Food additive "lauric acid" possess non-toxic profile on biochemical, haematological and histopathological studies in female Sprague Dawley (SD) rats

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Background: Lauric acid (LA), a common constituent of coconut oil is used as food additives and supplements in various formulations. Despite various potential pharmacological properties, no scientific evidence on its dose-related toxicity and safety is available till date.

Objective: The current study was conducted to evaluate acute oral toxicity of LA on normal rats.

Methods: Study was conducted in accordance with the Organization for Economic Co-operation and Development guidelines (OECD 423) with slight modifications. LA was administered orally to female Sprague Dawley (SD) rats (n = 6/group) at a single dose of 300 and 2000 mg/kg body weight respectively, while normal control received vehicle only. Animals from all the three groups were monitored for any behavioural and toxicological changes and mortality for two weeks. Food and fluid consumption, body weight was monitored on daily basis. At the end (on day 15th) of the experimental period, blood was collected for haematological and biochemical analysis. Further, all the animals were euthanized, and internal organs were harvested for histopathological investigation using four different stainings; haematoxylin and eosin, Masson trichrome, Periodic Acid Schiff and Picro Sirius Red for gross pathology through microscopical observation.

Results: Study result showed no LA treatment-related mortality and morbidity at two different dosages. Daily food and water consumption, body weight, relative organ weight, haematological, and biochemical analysis were observed to be normal with no severe alterations to the internal tissues.

Conclusion: Current finding suggests that single oral administration of LA, even up to 2000 mg/kg body weight did not exhibit any signs of toxicity in SD rats, thus, safe to be used on disease model in animals.

1 **Food additive "lauric acid" possess non-toxic profile on biochemical,**
2 **haematological and histopathological studies in female Sprague**
3 **Dawley (SD) rats**

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37 **Abstract**

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40 properties, no scientific evidence on its dose-related toxicity and safety is available till date.

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43 and Development guidelines (OECD 423) with slight modifications. LA was administered orally

44 to female Sprague Dawley (SD) rats ($n = 6$/group) at a single dose of 300 and 2000 mg/kg body

45 weight respectively, while normal control received vehicle only. Animals from all the three groups

46 were monitored for any behavioural and toxicological changes and mortality for two weeks. Food

47 and fluid consumption, body weight was monitored on daily basis. At the end (on day 15th) of the

48 experimental period, blood was collected for haematological and biochemical analysis. Further,

49 all the animals were euthanized, and internal organs were harvested for histopathological

50 investigation using four different stainings; haematoxylin and eosin, Masson trichrome, Periodic

51 Acid Schiff and Picro Sirius Red for gross pathology through microscopical observation. **Results**:

52 Study result showed no LA treatment-related mortality and morbidity at two different dosages.

53 Daily food and water consumption, body weight, relative organ weight, haematological, and

54 biochemical analysis were observed to be normal with no severe alterations to the internal tissues.

55 **Conclusion**: Current finding suggests that single oral administration of LA, even up to 2000 mg/kg

56 body weight did not exhibit any signs of toxicity in SD rats and safe to be used on disease model

57 in animals.

58 **Keywords**: Lauric acid, acute oral toxicity, haematological parameters, biochemical parameters.

59 **1. Introduction**

60   Plant-based products are considered as the primary source for food additives and ingredients

61   in various natural products. Natural substances are added in the food to enhance its flavour,

62   taste and appearance. Various plant additives are being used for centuries to preserve various

63   health care products and supplements (Amen et al. 2019; Arya et al. 2012a). According to the

64   world health organization (WHO) recommendation, food additives must pass through the

65   safety screen prior to use in any products. Additives in the form of crude plant or extracts are

66   needed to ensure processed food remains safe, and in good condition, a variety of plants

67   extracts contain specific phytochemicals whose biological activities is not known. Possibly,

68   some unwanted phytocompounds might lead to toxic effects or effects products preserving

69   capacity, stability and even the nutritional value of the products (Organization 2015; Yakob et

70   al. 2012). However, chemical characterization, proper dosing regimen, and insufficient

71   knowledge of the toxicity data constraint limit the usage of additives and herbal medicine

72   (Deng et al. 2013). Thus, it is the current need to evaluate the possible toxicity of the plant

73   extract and phytocompounds before its therapeutic use.

74   Lauric acid (LA) is the primary (> 50%) composition of coconut oil (Sheela et al. 2016). It is

75   a medium-chain fatty acid (MCFA) found abundantly in the coconut oil, palm kernel oil and

76   laurel oil, which constitutes about half of the fatty acid (Beare-Rogers et al. 2001; Ubgogu et

77   al. 2006). It is also found in various kinds of foods, such as fruits, seeds, and breast milk (Alves

78   et al. 2017; Silberstein et al. 2013; Uday Kumar 2014). It possesses pleiotropic biological

79   activities, including potent anti-microbial effects (Dayrit 2015), beneficial effect on the

80   cardiovascular system (CVS) by increasing high-density lipoproteins (HDL) and reducing

81   blood pressure and heart rate in hypertensive rats (Ahola et al. 2017). Moreover, it also prevents

82   testosterone-induced prostatic hyperplasia (Babu et al. 2010), and promote apoptosis in breast

83   and cancerous endometrial cells (Lappano et al. 2017).

84   Despite various pharmacological properties, scientific evidence on LA's toxicological, and

85   safety profile is not available. This is the first study aimed to investigate the acute toxicity of

86   LA following the Organization of Economic Cooperation and Development (OECD 423)

87   guideline in female Sprague Dawley (SD) rats with slight modifications. In this study, we

88   evaluated numerous parameters, like body weight, food and water intake, haematology,

89   biochemistry, and the histopathological changes in various tissues as shown in **Figure 1**.

90   **2.Materials & Methods**

91   *2.1. Ethical Consideration*

92   The acute toxicity study of LA was accomplished in accordance with the guidelines stipulated by

93   the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University of

94   Malaya, Kuala Lumpur, Malaysia with animal ethics approval no: 2018-210605/TAY/R/AA

95   (2018295).

96   *2.2. Reagents*

97   Lauric acid (MKCF8837, purity more than 98 %) Sigma-Aldrich (St Louis, MO 63103 USA 314-

98   77-5765), Tween-20 (C01-TW121-00, Systerm, UK), normal saline 0.9% (B Braun),10% neutral

99   buffered formalin solution pH 7.4 (R&M, UK), Haematoxylin (Merck, USA) and Eosin (Systerm,

100   UK), Masson trichrome (Abcam150686), Periodic Acid Schiff (Abcam150680) and Picro Sirius

101   Red stain kit (Abcam 150681).

102   All other chemicals and solvents were of analytical grade.

103   *2.3. Experimental animals*

104   In this study, eighteen (18) healthy specific pathogens-free (SPF) female   SD rats, weighing 240-

105   250 gram (g), age 8-10 weeks old were procured from the Animal Experimental Unit (AEU),

106   Faculty of Medicine, University of Malaya. The AEU facility at the University of Malaya is

107   certified by the Association for Assessment and Accreditation of Laboratory Animal Care

108   International (AAALAC International). All the animals were randomly segregated in three

109   different groups (six animals in each group) and housed in polypropylene cages for seven days to

110   acclimatise laboratory environment prior to the experiments. A consistent environment of 12-hour

111   light/dark cycle, temperature 22 ± 2 ^{o}C, and humidity of 50±10% were maintained. The animals

112   were allowed a standard rodent pellet diet and reverse osmosis (RO) water ad libitum throughout

113   the experimental period.

114   *2.4. Acute oral toxicity test*

115   Acute toxicity study was conducted to assess whether LA produce any mortality or other adverse

116   reactions upon oral administration of dose (300 and 2000 mg/kg body weight), according to

117   guideline 423 provided by the OECD for the acute toxicity class method procedure (OECD

118   2001)with minor amendments (Co-operation & Development 2002). Before the experiment, all

119   the animals were carefully weighed, marked, and grouped accordingly:

120   Group I (n=6): Vehicle (Tween 20/normal saline)-treated

121   Group II (n=6): Lauric acid-treated, dose 300 mg/kg body weight

122   Group III (n=6): Lauric acid-treated, dose 2000 mg/kg body weight.

123   The LA was dissolved in Tween 20 and diluted with normal saline 0.9% to get the desired dose

124   (Alves et al. 2017). Animals fasted overnight before dosing, and treatment groups received freshly

125   prepared single doses of LA suspension at 300 and 2000 mg/kg body weight, while animals in the

126   control group received Tween 20 or normal saline 0.9% suspension equally based on individual

127   body weight. Each gavage volume was 10 mL/kg body weight. All efforts were made to lessen

128   experimental animals' suffering, especially in handling and oral gavage.

129   *2.5. Daily observations*

130   After oral gavage, diet was withheld except water for 2–3 hours while animals were monitored

131   individually with special attention in the first half-hour till completion of first 24 hours followed

132   by daily cage-side examination for fourteen days for any signs of toxicity including general

133   behavioural in skin, eyes, touch, activeness, and movement changes. All the changes observed

134   were well documented before and after the gavage.

135   *2.6. Body weight, food, and water consumption*

136   The body weight of the individual animal was carefully noted before and after oral administration

137   of the LA or vehicle, then weekly and lastly on day fifteen (euthanized day). Daily food (g/day),

138   and water (ml/ day) intake was noted on daily basis in all the three groups for fourteen days.

