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Toxicity and Teratogenic Potential of Piplartine from *Piper tuberculatum* Jacq. during Embryonic Development in Mice (*Mus musculus*)

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Abstract: Piplartine, also known as piperlongumine, is a natural and biologically active amide alkaloid found in various Piper species within the Piperaceae family. It possesses numerous beneficial properties that can be leveraged in the development of nanotechnological and pharmaceutical products. However, information on the effects of piplartine on mammalian embryonic development is scarce. This study aims to assess the general toxicity and teratogenic potential of piplartine during the embryonic development of mice. Pregnant mice received daily treatments of 25, 50, or 100 mg/kg of piplartine via gavage from the sixth day of gestation (implantation) to the eighteenth. On the eighteenth day, the mice were euthanized, and whole organs, blood samples (for hematological and biochemical analyses), and bone marrow cells (for DNA fragmentation and cell cycle assays) were collected. The uterus was examined for implantation sites and embryo resorptions. Additionally, fetuses were collected to assess for fetal anomalies. Piplartine did not result in maternal or embryo-fetal toxicity, induce fetal anomalies, cause hematological and biochemical alterations, or lead to DNA fragmentation. The oral administration of piplartine is safe and does not exhibit toxicity or teratogenic effects in mice. This finding opens avenues for the development of piplartine-based biotechnological products for therapeutic interventions in disease treatment.

Keywords: piplartine; piperlongumine; maternal toxicity; teratogenic potential; developmental biology; toxicology

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1. Introduction

Piplartine, also known as piperlongumine, is a naturally occurring and biologically active amide alkaloid found in species of the Piper genus (Piperaceae) [1]. It has been credited with various pharmacological properties. For instance, it demonstrates anti-inflammatory effects during lipopolysaccharide-induced sepsis [2] and monosodium urate-induced peritonitis [3] in mice. This activity is linked to its capacity to inhibit inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, reduce pro-inflammatory cell infiltration, and suppress inflammasome activation as well as the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) [2]. Other pharmacological properties of piplartine encompass antiproliferative activity through microtubule depolymerization against MCF-7 breast cancer cells [4], cytotoxic effects on U87MG glioblastoma cells [5], and antiulcer and gastroprotective actions via control of acid secretion in the intestinal mucosa [6]. Piplartine has been primarily researched for its potential as an anticancer agent [7–9].

Additionally, there is evidence supporting the antimicrobial activity of piplartine. In vitro studies have demonstrated that piplartine exhibits a schistosomicidal effect on adult *Schistosoma mansoni*, inducing morphological changes in the parasite's integument in a dose-dependent manner [10]. More recently, Mengarda et al. [11] investigated the antiparasitic activity of piplartine in a mouse model of schistosomiasis. Additionally, piplartine shows microbicidal activity against other protozoa, such as *Leishmania amazonensis* and *Plasmodium falciparum* [12–14]. In the case of other microorganisms, piperine and piperlongumine, when combined with conventional antibiotics such as rifampicin and tetracycline, exhibit synergistic effects against *Staphylococcus aureus*, enhancing antibiotic efficacy [15]. Moreover, piperine and piperlongumine, two piperamide compounds, exhibit activity across a range of bacteria, including Gram-positive and Gram-negative species [16]. Synthetic piplartine derivatives also exhibit antibiotic activity against *S. aureus* and *Pseudomonas aeruginosa* [17].

Given its promising properties, piplartine and its derivatives represent potential alternatives for developing novel therapeutic products to manage human diseases [11,18]. The molecule could significantly influence the bioeconomy through the creation of bio-based technological products [19]. However, to ensure its safety for human use alongside its efficacy, assessing its cytotoxicity to mammalian cells is crucial. Cell viability assays have shown that piplartine is highly toxic to NIH-3T3 murine fibroblasts in culture, reducing cell viability by more than 95% after a 24 h treatment with 25 μg/mL of piplartine [20]. Piplartine also displays genotoxic effects, as evidenced by the induction of DNA strand breakage, interruption of the cell cycle in the G2/M phase, induction of dose-dependent apoptosis, and internucleosomal DNA fragmentation in V79 cells [21,22]. Therefore, in addition to its notable pharmacological properties, piplartine exhibits significant toxicity toward mammalian cells.

In light of the results from cell-based assays, it is crucial to assess piplartine's in vivo toxicity to fully grasp its potential for treating human conditions. One method to address this need involves evaluating adverse effects linked to gestational, peri-, and neonatal exposure. This strategy examines the transgenerational implications of substances that can enter the maternal–fetal circulation through the placenta and exert long-term effects [23]. Observations of abnormal manifestations, such as piloerection, diarrhea, variations in body weight, vaginal bleeding, hematological and biochemical changes, and histological and anatomical anomalies, offer significant insights into the toxic and teratogenic effects of the compound [24]. Hence, this study was designed to assess the toxicity and teratogenic potential of piplartine during the embryonic development of mice (*Mus musculus*) by examining clinical signs of morbidity and mortality, organ weights, hematological and biochemical parameters, DNA fragmentation, and cell cycle status. These data are crucial for supporting (or opposing) the clinical studies of piplartine, enabling its pharmacological potential to be fully harnessed for societal benefit.

2. Results

2.1. Maternal and Embryo–Fetal Toxicity

The assessment of markers of maternal toxicity, as detailed in Table 1 and illustrated in Figure 1, showed no statistically significant differences in organ weight, maternal weight, or body weight gain among the experimental groups.

