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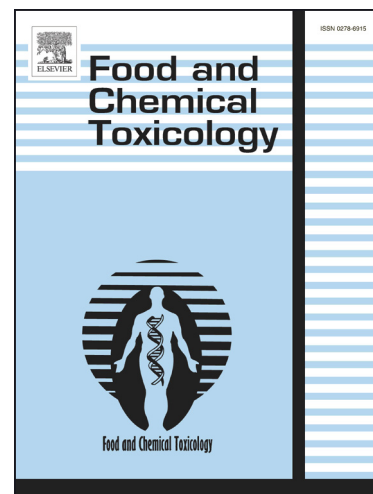
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Toxicological *in vitro* and subchronic evaluation of LASSBio-596

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1. INTRODUCTION

Asthma is defined as a chronic inflammation of the airways. Although combined treatments are commonly able to maintain most asthma conditions at asymptomatic levels, disease control is still quite poor (Souza et al., 2009). Many of the anti-asthmatic drugs that have been developed show some degree of toxicity (Cazzola et al., 2012; Quirce, 2012).

A 2001 report using symbiosis described the design, synthesis, and evaluation of an anti-inflammatory profile in a model of acute lung inflammation caused by the 2 - [4 - (1,4-tiazinan-4-ylsulfonyl) phenylcarbamoyl] benzoic acid, also known as LASSBio-596 (Lima et al., 2002). This original analysis was described as capable of preventing and treating acute respiratory distress syndrome using a murine model at 10 mg/kg (Rocco et al., 2003).

Designed as a thalidomide analog, LASSBio-596 is an achiral compound containing a subunit carboxylic amide rather than the phthalimide group (Lima et al., 2002). Several reports have discussed the molecular basis of thalidomide toxicity (Koch and M.J. Czejka, 1986; Stephens, 1988; Arlen and Wells, 1996; Hansen and Harris, 2004; Ito et al., 2010) and, although there are controversies, the phthalimide group has been

considered a toxicophore group. For this reason, this molecular fragment should be avoided. It is essential to identify toxicities early in the developmental process for both safety and economic reasons. Progress in determining the mechanisms of specific toxicities will greatly aid in both risk assessment and the researcher's ability to predict and avoid these toxicities (Szczzech, 1996; Brannen et al., 2011).

Therefore, considering the absence of the phthalimide unit in the LASSBio-596 structure, this molecule showed good prospects of becoming a new therapeutical option for asthma.

LASSBio-596, when administered orally, is capable of preventing induced mechanical and morphological changes in the lungs that commonly cause the onset of asthma, in turn reducing bronchoconstriction, blocking the process, and decreasing fibroproliferative airway resistance. For this reason, LASSBio-596 configures as a legitimate candidate for an anti-asthma drug and as a potential option for the treatment of steroid-resistant asthma (Campos, 2006). From the chemical point of view, LASSBio-596 is an achiral molecule and class-carbamoyl benzoic acid obtained by means of linear synthesis in the fourth stage (Campos, 2006). Rocco *et al.* (2010) investigated the acute toxicity of LASSBio-596 in rats and showed that, at doses up to 200 times that estimated for *in vivo* pharmacological tests, the administration of the compound did not induce animal death.

In an attempt to further evaluate its safety, the LASSBio-596 was submitted to both *in vitro* and *in vivo* preclinical studies. The *in vitro* evaluation used renal hepatic, pulmonary, cardiac, neurological, and intestinal cell lines using neutral red (NR) and [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; (MTT) assays; as well as micronuclei tests. As regards the *in vivo* evaluations, a subchronic assay on Wistar rats (OECD 408), at doses of 10, 50, and 250 mg/kg, and a zebrafish test (OECD 210) were performed.

2 MATERIAL AND METHODS

2.1 Drugs

LASSBio-596 was obtained as described in the literature (Rocco et al., 2010) and was characterized by High Performance Liquid Chromatography (HPLC) and Differential Scanning Calorimetry (DSC).

2.2 *In vitro* tests

2.2.1 Cytotoxicity assays

To evaluate the cytotoxicity, renal (BGM, MDCK and LLC-PK1), hepatic (sK-Hep), pulmonary (pneumocytes A-549), cardiac (H9C2), neurological (Neuro 2A), and intestinal (Caco-2 cell) cell lines were used, all of which were evaluated using NR and

MTT assays. First, the solubility of the sample was evaluated as recommended by the OECD (2009). Dimethylsulfoxide (DMSO) was used as a solvent, and the solubility was found to be 0.2 g/mL. According to the OECD, when the sample is hydrophobic, the highest concentration to be tested in the cells should be 1/200 of the maximum concentration in the solubility test. Therefore, the highest concentration of the sample was 1000 µg/mL, and the final volume of DMSO at its final concentration was less than 0.5% (v/v). Seven concentrations (68, 100, 147, 214, 315, 463, and 680 µg/mL) were established from the highest concentration, using the dilution factor of 1.47 (recommended to cover a range of relevant concentration, thus avoiding many cytotoxic and non-cytotoxic concentrations).

2.2.2 Neutral Red cytotoxicity assay

The test with NR was performed as described in the studies of Borenfreund and Puerner (1984) and Babich and Borenfreund (1993). The cells were placed on 96-well plates in a concentration of 1.0×10^4 cells/well containing 200 µL of medium and were then incubated with 8 different concentrations of the tested drug (68, 100, 147, 214, 315, 463, 680, and 1000 µg/mL) for 48 hours at 37°C in humidified air supplemented with 5% CO₂. After, the medium containing the drug was removed and 200 µL of the solution of NR (40 µg/mL) was added. The medium was then incubated at 37°C for 3 hours. The NR solution was then removed, and the cells were set with a 0.5% (v/v) formaldehyde in a 1% (g/v) CaCl₂ solution. The NR dye was eluted from the living cells with 100 µL of acid-alcohol (1% v/v acetic acid in 50% ethanol). Absorbance was read on a Multiskan MCC/340P plate reader at 540 nm with a 690-nm reference filter

(Thermo Labsystems®). Mean absorbance for each concentration was expressed as a percentage of the value obtained for the blank wells and plotted against the gentamicin concentration.

2.2.3 MTT cytotoxicity assay

The MTT test was performed based on the studies from Mosmann (1983). The cells were placed on 96-well plates in a concentration of 1.0×10^4 cells/well containing 200 μ L of medium and were then incubated with 8 different concentrations of the tested drug (68, 100, 147, 214, 315, 463, 680, and 1000 μ g/mL) for 48 hours at 37 °C in humidified air supplemented with 5% CO₂. After, the medium containing the drug was removed, 20 μ L of MTT solution (5.0 mg/mL) was added, and the plates were incubated for one hour at 37°C in humidified air supplemented with 5% CO₂. The MTT solution was then removed, and 100 μ L of DMSO was added to each well. The absorbance was read at 570 nm (Thermo Plate model TP-READER), and the results were expressed as the percentage of the viability present in treated cells as compared to control cells.

