

Intramuscular compatibility of an injectable nanodispersion anti-inflammatory (Chronic®) from a standardized Bixa orellana oil: a toxicological study in Wistar rats

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Abstract

Bixa orellana L. is a plant popularly known as “ucurum”, “annatto”, and “achiote”. It is native to South America, and its seeds are an abundant source of geranylgeraniol and tocotrienols. Nanoencapsulation is a valuable technique that can decrease the drug needed to achieve an effect, decreasing potential toxicity and side effects. This study aimed to evaluate the acute toxicity of an intramuscular application of a nanodispersion containing a standardized extract (Chronic®) from the seeds of *Bixa orellana* (NBO) in Wistar rats. The chemical evaluation showed δ -tocotrienol at 0.725 ± 0.062 mg/ml ($72.6 \pm 0.9\%$). The stability study showed the nanoparticles had an average size from 53.15 ± 0.64 nm to 59.9 ± 3.63 nm, with a polydispersity index ranging from 0.574 ± 0.032 to 0.574 ± 0.32 , Zeta potential from 18.26 ± 0.59 mV to 19.66 ± 1.45 mV. After testing the intramuscular application of NBO with doses from 1 to 5 mg/kg in animals, it was observed that the acute treatment did not elicit any toxic effects within this range. The dose of 10 mg/kg, although not affecting hematological and biochemical parameters (CPK, LDH, myoglobin, AST, ALT), could induce some muscle tissue changes, including leukocyte infiltration, morphological changes, and potentially necrosis. In conclusion, the results showed that the treatments devoided toxicity between 1 mg/kg and 5 mg/kg.

Introduction

Bixa orellana is a plant species known as urucum, annatto, and achiote. Its seeds have geranylgeraniol and tocotrienols. Both compounds exert health-promoting effects, including improvement of the blood-lipids profile, prevention of metabolic syndrome, improvement of cardiovascular system health, neuroprotection, among others (Matias Pereira et al. 2021; Batista et al. 2022).

Tocotrienols are compounds that belong to the family of Vitamin E. There are four tocotrienols isomers: α , β , γ , and δ . Structurally, tocotrienols have a chromanol ring identical to those of tocopherols. However, the isoprenoid tail from tocotrienols has three unsaturations, which brings some benefits to them (Qureshi and Khan 2015; Peh et al. 2016).

Nanoencapsulation is a technological process in which bioactive compounds are coated with a protecting material; these formulations can release their content in a controlled manner under specific conditions. Some of the main advantages that can be achieved with nanoencapsulation are: controlling the content release, masking unpleasant flavors, improving the bioavailability, consequently decreasing the amount of drug needed to achieve an effect, saving drug, and decreasing potential toxicity (De Jesus and Zuhorn 2015; Apolinário et al. 2020).

Toxicity studies are performed to study compounds' potential toxicity in organs, their dose-toxicity relationship, the potential toxicity mechanisms of action, diagnosis and treatment of toxic reactions, consequences of exposures, among others (Silva et al. 2021). Acute toxicity is one of the required parameters to assess according to ANVISA. This organ gives information about administration frequency, period of exposure, local effects, and histopathological changes (Agência de Vigilância Sanitária 2013).

Considering the information given, this study aimed to test the safety of an injectable nanodispersion containing a standardized oil of *Bixa orellana* L. (Chronic®) using Wistar rats.

Materials And Methods

Bixa orellana oil obtention and chemical analysis

The material (Chronic®) was kindly provided by the company Ages Bioactive Compounds, lot 0012/2022. A stock solution of δ -tocotrienol was prepared in hexane (Sigma-Aldrich, MKCF5755, CG grade, Germany) in 10 mg/mL. From this stock solution, other solutions were prepared to build a concentration curve: 2.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.1 mg/mL. The chromatographic running (GC-MS) was performed as previously described by our group (Rodrigues et al. 2022).

Polymeric nanoparticle obtention

The nanoparticulate polymeric system containing the oil of *B. orellana* (Chronic®) was obtained using the nanoprecipitation method, adapted from (Gandhi et al. 2014). This method relies on the precipitation or interfacial deposition of a formed polymer. For 20 mL of nanodispersion of *B. orellana* oil (NBO) was used purified oil, polyethyleneglycol 4000 (PEG 4000), Tween-80, absolute ethanol, and saline solution (BR 10 2022 006416 4, Patent).

First was prepared the organic phase by solubilizing the oil with PEG 4000 on absolute ethanol under magnetic stirring (Fisatom, SP, Brazil) for 20 min. The aqueous phase was prepared with Tween-80 and saline solution under magnetic stirring for 20 min. Finally, the organic phase was added to aqueous phase under agitation for 20 minutes, and the nanodispersion obtained was characterized.

Animals and ethic aspects

This study used three weeks old male Wistar rats from the CEMIB department of the University of Campinas (UNICAMP). During the study the animals were kept in polyethylene cages (60 × 50 × 22 cm) on ventilated cabinets (5 animals/cage). The room had controlled temperature (22 ± 2 °C), 12h dark/light cycle, and the animals had free access to food and water. The study was approved by the Ethics Committee of Animals Use from the Federal University of Amapá (CEUA, UNIFAP), under n° 002/2022.

Acute toxicity assessment of NBO injection

In the acute toxicity screening, the animals (260 – 280g) were divided into five groups (n = 4 / group) and received a single intramuscular injection in the gastrocnemius. The groups were divided as follows:

CON: Control group. Injected with vehicle (4% Tween-80, 0.4 mL);

NBO 1: Injected with NBO at 1 mg/kg (0.04 mL);

NBO 2.5: Injected with NBO at 2.5 mg/kg (0.1 mL)

NBO 5: Injected with NBO at 5 mg/kg (0.2 mL);

NBO 10: Injected with NBO at 10 mg/kg (0.4 mL).