139   *2.7. Haematology and biochemical analysis*

140   On day fourteen, all the animals fasted overnight for 12 hours, only water was allowed. All the

141   animals were euthanized in the morning by an intraperitoneal (IP) injection of ketamine (80mg/kg)

142   & xylazine (7mg/kg), and blood was collected via micro-capillary tubes from the retro-orbital

143   plexus region into two different sets of test tubes, one containing anticoagulant, ethylene

144   diaminotetraacetic acid (EDTA) for haematological tests including red blood cells (RBC), white

145   blood cells (WBC), platelets count, haemoglobin (Hb), packed cell volumes (PCV), mean

146   corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular

147   haemoglobin concentration (MCHC), neutrophil, lymphocytes, monocytes, and eosinophils and

148   the other without additives for clinical biochemistry tests like urea, creatinine, calcium, inorganic

149   phosphate, uric acid, sodium, potassium, chloride, total cholesterol (TC), high-density lipoproteins

150   (HDL), low-density lipoproteins (LDL), triglycerides (TG), total (HDL) ratio, albumin, globulin,

151   albumin-globulin (A/G) ratio, ALP, AST and ALT levels. The collected blood samples were then

152   sent to an ISO-certified blood-testing laboratory.

153   *2.8. Measurement of relative organ weight*

154   Internal organs such as adrenal glands, brain, eyes, heart, kidneys, liver, lungs, ovaries, spleen,

155   stomach, urinary bladder, uterus, and cervix/vagina were isolated through a fine incision in the

156   abdomen at the midline of the euthanized animals. All the harvested tissues were cleaned with

157   phosphate buffer saline (PBS), wiped with clean blotting paper and weighed on a calibrated

158   weighing balance. Relative organ weight (ROW) of all the animals was noted in proportion to

159   body weight according to the following equation:

160    $$\text{ROW} = \frac{absolute\ organ\ weight}{body\ weight\ at\ the\ time\ of\ sacrifice} X100$$

161

162   *2.9. Histopathological analysis*

163   Histopathological examination was conducted on the harvested liver, kidney, heart, lungs, brain,

164   spleen, and pancreas in all the animals. Tissues were preserved in 10% neutral, buffered formalin

165   solution for 48 hours at 4°C for histopathological study. Paraffin-embedded specimens of selected

166   tissues were cut into 4-5 μm thick samples and stained with haematoxylin and eosin (H&E),

167   Masson trichrome, Periodic Acid Schiff (PAS) and Picro Sirius Red staining as per the

168   manufacturer protocol detailed in the kits. Later, all the stained tissues were sectioned and observed

169   under the fluorescence compound microscope (40X) (Eclipse Ni-U, Nikon Corporation, Japan).

170   *2.10. Statistical analysis*

171   All statistical results were expressed as the mean ± standard deviation (SD). The comparison

172   between the treated and control group was performed by a one-way analysis of variance (ANOVA)

173 using statistical software, SPSS package version 22 (IBM, SPSS Statistics, Inc., Chicago, IL, USA)

174 to evaluate the significant differences. The (p < 0.05) compared to the control group was

175 considered statistically significant.

176 **3.Results**

177 *3.1. Acute toxicological evaluation and daily observation*

178 Animals in LA (300 and 2000mg/kg body weight) treated group were normal and healthy during

179 fourteen days study period. No signs of mortality or sudden death were observed compared to the

180 untreated group animals. No significant changes in the temperature, skin, eye colour, food and

181 water intake were observed. Furthermore, no behavioural changes or signs of diarrhoea, coma,

182 sedation and tremors were noted as compared to the control group **(Table 1).** Experimental results

183 suggest that LD_{50} of LA should be higher than 2000 mg/kg body weight.

184

185 *3.2. Effect of LA on body weight, food and water consumption*

186 Body weight, food & water intake in the LA treated groups (300 and 2000 mg/kg body weight)

187 were noted to be non-significant compared to the control group. However, LA treated groups

188 showed slight changes in the body weight, food and water consumption on day fourteen, compared

189 to the untreated group, as shown in **Table 2 and 3**. The mean body weight of the control group

190 was noted as 244.71 ± 3.13 g, whereas treatment groups showed 247.53 ± 2.96, and 240.63 ± 4.76

191 g, respectively. On day fourteen, the mean body weight gained by the control group was 14.81 ±

192 0.064 g, while LA treated animals (300 and 2000 mg/kg body weight) displayed non-significant

193 gain in the body weight (14.93 ± 0.68 and 15.36 ± 0.76 g), respectively. The percentage mean in

194 the body weight of all the treated animals was non-significant in comparison to the control group

195 (*p > 0.05*), as shown in **Table 2**.

196   Moreover, LA administration (300 and 2000 mg/kg) showed non-significant changes ($p > 0.05$)

197   in the food and water consumption, compared to the control group, as shown in **Table 3**.

198   *3.3. Effect of LA on relative organ weight.*

199   The relative organ weight of the harvested tissues—adrenal glands, brain, eyes, heart, kidney, liver,

200   lungs, ovaries, spleen, stomach, urinary bladder, uterus and cervix/vagina, revealed non-significant

201   changes post-LA treatment (300 and 2000 mg/kg body weight) compared with the control group

202   animals ($p > 0.05$), as shown in **Table 4.**

203   *3.4. Effect of LA on haematology and clinical biochemistry*

204   The effect on haematological analysis is summarised in **Table 5**. Parameters including RBC,

205   WBC, platelets count, Hb, PCV, MCV, MCH, MCHC, neutrophil, lymphocytes, monocytes, and

206   eosinophils shows non-significant changes ($p > 0.05$) after oral administration of LA (300 and

207   2000 mg/kg body weight) when compared with the control animals. Importantly, all the values

208   were within the reference range, compared to the control group.

209   Similarly, the biochemical analysis of liver and renal function tests, including urea, creatinine,

210   calcium, inorganic phosphate, uric acid, sodium, potassium, chloride, TC, HDL, LDL, TG, total

211   HDL ratio, albumin, globulin, A/G ratio, ALP, AST and ALT levels for LA treatment groups were

212   not affected and observed to be normal (non-significant ($p > 0.05$)), when compared with the

213   control group as presented in **Table 6**.

214   *3.5. Effects of LA on Histopathology of internal organs*

215   The histopathological analysis involves macroscopic and microscopic examination of processed

216   and fixed tissues to visualise various components and structural alterations in the tissues (Greaves

217   2011; Khoo et al. 2018). H&E staining is the most common and widely used technique to

218   essentially identify morphological changes during diagnosis and screening of any malignancies in

219   the tissues (Fischer et al. 2008). However, Masson trichrome staining remains reliable for the

220   cytoplasm, which gives in-depth and selective detail about the connective tissue (Tsounapi et al.

221   2017). PAS staining assesses the changes in glycogen content within muscle fibre sections (Arya

222   et al. 2015; Fairchild & Fournier 2004). Moreover, the collagen content is analysed with the help

223   of Picro Sirius Red staining, which provides a clear understanding of collagen accumulation in

224   the tissues (Lattouf et al. 2014).

225   In brief, macroscopic analysis of liver sections (**Figure 2**) in LA-treated rats (300 and 2000 mg/kg

226   body weight) displays no alterations in the colour or texture, importantly no changes to

227   hepatocytes, hepatic sinusoids, central vein, and portal triad were observed when compared with

228   the control group (**Figure 3**). Kidney showed no structural degeneration, and colour changes in

229   the renal cortex and tubules, without any tubular necrosis and inflammation in LA-treated animals

230   compared to untreated animals, as displayed (**Figure 4** and **5**). The photomicrographs of the heart

231   (**Figure 6** and **7**) tissue showed a normal size of myocardial fibre and filaments when compared

232   with the control group. Lung tissues (**Figure 8** and **9**) appeared to be normal, with no abnormal

233   changes noted in colour. Moreover, microscopic observation displayed thin inter-alveolar septa,

234   clear alveoli and alveolar sacs with the absence of inflammatory cells, including neutrophils, or

235   thickening of alveolar septa, when compared with the untreated group. Furthermore, brain (**Figure**

236   **10** and **11**) tissues showed normal morphology with the regular shape of the pyramidal neuron and

237   uniform distribution of glial cells in LA-treated and control animals. Macroscopic texture (**Figure**

238   **12** and **13**) exhibited normal and regular appearance with white and red pulp without any sign of

239   apoptotic, necrotic lesions or megakaryocytosis compared with the control group. Microscopic

240   appearance of the pancreas (**Figure 14** and **15)** appeared with healthy acinar, clear round or oval-

241   shaped Islets cells in cluster form, with lightly stained cytoplasm in the LA-treatment and control

242   animals. Thus, the study results suggest that treatment with minimum and maximum doses of LA

243   (300 & 2000 mg/kg body weight) did not cause any tissue damage or morphological alteration at

244   the cellular level.

245   *3.6 Discussion*

246   Toxicological studies of plant-based ingredients or food additives are important to ensure

247   processed food remains safe and in good condition throughout its preservation to avoid any disease

248   conditions. Herbs or plants additives used in dietary supplements, nutraceuticals, should be

249   investigated for toxicity, despite their traditional claims (Figueredo et al. 2018; Lulekal et al.

250   2013).

251   Lauric acid revealed various pharmacological activities, but its safety profile still remains

252   unevaluated. The current study is undertaken to screen the safety, and toxicity of LA at two

253   different dose levels in normal rats, following OECD 423 guidelines.

