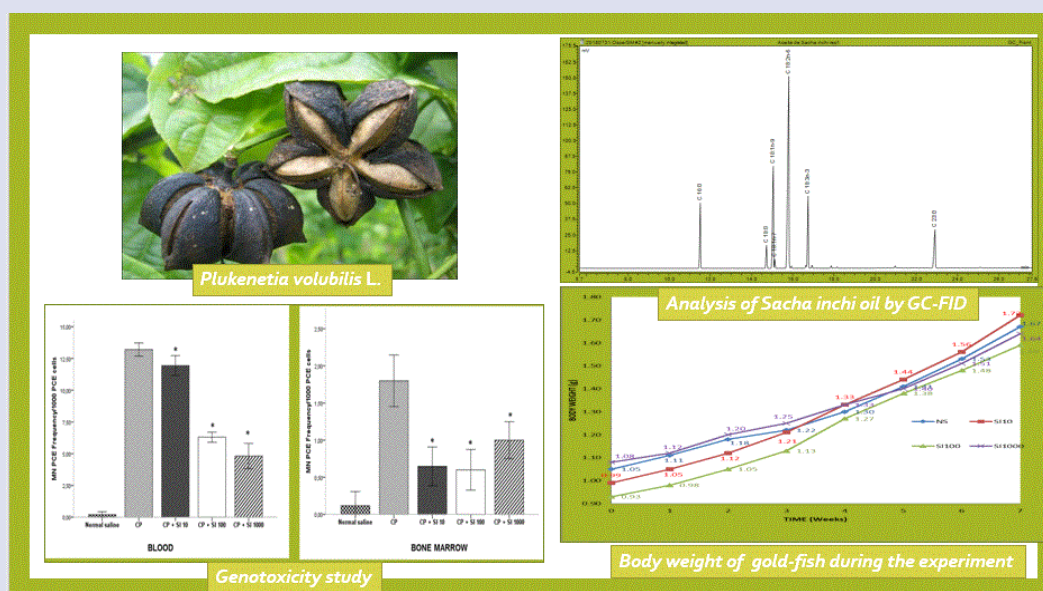
The authors declare no conflicts of interest.

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## GRAPHICAL ABSTRACT



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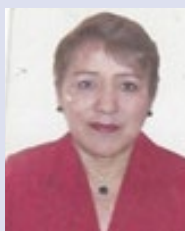
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