Table 1. Variations in maternal weight, maternal organ weights, and corrected body weight gain (Δ weight) across different doses from the 6th to the 18th day of the experiment.

Variable	Group				*p*-Value
	Control	25 mg/kg	50 mg/kg	100 mg/kg	
Organ weight					
Uterus	1.8 ± 0.6 (*n* = 8)	1.2 ± 0.6 (*n* = 6)	1.2 ± 0.8 (*n* = 7)	1.1 ± 1.1 (*n* = 3)	0.8872
Heart	0.1 ± 0.9 (*n* = 8)	0.2 ± 0.1 (*n* = 8)	0.1 ± 0.0 (*n* = 8)	0.1 ± 0.1 (*n* = 7)	0.9983
Liver	1.4 ± 0.7 (*n* = 8)	1.5 ± 1.0 (*n* = 8)	1.1 ± 0.7 (*n* = 8)	1.2 ± 1.0 (*n* = 7)	0.9864
Spleen	0.1 ± 0.1 (*n* = 8)	0.2 ± 0.1 (*n* = 7)	0.1 ± 0.0 (*n* = 8)	0.1 ± 0.0 (*n* = 7)	0.8695
Intestine	0.1 ± 0.1 (*n* = 8)	0.2 ± 0.2 (*n* = 7)	0.2 ± 0.2 (*n* = 8)	0.1 ± 0.1 (*n* = 6)	0.9473
Right kidney	0.2 ± 0.0 (*n* = 7)	0.2 ± 0.0 (*n* = 7)	0.2 ± 0.1 (*n* = 8)	0.2 ± 0.0 (*n* = 7)	0.4092
Lungs	0.2 ± 0.3 (*n* = 9)	0.2 ± 0.0 (*n* = 7)	0.3 ± 0.2 (*n* = 8)	0.1 ± 0.1 (*n* = 7)	0.9346
Maternal weight (g)					
Initial	31.0 ± 5.8 (*n* = 10)	31.7 ± 4.4 (*n* = 10)	30.3 ± 2.2 (*n* = 9)	32.0 ± 2.2 (*n* = 7)	0.9931
Final	45.6 ± 8.8 (*n* = 10)	44.4 ± 8.3 (*n* = 9)	46.6 ± 5.2 (*n* = 9)	46.8 ± 8.9 (*n* = 7)	0.9965
Maternal weight gain (g)					
6th day–Day 0	31.1 ± 5.9	31.0 ± 4.1	30.4 ± 2.1	31.8 ± 2.2	0.9970
9th day–6th day	31.9 ± 6.6	32.7 ± 3.6	31.7 ± 1.7	32.5 ± 3.3	0.9983
12th day–9th day	34.8 ± 6.4	35.4 ± 5.7	34.9 ± 2.5	35.3 ± 2.5	0.9997
15th day–12th day	39.1 ± 6.9	41.3 ± 7.1	39.1 ± 4.0	41.0 ± 3.5	0.9889
18th day–15th day	45.6 ± 9.9	45.4 ± 10.3	46.3 ± 5.4	48.5 ± 6.2	0.9946
Δ weight	11.9 ± 6.85	17.7 ± 8.01	13.7 ± 5.28	12.2 ± 7.78	0.9296

Data are presented as mean ± standard deviation. Differences were deemed statistically significant at $p < 0.05$, indicated by different letters. Parameters, including maternal weight, weight of the pregnant uterus, heart weight, liver weight, spleen weight, intestine weight, right kidney weight, and lung weight, were analyzed using the non-parametric Kruskal–Wallis test. The Δ Weight result was evaluated using the parametric ANOVA test. Δ Weight is calculated as [(final weight–initial weight)–weight of the uterus].

Similarly, no significant alterations were observed for markers of embryo–fetal toxicity (Table 2), including the number of implantations, number of resorptions, average placental weight, number of fetuses, and average weight of the fetuses among the experimental groups.

Table 2. Markers of embryo–fetal toxicity in mice treated with piplartine at different concentrations.

Variable	Group				*p*-Value
	Control	25 mg/kg	50 mg/kg	100 mg/kg	
Implantations	16.7 ± 4.7 (*n* = 10)	18.3 ± 7.5 (*n* = 10)	20.2 ± 5.9 (*n* = 9)	18.8 ± 9.7 (*n* = 7)	0.9296
Resorptions	8.4 ± 3.7 (*n* = 10)	10.7 ± 4.6 (*n* = 9)	12.3 ± 3.5 (*n* = 9)	10.6 ± 4.1 (*n* = 6)	0.9108
Placental weight	0.8 ± 0.3 (*n* = 8)	0.8 ± 0.5 (*n* = 8)	1.1 ± 1.0 (*n* = 7)	0.6 ± 0.4 (*n* = 3)	0.9710

Table 2. *Cont.*

Variable	Group				*p*-Value
	Control	25 mg/kg	50 mg/kg	100 mg/kg	
Number of fetuses	7.8 ± 2.5 (*n* = 10)	8.6 ± 4.3 (*n* = 10)	8.1 ± 3.3 (*n* = 9)	9.7 ± 5.3 (*n* = 7)	0.9883
Fetus weight	1.6 ± 0.8 (*n* = 10)	1.3 ± 0.2 (*n* = 9)	1.3 ± 0.1 (*n* = 7)	1.5 ± 0.2 (*n* = 4)	0.9694

Data are presented as mean ± standard deviation. Differences were deemed statistically significant at $p < 0.05$. The results for the number of resorptions, number of live fetuses, number of dead fetuses, average placental weight, and average fetal weight were analyzed using the non-parametric Kruskal–Wallis test. The ANOVA parametric test was used for the number of implantations.