254   No morbidity or mortality was noticed in LA-treated animals throughout fourteen days of the

255   experimental period. Moreover, in line with previous study of (Arya et al. 2012b), no changes

256   were observed in general behaviour of treated animals when compared with the control group,

257   suggesting that oral ingestion of LA neither cause any alterations nor produced any internal

258   changes in female SD rats with doses up to 2000 mg/kg body weight.

259   Body weight changes are the basic parameter which indicates the health status of animals.

260   However, abrupt variations in the body weight indicate the toxic nature of the chemicals or drugs

261   (Dongmo et al. 2019). In this study, LA treatment and control group recorded a normal gain in

262   body weight, indicating, LA does not cause any physiological alterations that affects the normal

263   growth in the animals, which is in agreement with the previous study published by (Clemente et

264   al. 2019). Similarly, organ weight variation is the fundamental and perceptive factor correlating

265   organ to body weight index, which is important and critical in determining drug toxicity linked to

266   treatment-related effects (Sellers et al. 2007). It is believed that medicinal plants in high dose could

267   cause toxic or even lethal effects when administered in vivo (Silveira et al. 2008; Siwe et al. 2015).

268   Results from this study showed no difference in ROW in the treatment group when compared to

269   control group, which indicates that LA is safer to the internal organs, supported by an earlier study

270   published by (Porwal et al. 2017). Studies have proven that ingesting toxic substances could

271   suppress appetite, thus leading to less caloric intake (Madingou et al. 2016). During fourteen days

272   observation period, no significant changes were noted in water and food consumption **(Table 5),**

273   which shows that LA has no effect on the appetite of the animals, which is in line with the previous

274   findings published by (de Oliveira et al. 2020; Tonholo et al. 2020).

275   The haematopoietic system is crucial in the production of blood cells that accelerate metabolic

276   functions and enhance normal proliferation in the living organism which act and delineate as a

277   vital tool. This system is profound and directly or indirectly susceptible to chemical-induced

278   injury, affecting physiological and pathological status in both humans and animals (da Silva et al.

279   2018; Traesel et al. 2014). In the current findings, LA administration indicate non-significant

280   changes in the essential blood cells and related parameters RBC, WBC, platelets count, Hb PCV,

281   Mean MCV, MCH, MCHC, Neutrophil, Lymphocyte, Monocytes and Eosinophils, suggesting

282   non-toxic nature of LA on haematological parameters and substantiating an understanding of

283   normal haematopoiesis, which is in line with the previous findings published by (Deyno et al.

284   2020; Thai et al. 2020).

285   The liver is the central metabolic organ playing a key role in body metabolic functions where drug

286   and chemical metabolism, biotransformation and detoxification occurs (Doherty 2016). When

287   hepatic cellular damage occurs due to hepatotoxic substances, the sensitive markers including

288 transaminase serum enzymes ALT & AST start releasing from the cytosol and translocate into the

289 bloodstream, leading to mild or severe liver injury (Figueredo et al. 2018; Hazarika et al. 2019;

290 Ramaiah 2011). In our study, LA did not elevate these transaminase enzymes, considering non-

291 toxic nature of LA at different dose levels. Similar findings were noted by (Moorthy et al. 2019),

292 where oral administration of the geraniin-enriched extract at 2000mg/kg body weight did not

293 affect the biochemical parameters. Similarly, LA treatment did not change renal functions

294 including serum creatinine and urea levels, which are used as an essential index to estimate renal

295 damage (Arya et al. 2014; Mariappan et al. 2011; Meguellati et al. 2019). The elevated level of

296 creatinine and urea in the blood displays malfunctioning of the kidney (Brondani et al. 2017;

297 Feriani et al. 2017; Kifayatullah et al. 2015). In addition, serum electrolyte levels of LA treated

298 animals observed to be in normal ranges. Overall, biochemical parameters are in line with previous

299 reports of (Bedi & Krishan 2019; Thinkratok et al. 2014).

300 Histopathological investigation plays a significant role in determining tissue morphology through

301 microscopical evaluations. The analysis of internal organs are identical and consider as the prime

302 safety tool to determine any histological perturbations or deformities in the tissues (Chitra et al.

303 2015; Prabu et al. 2013). Therefore, the histopathological judgements concrete the haematological

304 and biochemical findings and further substantiate the biological response factors (Jaganathan et al.

305 2012). Histopathological examination (macroscopic and microscopic) of internal organs including

306 liver, kidney, heart, lungs, brain, spleen, and pancreas revealed a regular architecture, no

307 morphological alterations were observed in LA treatment groups compared to the control group.

308 Tissue degeneration or anomalies leading to necrosis, apoptosis or fibrosis are the basic parameters

309 to determine toxicity in internal organs (de Lima et al. 2017).

310   The liver is the metabolic connective tissue which controls biliary and vascular traffic.

311   Administration of LA at the minimum and maximum dose did not cause structural degeneration

312   or accumulation in the liver tissues, displaying normal clusters of hepatocytes and parenchymal

313   cells housed in cord-like arrangements. In this study, microscopical features of liver tissue is in

314   agreement with the published data by (Khoo et al. 2018) who postulated that changes to these

315   cellular events might potentially be associated with the accumulation of exogenous chemicals and

316   their metabolites may cause dose-dependent toxicity or damage to the liver (Debelo et al. 2016).

317   Similarly, LA administration did not alter kidney tissue, which showed organised pyramidal

318   structure without any tubular necrosis and inflammation in the cortex and inner portion of the

319   medulla region. However, the toxic effect of any compound on the kidney is demonstrated by

320   changes in the serum creatinine or urea level (Gopi et al. 2016). Finding from this study reveals

321   the harmless nature of LA on renal function by establishing the outcomes from histopathological

322   analysis, which did not notice lesion or necrotic marks upon LA administration. Likewise, LA

323   treatment on heart tissues displayed normal architecture of myocardial fibre and filaments which

324   is in line with (Greaves 2011) findings, who reported no morphological changes in heart tissues

325   upon treatment with the phytocompounds. In a similar way, lungs of LA-treated animals showed

326   no treatment-related morphological alterations, and our findings are in agreement with (Aamir et

327   al. 2019) who reported that no toxicological alteration was noted upon Arjunolic acid treatment in

328   normal female SD rats. Furthermore, brain tissues showed normal morphology upon LA treatment,

329   with no alterations to the cell types approaching through normal characteristics based on tissue

330   pattern recognition, including neurons, glia, and microglia cells without any modulation to

331   perivascular region. Thus, our study is supported by previous findings by (Pravallika et al. 2019)

332   who reported no structural and morphological changes in brain tissues following treatment with

333 ethanolic extract of *Centella asiatica* plant. Moreover, LA treatment did not alter pancreas and

334 spleen tissue which shows normal architecture, as we know rodent pancreas and spleen are diffused

335 and soft compare to human tissues, which is in line with the previously published study by (Prabu

336 et al. 2013) that reveals no morphological variation in tissues of control and hydroalcoholic extract

337 of polyherbal treatment groups.

338 The present study expressed that oral administration of LA did not cause any morbidity or

339 mortality at the selected doses, and LD50 values were greater than 2000 mg/kg body weight. Based

340 on the current findings, LA should remain in toxicity class 5, which exhibited the lowest toxicity

341 (LD50 > 2000 mg/kg body weight) as per the OECD 423 guideline, under chemical labelling and

342 classification (OECD 2001).

343 *4.Conclusions*

344 The findings of the current study showed that LA did not cause any significant changes upon oral

345 administration of LA up to 2000 mg/kg body weight in female SD rats, no treatment-related signs

346 of toxicity or mortality were noted. Moreover, histopathological examination exhibits no

347 remarkable changes in the harvested tissues. Thus, it provides basic information on the toxicity

348 and safety profile of LA, indicating low toxicity in the analysed parameters. This study could be

349 helpful in the selection of dosages for future subacute or chronic disease model. However, in

350 future, well-designed treatment strategies on pharmacological aspects should be carried out to set

351 a clear understanding of safety and efficacy. Therefore, further validation on LA would enable to

352 decide effective therapeutic dose prior to its long-term treatment as well as before the development

353 of any health care products.