2.2.4 Micronuclei *in vitro*

Micronuclei *in vitro* testing was performed following the OECD 487 protocol (OECD, 2004), using cultured primary human peripheral blood lymphocytes at 3.18×10^4 mg/mL of LASSBIO-596. The cells were grown for one complete cell cycle (24 h) and treated for a period of 30 h with LASSBIO-596, simultaneously with cytochalasin-B, and immediately harvested. The cell suspension was centrifuged at 1,000 rpm for 5 min and

cells were then resuspended in a KCl 0.075 M solution maintained at room temperature for 10 min (mild hypotonic treatment). The fixation step with methanol/acetic acid (3:1) solution was repeated twice and, finally, the cells were resuspended in a small volume of methanol/acetic acid and dropped onto clean slides. The slides were then stained with 10% Giemsa (pH 6.8), and the micronuclei were manually counted in 2,000 cells with well-preserved cytoplasm. To produce a valid test, two positive controls – Colchicine (to detect numerical abnormalities, known as Aneugenic) and cyclophosphamide (to detect structural abnormalities, known as Clastogenic) were used in the presence or absence of a metabolic activator (S9). The identification of micronuclei was carried out according to Fenech (2000).

2.3 In vivo test

2.3.1 Subchronic test

2.3.1.1 Animals

The animals (Wistar rats) were provided by the Animal Shelter of the School of Pharmacy from the Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. Rats were maintained under controlled conditions of temperature ($22 \pm 3^{\circ}\text{C}$) and humidity (50 to 60%), with a 12 h light-dark cycle (07:00 am - 7:00 pm), free access to water, and standard rat chow. The experimental protocols were approved by the Ethics Committee on Animal Experimentation (protocol number 245/11).

2.3.1.2 Preparation of solutions

The solutions were prepared just before the administration of the doses. The administration was carried out through gavage in constant volumes. The drugs were suspended in a solution of carboxymethylcellulose (CMC) (Galena Ltda., São Paulo, Brazil) as a vehicle. The control group received only the CMC solution. Both the test and the control groups received the same vehicle so that the differences between the effects observed in both groups could be attributed only to the use of the drug.

2.3.1.3 Procedure

Subchronic toxicity tests were based on the OECD 408 guideline (OECD, 1998). Male (180 - 230 g) and female (170 - 200 g) Wistar rats, aged 8 to 9 weeks, were used in this model. The animals were randomly divided into six groups: control group (with 10 males and 10 females), three test groups (with 10 males and 10 females in each group), and two satellite groups (with 6 males and 6 females in each group). A constant volume of the test product was administered daily (7:00 a.m. to 9:00 a.m.) by gavage, for 90 days, with one dose for each group. During the administration period, the animals were observed daily. The animals were weighed weekly, so that the dose (mg/kg body weight) could be adjusted if necessary. The consumption of food and water was evaluated weekly. Three doses (10, 50 and 250 mg/kg) and a control solution (CMC) were administered. The selection of the proper dose to perform the subchronic toxicity test was based on the therapeutic dose of LASSBio596, which was 10 mg/kg [16]. Therefore, the doses chosen were 10, 50, and 250 mg / kg, in other words, the factor of 5 was used for the remaining doses, considering the therapy as a reference. The

satellite control group received the control solution, while the satellite highest-dose group received the dose of 250 mg/kg.

After treatment, the animals were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (15 mg/kg), and approximately 5 mL of blood was removed by direct puncture of the abdominal vena cava (Melo et al., 2008). The blood samples were divided into two aliquots: one was transferred to an empty tube and the other to a tube containing EDTA for biochemical and hematological assays. The animals had been subjected to fasting for 12 h prior to this procedure (OECD, 1998). The animals were then euthanized by an overdose of anesthesia, and the organs were removed for future macroscopic and histopathological analyses.

2.3.1.4 Laboratory Analyses

Laboratory analyses were performed for subchronic toxicity (biochemical, hematological, microscopic, and macroscopic analyses of the organs). At the end of the subchronic toxicity test, the animals were weighed, subjected to fasting for 12 h, anesthetized to collect blood, and euthanized for necropsy. Hematological analyzes were performed on the full amount of blood collected in tubes with EDTA, using the Abacus Junior Vet apparatus (Veterinary School, UFMG, Brazil).

Biochemical analyses were performed in serum obtained after centrifugation (2500 rpm/15 minutes) of whole blood samples without anticoagulants. Standardized Ortho-

Clinical Diagnostics[®] (Johnson & Johnson company) and Vitros[®] equipment with dry slide technology were employed to determine the following biochemical parameters: uric acid (AUR), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (COLT), creatinine (CREA), alkaline phosphatase (ALP), glucose (GLU), total proteins (PROT), urea (URE), gamma-glutamyl transferase (GGT), creatine kinase (CK), triglycerides (TRI), total bilirubin (BiIT), direct bilirubin (BiID), amylase (AML), calcium (CA), chloride (Cl), and phosphorus (P).

Hearts, lungs, kidneys, adrenal glands, spleens, livers, stomachs, pancreas, small intestines, uterus, ovaries, testes, epididymides, brains, and thymuses were removed for macroscopic examination. Fragments of these organs were fixed in 10% formalin for histopathological examination (Palmeiro et al., 2003; Kanjanapothi et al., 2004).

2.3.2. Zebrafish test

The zebrafish test was performed according to OECD 210 guidelines (OECD, 2013). Embryos were collected and enzymatically dechorionated with pronase (1 mg/mL) to allow for efficient transdermal exposure. The two dechorionated embryos were exposed to 1, 10, or 25 μ M LASSBio-596 or 0.5% DMSO and maintained at 28-30°C in 6-well plates with embryo medium (Instant Ocean and methylene blue). The medium with treatments was renewed every 24 h. The absence of any added treatment was considered the control. At 72 hpf, the embryos were viewed, and cardiac frequency was measured using an Olympus SZ6145TR microscope (Olympus[®] Japan). Images were acquired with an Olympus DP72 digital camera (Olympus[®] Japan), using an Axiovert

100 microscope (Carl Zeiss® Germany). Heart frequency was also analyzed from the zebrafish movies using a re-slice, which generates a kimogram. The frequency of the kimogram image was analyzed using Fast Fourier Transform. This analysis confirms the heart beat alteration measured manually.

2.4 Statistical analysis

Results are expressed as means $\pm$ standard deviation of the mean (SDM). For *in vitro* tests, statistical analyses were performed using ANOVA, followed by the Student's t and Student-Newman-Keuls or Bonferroni's tests to evaluate significant differences between the groups. For *in vivo* tests, statistical analyses were also performed using ANOVA, followed by the Student's t and Student-Newman-Keuls or Bonferroni's tests to evaluate significant differences between the groups. Differences were considered significant for $p < 0.05$. Results that did not present a normal distribution were analyzed using the Non-Parametrical Kruskal-Wallis test (Sampaio, 2002).