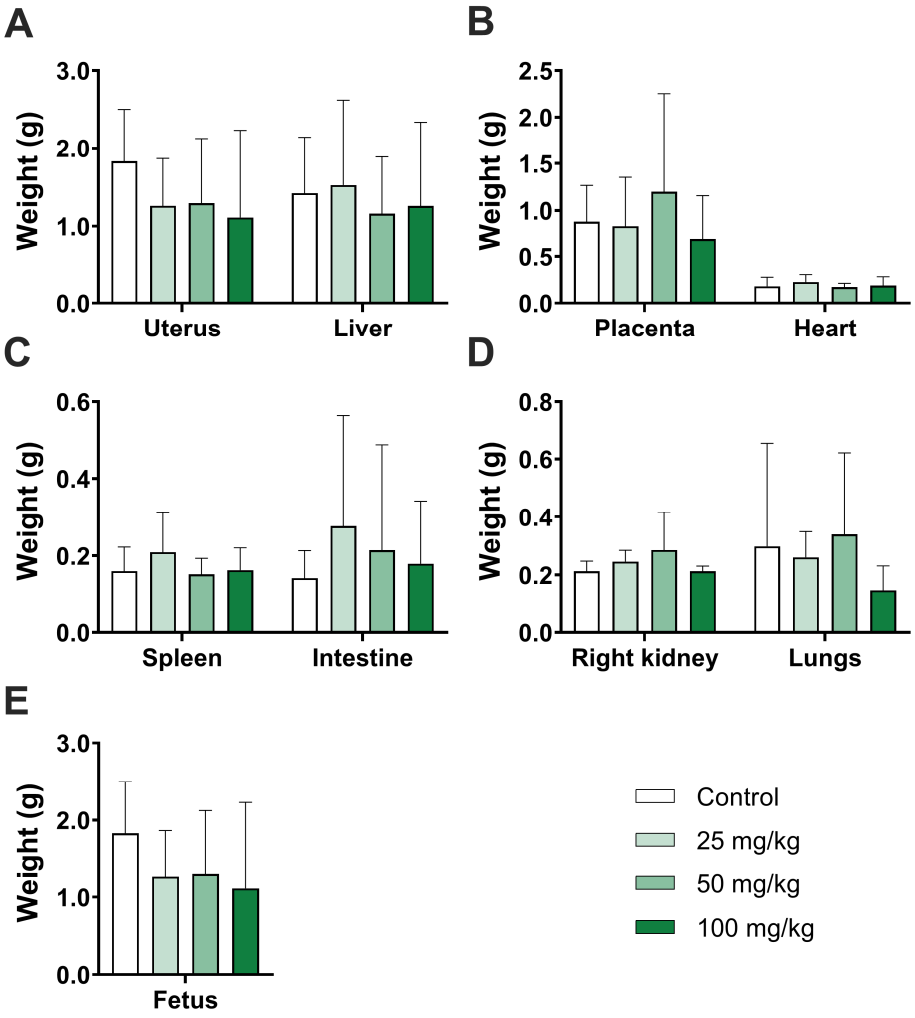

Figure 1. Mass (in grams) of maternal organs across experimental groups treated with 25, 50, and 100 mg/kg piplartine and the control group (2% DMSO). (**A**) Uterus and liver. (**B**) Placenta and heart. (**C**) Spleen and Intestine. (**D**) Kidney and lungs. (**E**) Fetus.

2.2. Fetal Visceral and Skeletal Anomalies

There were no significant differences in the frequencies of visceral (Table 3) or skeletal (Table 4) anomalies in fetuses from mice treated with piplartine compared to those from the control group. Furthermore, no differences were observed between the experimental groups.

Table 3. Visceral anomalies observed in mice treated with piplartine at different concentrations.

Visceral Anomaly	Present	Group			
		Control	25 mg/kg	50 mg/kg	100 mg/kg
Palate	Yes	1	3	0	0
	No	55	56	45	61
Heart ventricle	Yes	1	3	0	1
	No	55	56	44	60
Heart atrium	Yes	0	5	0	2
	No	56	54	44	59
Intestinal loop	Yes	0	1	0	0
	No	57	58	44	61
Kidney	Yes	0	2	0	1
	No	57	57	44	60
Adrenal	Yes	0	0	0	2
	No	57	59	44	59
Thymus	Yes	0	0	2	0
	No	56	59	42	61
Lung	Yes	0	0	1	0
	No	56	59	43	61
Liver	Yes	0	0	1	0
	No	56	59	44	61

Table 4. Skeletal anomalies observed in mice treated with piplartine at different concentrations.

Skeletal Anomaly	Present	Dose			
		Control	25 mg/kg	50 mg/kg	100 mg/kg
Sternebrae	Yes	0	9	6	4
	No	32	18	12	13

2.3. Hematological and Biochemical Analyses

Treatment with piplartine did not lead to significant changes in the parameters of the erythrogram, leukogram, and plateletogram (Table 5) when compared with the control group. Similarly, the levels of circulating biochemical markers (Table 6) remained unaffected by piplartine treatment.

Table 5. Hematological parameters in pregnant mice treated with 25, 50, or 100 mg/kg of piplartine (or vehicle for the control group) from the sixth (implantation) to the eighteenth day of gestation via gavage.