This study followed the guidelines of Organisation for Economic Cooperation and Development (OECD 2001) and ANVISA (Agência de Vigilância Sanitária 2013). After performing the treatments, the animals were subjected to observational screening to assess physical activity, reflexes, and SNC function. The following parameters were evaluated: vocal fremitus, irritability, touch response, tail touch response, contortion, body tonus, grip strength, ataxia, auricular reflex, corneal reflex, tremors/convulsions, anesthesia, lacrimation, urination and defecation, piloerection, cianosis/hyperemia, and death. These parameters were assessed 1h, 2h, 4h, and 8h post injection and then daily until the fourth day.

Other parameters observed were water, food intake, and weight gain until the fourth-day post-treatment. After this period, the animals were anesthetized with sodium thiopental at 45 mg/kg (Cristália, São Paulo, Brasil) to gather 1.5 mL of blood from the retroorbital plexus for biochemical and hematological analysis.

Biochemical and hematological analysis

The blood collected was centrifuged for 10 min at 5,000 rpm. All analysis was performed using kits (Doles[®], Goiânia – GO, Brazil) and a spectrophotometer UV-VIS model UV-mini 1240 (Shimadzu Corporation, Kyoto, Japan). The markers assessed were aspartate aminotransferase (AST), alanine aminotransferase (ALT), myoglobin, creatine kinase (CPK), and lactate dehydrogenase (LDH).

The hematological analysis was performed using an automatic counter HumaCount Plus (Human GMBH, Germany). The following parameters were observed: blood cells, hemoglobin, hematocrit, platelets, mean corpuscular size (MCS), mean corpuscular hemoglobin (MCH), and leucocytes (total, segmented, eosinophils, basophils, lymphocytes, monocytes) (Silva Lima et al. 2017)

Muscle histopathology

After euthanization, the gastrocnemius that received an injection from the animals was removed to be evaluated macroscopically and microscopically. For histopathological analysis, the muscle tissue was fixed in buffered formaldehyde (10%) for 24 hours and then dehydrated in crescent concentrations of ethanol (70%, 80%, 90%, 100%), clarified in xylene and embedded in paraffin at 60 °C. The sections were cut using a rotary microtome CUT 5062 (SLEE) in 5 µm and stained with Hematoxylin and Eosin (Vasconcelos et al. 2011; Carvalho et al. 2018). The slides were observed with 400x magnitude.

Statistical Analysis

The data was presented as a mean $\pm$ SD, and the groups were compared using One-Way ANOVA followed by the Tukey test in case of statistical significance, considering $p < 0.05$ statistically significant.

Results And Discussion

Δ -Tocotrienol quantification in *B. orellana* purified oil

The calibration curve of δ -tocotrienol had R^2 equal to 0.9973 (Fig. 1), and the line equation achieved was $y = 8,30 \times 10^6 x - 2,818 \times 10^6$. The chromatogram shown in Fig. 2 shows δ -tocotrienol at a retention time of 28.6 min. The fragment pattern read by the library and compared to the external standard confirms this peak as δ -tocotrienol, with an average concentration of 0.725 ± 0.062 mg/ml (72.6 ± 0.9 % of the oil). This is in accordance with other studies reporting this molecule as the major compound from *B. orellana* oil (Aggarwal et al. 2010; Mohamad et al. 2018).

Nanodispersion of *B. orellana* oil characterization

The method used to produce the nanoparticles in this study is known as the solvent displacement method. It consists of the interfacial deposition of a polymer after the displacement of an organic solvent from a lipophilic solution to an aqueous phase. Due to the spontaneous diffusion of the polymer to the aqueous phase, the nanoparticles are formed instantly (Martins et al. 2010; Souto et al. 2012).

To form the nanoparticulate formulation containing the oil, nanoprecipitation was used, adapted from (Gandhi et al. 2014). During the tests, no instability-related signs, such as phase separation, were observed, and the visual aspect was visibly stable among the formulation components. The formulation had a slightly yellow color and pH of 5.8.

Nanoparticles produced through nanoprecipitation typically have a well-defined size and narrow size distribution range; they are often more efficient than those produced through emulsification and evaporation (Crucho and Barros 2017). The nanoparticles used in this study were stable, with 53.15 ± 0.64 nm size and 0.574 ± 0.032 PDI on the first day and 59.90 ± 3.63 nm with 0.574 ± 0.032 PDI on the 30° day. The zeta potential was 18.26 ± 0.59 mV on the first day and 19.66 ± 1.45 mV on the 30° day (Table 1; Fig. 3).

Table 1
Nanoparticles' Zeta potential (mV), average size (nm), and polydispersity index.
Three replicates analyzed on days 0, 7 and 30.

		Zeta potential (mV)	Average size (nm)	PDI
Day 0	1	19.3	53.45	0.533
	2	20.5	53.6	0.541
	3	19.8	52.42	0.525
	Mean	19.86 ± 0.60	53.15 ± 0.64	0.533 ± 0.008
Day 7	1	15.5	57.33	0.592
	2	14.9	58.01	0.588
	3	15.4	58.86	0.603
	Mean	15.26 ± 0.32	58.06 ± 0.76	0.594 ± 0.007
Day 30	1	18.5	58.31	0.593
	2	19.2	64.06	0.61
	3	21.3	57.34	0.581
	Mean	19.66 ± 1.45	59.90 ± 3.63	0.594 ± 0.014

In general, studies suggest that nanoformulations with particles smaller than 150 nm have higher stability (de Oliveira Carvalho et al. 2021), which can be observed in our study as well. The qualitative and quantitative composition of the formulations are crucial to affect the resulting particles' average size and size distribution (Apolinário et al. 2020). It is essential noticing the presence of a polymer can lead to decreased particle size compared to emulsion due to its stabilizing effects surrounding the droplets.

The components of the formulation can also affect the zeta potential. Zeta potential is a measure of charge at the particles' surface and is influenced by interfacial changes, which could occur due to the dissociation of functional groups at the particles' surface or ion adsorption from the aqueous medium (Honary and Zahir 2013). High Zeta potential values (> 15 mV) are an indicator of good physicochemical stability because the repulsive charges at the droplets' surface prevent particles' aggregation (Zielińska et al. 2020). The nanoparticles produced in our study had a zeta potential higher than 15 mV, which could explain the stability during the observed period.