3 RESULTS

3.1 *In vitro* assays

LASSBio-596 produced no toxicity in any of the cell lines (renal: BGM, MDCK, and LLC-PK1; hepatic: Sk-Hep; pulmonary: pneumocytes A-549; cardiac: H9C2; neurological: Neuro 2A; intestinal: Caco-2) when submitted to NR and MTT cytotoxicity assays. In a similar manner, as that found in micronuclei tests, the combined analysis, both qualitative and quantitative, presented no DNA damage or toxicity, either in the absence or in the presence of an external metabolic activator (figure 1A and 1B).

<insert figure 1A and 1B here>

3.2 *In vivo* assay

3.2.1 Subchronic test

No deaths occurred during the subchronic toxicity test. No clinical signs of toxicity or changes in animal behavior were noted during this study. Significant suppression of body weight gain was observed at 250 mg/kg from week 4 for males (figure 2). Food and water consumption was statistically equal to the control group in all tested doses.

<insert figure 2 here>

Hematology data are summarized in Table 1. In both sexes, platelets (PLT) decreased in males and females at all doses. Meanwhile an increase was observed in lymphocytes for males (relative and absolute) and for females (relative only) at all doses. Another observed decrease was in granulocytes in both sexes at all doses. Analysis of the satellite at a dose of 250 mg/kg showed that these effects were not reversible for

absolute lymphocytes (males) and PLT, relative lymphocytes, and relative granulocytes (females).

<insert table 1 here >

In the serum biochemical analysis (Table 2), a significant increase in creatine kinase (CK) and aspartate aminotransferase (AST) could be observed in the male and female rats at doses of 10, 50, or 250 mg/kg. A significant increase in triglycerides (TG) could be observed for males and females at the highest dose (250 mg/kg). A significant decrease in the levels of glucose could also be observed in male and female rats at 50 or 250 mg/kg. An increase in the levels of amylase could also be observed in female rats at a dose of 50 or 250 mg/Kg and in males rats at a dose of 250 mg/kg. Analysis of the satellite at a dose of 250 mg/kg showed that these effects were not reversible for CK and TG. Analysis of the satellite at a dose of 250 mg/kg showed that these effects were reversible for AST and CK. As for glucose and amylase, the effects proved to be irreversible. Other biochemical parameters presented no statistically significant changes.

<insert table 2 here >

Organ weights (Table 3) showed statistical significance for the heart, kidneys, liver, and stomach. Absolute and relative weights of the heart decreased for males and females at all doses. The absolute weight of the liver was found to have decreased at all doses for both males and females. As regards the kidneys, decreases were only found in males at all doses for both absolute and relative weights. As for the stomach, there was an increase for both males and females at a dose of 250 mg/kg. Analysis of the satellite

250 mg/kg showed that these effects were not reversible for the kidneys (relative) or for the stomach (absolute). Other organ weights showed no statistically significant changes. Macroscopic and histopathological evaluation of organs also showed no alterations.

<insert table 3 here >

3.2.2 Zebrafish test

After exposure to different concentrations of LASSBio-596 (1, 10, and 25 μ M), the evaluation of the heart rate of 72 hpf zebrafish embryos, showed that the compound promotes a significant reduction in heart frequency to an average of 85% at all the concentrations used, when compared to the control (Figure 3A). In addition, the morphological analysis showed the occurrence of pericardial edema and cardiac malformation (Figure 3B-D). Changes in the pattern of cardiac rhythmicity could also be observed in most of the analyzed embryos (data not shown).

<insert figure 3A-D here >

4 DISCUSSION

LASSBio-596 caused no alterations in either the MTT cytotoxicity assay or the NR using renal, hepatic, pulmonary, cardiac, neurological, and intestinal cell lines. MTT and NR

are among the most common assays used to detect cytotoxicity or cell viability after exposure to toxic substances. The MTT test is based mainly on the enzymatic conversion of MTT in mitochondria, while the NR assay is a colorimetric assay that measures the uptake ability of the dye using functional lysosomes (Fotakis & Timbrell, 2006). The methods are most commonly used *in vitro* to evaluate the metabolic and functional activities of subcellular organelles (Sovcikova et al, 2002), mitochondria, and lysosomes, respectively.

LASSBio-596, when compared to two positive controls (colchicine and cyclophosphamide), showed no non-induction of micronuclei by LASSBio-596 when in the presence or absence of a metabolic activator (S9). These data suggest that this substance did not cause DNA damage in terms of numerical (aneugenic) or structural (clastogenic) abnormalities. As such, future studies are warranted to identify biological markers with the potential to elucidate pathways of carcinogenesis (Demetriou et al., 2012). Therefore, LASSBio-596 presented good results in the genotoxicity test, demonstrating that this drug has a low probability of inducing DNA damage. However, further tests to evaluate genotoxicity are warranted.

Since *in vitro* assays have their own limitations (Bhanushal et al., 2010), further *in vivo* testing was performed to confirm the safety of LASSBio-596. A previous study of acute toxicity using oral LASSBio-596 (2000 mg/kg), following OECD 423 guidelines, induced no deaths in any of the animals; however, its histopathological analysis did reveal alterations in the livers, kidneys, and stomachs of the rats (Rocco et al., 2010).

Nevertheless, 2000 mg/kg is equal to two hundred times the therapeutic dose (10 mg/kg). Therefore, this much higher dose may well explain the presence of organ damage in the histopathological analysis.

In the subchronic toxicity study (10, 50, and 250 mg/kg), a slight suppression of body weight gain could be observed at the higher dose, but only for the male rats. In addition, as no differences in water and food could be observed, this suppression of body weight gain was considered to have been caused by the administration of the drug and not by malnutrition or dehydration (Campbell et al., 2009; Rao et al. 2002).

Regarding the biochemical parameters, glucose levels decreased, while AST and CK increased. Considering the glucose levels, one could suggest that this mechanism is triggered by the inhibition of the phosphodiesterase-4 and phosphodiesterase-5 (PDE) enzymes by Lassbio-596 (Lima et al. 2002), as is the mechanism of action related to xanthenes (Banner et al., 1997). It is well-known that the inhibition of PDE family members increases intracellular cAMP and cGMP levels (Essayan, 2001). Increased cAMP levels activate Protein Kinase A (Taylor et al., 2008). Likewise, the protein Kinase A (PKA) induces phosphorylation of NO synthase and stimulates nitric oxide (NO) production (Tanano et al., 2013). Thus, prolonged exposure to a large amount of NO can inhibit the activity of several enzymes, such as aconitase and cytochrome c oxidase, resulting in potential cytotoxicity and the development of pathogenesis (Nathan and Hibbs 1991).

Peroxynitrite (ONOO-) is a mediator of the cytotoxic effects of NO (Guzik, 2003). This product can target numerous proteins and enzymes that are critical for cell survival and signaling. In addition, these metabolites can inhibit the cytosolic and mitochondrial aconitase, the latter being a component of the Krebs cycle and thus required for cellular respiration (Brown, 2007). Therefore, a decrease in ATP production and an increase in the consumption of glucose (Silveira et al., 2007) could be observed. Inhibition of metabolic pathways, such as Krebs' cycle, will impair ATP production (Dyken et al., 2007). Therefore, as mitochondrial function declines, the cell finds another means through which to respond to the loss of ATP, which is the enhancement of production capacity by accelerating glycolysis (Dyken et al., 2007), in turn reducing glucose levels.