Hematological Analysis	Control	25 mg/kg	50 mg/kg	100 mg/kg	*p*-Value
RBC ($\times 10^6/\mu L$)	(n = 10) 8.75 ± 1.16	(n = 10) 9.40 ± 1.19	(n = 9) 8.79 ± 0.49	(n = 7) 9.28 ± 1.60	0.5649
HGB (g/dL)	13.16 ± 1.71	13.86 ± 1.79	13.29 ± 0.90	13.80 ± 2.30	0.334
HCT (%)	34.32 ± 4.39	36.14 ± 4.49	34.73 ± 2.28	36.47 ± 6.25	0.446
MCV (fL)	39.27 ± 1.49	38.48 ± 1.69	39.50 ± 1.39	39.31 ± 1.03	0.3526
MCH (pg)	15.07 ± 0.71	14.78 ± 0.95	15.13 ± 0.73	14.91 ± 0.52	0.7469
MCHC (g/dL)	38.36 ± 1.07	38.34 ± 1.31	38.28 ± 1.58	37.92 ± 1.16	0.8644
RDW-CV (%)	18.24 ± 1.06	19.79 ± 2.14	18.22 ± 1.85	18.14 ± 1.09	0.1523

Table 5. *Cont.*

Hematological Analysis	Control	25 mg/kg	50 mg/kg	100 mg/kg	*p*-Value
WBC ($\times 10^3/\mu L$)	3.13 ± 1.78	3.32 ± 1.68	4.50 ± 4.95	3.01 ± 1.00	0.9791
W-SCR (%)	61.79 ± 14.29	64.05 ± 9.69	68.24 ± 2.42	66.89 ± 8.88	0.4596
W-MCR (%)	36.59 ± 14.34	34.60 ± 8.79	29.84 ± 1.45	32.32 ± 9.01	0.3237
W-LCR (fL)	1.62 ± 1.37	1.35 ± 1.71	2.11 ± 1.82	0.79 ± 0.73	0.2281
PLT ($\times 10^3/\mu L$)	1162.27 ± 443.11	1373.23 ± 542.46	1179.44 ± 491.07	1208.44 ± 454.24	0.7446

RBC: Red Blood Cells; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW-CV: Red Blood Cell Distribution Width presented as the Coefficient of Variation; WBC: Total White Blood Cells; W-SCR: Small Cell (Lymphocyte) Rate; W-MCR: Medium Cell Ratio (Monocytes, Basophils, and Eosinophils); W-LCR: Large Cell (Neutrophil) Rate; PLT: Platelet Count. Units are g/dL for grams per deciliter, fL for femtoliters, and pg for picograms. Data are presented as mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were conducted using One-way ANOVA and Tukey post hoc test (for MCV and MCHC) and Kruskal–Wallis and Dunn post hoc test (for the other parameters).

Table 6. Biochemical markers in in pregnant mice treated with 25, 50, or 100 mg/kg of piplartine (or vehicle for the control group) from the sixth (implantation) to the eighteenth day of gestation via gavage.

Biochemical Marker	Control	25 mg/kg	50 mg/kg	100 mg/kg	*p*-Value
	(*n* = 10)	(*n* = 10)	(*n* = 9)	(*n* = 7)	
ALT (U/L)	50.82 ± 26.17	34.00 ± 10.39	41.50 ± 23.55	33.43 ± 13.26	0.2699
AST (U/L)	91.00 ± 41.15	61.56 ± 18.63	74.14 ± 22.42	73.29 ± 28.82	0.3415
Albumin (g/dL)	1.14 ± 0.44	1.44 ± 0.43	1.60 ± 0.43	1.38 ± 0.46	0.1513
Creatinine (mg/dL)	0.44 ± 0.13	0.42 ± 0.08	0.37 ± 0.06	0.39 ± 0.08	0.3976
Urea (mg/dL)	48.27 ± 10.64	54.64 ± 20.08	54.67 ± 5.77	43.86 ± 9.55	0.0864
LDH (mg/dL)	893.38 ± 521.70	698.70 ± 260.61	933.38 ± 413.48	724.33 ± 214.89	0.4954

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; LDH: Lactate Dehydrogenase. Units are U/L for units per liter, mg/dl for milligrams per deciliter, and g/dl for grams per deciliter. Data are presented as mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were conducted using One-way ANOVA and Tukey post hoc test (for albumin, creatinine, and LDH) and Kruskal–Wallis and Dunn post hoc test (for ALT, AST, and urea).

2.4. DNA Fragmentation and Cell Cycle

The DNA fragmentation levels in bone marrow cells of mice treated with piplartine were less than 22%, which is similar to the approximately 19% observed in the control group (Figure 2A). However, cell cycle analysis (Figure 2B) indicated that the relative number of cells in the S phase was different between the mice treated with 50 mg/kg piplartine (4.4%) and those in the control group (6.8%).