NBO intramuscular injection acute toxicity

During four days of observational screening, there were no evident signs of toxicity in all doses of NBO related to the motor system, reflexes, or central nervous system disturbances (Table 2). Other parameters commonly observed that could be a sign of toxicity are altered ponderal growth, food and water intake, altered behavior and apathy, and poor hair quality (Cunha et al. 2009; Júnior et al. 2012; Tubesha et al. 2013). Here, these parameters were unaffected as well (Table 3)

Table 2
Results observed from the observational screening four days after the injections.

Groups					
Parameter	CON	NBO 1	NBO 2.5	NBO 5	NBO 10
Vocal fremitus	Normal	Normal	Normal	Normal	Normal
Irritability	Absent	Absent	Absent	Absent	Absent
Touch response	Normal	Normal	Normal	Normal	Normal
Tail touch response	Normal	Normal	Normal	Normal	Normal
Contortions	Absent	Absent	Absent	Absent	Absent
Body tonus	Normal	Normal	Normal	Normal	Normal
Grip strength	Normal	Normal	Normal	Normal	Normal
Ataxia	Absent	Absent	Absent	Absent	Absent
Auricular reflex	Normal	Normal	Normal	Normal	Normal
Tremors	Absent	Absent	Absent	Absent	Absent
Hypnosis/anesthesia	Absent	Absent	Absent	Absent	Absent
Lacrimation	Absent	Absent	Absent	Absent	Absent
Urination/defecation	Normal	Normal	Normal	Normal	Normal
Piloerection	Absent	Absent	Absent	Absent	Absent
Cyanosis/hyperemia	Absent	Absent	Absent	Absent	Absent
Death	Absent	Absent	Absent	Absent	Absent

Table 3

Food intake, water intake, and weight after four days of the intramuscular injection of NBO. The results are represented as a Mean $\pm$ SD; *: $p < 0.05$ (ANOVA followed by Tukey test).

	CON	NBO1	NBO2.5	NBO5	NBO10
Water intake (mL)	27,59 $\pm$ 5,60	25,52 $\pm$ 3,45	24,39 $\pm$ 6,12	26,35 $\pm$ 3,58	36,6 $\pm$ 4.35
Ration intake (g)	17,01 $\pm$ 3.88	22,28 $\pm$ 3.48	22,70 $\pm$ 2.65	29,34 $\pm$ 3.35	22.56 $\pm$ 3.45
Weight (g)	261.35 $\pm$ 5.08	263.67 $\pm$ 4.34	272,75 $\pm$ 6.21	271,68 $\pm$ 5.81	269.35 $\pm$ 5.62

After observing the animals to assess general signs of toxicity, they were euthanized to observe the muscle injected (Fig. 4). Even after four days of the administration, we observed that the formulation could still be observed, identified by its slightly yellow color (see asterisks in Fig. 4); this suggests that the NBO has a slow absorption rate. At the dose of 10 mg/kg was observed mild muscular edema and hemorrhage.

Using the animals' blood, we assessed biochemical markers of muscle toxicity (Fig. 5). The results show that doses from 1 to 5 mg/kg did not significantly affect CPK, LDH, or myoglobin levels. The group treated with NBO at 10 had slightly higher levels of these markers, but the difference was not statistically significant. Highly altered levels of these markers are an indicator of muscle damage since these proteins are involved in this tissue maintenance. Intramuscular injection of drugs at high concentrations is expected to increase the amount of these markers in the blood (Ely et al. 2000; de Oliveira Carvalho et al. 2021).

CPK is a cytoplasmic enzyme highly abundant in skeletal muscle, myocardium, and brain. Studies show that this enzyme has increased concentration proportionally to the degree of muscle damage two hours after the injury. Exceedingly high CPK and LDH can indicate muscle necrosis (Ely et al. 2000; Alves et al. 2019). According to (Moniz et al. 2017), myoglobin should also be evaluated, considering that this marker also is increased during muscle necrosis. Overall, our results show that applying NBO from 1 to 5 mg/kg did not induce any observed changes, while 10 mg/kg could induce slight tissue bleeding.

AST and ALT are common biochemical markers of hepatotoxicity (Rodrigues et al. 2022). The values obtained are shown in Fig. 6. It is observed that the values were all statistically similar, which indicates a lack of toxicity in the liver.

No changes were observed in the erythrogram as well (Table 4); there was a slight but statistically significant ($p < 0.05$) increase in the number of segmented neutrophils in 2.5 and 5 mg/kg, and increased eosinophils in 1 mg/kg. However, considering the lack of dose dependence and the results from others parameters, this does not necessarily mean a sign of toxicity. There is a natural variation among animals

according to their gender, strain, genotype, among other factors. Also, all the values observed are within the acceptable range of the species (Silva Lima et al. 2017; Villas Boas et al. 2018).

Table 4. Results from blood parameters (erythrogram, leukocytes, platelets). The values are shown as Mean $\pm$ SD; *: $p < 0.05$ (ANOVA followed by Tukey test).