In addition, the inhibition of aconitase proved to enhance the AST levels shown in the animals in the present study. AST is an enzyme responsible for catalyzation through the reversible transformation reaction of the amino acid aspartate into oxaloacetate, which is used in the Krebs cycle. When the tissue is damaged, AST is released into the blood (Henry, 1999). Other studies report that the inhibition of aconitase can cause an increase in AST to cause the formation of the Krebs Cycle intermediates (Silveira et al., 2007).

The CK enzyme plays a key role in energy metabolism, mainly for tissues with a high energy demand, such as the brain, the heart, and skeletal muscles, where CK functions as an effective buffer system for cell levels of ATP (Pilla et al. 2003). The upregulation of MtCK has appeared in creatine-depleted muscles as well as in patients with

mitochondrial cytopathies, which may explain the compensation mechanism for the functional impairment of the control of the state of energy (Kwon et al. 2010).

Since LASSBio-596 was designed as a structural analog of thalidomide, it was crucial to evaluate the potential embryo toxicity of this compound. Since the increase of CK activity indicates some degree of cardiac interference, and it has been reported that thalidomide can in fact disrupt the development of the cardiac system (Smithells and Newman, 1992), the present study focused on the effect of LASSBio-596 on the cardiac system. It is well-known that thalidomide does not affect the development of murines. It does, however, present similar teratogenic effects on primates, rabbits, chicken, and fish (Ito et al., 2011). In this sense, and taking into account other advantages of this species, such as low cost, fast development, and embryo and larvae transparency (Zon and Peterson, 2005), the zebrafish model was chosen to evaluate the effect of LASSBio-596 on cardiac development and function.

CK, along with other biomarkers, is used to evaluate cardiotoxicity (Kehl et al., 2012). In the subchronic study, CK proved to be altered in rats; however, this finding was not found in the histopatological evaluation. On the other hand, the zebrafish test demonstrated cardiotoxicity in embryos, demonstrating that this type of study is a reliable and sensitive method to determine cardiac damage and is more effective than multiple doses in rats and *in vitro* tests, where histopathological evaluations demonstrated no changes. In addition, the absence of histological alterations in adult

rats, and the observation of a high degree of cardiac disruption in zebrafish embryos, suggests a specific effect of LASSBio-596 in the early stages of embryo development.

Regarding the hematological evaluation, a non-reversible increase in lymphocytes and a decrease in granulocytes could be identified, although no histopathological changes could be observed. According to these findings, one could suggest an effect similar to that of lenalidomide, a thalidomide analog that causes the suppression of bone marrow. The suppression would decrease lymphocytes and inhibit leukocyte infiltration into the tissue. Therefore, an increase in lymphocytes and a decrease in granulocytes could be identified in the peripheral blood; however, lymphocytes were suppressed in the bone marrow, and histopathological changes were identified (List et al. 2006; Caceres et al. 2013; Rosenzweig et al., 2013; Bjorklund et al. 2014).

Moreover, there is evidence in the literature to support a major role of lymphocytes in asthma (Kay, 2005). After stimulus, peripheral type 0 lymphocytes (Th0) (also known as Th naïve) could differentiate into either type 1 (Th1) or type 2 (Th2) (Sad, Mosmann, 1994). Th2 produces cytokines, such as IL-4, IL-5, IL-9, and IL-13, which are responsible for recruiting a type of granulocyte: eosinophils (D'Ambrosio et al., 2001; Van Rijt, Lambrecht, 2001). Th1 are responsible for recruiting other types of cells: macrophages and neutrophils (D'Ambrosio et al., 2001).

Activation of Th2 appears to be specific for asthma, leading to local tissue eosinophilia and IgE-dependent mast-cell activation (Durham et al., 2000). Eosinophils are the most characteristic inflammatory cells in the airway mucosa in asthma. In addition, their influx and the degree of activation of these cells in the airway mucosa are related to the severity of asthma and appear to play a key role in some cases of asthma deaths (Dusser, 2000). The reduction of granulocytes after the subchronic intake of LASSBio-596 could also be an important factor in the efficacy shown in the asthma treatment. Although further testing is warranted, the results indicate that LASSBio-596 may well be an effective stimulus to differentiate Th0 into Th1.

In addition to the histopathological evaluation of the heart, no changes could be found in any of the analyzed tissues. These results were different from acute toxicity tests, given that this study used major doses for a short period of time, while the subchronic test used minor doses over the long term.

The findings from the present study demonstrate that *in vivo* testing can detect details which *in vitro* testing is still unable to determine (Alder et al., 2010). To date, no *in vitro* model of long-term toxicity can predict the *in vivo* model (Alder et al., 2010; Hartung et al., 2011). As the *in vitro* study reflects the acute toxicity profile, which evaluates toxicity over the short term (Vitalone et al., 2011), it was impossible to detect any cardiac injury in the present study's *in vitro* model.

Moreover, a comparative study of the *in vitro* (heart cells) and *in vivo* (acute and long-term) methods proved not to cause DNA damage in the *in vitro* model, but did show toxicity in *in vivo* models (Wallace et al., 2000).

In conclusion, although the *in vitro* evaluations, including cardiac, pulmonary, hepatic, as well as renal cell and micronuclei testing, had demonstrated no significant levels of toxicity, *in vivo* tests in rats after multiple doses identified details which *in vitro* testing is unable to determine. However, the results found in the zebrafish test indicated that LASSBio-596 promotes a cardiac toxicity most likely by interfering in signaling pathways that are crucial to heart development. Cardiovascular toxicity is a major limiting factor in drug development and requires multiple cost-effective models to perform toxicological evaluations. Therefore, considering the changes in CK within the three evaluated doses and the cardiac toxicity's impact on the zebrafish test, it was impossible to estimate the no-observed-adverse-effect-levels (NOAEL) and lowest-observed-adverse-effect-levels (LOAEL).