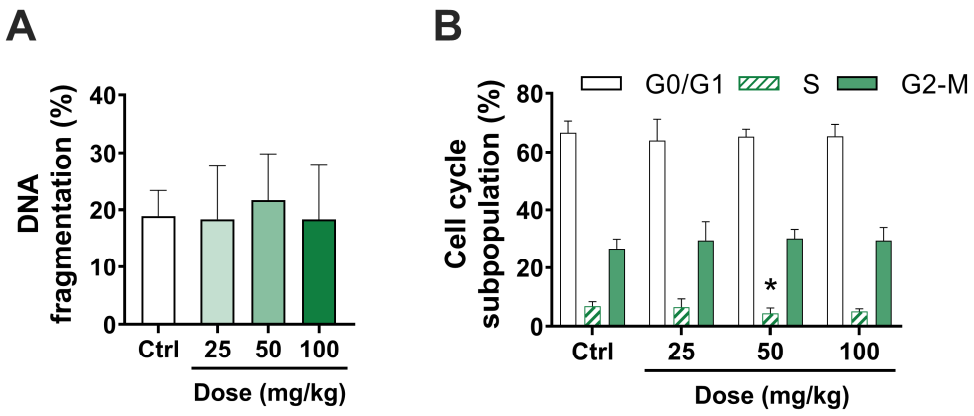

Figure 2. Effect of piplartine on bone marrow cells: (**A**) percentage of DNA fragmentation and (**B**) distribution of cell population in the G0/G1, S, and G2/M phases of the cell cycle. Statistical analysis was conducted using Kruskal–Wallis and Dunn post hoc tests. * Indicates a significant difference compared to the control group ($p = 0.0095$).

3. Discussion

3.1. Maternal Toxicity

Among the organs examined, only the heart exhibited a notable weight increase in the 100 mg/kg experimental group. Conversely, in organs like the liver, spleen, and intestine, the average weight was elevated in the group treated with a 25 mg/kg dose. The weight gain in the right kidney and lung was more pronounced in the group subjected to 50 mg/kg. Unlike other experimental conditions, the average uterus weight in the control group was higher than in those exposed to piplartine, indicating a potential connection to implantation and resorption sites.

The corrected maternal weight gain in the 25 mg/kg group was higher on average compared to the other groups. The variations noted in these parameters may not necessarily be attributable to piplartine maternal toxicity at the evaluated doses. The changes in maternal organ weight, whether increases or decreases, were insufficient to confirm piplartine toxicity. This possibly implies that there is no direct association with exposure to piplartine.

Consequently, the exposure of female *Mus musculus* mice to experimental doses of 25, 50, and 100 mg/kg did not significantly impact the overall assessment of maternal toxicity.

3.2. Embryo–Fetal Toxicity

Maternal exposure to chemical agents during pregnancy can lead to implantation failures and affect embryonic and fetal viability through various mechanisms, such as morphological changes in the embryo, alterations in maternal hormone levels, changes in uterine morphology, or disturbances in tubal transport [25].

In our study, it was not possible to calculate preimplantation losses since the corpus luteum in mice involutes very easily [26]. However, we observed that the average number of implantations, resorptions, and dead fetuses was higher at the experimental dose of 50 mg/kg. Conversely, at the dose of 100 mg/kg, implantations and fetal resorptions nearly leveled off, while the average number of dead fetuses decreased. The average weight of placentas and fetuses, as well as the number of live fetuses, was higher in the group exposed to 100 mg/kg. These aspects of embryo–fetal toxicity can be compared in Table 2.

Although there were variations among the different groups, no significant differences were found, leading us to conclude that the exposure of female mice to experimental concentrations of 25, 50, and 100 mg/kg did not alter the evaluated parameters significantly.

3.3. Fetal Visceral Analysis

In all the viscera analyzed, the likelihood of fetal malformations was higher with exposures to concentrations of 25 and 100 mg/kg. Specifically, in the ventricle and atrium,

the expected frequency of abnormalities at the 100 mg/kg concentration was slightly higher than at 25 mg/kg. However, when abnormalities were observed, they appeared at the lower concentration for both variables.

The expected frequency of abnormalities in the palate, intestinal loop, kidney, and adrenal gland was the same for these two doses. Except for the adrenal gland, there was a consistent pattern of more frequent visceral alterations at the 25 mg/kg concentration. In the case of the adrenal gland, changes were more common with exposure to 100 mg/kg/day.

Therefore, the exposure of female mice to experimental concentrations of 25, 50, and 100 mg/kg did not significantly impact the analysis of visceral anomalies.

3.4. Fetal Skeletal Analysis

The findings suggest that initial bone formation was not significantly altered by exposure to piplartine, meaning that disruptions in the formation of the chest wall were insufficient to indicate a potential restriction in fetal development due to the studied doses of the substance.

The anomalies identified in the fetal skeletal analysis might indicate a delay in intrauterine development, specifically related to ossification. Notably, incomplete ossification of the sternebrae was more evident in the 25 mg/kg experimental group, with the prevalence decreasing as the dose increased.

In the 25 mg/kg group, the primary anomaly was the reduced size of the fifth sternebra. Additionally, the absence of these primitive elements was observed in the first and fifth formation positions, along with irregularities in shape, including the appearance of a butterfly shape. In the 50 mg/kg group, the reduced fifth sternebra was again the most common change, accompanied by either the lack of formation of certain segments or the addition of an element. For fetuses indirectly exposed to a dose of 100 mg/kg, the absence of one of the sternebrae was predominant, along with reductions and irregularities in formation.

During the acute phase of piplartine administration via gavage, there were no observations of animals displaying lethargy, piloerection, vocalization, or tremors. There was a loss of five animals due to complications from gavage, but no deaths were directly related to the dosages. Thus, the exposure of female Mus musculus mice to experimental concentrations of 25, 50, and 100 mg/kg did not significantly impact the skeletal analysis.

3.5. Hematological and Biochemical Analyses

Blood analyses are instrumental in identifying various pathologies or physiological changes induced by a biological entity. Organs responsible for excretion, like the kidneys, and those involved in detoxification, such as the liver, are particularly vulnerable and can reflect significant shifts through biochemical markers. Accordingly, the outcomes related to erythrocytes, hemoglobin, RBC indices, and biochemical tests in our study did not reveal any hematological and metabolic changes indicative of maternal toxicity.