Parameter	COM	NBO1	NBO2.5	NBO5	NBO10
Erythrogram					
Red cells ($10^6/\text{mm}^3$)	8.17 $\pm$ 0.41	9.33 $\pm$ 0.282	9.36 $\pm$ 0.361	9.50 $\pm$ 0.276	8.28 $\pm$ 0.239
Hemoglobin (g%)	14.77 $\pm$ 0.74	15.52 $\pm$ 0.70	15.15 $\pm$ 0.50	15.67 $\pm$ 0.25	14.92 $\pm$ 0.66
Hematocrit (%)	42.25 $\pm$ 2.05	44.4 $\pm$ 2.30	42.0 $\pm$ 1.41	44.1 $\pm$ 0.81	42.2 $\pm$ 1.70
MCV (μ^3)	51.68 $\pm$ 0.20	51.83 $\pm$ 1.14	44.97 $\pm$ 1.56	46.32 $\pm$ 1.20	41.45 $\pm$ 15.8
MCH (pg)	18.07 $\pm$ 0.29	16.61 $\pm$ 0.27	16.18 $\pm$ 0.58	16.50 $\pm$ 0.47	17.02 $\pm$ 0.93
MCHC (%)	34.97 $\pm$ 0.65	32.06 $\pm$ 0.54	35.97 $\pm$ 0.24	35.62 $\pm$ 0.15	35.31 $\pm$ 0.24
RDW (%)	16.47 $\pm$ 0.42	18.06 $\pm$ 0.54	22.02 $\pm$ 1.01	21.20 $\pm$ 0.63	19.02 $\pm$ 1.55
Leucogram					
Leucocytes (/mm ³)	3.580 $\pm$ 882	4.310 $\pm$ 578	4.778 $\pm$ 800	5.708 $\pm$ 632	3.100 $\pm$ 637
Segmented (/mm ³)	430 $\pm$ 109.1	503 $\pm$ 243	1.555 $\pm$ 205*	1.125 $\pm$ 271*	334 $\pm$ 120
Eosinophils (/mm ³)	77.7 $\pm$ 10.3	264.0 $\pm$ 130.8*	81.5 $\pm$ 37.42	68 $\pm$ 15.53	50.2 $\pm$ 14.5
Basophils (/mm ³)	436 $\pm$ 100.9	208 $\pm$ 137	384 $\pm$ 172	120 $\pm$ 51.3	345 $\pm$ 12.9
Linfocytes (/mm ³)	2.454 $\pm$ 650	3.700 $\pm$ 575	3.225 $\pm$ 553	4.971 $\pm$ 2.164	2.355 $\pm$ 555
Monocytes (/mm ³)	182 $\pm$ 37.7	235 $\pm$ 100.7	158 $\pm$ 97	227 $\pm$ 75	177 $\pm$ 80.6
Platelets					
Platelets ($10^6/\text{mm}^3$)	0.86 $\pm$ 0.20	0.89 $\pm$ 0.13	0.92 $\pm$ 0.11	0.76 $\pm$ 0.17	0.74 $\pm$ 0.16
MPV (/mm ³)	8.8 $\pm$ 0.21	8.9 $\pm$ 0.12	8.3 $\pm$ 0.09	9.1 $\pm$ 0.31	8.9 $\pm$ 0.14

In the histopathological analysis, the groups treated with NBO at 1, 2.5, and 5 mg/kg had a healthy muscle structure. In NBO at 10 was observed necrotic fibers and high leukocyte infiltration, fiber diameter variation, and increased connective tissue (Fig. 7). According to (Paulsen et al. 2012), tissue changes caused by inflammatory cells in the muscle can affect its function with the occurrence of edema and

increased protein expression, which will be responsible for the increased values of muscle damage such as CPK (Ribeiro et al. 2006). No inflammatory cells were observed in the muscle of NBO from 1 to 5 mg/kg.

Conclusion

The toxicological results of this study show that the acute intramuscular injection of NBO did not induce toxicity signs up to 5 mg/kg and was considered safe. The injection of 10 mg/kg, despite not inducing biochemical (CPK, LDH, myoglobin, AST, ALT) or hematological changes, had histopathological alterations, with infiltrated leukocytes, muscle fiber morphological variation, and necrosis.

Abbreviations

ALT - Alanine aminotransferase

AST – Aspartate aminotransferase

CPK – Creatine kinase

LDH – Lactate dehydrogenase

MCH - Mean corpuscular hemoglobin

MCS - Mean corpuscular size

NBO - Nanodispersion of *B. orellana* oil

OECD - Organisation for Economic Cooperation and Development (OECD)

Declarations

Author contributions HOC: performed experiments and assays, analyzed data, prepared manuscript. ALRS, ALN, AVTLT and DESG: performed experiments and assays, interpreted data, edited manuscript. LG, GCF, HRS and GCS: performed experiments and assays, interpreted data, edited manuscript. JCTC: conceptualization and design of study, assisted with statistical analyses, interpreted data. SS, ACMP and JCTC: conceptualization and design of study, assisted with statistical analyses, interpreted data, troubleshooted assays, edited manuscript.

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Data availability Data will be made available for review as needed upon legitimate request.

Conflict of interests The authors have no relevant financial or no-financial interests to disclose.

Ethical approval Animal manipulations and handling processes for all experiments were approved by the Ethics Committee of Animals Use from the Federal University of Amapá (CEUA, UNIFAP), under n° 002/2022.