354

355

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360

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547

The behavioural and general response of animals after oral administration of LA

(n= normal, no=not observed)

1 **Table 1**

2 The behavioural and general response of animals after oral administration of LA

Observation	Control	LA 300 mg/kg	LA 2000 mg/kg
Temperature	n	n	n
Skin changes	no	no	no
Change eyes colour	no	no	no
General physique	n	n	n
Diarrhoea	no	no	no
Coma	no	no	no
Drowsiness	no	no	no
Breathing difficulty	no	no	no
Sedation	no	no	no
Tremor	no	no	no
Death	no	no	no

3 (n= normal, no=not observed)

Effect of LA on body weight (g) assessment

Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-

way ANOVA *p < 0.05) significant value

1   **Table 2**

2   Effect of LA on body weight (g) assessment.

Week	Control	LA 300mg/kg	LA 2000 mg/kg	*F-value*	*P-value*
Day 0	244.71 ± 3.13	247.53 ± 2.96	240.63 ± 4.76	1.464	0.263
Week 1%	7.64 ± 0.46	7.52 ± 0.22	7.87± 0.35	0.149	0.863
Week 2%	14.81±0.064	14.93 ± 0.68	15.36± 0.76	0.113	0.894

3   Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA

4   *$^{*}p < 0.05)$ significant value*

Effect of LA on food and water consumption

Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-

way ANOVA $^*p < 0.05)$ significant value

1 **Table 3**

2 Effect of LA on food and water consumption.

Week	Control	LA 300 mg/kg	LA 2000 mg/kg	*F-value*	*P-value*
Food (g)					
Week 1st	136.35 ± 5.55	150.38 ± 19.65	133.84 ± 9.18	0.265	0.570
Week 2nd	139.50 ± 13.13	123.07 ± 54.79	142.61 ± 12.82		
Water (ml)					
Week 1st	204.28 ± 23.17	211.42 ± 31.58	200.71 ± 67.23	2.609	0.071
Week 2nd	184.28 ± 13.36	172.85 ± 7.55	187.14 ± 14.67		

3 Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA

4 *$p < 0.05$) significant value

Effect of LA on relative organ weight (g)

Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA $^*p < 0.05)$ significant value

1 **Table 4**

2 Effect of LA on relative organ weight (g).

Organ	Control	LA 300 mg/kg	LA 2000 mg/kg	*F-value*	*P-value*
Adrenal gland (left)	0.018 ± 0.001	0.019 ± 0.002	0.019 ± 0.002	0.638	0.698
Adrenal gland (right)	0.020 ± 0.004	0.018 ± 0.003	0.019 ± 0.001	0.537	0.595
Brain	0.643 ± 0.032	0.626 ± 0.069	0.632 ± 0.051	0.162	0.852
Eye (left)	0.041 ± 0.002	0.042 ± 0.003	0.044 ± 0.003	1.632	0.228
Eye (right)	0.043 ± 0.001	0.043 ± 0.002	0.045 ± 0.004	1.218	0.323
Heart	0.277 ± 0.016	0.290 ± 0.019	0.286 ± 0.012	0.960	0.405
Kidney (left)	0.285 ± 0.012	0.300 ± 0.024	0.280 ± 0.025	1.480	0.259
Kidney(right)	0.288 ± 0.014	0.297 ± 0.020	0.275 ± 0.013	2.640	0.104
Liver	2.981 ± 0.254	2.918 ± 0.367	2.931 ± 0.344	0.062	0.940
Lungs	0.476 ± 0.038	0.469 ± 0.028	0.502 ± 0.029	1.693	0.217
Ovary (left)	0.032 ± 0.008	0.031 ± 0.005	0.033 ± 0.010	0.062	0.940
Ovary (right)	0.033 ± 0.005	0.033 ± 0.009	0.033 ± 0.006	0.003	0.997
Pancreas	0.292 ± 0.043	0.359 ± 0.074	0.306 ± 0.056	2.117	0.115
Spleen	0.220 ± 0.018	0.209 ± 0.027	0.195 ± 0.013	2.284	0.136
Stomach	0.754 ± 0.111	0.762 ± 0.184	0.939 ± 0.238	1.890	0.185
Urinary bladder	0.037 ± 0.003	0.041 ± 0.007	0.037 ± 0.004	0.922	0.419
Uterus	0.398 ± 0.054	0.445 ± 0.211	0.258 ± 0.315	2.579	0.109
Cervix/Vagina	0.081 ± 0.017	0.075 ± 0.011	0.074 ± 0.008	0.445	0.445

3 Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA

4 *$p < 0.05$) significant value

Effect of LA on haematology

Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-

way ANOVA ($^*p < 0.05$) significant value

1 **Table 5**

2 Effect of LA on haematology

Parameters	Control	LA 300 mg/kg	LA 2000 mg/kg	*F*-value	*P*-value
RBCs (10^{12}/L)	7.350 ± 0.187	7.616 ± 0.584	7.666 ± 0.216	1.232	0.320
WBCs (10^9/L)	3.883 ± 0.491	3.866 ± 1.237	3.533 ± 0.960	0.163	0.851
Platelets (10^9/L)	953.333 ± 68.677	865.000 ± 356.083	846.666 ± 154.749	1.091	0.361
Hb (g/dl)	13.716 ± 0.183	13.783 ± 0.523	13.833 ± 0.265	0.553	0.587
PCV (HCT%)	44.666 ± 1.210	36.833 ± 18.082	44.500 ± 1.378	0.393	0.682
MCV fL	52.333 ± 16.366	57.500 ± 2.345	57.333 ± 2.732	0.259	0.775
MCH pg	18.000 ± 0.894	17.666 ± 1.211	17.500 ± 0.836	0.377	0.692
MCHC g/dL	31.333 ± 0.816	31.500 ± 0.836	31.166 ± 0.752	0.260	0.775
Neutrophil (%)	24.000 ± 6.131	25.500 ± 7.582	32.500 ± 5.319	3.002	0.080
Lymphocyte (%)	64.000 ± 5.966	67.666 ± 7.004	61.833 ± 4.215	1.528	0.249
Monocytes (%)	4.333 ± 1.632	3.500 ± 1.760	3.500 ± 1.643	0.492	0.621
Eosinophils (%)	2.500 ± 0.547	2.333 ± 0.516	2.33 ± 0.516	0.200	0.821

3 Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA

4 (*$p < 0.05$) significant value

Effect of LA on clinical biochemistry

Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-

way ANOVA $^*p < 0.05)$ significant value

1   **Table 6**

2   Effect of LA on clinical biochemistry

Clinical biochemistry	Control	300 mg/kg	2000 mg/kg	*F-value*	*P-value*
Urea (mmol/L)	6.633 ± 1.040	6.966 ± 0.977	8.200 ± 1.240	3.427	0.059
Creatinine (Umol/L)	36.000 ± 4.898	28.833 ± 13.044	37.000 ± 5.656	1.579	0.239
Calcium (mmol/L	2.530 ± 0.062	2.500 ± 0.109	2.403 ± 0.095	3.126	0.073
Inorganic Phosphate (mmol/L)	3.195 ± 0.350	3.401 ± 0.642	3.900 ± 0.536	2.871	0.088
Uric Acid (mmol/L)	0.211 ± 0.290	0.106 ± 0.048	0.098 ± 0.041	0.813	0.462
Sodium (mmol/L)	145.00 ± 2.097	145.833 ± 1.471	145.333 ± 2.338	0.263	0.772
Potassium (mmol/L)	5.566 ± 0.163	5.900 ± 0.433	5.616 ± 0.248	2.105	0.156
Chloride(mmol/L)	98.500 ± 1.048	99.000 ± 2.449	100.500 ± 2.073	1.711	0.214
TC (mmol/L)	2.033 ± 0.436	2.300 ± 0.517	1.966 ± 0.307	1.012	0.387
HDL(mmol/L)	0.62 3± 0.082	0.640 ± 0.089	0.590 ± 0.098	0.474	0.632
LDL (mmol/L)	0.868 ± 0.198	0.933± 0.377	0.733 ± 0.398	0.550	0.588
TG (Umol/L)	1.783 ± 0.231	1.483 ± 0.487	1.116 ± 0.636	2.879	0.087
Total HDL Ratio	3.383 ± 0.231	3.516 ± 0.444	3.400 ± 0.236	0.309	0.739
Total protein (g/dL)	70.833 ± 2.926	71.500 ± 3.507	66.833 ± 3.816	3.236	0.068
Albumin (g/dL)	42.166 ± 4.445	44.500± 4.888	43.333 ± 3.076	0.461	0.639
Globulin(g/dL)	24.333 ± 1.211	25.333 ± 1.211	23.666 ± 0.816	3.519	0.056
A/G ratio	1.800 ± 0.894	1.800 ± 0.109	1.833 ± 0.121	0.192	0.827
AP (IU/L)	53.666 ± 5.750	57.166 ± 11.669	68.33 ± 25.453	1.293	0.303
SGOT /AST (IU/L)	165.166±10.703	200.166 ± 35.204	174.33 ± 18.250	3.315	0.056
SGPT /ALT(IU/L)	36.666 ± 1.966	36.000 ± 3.741	40.500 ± 19.065	0.278	0.761