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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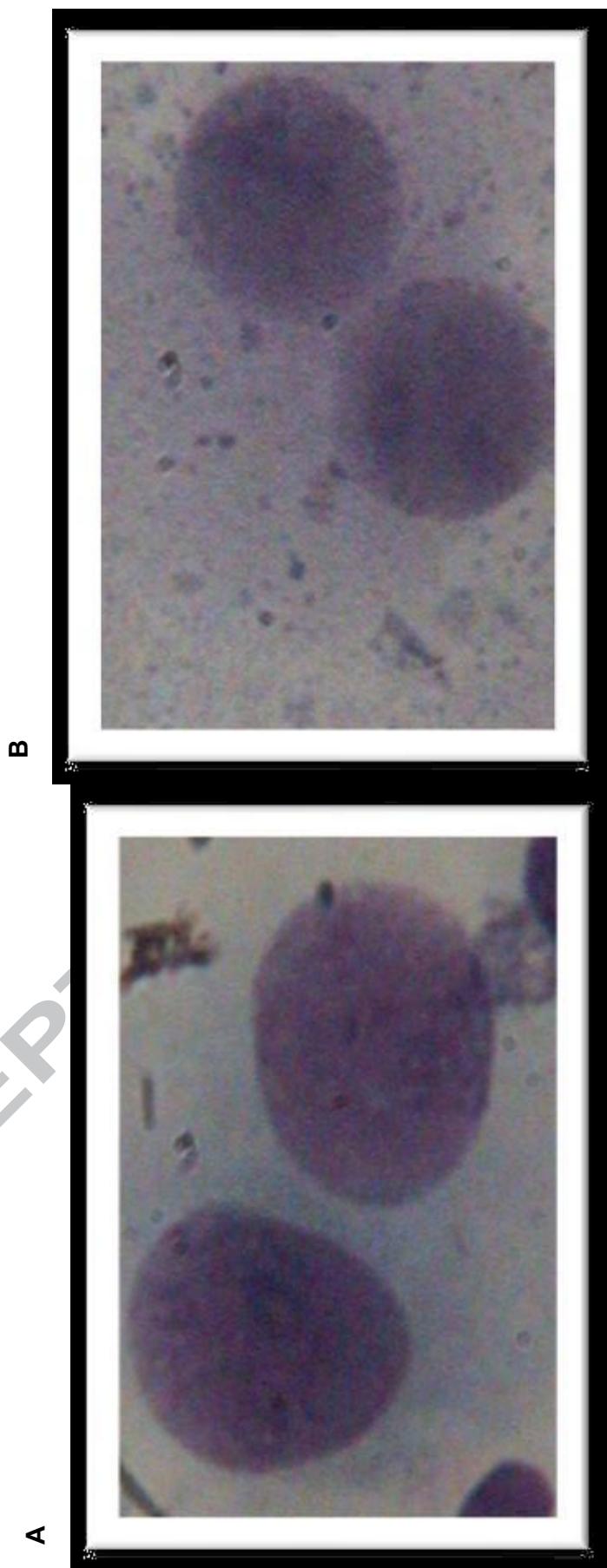
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Figure 1: Qualitative Analysis of micronuclei production by substance LASSBio 596 **A:** Binucleated cell without micronucleus. **B:** Cellula binucleated without micronucleus.



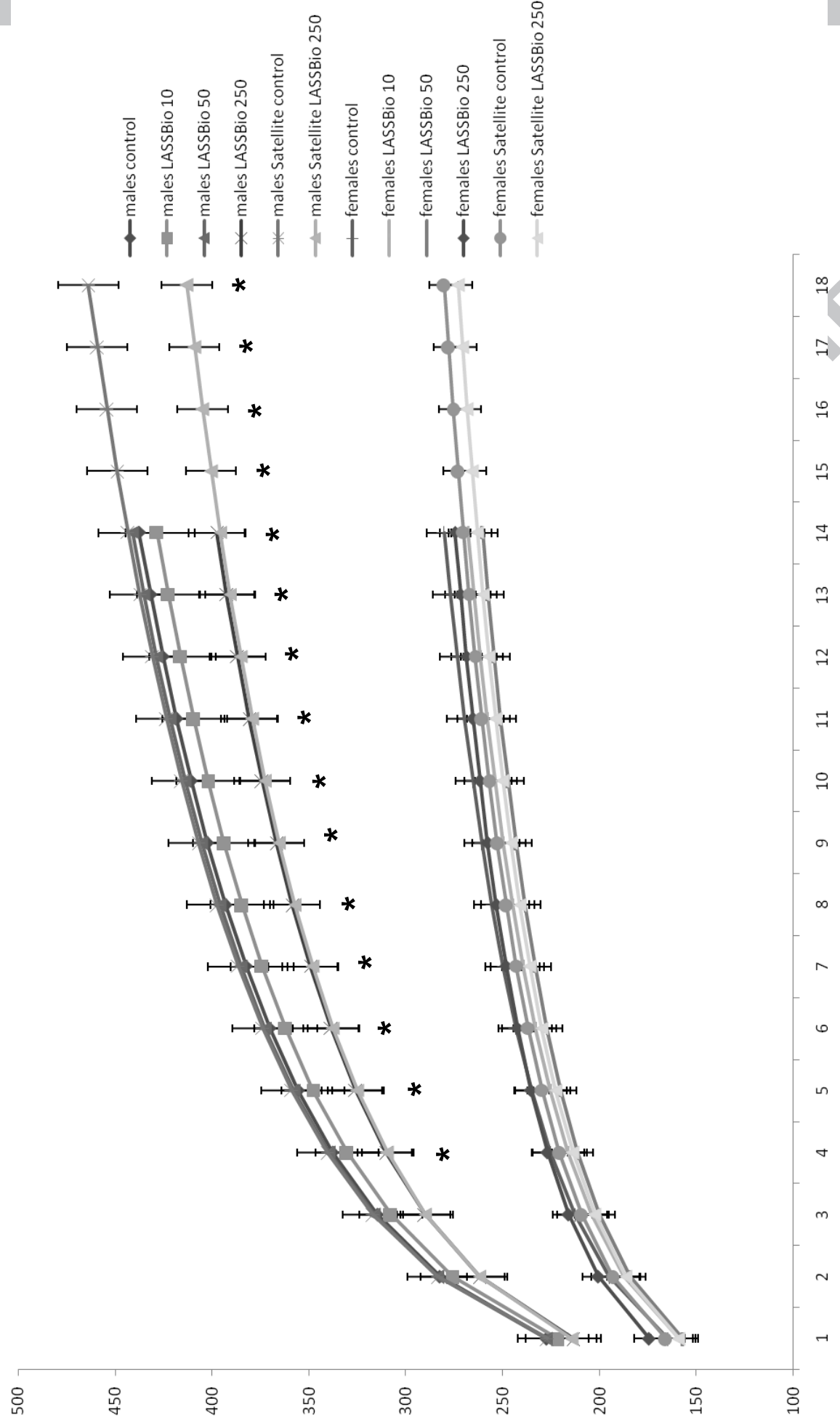


Figure 2. Evaluation of the body weight of male and female Wistar rats weekly, over the 90-day period, with the administration of the control solution and LASSBio-596 suspensions at 10mg/kg or 50 or 250 mg/kg. Males (n=10). Females (n = 10). Satellite (n=6). The first week correspond to the acclimatization of the animals. * p<0.05 versus control group.