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Slides

Slide 1 is available in the Supplementary Files section

Figures

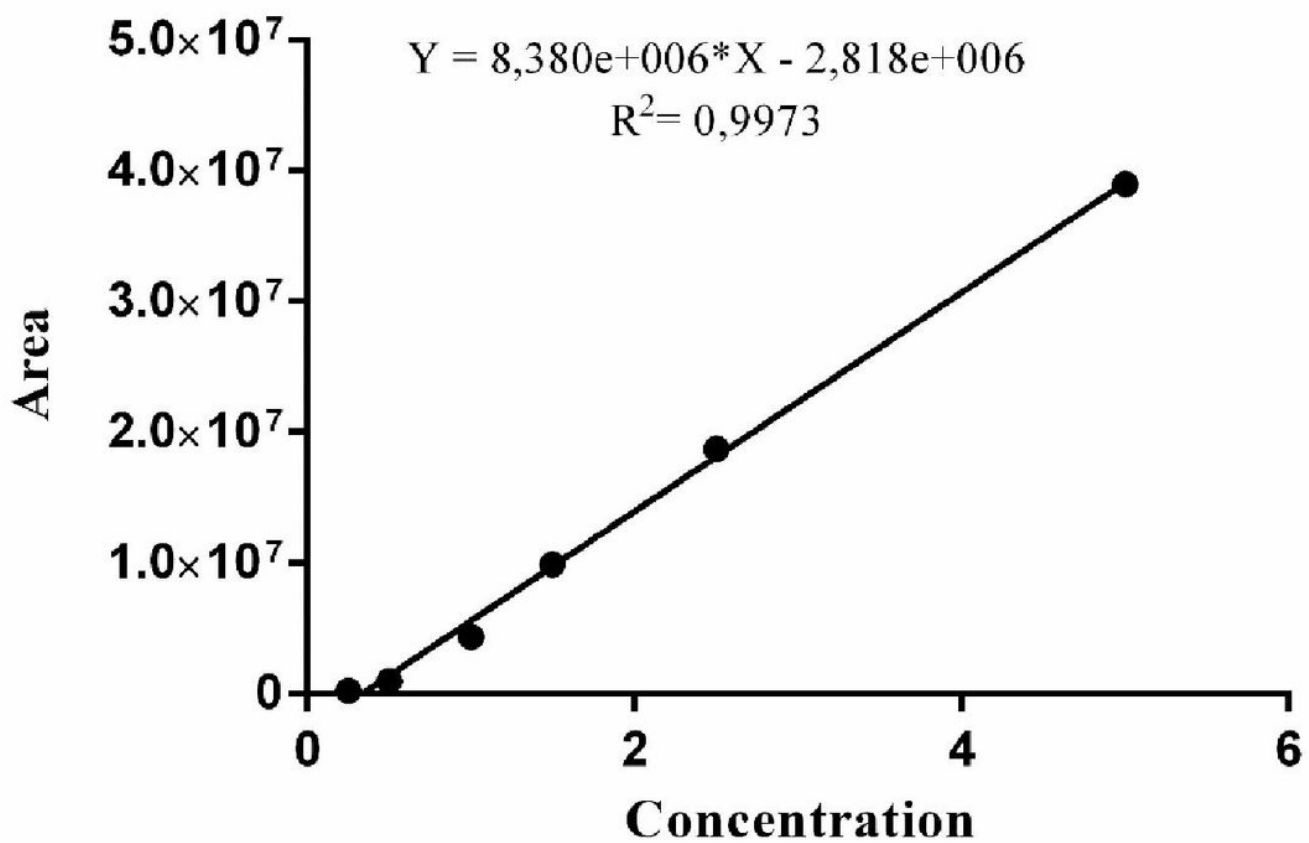


Figure 1

Calibration curve of δ -tocotrienol.

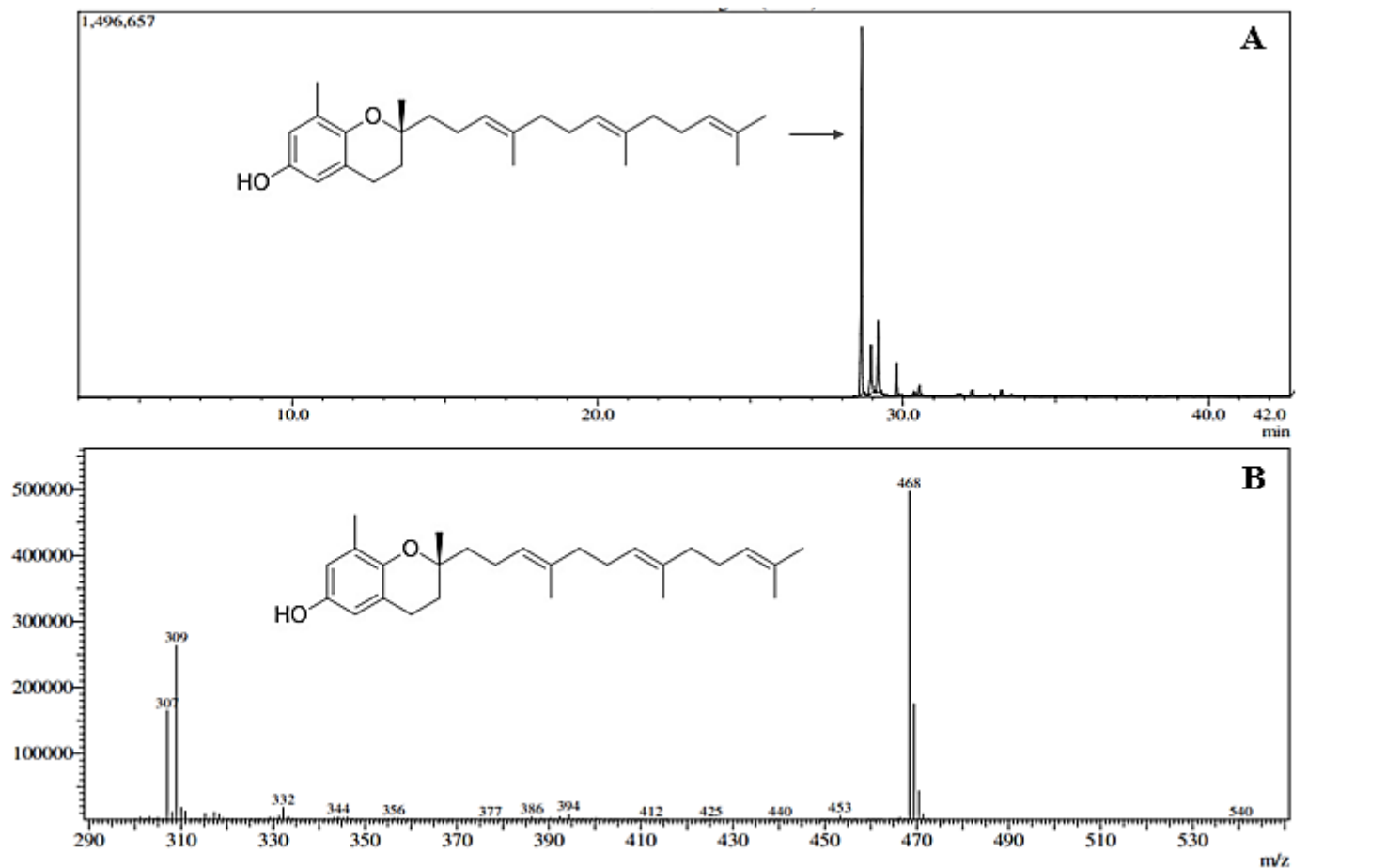


Figure 2

A: Chromatographic profile of *B. orellana* oil; the major peak represents δ -tocotrienol ($C_{27}H_{40}O_2$). B: Fragmentation pattern of δ -tocotrienol.