In comparison with similar studies [27,28] that evaluated toxicity in mice due to exposure to piplartine, we can affirm, based on the correlation with published results, the safety of piplartine exposure in terms of hematological and biochemical parameters. This demonstrates that the treatment did not modify the enzymatic activity of transaminases, thereby excluding potential harm to the liver and kidneys, as well as preventing inflammatory processes, anemia, and bone marrow dysfunction.

3.6. DNA Fragmentation and Cell Cycle

Alongside the hemogram, the evaluation of DNA damage and changes in the proliferation of oral mucosa (OM) cells served as indicators of hematopoietic toxicity. The impact of DNA damage on cell progression is well documented. When damage occurs, checkpoint pathways that regulate DNA repair mechanisms are activated, leading to a halt in cell cycle progression until the damage is addressed. If the damage is beyond repair, cells either

permanently cease cycling or undergo apoptosis [29]. No significant differences in DNA fragmentation rates were observed between the treated and control groups.

In the group treated with 50 mg/kg, the subpopulation of S-phase cells was marginally lower (4.4 ± 1.82%) than in the control group (6.8 ± 1.56%), achieving statistical significance ($p < 0.05$). Despite this, the profiles of cells in the G0/G1 and G2/M phases remained similar between the groups, implying that piplartine does not induce genotoxicity, a cytostatic effect, or unregulated multiplication of blood progenitor cells. In line with these observations, piplartine administered intraperitoneally at 50 mg/kg did not lead to micronucleus formation in mouse OM cells [1].

3.7. Limitations

This study, while providing valuable insights into the safety and therapeutic potential of piplartine, has several limitations that warrant consideration. The investigation was conducted using a specific animal model (*Mus musculus*), which, although informative, may not fully replicate human physiological responses, potentially limiting the direct translatability of our findings to human clinical scenarios [30–32]. The scope of piplartine doses examined was restricted to 25–100 mg/kg, leaving the safety profile at other doses or with prolonged exposure unexplored. Our assessment focused predominantly on maternal and embryo–fetal toxicity, alongside selected biochemical markers of liver and kidney function, omitting comprehensive evaluations of other critical aspects such as neurotoxicity, immunotoxicity, or carcinogenic potential [33], as performed for other potentially harmful molecules [34,35]. Moreover, the study did not delve into the pharmacokinetics and pharmacodynamics of piplartine, factors essential for understanding its systemic behavior, optimizing dosing, and foreseeing drug–drug interactions [36,37]. Similarly, assessments of bioavailability and biodistribution were absent, crucial for evaluating efficacy and safety, especially concerning target tissue reach and effect [38,39]. Addressing these limitations in subsequent research will be essential for a more comprehensive understanding of piplartine safety and efficacy, facilitating its progress toward clinical application.

4. Materials and Methods

4.1. Piplartine Extraction and Isolation

Extracts of *Piper tuberculatum* Jacq., gathered from the campus of the University of São Paulo, were prepared by mixing 350 g of dried roots with 1.5 L of ethyl acetate. This mixture was incubated under agitation for two days, with the procedure repeated three times. *Piper tuberculatum*, natively found from Mexico to Tropical America, typically grows as a shrub or tree primarily in the wet tropical biome [40]. The samples were collected and cataloged in the Herbarium of the University of São Paulo under the voucher number K-163. The resulting extraction solution was concentrated using a rotary evaporator to yield 56 g of crude extract. This crude extract was then solubilized in hot methanol, filtered through a paper filter, allowed to stand for four days at room temperature, and subsequently vacuum-filtered. The initially obtained solid (6 g) was recrystallized from methanol, resulting in 1.5 g of piplartine (Figure 3). The identity and purity of piplartine were verified by HPLC and ^{1}H NMR (500 MHz, $CDCl_3$), with the spectral signals compared against a standard sample. The NMR spectrum was obtained using Inova 300 MHz equipment (Varian, Palo Alto, CA, USA); the samples were dissolved in deuterated chloroform (Sigma-Aldrich, St. Louis, MO, USA) with TMS serving as the internal standard. The liquid chromatography setup (Shimadzu, Kyoto, Japan) included two LC-20AD analytical pumps, an SIL-20AHT automatic injector, an SPD-20A UV/Vis detector, a CTO-20A column oven, and a CBM-20A controller, using a Luna 5 µm PFP(2) 100 Å, 150 × 2 mm column (Phenomenex, Torrance, CA, USA). The flow rate was set at 0.2 mL/min, with mobile phase A being H_2O (0.1% formic acid) and B being Acetonitrile (0.1% formic acid). The gradient started at 0 min with 20% B, holding until 5 min, then from 5 to 50 min, it shifted from 20 to 100% B, maintained at 100% until 55 min. The oven temperature was kept at 40 °C, analyzing wavelengths at 254 and 330 nm (Figure S1).

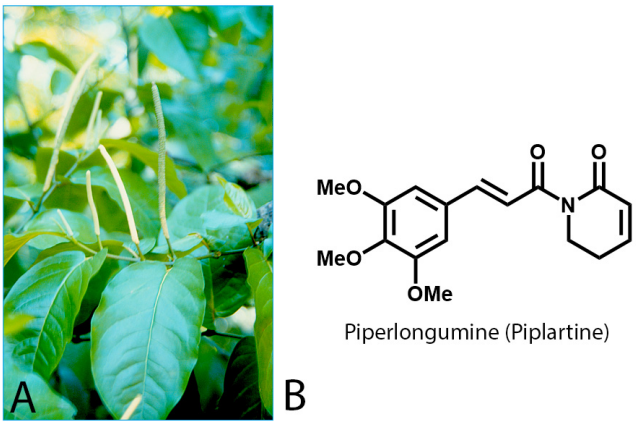

Figure 3. (**A**) *Piper tuberculatum* Jacq. ("Pimenta de Macaco"). (Photo: M. J. Kato) growing in the *Campus* of University of São Paulo, USP, São Paulo, Brazil. (**B**) Molecular structure of piplartine (1-[(*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoyl]-2,3-dihydropyridin-6-one, IUPAC name).