3   Values are expressed as the mean ± standard deviation (n=6 for each group, female). One-way ANOVA

4   *$p < 0.05$) significant value

Figure 1

Study design
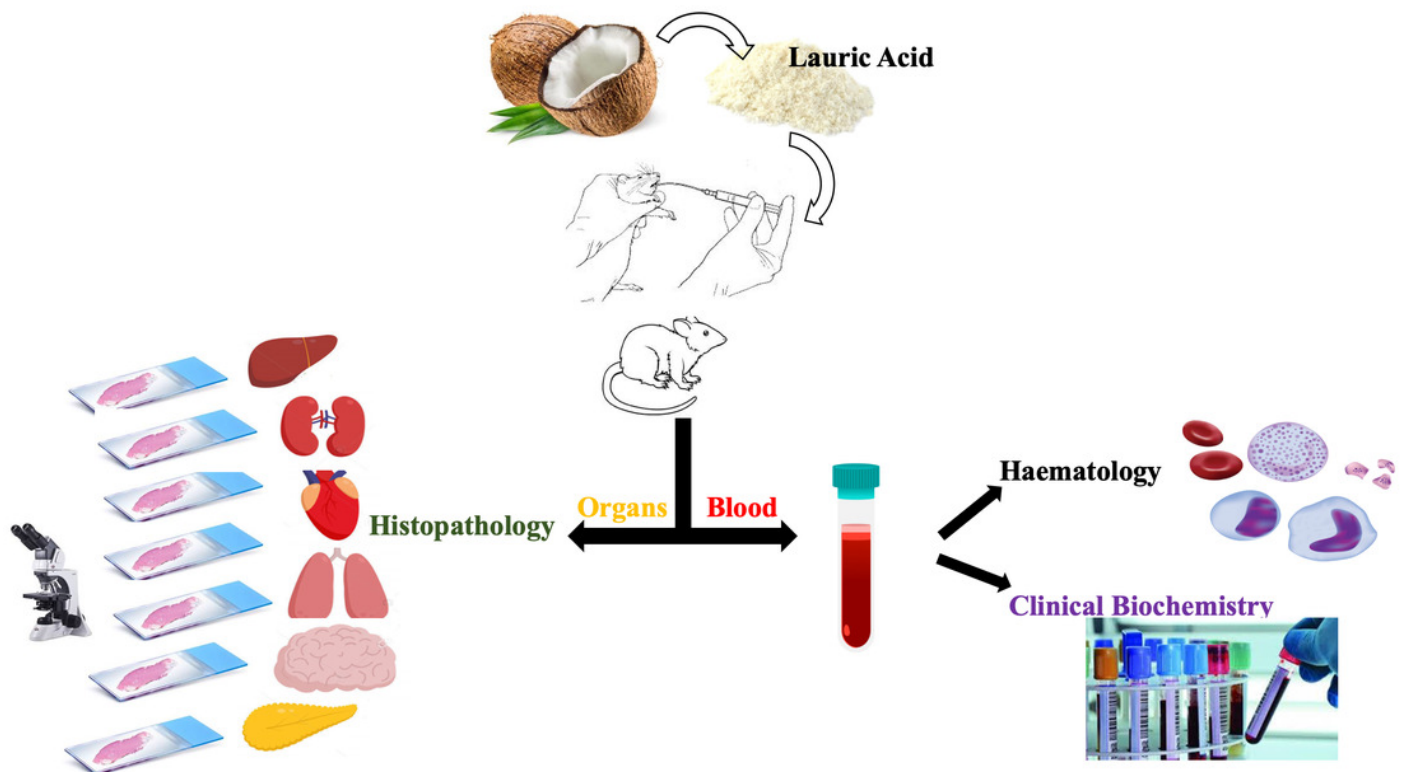
Figure 2

Macroscopic view of liver

Macroscopic view of liver tissues of female SD rats in acute oral toxicity study, LA treated animals presenting normal reddish colour closed to the normal control group, after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
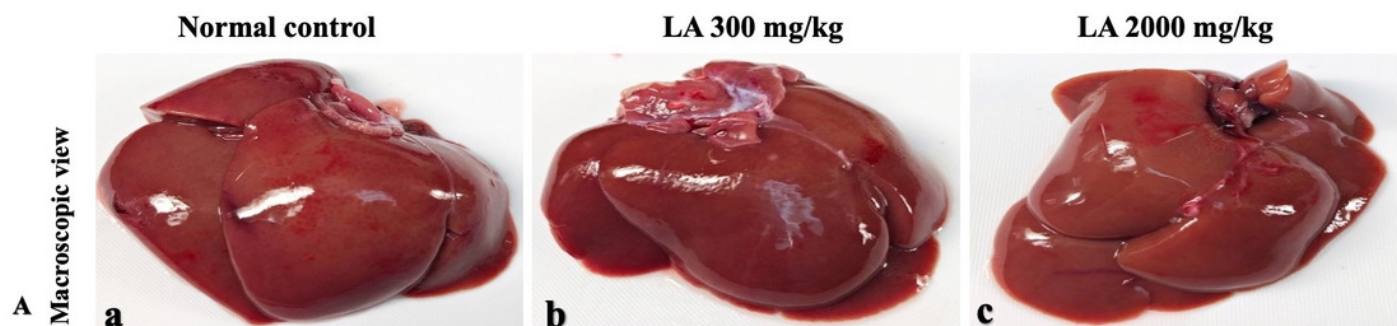
Figure 3

Microscopic analysis of liver

Microscopic analysis of liver tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg).H&E (A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40× original magnification
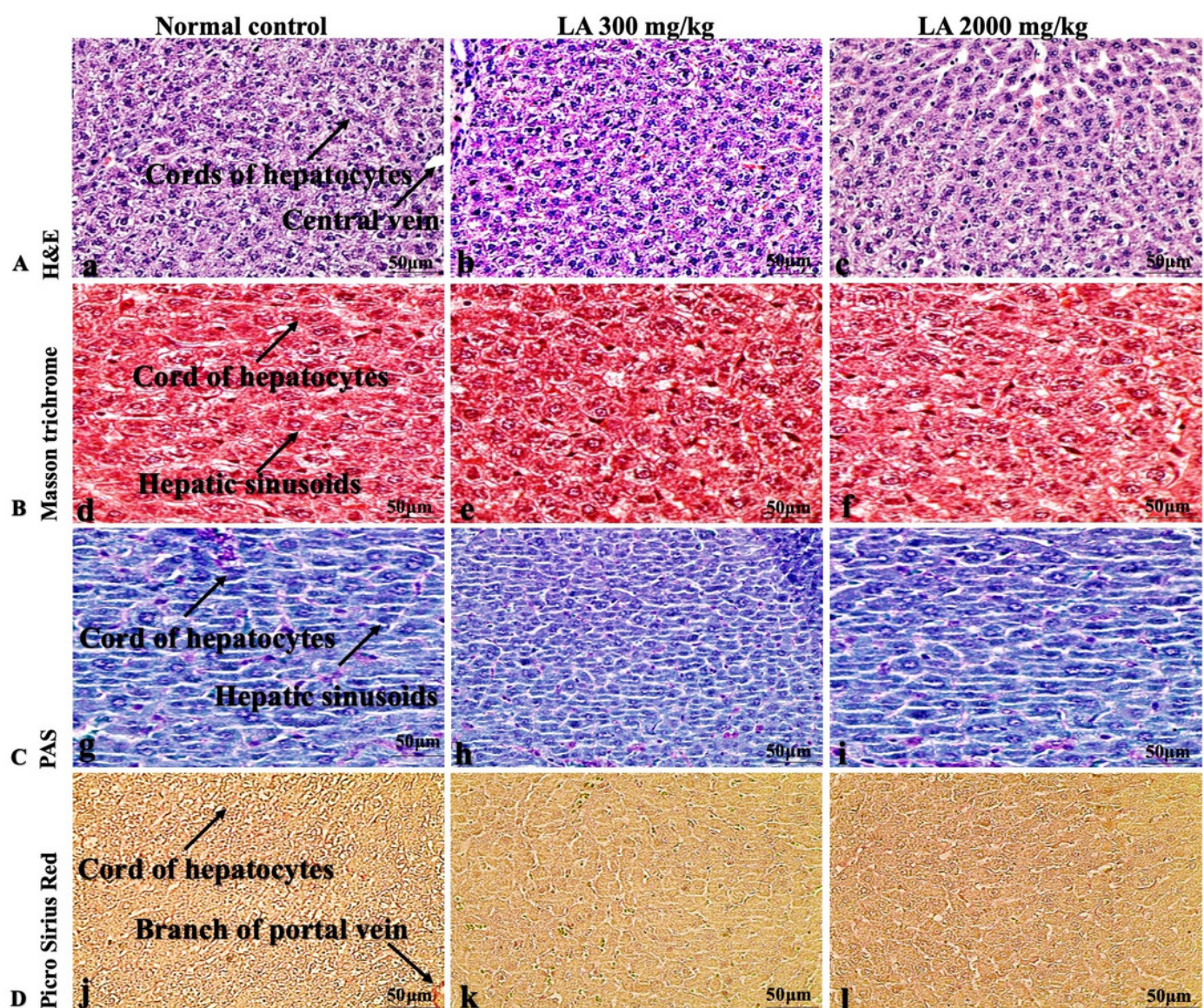
Figure 4

Macroscopic view of Kidney

Macroscopic view of kidneys of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
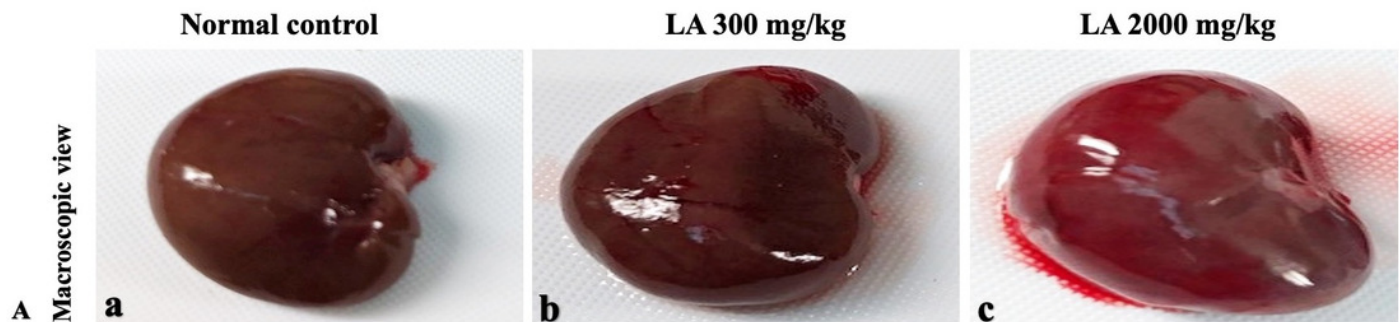
Figure 5