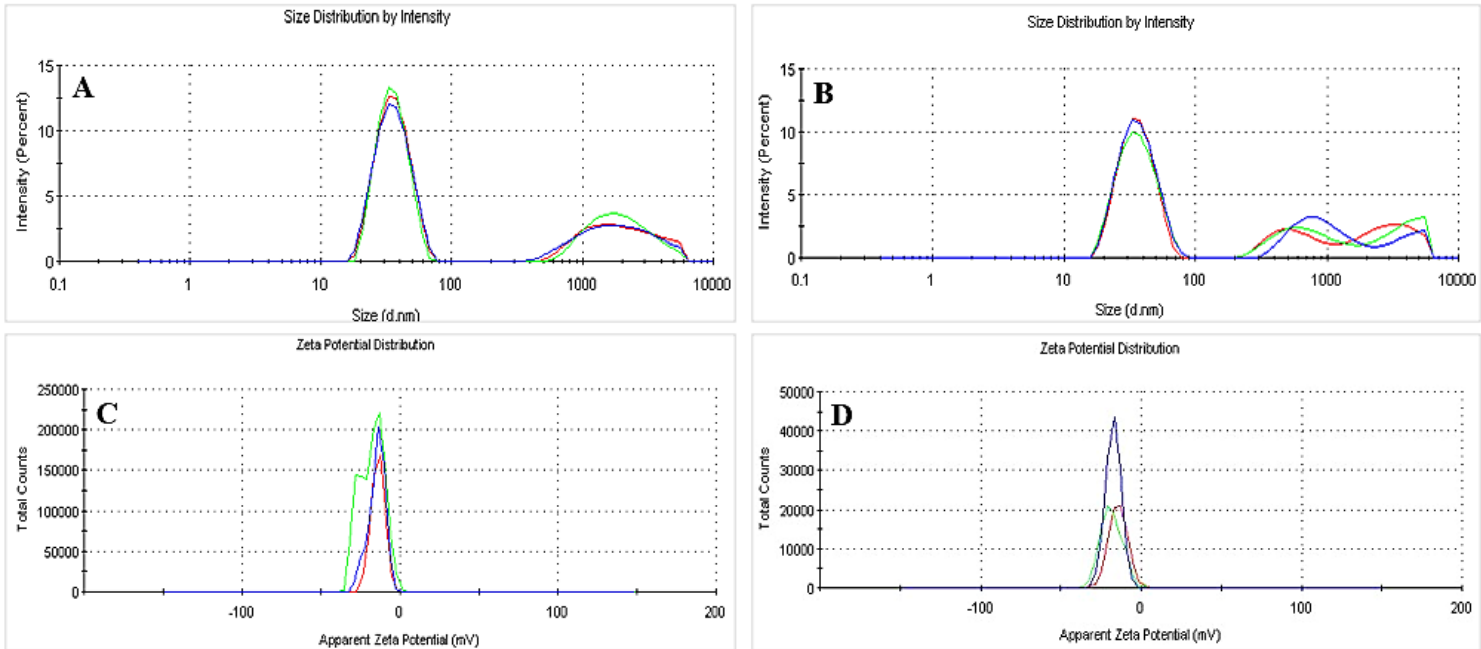


Figure 3

Assessment of the nanodispersion stability. The particle size is shown in A (1st day) and B (30th day); the zeta potential is shown in C (1st day) and D (30th day).

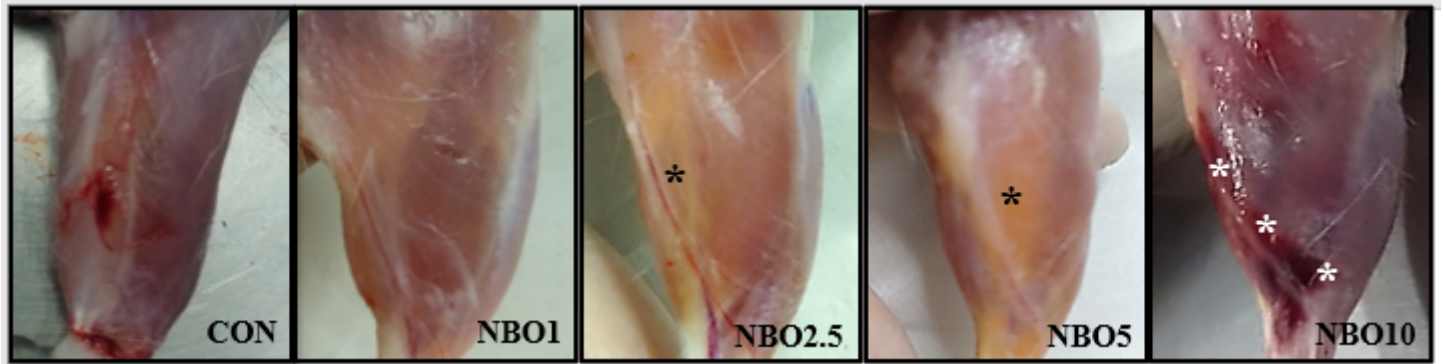


Figure 4

Representative pictures of the macroscopical aspect of the injected muscle (gastrocnemius). Note that in NBO 2.5 and 5, the formulation can still be observed in a yellowish color (asterisks). In NBO 10 can be observed hemorrhage (white asterisks).