A

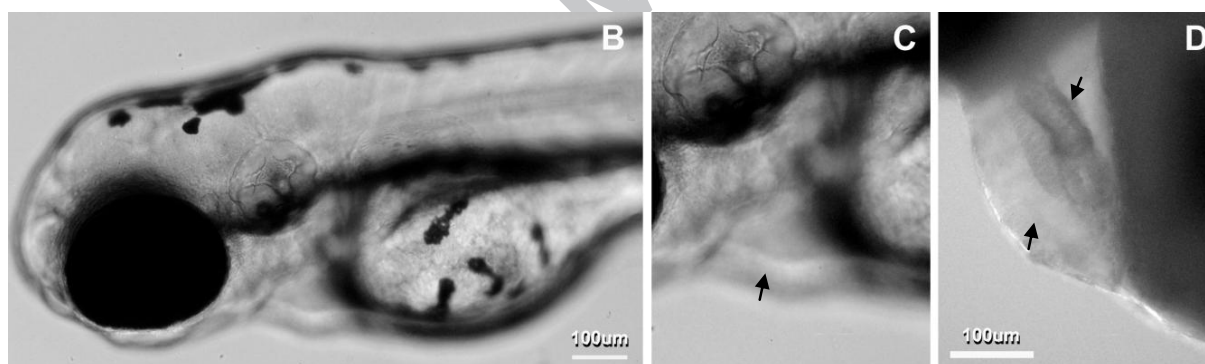
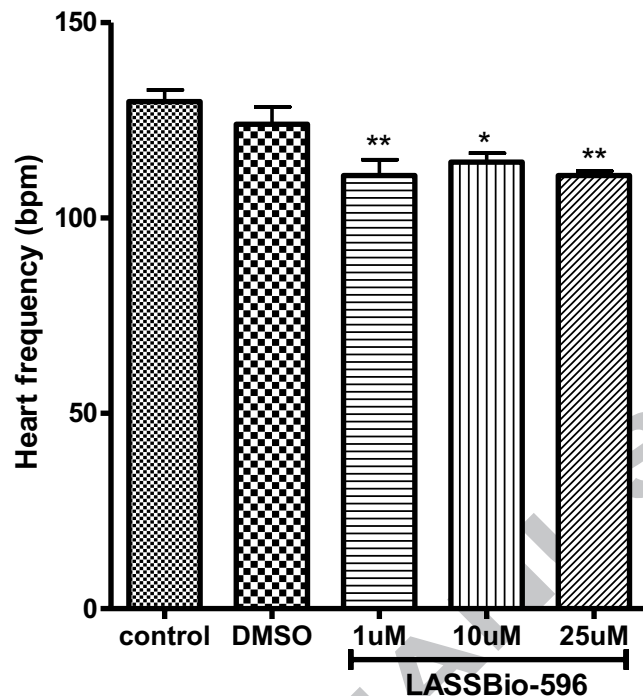


Figure 3. Effect of LASSBio-596 on cardiac system of zebrafish embryos. (A)

Measure of 72 hpf zebrafish embryos heart rate after treatment with LASSBio-596 (1, 10 and 25 μ M) or 0.5% DMSO (vehicle). Control represents the absence of any treatment. The embryos were treated at 2 hpf stage and the analysis was performed at 72 hpf. The medium with treatments was renewed each 24 hours. The statistical analysis was performed using One-way ANOVA followed by Bonferroni's multiple test comparison (Treatment vs. control. * $P < 0.1$; ** $P < 0.01$; $n=10$). **(B-D)** Representative images of 72 hpf zebrafish normal embryo (B), with detail of heart in (C), black arrow points the pericardio region. The heart area of a 72 hpf zebrafish embryo treated with 1 μ M of LASSBio-596 for 3 days (D). Note the heart in a tube shape with a conspicuous pericardial edema (black arrows). Scale bars = 100 μ m.

Table 1. Evaluation of haematological parameters after *per ost* treatment of Wistar rats with LASSBio-596 during 90 days

		Control	10 mg/kg	50 mg/kg	250 mg/kg	Satellite control	Satellite 250mg/kg
Number of examined animals		10	10	10	10	6	6
Males							
	RBC	10.52 ± 0.76	9.31 ± 0.53	10.04 ± 0.62	9.38 ± 0.60	10.28 ± 0.93	10.15 ± 0.71
	Hb	14.87 ± 1.00	14.94 ± 0.69	15.88 ± 1.09	16.17 ± 1.15	15.93 ± 1.05	15.56 ± 1.23
	Ht	50.89 ± 3.18	50.43 ± 3.87	49.78 ± 2.6	49.44 ± 2.79	48.18 ± 2.18	47.53 ± 2.76
	Plt	849.30 ± 113.10	675.10 ± 114.30 *	506.1 ± 156.4 *	798.80 ± 81.10 *	738.00 ± 74.05	634.00 ± 80.10
	WBC	5448.57 ± 465.02	6280.00 ± 2645.25	6560.00 ± 1695.49	6090.00 ± 1650.22	6350.00 ± 1426.00	5400.00 ± 1113.55
	Lymphocytes (x10 ⁻³)	3542.71 ± 278.38	6880.80 ± 239.63 *	5389.10 ± 181.75 *	5546.00 ± 159.01 *	5595.67 ± 25.44	5799.00 ± 24.27 **
	Lymphocytes%	65.29 ± 6.26	83.00 ± 4.24 *	81.50 ± 11.03 *	91.60 ± 8.44 *	6069.00 ± 979.00	3830.67 ± 2004.63
	Monocytes (x10 ⁻³)	156.43 ± 44.24	164.00 ± 101.00	119.30 ± 87.19	169.60 ± 101.20	173.30 ± 81.73	140.67 ± 23.69
	Monocytes%	2.86 ± 0.69	2.10 ± 0.88	1.80 ± 1.14 *	2.80 ± 1.48	2.50 ± 0.50	2.67 ± 0.58
	Granulocytes (x10 ⁻³)	1645.14 ± 373.66	1122.60 ± 518.82 *	996.70 ± 728.05 *	959.80 ± 620.55 *	1223.50 ± 523.50	1396.00 ± 252.31
	Granulocytes%	30.00 ± 5.57	13.70 ± 4.92 *	15.80 ± 11.27 *	5.40 ± 8.33 *	27.83 ± 1.73	27.67 ± 3.16
		Control	10 mg/kg	50 mg/kg	250 mg/kg	Satellite control	Satellite 250
Number of examined animals		10	10	10	10	6	6
Females							
	RBC	8.59 ± 0.53	7.95 ± 0.51	8.52 ± 0.54	8.29 ± 0.30	7.83 ± 0.41	8.39 ± 0.34
	Hb	13.09 ± 0.71	13.86 ± 0.79	14.31 ± 1.05	13.95 ± 0.72	14.35 ± 0.77	14.22 ± 0.54
	Ht	44.63 ± 2.49	41.16 ± 1.96	45.96 ± 4.16	42.05 ± 1.82	40.28 ± 2.23	44.92 ± 3.71
	Plt	870.30 ± 115.30	682.20 ± 109.50 *	609.20 ± 199.00 *	712.50 ± 175.15 *	785.67 ± 110.80	628.00 ± 77.90 **
	WBC	5452.00 ± 2002.09	4040.00 ± 783.40	5313.00 ± 1758.00	4710.00 ± 994.93	4216.67 ± 1187.29	4050.00 ± 580.23
	Lymphocytes (x10 ⁻³)	3828.30 ± 1493.19	3371.20 ± 772.50	3574.20 ± 2015.00	4480.20 ± 994.62	2141.33 ± 643.06	2434.50 ± 525.50
	Lymphocytes%	69.80 ± 7.25	83.10 ± 5.11 *	82.00 ± 10.87 *	95.00 ± 3.09 *	50.67 ± 5.16	59.75 ± 7.27 **