Microscopic analysis of kidney

Microscopic analysis of kidney tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E (A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40x original magnification.
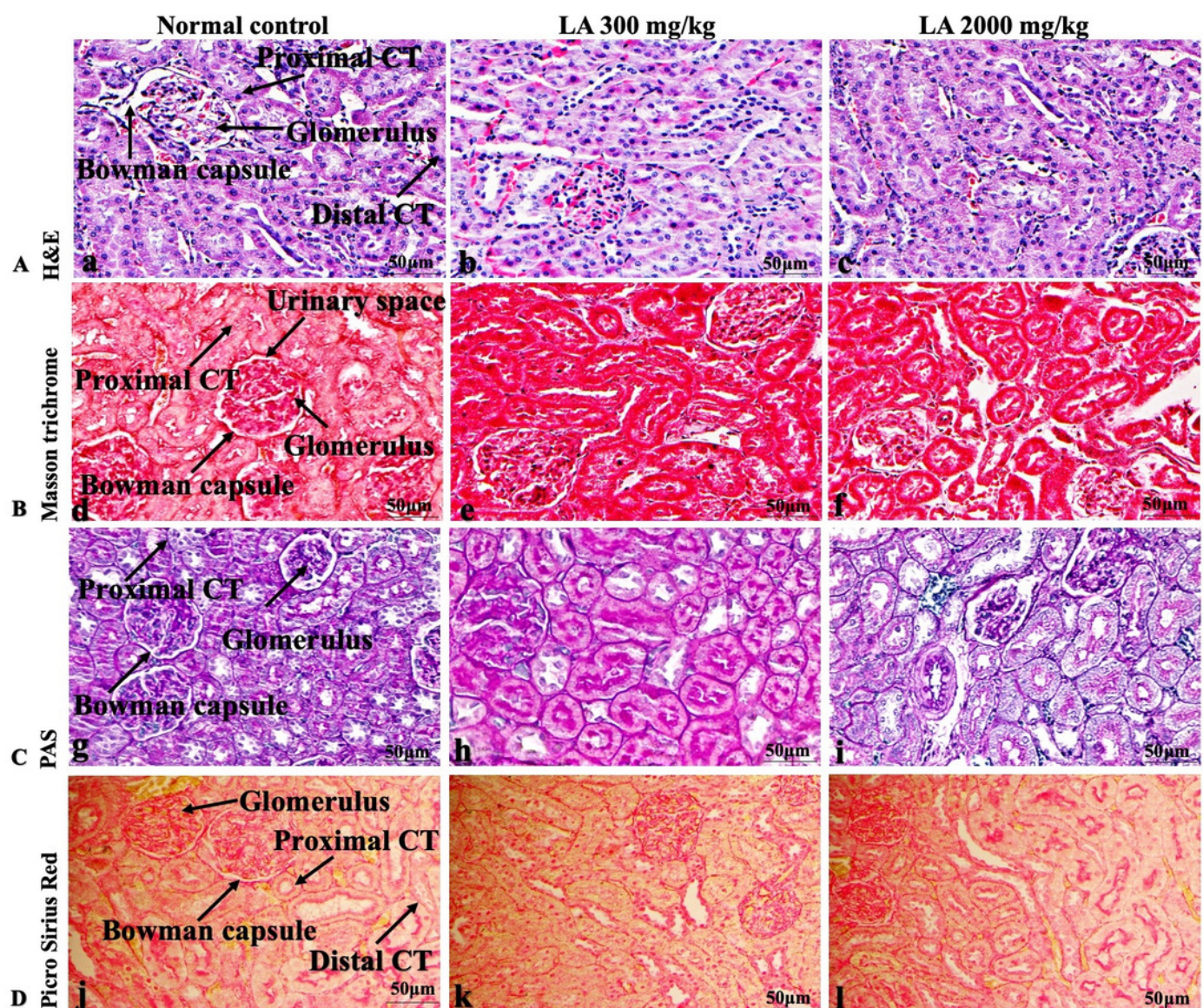
Figure 6

Macroscopic view of heart

Macroscopic view of heart of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
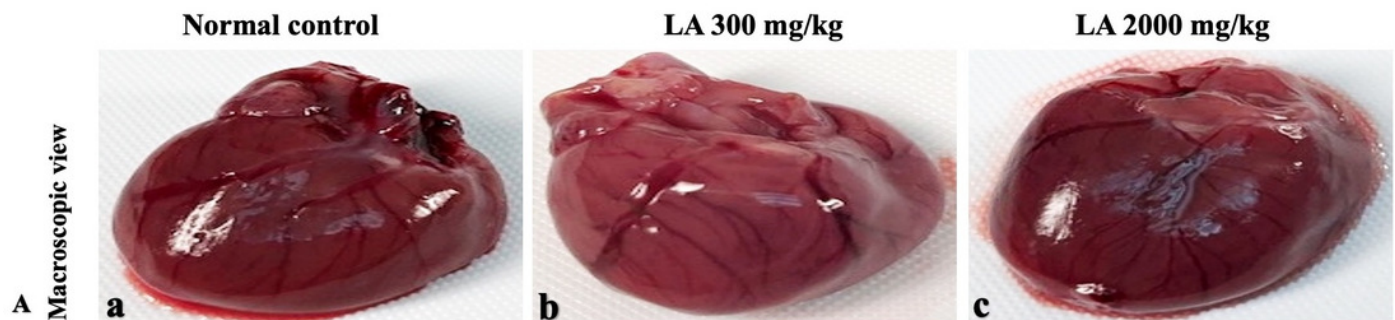
Figure 7

Microscopic analysis of heart

Microscopic analysis of heart tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E (A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40× original magnification.
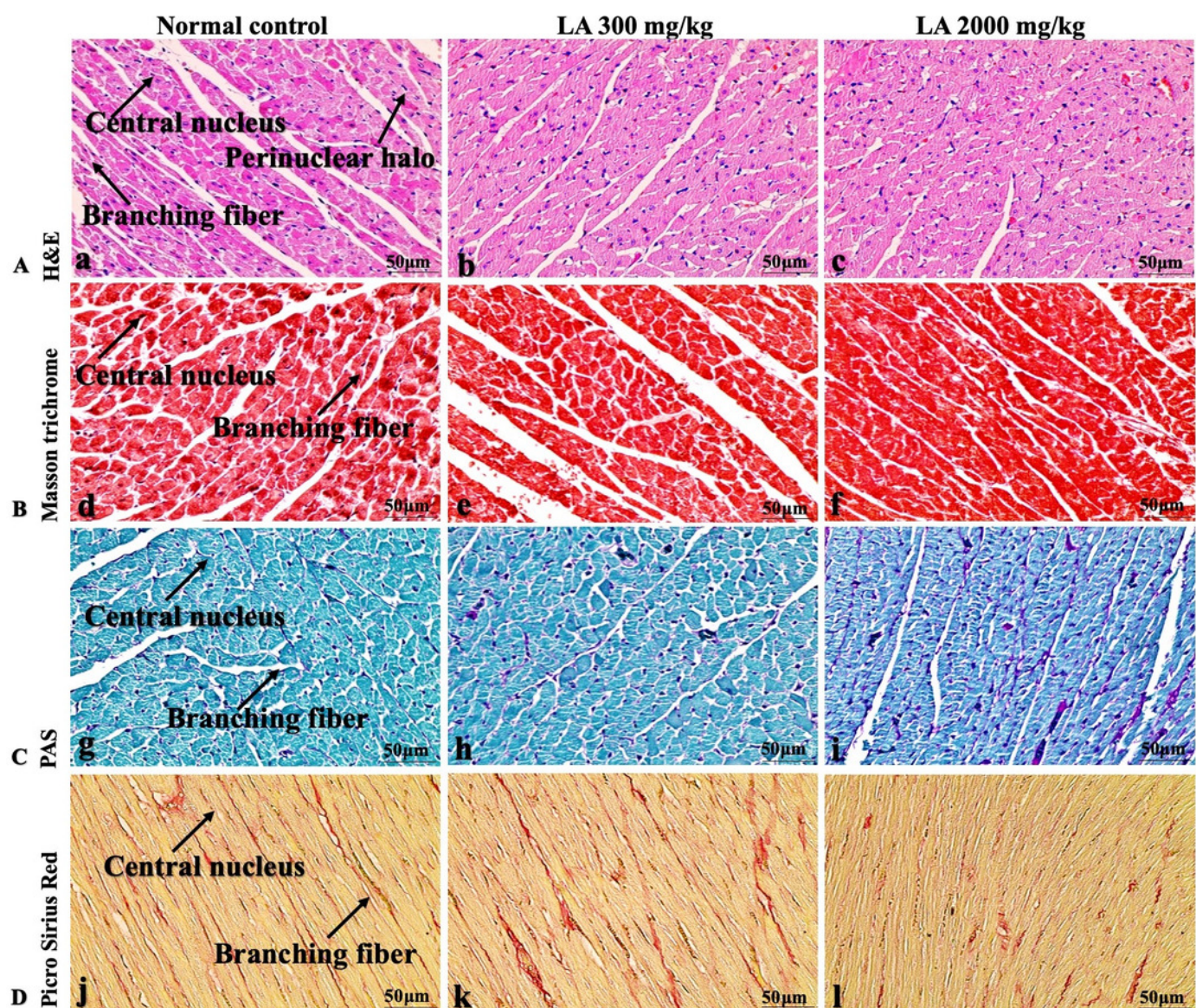
Figure 8