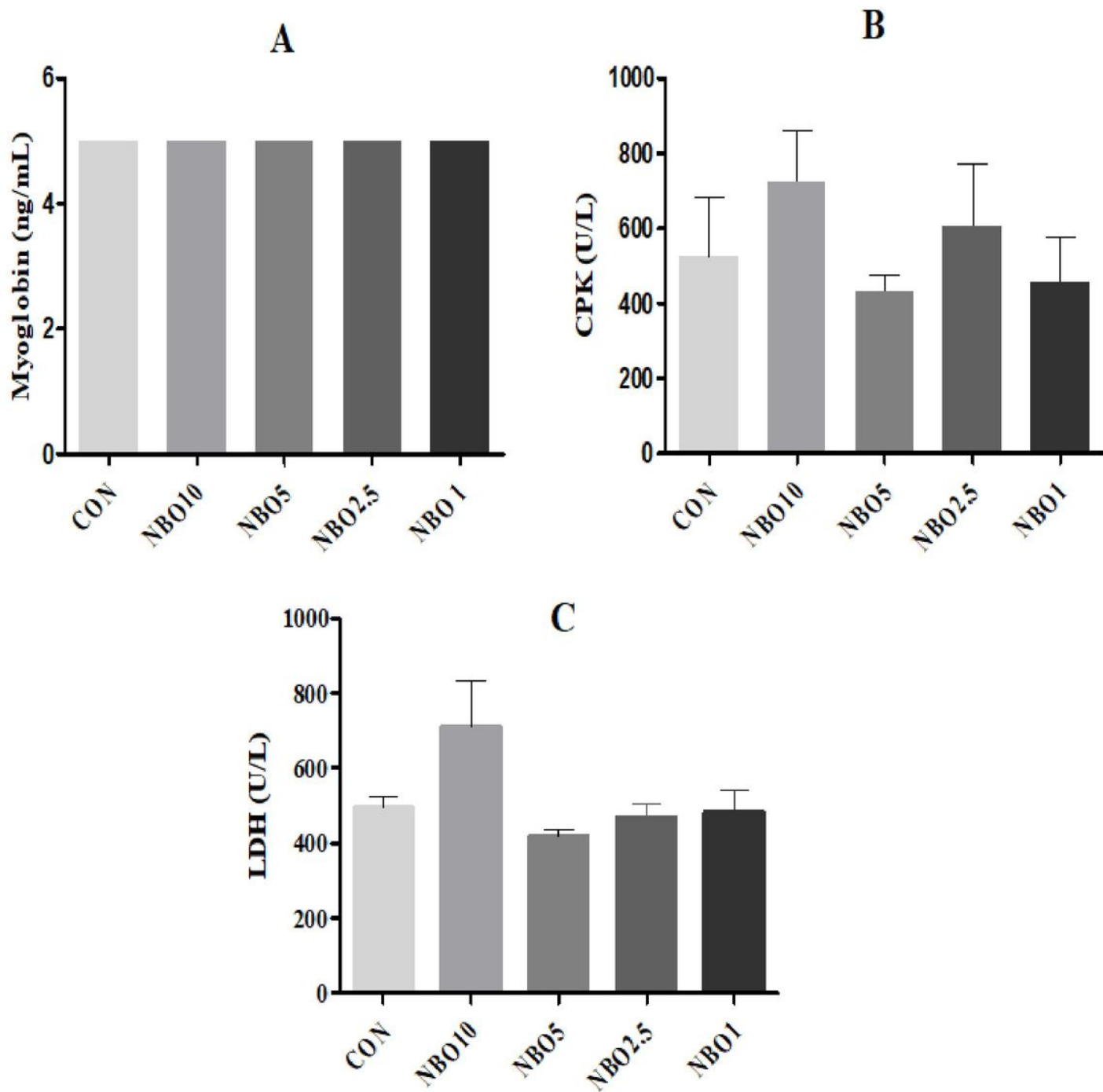


Figure 5

Muscle damage markers biochemical results. A: myoglobin; B: Lactate dehydrogenase; C: creatine kinase. The results are shown as a Mean $\pm$ SD; no differences among the groups ($p > 0.05$; ANOVA).