	Monocytes($\times 10^{-3}$)	125.20 ± 46.84	60.70 $\pm$ 42.69*	83.30 $\pm$ 46.70	87.70 ± 29.68	82.00 $\pm$ 59.42	88.75 $\pm$ 34.33
	Monocytes%	2.40 $\pm$ 0.70	1.50 $\pm$ 0.97*	1.70 ± 0.95	1.90 ± 0.57	1.83 $\pm$ 0.98	2.25 $\pm$ 0.96
	Granulocytes($\times 10^{-3}$)	1415.80 ± 615.34	551.10 $\pm$ 221.70*	744.80 $\pm$ 439.00*	732.70 $\pm 101.31^*$	1939.00 $\pm$ 578.50	1507.50 $\pm$ 309.57
	Granulocytes%	26.30 $\pm$ 7.02	14.00 $\pm$ 5.37*	15.20 $\pm$ 10.74*	2.60 $\pm$ 2.32*	46.00 $\pm$ 4.34	37.50 $\pm$ 7.14**

Abbreviations: LASSBio-596 - 2 - [4-(1.4-tiazinan-4-ylsulfonyl) phenylcarbamoyl] benzoic acid; RBC – red blood cell; Hb – hemoglobin; Ht – hematocrit; MCV – mean corpuscular hemoglobin concentration; MCH - Mean Corpuscular Hemoglobin; MCHC - Mean Corpuscular Hemoglobin Concentration; RDW - Red Cell Distribution Width; Plt. platelet; WBC – white blood cell. The data are expressed as mean $\pm$ SD. * p < 0.05 versus control group. [§]p < 0.05 versus satellite control group.

Table 2. Evaluation of biochemical parameters after *per ostreatment* of male and female Wistar rats with LASSBio-596 during 90 days

		Control	10 mg/kg	50 mg/kg	250 mg/kg	Satellitecontr ol	Satellite 250
Number of examinat ed animals		10	10	10	10	6	6
Males							
	AST	85.75 ± 22.19	95.32± 34.72*	108.93±47.91*	108.17 ± 29.70*	97.50±58.07	95.3 +21.50
	ALT	49.96± 9.48	55.01± 11.68	56.55±8.05	53.58±4.27	53.40±6.95	56.6 +4.92
	CK	395.29±756. 60	1386.61±1246. 12*	1683.41± 1170.87*	1069.06 ±779.14*	350.93±1991. 82	1447.2 ±163.90 **
	Amylase	1567.12± 257.74	1591.89±165.6 9	1671.81±466. 70*	1783.45 ±383.64*	1942.15±538. 60	1965.02 ± 442.87
	Glucose	241.98±83.0 5	236.75±49.28	106.62± 36.90*	102.25± 44.50*	258.10±51.71	262.1 +24.84
	BUN	42.61± 2.82	41.11±5.74	42.62± 2.90	42.32±5.42	40.70±4.74	42.80± 2.88
	CRN	0.55±0.10	0.57± 0.09	0.58± 0.07	0.56 ± 0.08	0.53± 0.03	0.51 +0.04
	BiIT	0.21± 0.07	0.16± 0.05	0.16± 0.07	0.22 ± 0.14	0.17± 0.09	0.16 +0.10
	TP	6.05± 0.30	6.08±0.31	6.21± 0.28	6.15± 0.36	6.06± 0.25	6.78 +0.29
	Alb	2.97± 0.29	3.01±0.21	3.11± 0.16	3.15± 0.29	2.94± 0.22	2.78 +0.27
	TG	64.71±17.43	62.49±17.54	68.84±15.48	83.23±20.0 9*	60.20±20.70	82.8 ±18.31* *
	Cholesterol	110.97±19.1 4	114.29±28.72	114.05±21.46	108.37±17. 93	104.30±39.06	115.0 +20.40
	Ca	10.13± 0.84	9.79±0.42	10.12±0.22	10.16±0.72	10.11±0.26	10.73 +0.53
	Cl	105.04± 1.97	104.60±3.04	103.68±2.48	105.39± 2.22	104.00± 1.08	107.3 +2.54
	IP	10.32± 2.47	9.48±1.16	9.99± 0.71	9.76± 0.86	9.40± 0.46	9.76 +0.31
	ALP	121.72 +42.12	113.68 ± 37.23	119.61 ± 30.09	117.56 ± 19.03	127.37 ± 26.82	130.44± 17.40
	GGT	-	-	-	-	-	-
	Uric acid	1.26 ± 2.58	1.36 ± 0.67	1.62 ± 0.25	1.75 ± 1.37	1.38± 0.20	1.29 ± 0.44
Females							
	AST	67.00 ± 9.49	86.56 ± 9.84*	95.90 ± 31.11*	94.71 ± 34.62*	79.13 ± 10.06	75.3 +10.87
	ALT	45.37 ±5.80	47.03 ±3.76	52.27 ± 11.38	54.40 ± 4.38	59.30 ± 7.63	56.6 +2.94
	CK	410.87 ± 205.43	836.21 ± 238.62*	1362.95 ± 1118.01*	1013.77± 567.05*	531.71 ± 361.33	2447.2 ± 299.30* *
	Amylase	1010.71 ± 182.90	1154.96 ± 240.72	1115.05 ±126.78	1252.00 ± 80.49*	979.43 ± 192.70	939.4 +90.90

	Glucose	205.77 ± 24.30	194.22 ± 54.13	132.10 ± 21.64**	135.43 ± 34.04*	159.43 ± 51.41	322.10 ± 22.03
	BUN	41.75 ± 3.07	41.37 ± 3.15	43.95 ± 2.62	38.89 ± 1.92 *	38.15 ± 3.00	32.8 ± 2.44
	CRN	0.61 ± 0.05	0.59 ± 0.05	0.51 ± 0.06	0.56 ± 0.05	0.58 ± 0.06	0.51 ± 0.04
	BilT	0.16 ± 0.05	0.17 ± 0.09	0.24 ± 0.11	0.22 ± 0.15	0.28 ± 0.11	0.26 ± 0.12
	TP	6.25 ± 0.38	6.54 ± 0.29	6.30 ± 0.01	6.43 ± 0.28	6.31 ± 0.24	6.78 ± 0.25
	Alb	3.10 ± 0.28	3.53 ± 0.27	3.45 ± 0.04	3.42 ± 0.29	3.43 ± 0.24	3.60 ± 0.32
	TG	70.20 ± 27.63	75.62 ± 15.63	72.15 ± 17.61	90.48 ± 13.62*	70.73 ± 3.61	92.86 ± 12.69**
	Cholesterol	116.60 ± 8.38	107.82 ± 4.98	97.90 ± 14.81	114.50 ± 13.58	100.08 ± 21.69	115.00 ± 4.68
	Ca	9.82 ± 0.26	9.95 ± 0.29	10.33 ± 0.95	10.20 ± 0.86	10.10 ± 0.17	10.40 ± 0.13
	Cl	105.37 ± 1.32	107.25 ± 2.12	106.80 ± 0.85	104.52 ± 2.42	108.05 ± 1.74	107.3 ± 0.99
	IP	6.78 ± 0.77	6.61 ± 0.73	7.37 ± 0.35	7.10 ± 1.30	6.94 ± 0.98	7.76 ± 0.75
	ALP	104.72 ± 46.30	76.89 ± 29.94	82.90 ± 60.10	73.47 ± 27.27	80.95 ± 43.23	79.52 ± 32.61
	GGT	-	-	-	-	-	-
	Uricacid	1.64 ± 0.74	1.56 ± 0.20	1.54 ± 0.47	1.60 ± 0.54	1.59 ± 0.22	1.63 ± 0.31