Macroscopic view of lungs

Macroscopic view of lungs of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
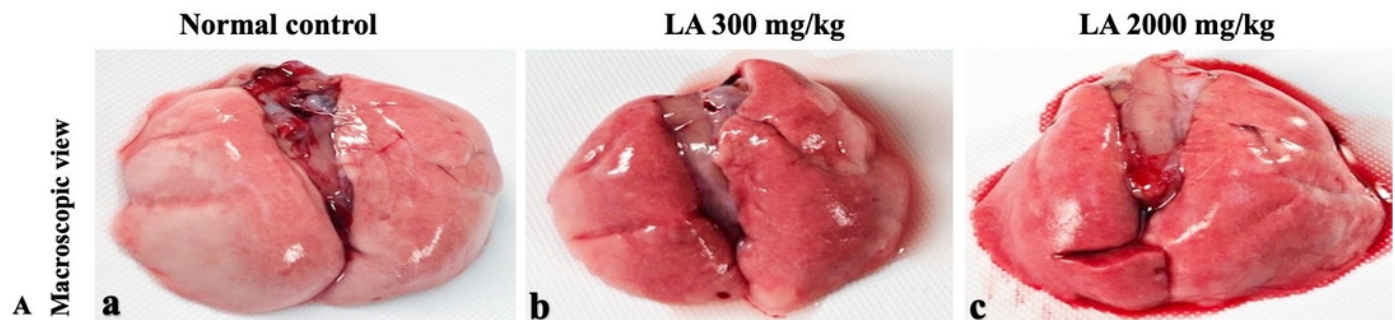
Figure 9

Microscopic analysis of lungs

Microscopic analysis of lungs tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40× original magnification.
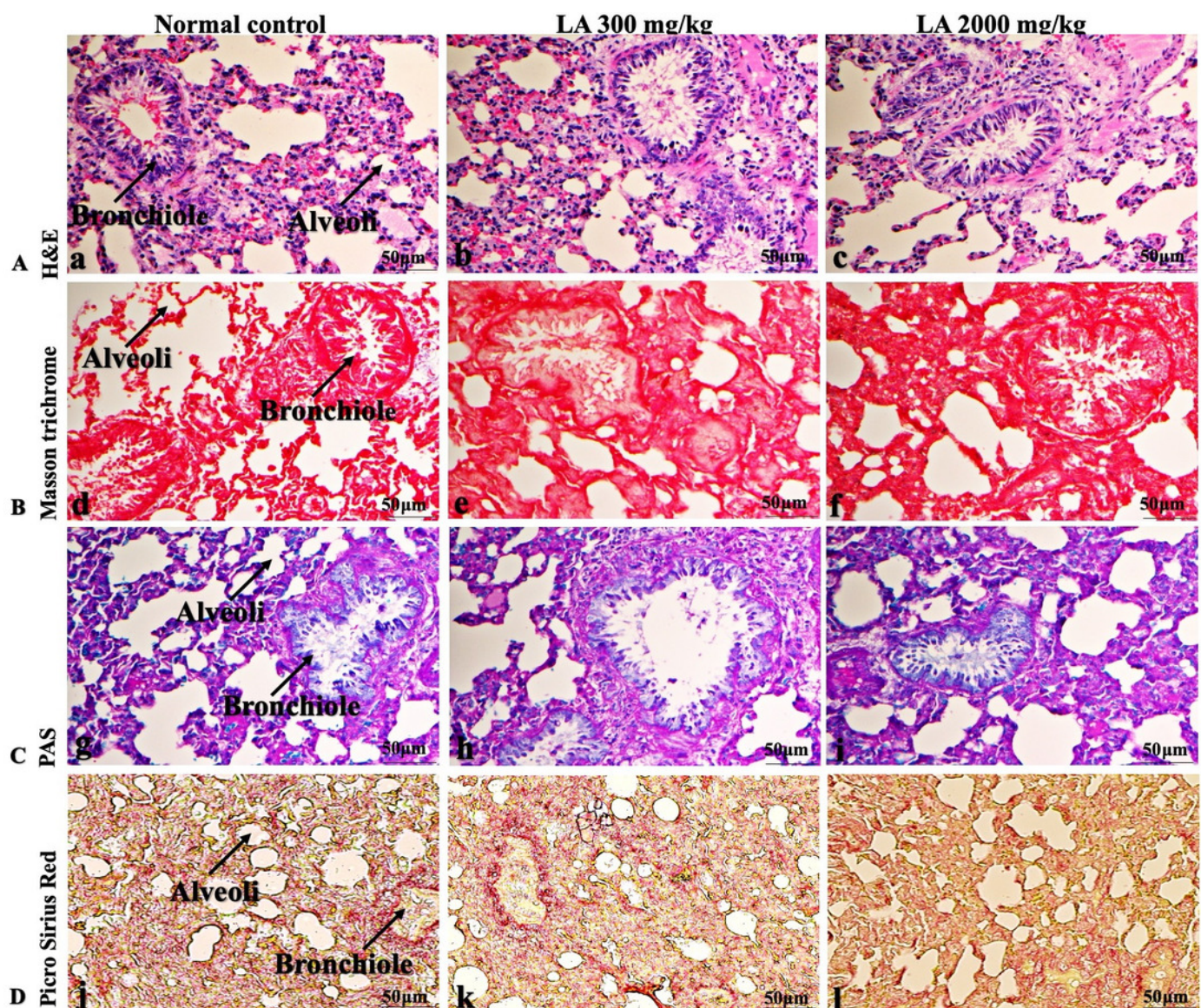
Figure 10

Macroscopic view of brain

Macroscopic view of brain of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
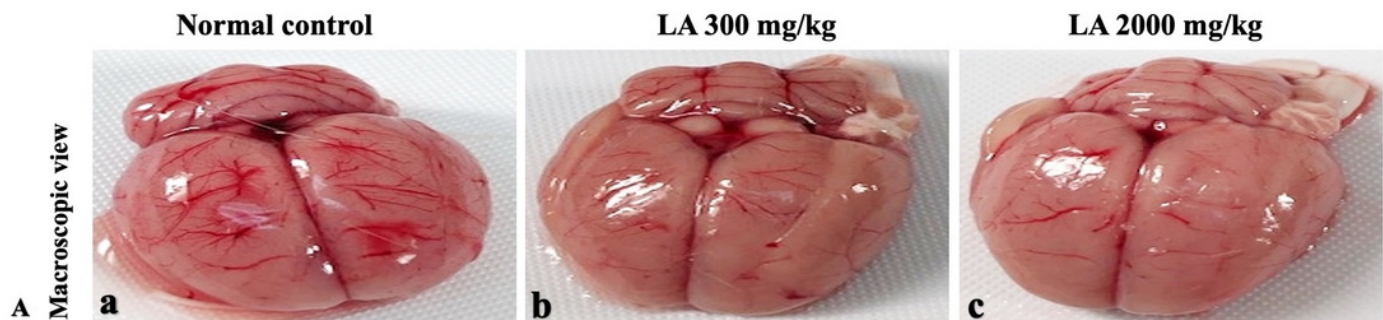
Figure 11

Microscopic analysis of brain

Microscopic analysis of brain tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40x original magnification.
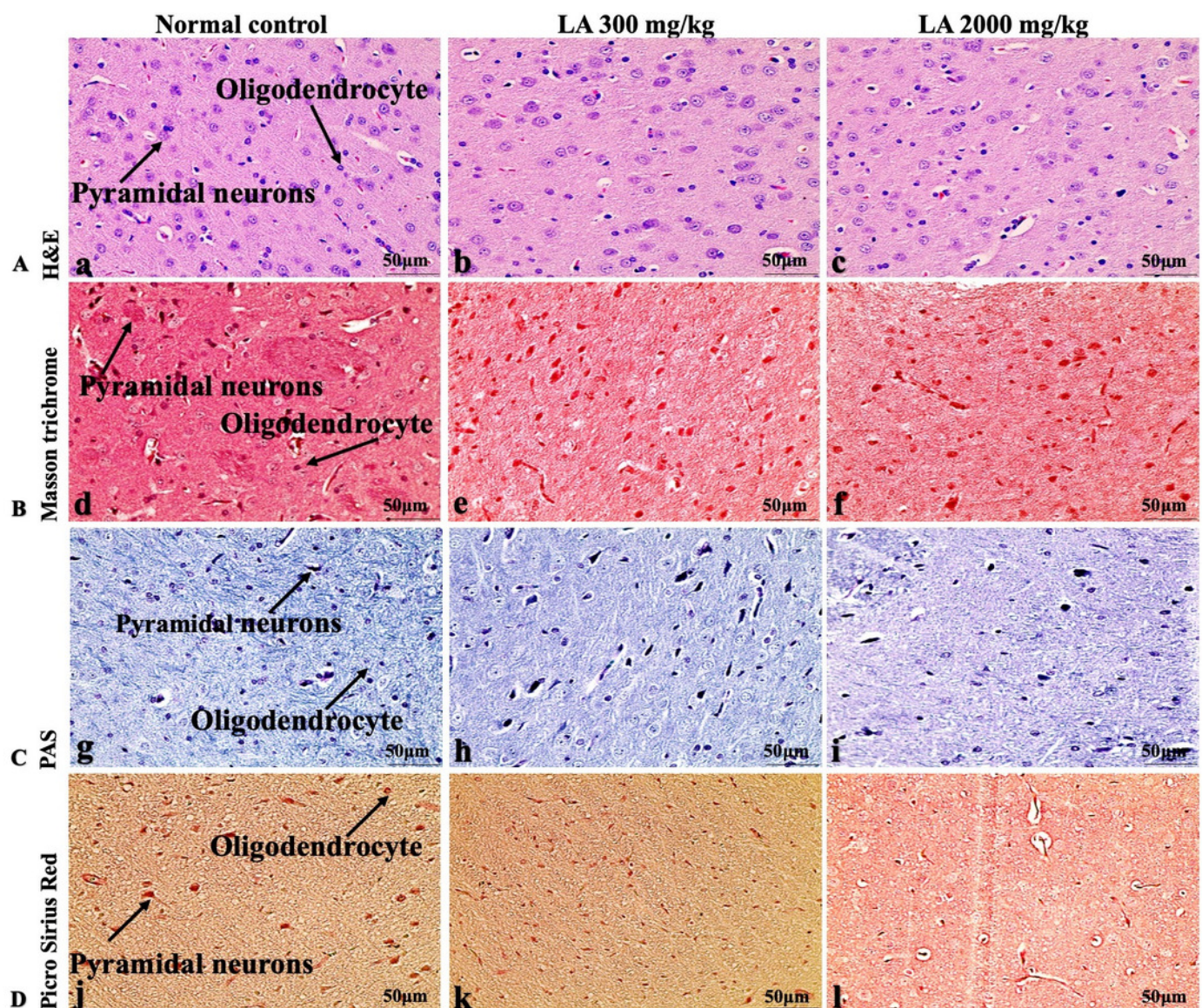
Figure 12