4.2. Animals

Mus musculus mice, weighing approximately 30 ± 5 g and aged 60 days, were acquired from the Multidisciplinary Center for Biological Research in the Field of Science in Laboratory Animals—CEMIB/UNICAMP. Before the experiment began, the animals were allowed a fifteen-day acclimatization period. They were housed in polypropylene cages with zinc-plated wire lids, situated in air-conditioned racks maintained at 22 °C within the Animal Housing Facility of the Faculty of Medicine at the University of Brasília, under a twelve-hour light–dark cycle. All experimental procedures were conducted following a protocol that received approval from the Committee on Ethics in Animal Use (CEUA) of the University of Brasília, under protocol No. 47/2019.

4.3. Animal Mating

Mice were arranged in a setup of three females to one male to facilitate mating. The presence of copulation was checked daily by inspecting females for a vaginal plug. Females with a vaginal plug were weighed, labeled, and sorted into one of the four experimental groups. Potentially pregnant mice underwent daily gavage treatment in the morning from the sixth day of implantation to the eighteenth day of gestation. Piplartine, dissolved in 2% (*v*/*v*) dimethyl sulfoxide (DMSO), was administered to the pregnant mice in dosages of 25, 50, and 100 mg/kg. These dosages were chosen based on findings from prior research [1]. A control group received the vehicle (i.e., 2% DMSO) following the same treatment regimen. Each group was initially intended to include 10 pregnant mice. However, the groups treated with 50 and 100 mg/kg of piplartine ended up with only 9 and 7 pregnant mice, respectively, due to some animals in these groups not becoming pregnant.

4.4. Maternal Toxicity

General toxicity was assessed by daily observations of clinical signs of morbidity and mortality, including death, piloerection, changes in locomotion, diarrhea, vaginal bleeding, and variations in body weight. These evaluations were conducted once a day, following the administration of piplartine to pregnant females throughout their pregnancy period.

4.5. Laparotomy

On the eighteenth day of gestation, approximately one day before the expected natural birth, mice were anesthetized with isoflurane, euthanized via CO_2 exposure, and subsequently subjected to a laparotomy. Vital organs including the liver, kidneys, spleen, heart, intestine, lung, and placenta were extracted and their weights recorded. The uterus was then exposed and weighed after the removal of the fetuses. Additionally, the average fetal weight, the count of live and dead fetuses, the total number of implantation sites, the

number of embryo resorptions [41], and the corrected body weight gain, alongside the initial and final maternal weights, were meticulously calculated and documented.

4.6. Fetal Anomalies

The fetuses were examined using stereomicroscopes to identify any external structural malformations (Figure 4). They were divided into two groups for further analysis: one group was fixed in Bodian solution for visceral examination, while the other was fixed in acetone, eviscerated, bleached with potassium hydroxide, and stained with alizarin to facilitate the analysis of skeletal anomalies [42]. The assessment of visceral anomalies followed established standard protocols [42,43]. The fetuses designated for skeletal analysis were evaluated using a technique previously described [44].