Abbreviations: LASSBio-596 - 2 - [4-(1,4-tiazinan-4-ylsulfonyl) phenylcarbamoyl] benzoic acid;; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT – Gamma-glutamyltransferase; ALP – alkaline phosphatase; CK – creatine kinase; BUN – blood urea nitrogen; CRN – creatinine; TP – total protein; Alb – albumin; TG – triglyceride; BilT – total bilirubin; Ca – calcium; Cl – chloride; IP – inorganic phosphate. The data are expressed as mean ± SD. * p < 0.05 versus control group. ** p < 0.05 versus satellite control group.

Table 3. Effects on organ weight of Wistar rats administered *per os* with LASSBio-596 during 90 days

		Control	10 mg/kg	50 mg/kg	250 mg/kg	Satellitecontrol	Satellite 250
Number of examined animals		10	10	10	10	6	6
Males							
	Heart	3.55 ± 0.34	3.20 ± 0.58*	3.10 ± 1.09*	3.10 ± 0.25*	3.39 ± 0.24	3.47 ± 0.37
	(g%)	0.50±0.06	0.47±0.09*	0.49±0.07*	0.49±0.05*	0.49±0.04	0.46 ± 0.02
	Lung	4.59 ±0.59	4.71±0.90	4.85±0.66	4.87±0.53	4.85 ±0.41	4.66±0.64
	(g%)	0.20±0.02	0.19±0.03	0.21±0.02	0.21±0.01	0.21±0.03	0.20 ± 0.02
	Kidneys	7.86 ± 0.55	6.93 ± 0.51*	7.24 ± 0.33*	6.91 ± 0.47*	7.74 ± 0.48	6.96 ± 0.97
	(g%)	0.79±0.06	0.69±0.05*	0.72±0.03*	0.69±0.05*	0.77±0.05	0.70 ± 0.09**
	Spleen	1.95±0.16	1.89±0.32	2.06±0.21	2.04±0.15	2.11 ± 0.35	2.05±0.35
	(g%)	0.52±0.04	0.49±0.08	0.53±0.04	0.54±0.05	0.48±0.05	0.50 ± 0.08
	Liver	27.80 ± 1.36	25.65 ± 2.28*	26.11 ± 1.18*	25.91 ± 2.02*	25.69 ± 1.34	25.29 ± 1.29
	(g%)	2.78±0.14	2.64±0.77	2.61±0.12	2.33±0.08	2.57±0.13	2.31 ± 0.10
	Testis	14.12±1.26	13.63±1.48	13.54±2.63	13.43±0.60	14.72±1.46	11.20±4.31
	(g%)	1.41±0.13	1.36±0.15	1.35±0.26	1.34±0.06	1.47±0.15	1.34 ± 0.22
	Brain	5.07 ±0.51	4.99±0.28	4.94±0.51*	4.89±0.25*	4.98±0.46	5.16±0.38
	(g%)	0.51±0.05	0.48±0.03	0.47±0.05	0.47±0.03	0.50±0.05	0.51±0.03
	Stomach	5.17 ± 0.42	4.89 ± 0.82	5.42 ± 0.55	5.61 ± 0.44*	4.84 ± 0.52	5.62 ± 0.32**
	(g%)	0.32±0.12	0.34±0.06	0.31±0.02	0.31±0.02	0.34±0.04	0.33 ± 0.03
Females							
	Heart	4.11 ± 0.44	3.49 ± 0.57*	3.47 ± 0.28*	3.24 ± 0.39*	3.78 ± 1.07	3.89 ± 0.77
	(g%)	0.69±0.10	0.60±0.05*	0.59±0.13*	0.62±0.10*	0.67±0.09	0.69 ± 0.05
	Lung	6.42±0.79	5.95±0.49	6.90±1.34	6.25±0.96	5.82±1.30	5.96±0.77
	(g%)	0.26±0.05	0.24±0.02	0.27±0.02	0.27±0.02	0.26±0.02	0.27 ± 0.01
	Kidneys	7.97 ±0.60	7.65 ± 0.55	7.77 ± 0.33	7.60 ± 0.40	7.00 ± 0.47	7.41 ± 0.97
	(g%)	0.73±0.06	0.75±0.08	0.68±0.08	0.71±0.10	0.72±0.07	0.74 ± 0.09
	Spleen	2.52±0.22	2.38±0.22	2.66±0.24	2.67±0.24	2.68±0.56	2.61±0.33
	(g%)	0.65±0.07	0.72±0.08	0.68±0.08	0.71±0.10	0.72±0.07	0.65 ± 0.05
	Liver	29.60 ± 2.03	27.78 ± 1.95*	27.25 ± 2.33*	27.63 ± 2.12*	29.12 ± 1.95	27.65 ± 3.38
	(g%)	2.55±0.26	2.50±0.39	2.62±0.23	2.66±0.21	3.02±0.20	2.99 ± 0.34
	Uterus	2.88±0.48	2.87±0.64	2.93±0.90	3.02±1.16	2.33±0.51	3.12±1.24
	(g%)	0.25±0.05	0.29±0.06	0.29±0.06	0.30±0.12	0.29±0.05	0.29 ± 0.10
	Brain	7.53±0.47	7.26±0.74	7.97±0.47	7.59±0.53	7.16±0.29	7.72±0.59
	(g%)	0.72±0.04	0.73±0.07	0.80±0.05	0.76±0.05	0.77±0.02	0.74 ± 0.10
	Stomach	6.75 ±0.79	7.05±1.03	6.84 ± 0.85	7.22 ± 0.79*	6.99±0.80	7.82 ± 1.21**
	(g%)	0.39±0.09	0.40±0.06	0.35±0.03	0.32±0.04	0.41±0.05	0.46 ± 0.16

The data are expressed as mean±SD.* significantly different from group 0 at p <0.05.

§significantly different from group satellite 0 at p<0.05.

Highlights

Relevant changes in the blood parameters of CK, amylase, ALT, and glucose. > Zebrafish test demonstrated cardiac damage. > LASSBio-596 evaluation by Zebra fish test shown cardiac damage.