Macroscopic view of spleen

Macroscopic view of spleen of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
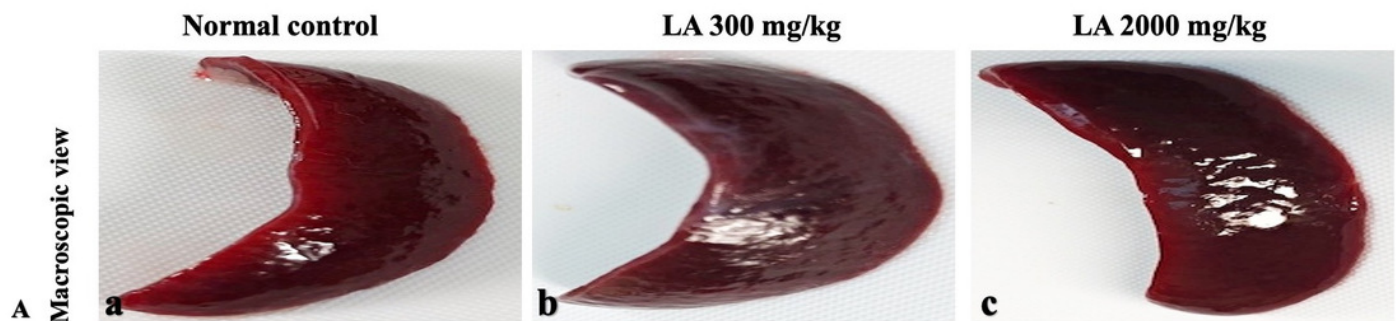
Figure 13

Microscopic analysis of spleen

Microscopic analysis of spleen tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40´ original magnification.
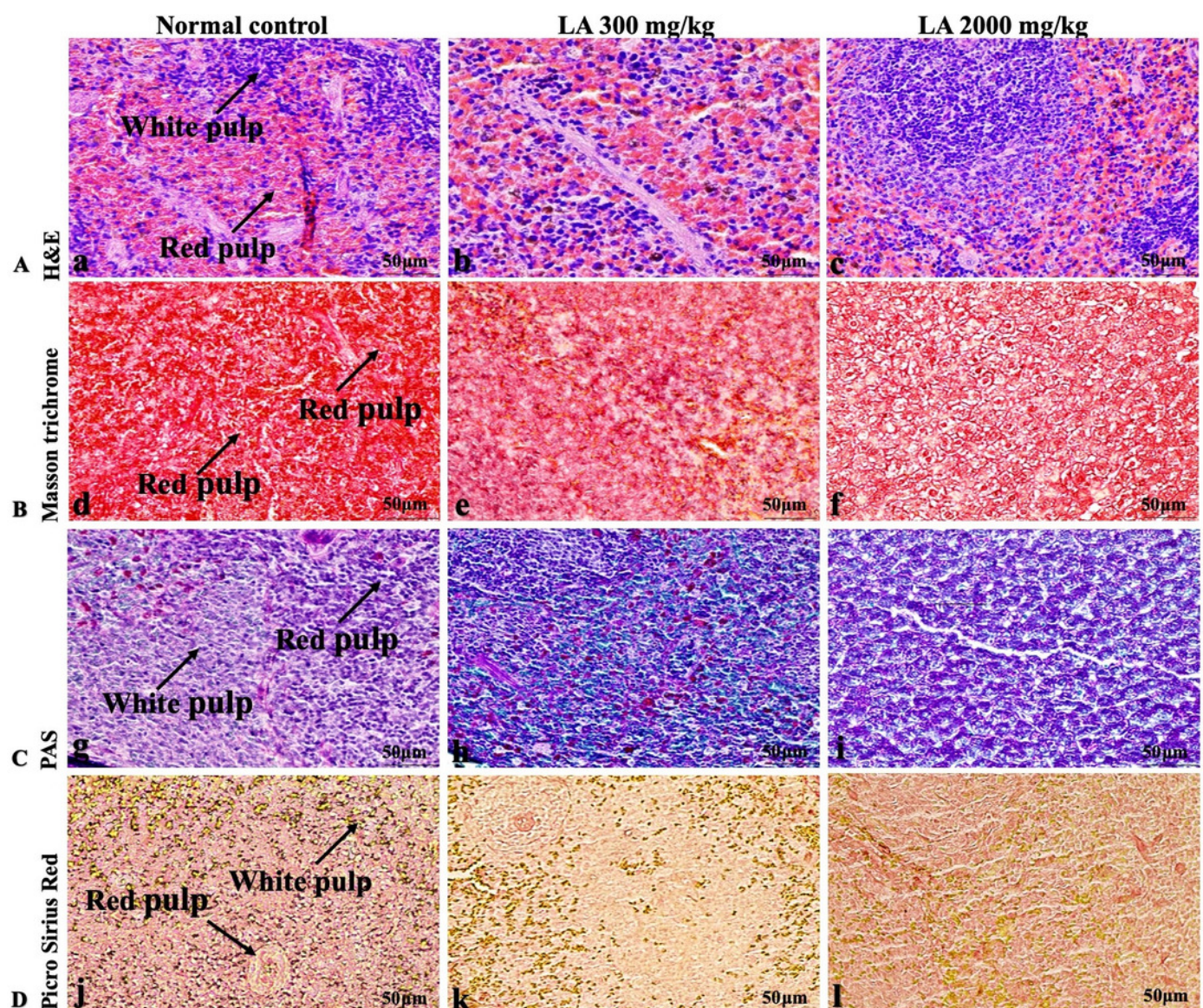
Figure 14

Macroscopic view of pancreas

Macroscopic view of pancreas of female SD rats in acute oral toxicity study, presenting normal shape and appearance after a single oral doses of LA (300 mg/kg and 2000 mg/kg).
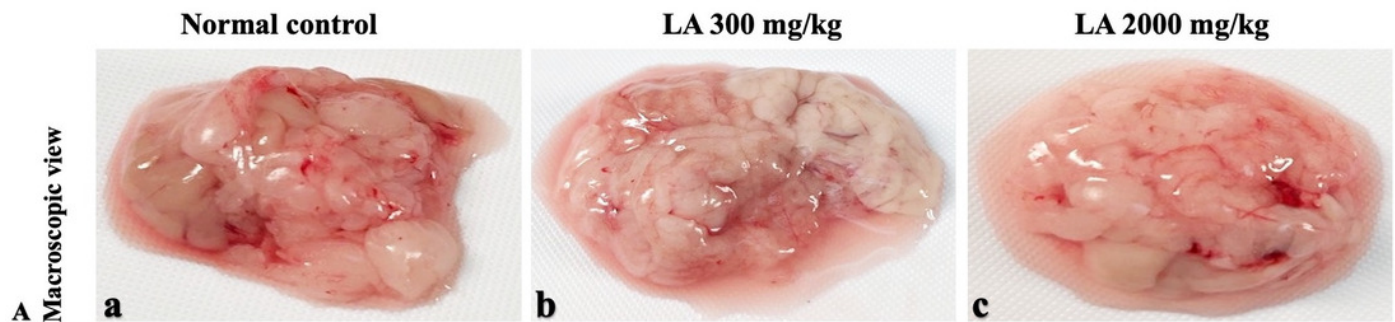
Figure 15

Microscopic analysis of pancreas

Microscopic analysis of pancreas tissues of female SD rats in acute oral toxicity study, presenting normal morphology after a single oral doses of LA (300 mg/kg and 2000 mg/kg). H&E A), Masson trichrome (B), PAS (C), and Picro Sirius Red (D). 40× original magnification.