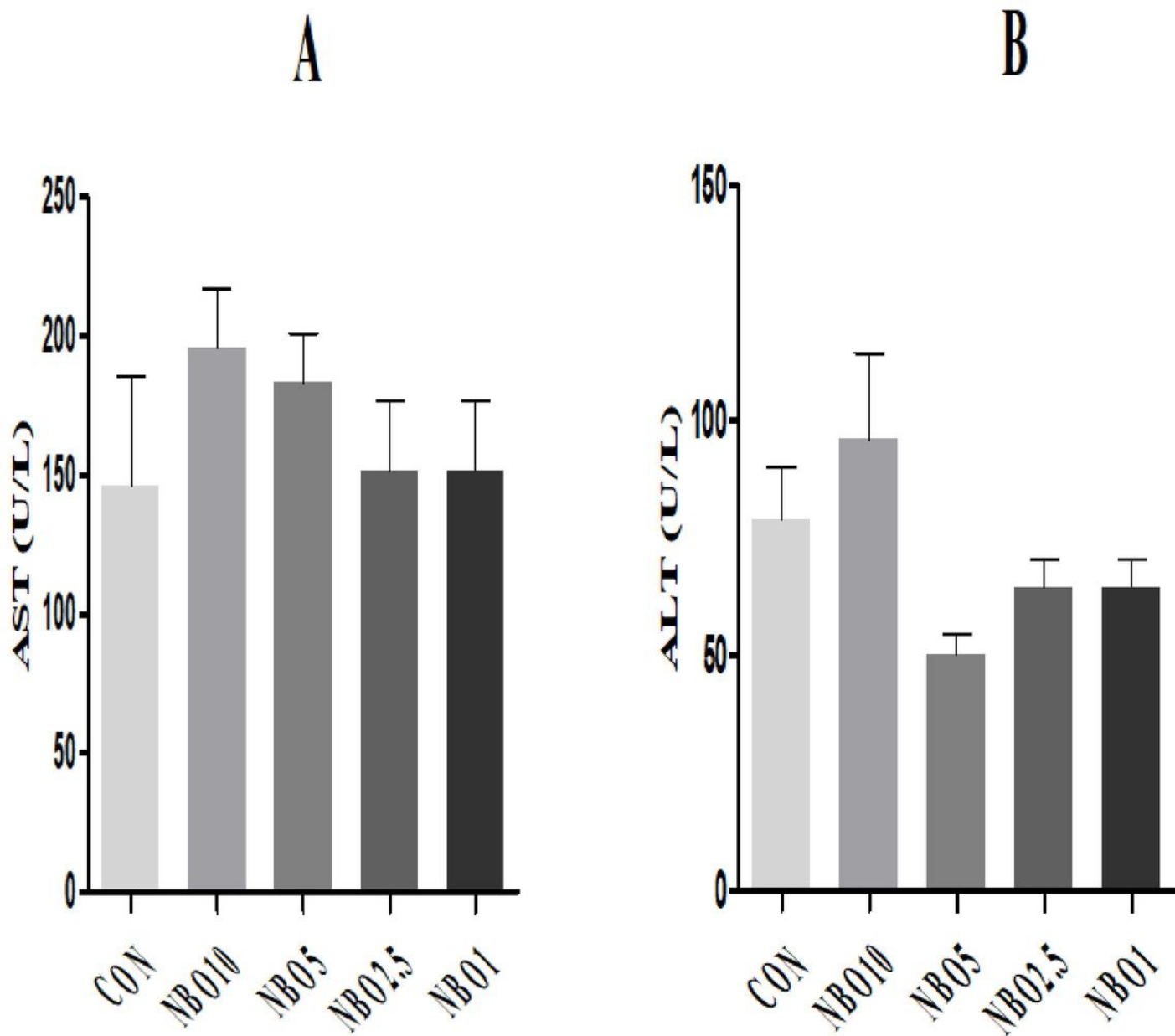


Figure 6

Hepatotoxicity markers biochemical results. A: aspartate aminotransferase; B: alanine aminotransferase. The results are shown as a Mean $\pm$ SD; no differences among the groups ($p > 0.05$; ANOVA).

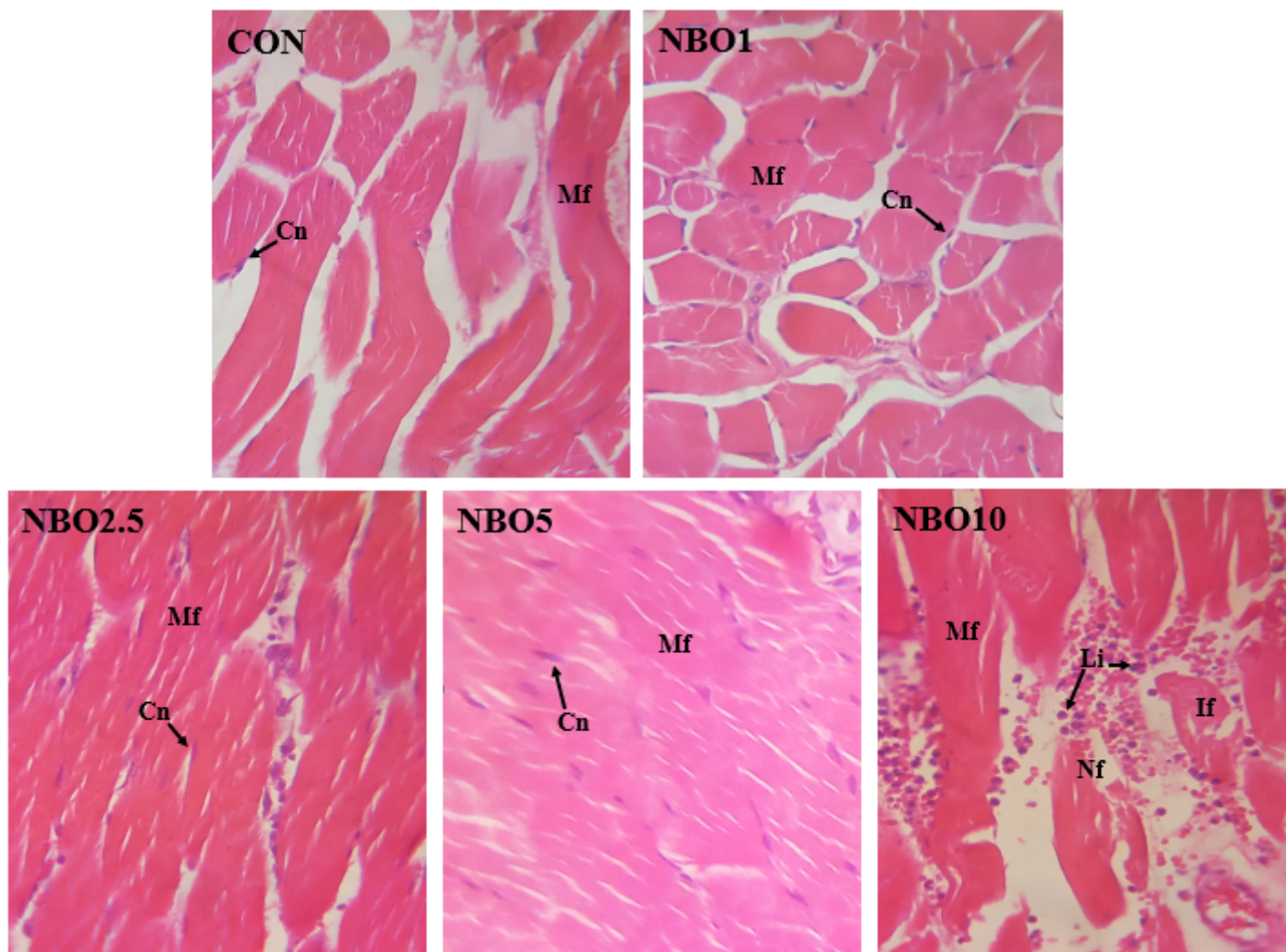


Figure 7

Representative slides of the animals' muscles. In the control group and NBO from 1 to 5, healthy muscle fibers (Mf) and cell nuclei (Cn) can be observed without damage. On the other hand, In NBO 10 can be observed irregular muscle fibers (If), necrotic muscle fibers (Nf), and high leucocytes infiltration (Li). H&E.

Supplementary Files

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- [Slide1.jpeg](#)