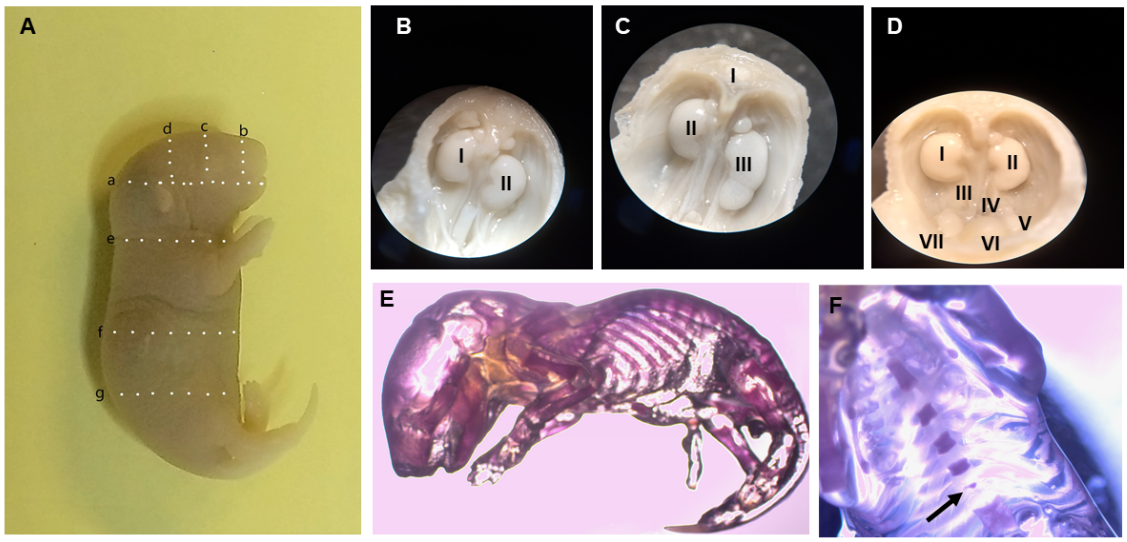

Figure 4. Representative images of biological material processed for toxicological studies: (**A**) Planes of fetal sections for visceral analysis: a. Cross section at the height of the oral cavity. b. Frontal section in the preglabellar region; c. Frontal section in the orbital region; d: Frontal section in the vertex region; e. Cross section in the cervical region; f. Cross section in the abdominal region, just below the diaphragm; g. Cross-section in the pelvic region, passing through the navel. (**B**): Right kidney with adrenal gland attached to the upper portion and enlarged left ureter (I) and left kidney with adrenal gland not attached to the upper portion (II). (**C**): Spinal cord (I); right kidney (II) and left kidney (III) with their respective adrenal glands attached to the upper portion. The left kidney is bilobed and abnormally shaped compared to the right kidney. Over the right kidney, there are two adrenals. (**D**) Absence of the adrenal gland in the right kidney (I); left kidney with normal appearance and adrenal gland attached to the upper portion (II); right (III) and left (IV) ureters; right (VII) and left (V) male gonads; bladder (6). (**E**) Alizarin-stained mouse fetus, left lateral view. (**F**) Reduced sternebra.

4.7. Hematological and Biochemical Analysis

Whole-blood samples were extracted from pregnant mice via cardiac puncture. A portion of these samples (50 µL) was placed into a microtube with EDTA for automated complete blood count analysis using the pocH-100iV Diff system (Sysmex, Kobe, Japan) [45]. Additionally, markers indicative of liver and kidney function were assessed. This involved measuring serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, creatinine, urea, and lactate dehydrogenase (LDH). To separate the serum, about 700 µL of whole blood was deposited into a microtube equipped with separator gel and clot accelerator, then centrifuged at 5000 rpm for 5 min. The measurements were carried out on an automated chemical analyzer, ChemWell-T® (Labtest, Lagoa Santa, Brazil), using commercial kits provided by Labtest® in accordance with the manufacturer's guidelines.

4.8. DNA Fragmentation and Cell Cycle

Bone marrow cells were harvested from the femur for DNA integrity analysis and cell cycle profiling. The procedure began with the removal of soft tissues surrounding the femur, followed by cutting the epiphyses to open the bone marrow cavities. Fetal bovine serum (FBS) was then injected into one end of the femur, allowing it to flow through and collect into a microtube at the other end. The cell suspension in FBS was centrifuged at 2000 rpm for 5 min at 4 °C, after which the cell pellet was washed with phosphate-buffered saline (PBS). The bone marrow cells were then fixed in ice-cold 70% ethanol and stored at −20 °C for at least 24 h. Following another wash with PBS and removal of the supernatant, the cells were treated with 200 µL of lysis buffer containing propidium iodide (20 µg/mL) and RNAse A (50 µg/mL) for 30 min at room temperature. The analysis was conducted using flow cytometry (FACS Verse, BD Biosciences, Franklin Lakes, NJ, USA) with Flow Jo®vX 0.7 software, evaluating a total of 10,000 events per sample. Parameters measuring the size and granularity of the detected events (FSC—forward scatter and SSC—side scatter, respectively) were recorded concurrently, and cellular debris was excluded. The distribution of cells across the cell cycle phases (G0/G1, S, and G2/M) was calculated.

4.9. Statistical Analysis

The results are presented as mean ± standard deviation. The normality of the data was assessed using the Shapiro–Wilk test. For variables that did not deviate from normality, ANOVA was employed for analysis; these included the number of implantations and corrected body weight gain (Δ weight). The non-parametric Kruskal–Wallis test was used for variables where at least one group did not adhere to the normality assumption. These variables encompassed maternal weight, uterine weight, average fetal weight, number of live fetuses, number of dead fetuses, number of resorptions, average placenta weight, heart weight, liver weight, spleen weight, intestine weight, right kidney weight, and lung weight. Fisher's Exact test was utilized for nine variables associated with fetal visceral alterations: palate, ventricle, atrium, intestinal loop, kidney, adrenal, thymus, lung, and liver. These were subjected to Fisher's Exact test due to all having an expected frequency of malformations below five, justifying the use of this test. The sternebra, a skeletal variable in fetuses, was analyzed using the Chi-square test as it met the criterion of a minimum of five expected observations. A p-value below 0.05 was considered indicative of statistical significance in all analyses, which were performed using SPSS—Statistical Package for the Social Sciences, version 20.

5. Conclusions

The reported findings affirm the safety of administering piplartine to the evaluated animal model within the dosage range of 25–100 mg/kg. A notably promising balance between efficacy and toxicity was observed, as our results do not indicate significant toxic effects associated with the exposure levels used in the experimental groups.

Nonetheless, further research on piplartine exposure is necessary to deepen scientific understanding in vivo, focusing on its pharmacokinetics, pharmacodynamics, bioavailability, biodistribution, and any other potential side effects in different animal models. This step is crucial before asserting its complete safety for clinical studies in humans. This study showcases the innovative potential of the molecule, offering encouraging outcomes for subsequent research and supporting its application in biomedical, nanotechnological, and pharmaceutical fields [46]. For example, the study by Giacone et al. [47] underlines the potential use of chitosan-modified nanoemulsion containing piplartine as a novel approach for the local treatment of skin cancer, highlighting the broader applicability and promise of this compound.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ddc3020021/s1, Figure S1: NMR and HPLC data.

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Informed Consent Statement: Not applicable.

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