

# Pharmacotoxicological screening on new derivatives of beta-phenylethylamine, potential agonists of beta3-adrenergic receptors

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## Abstract

**Background and Aims:** Beta3-adrenergic receptors (beta3-ARs) have been initially characterized in 1989. Afterwards, their tissue distribution was established: white and brown adipose tissue, central nervous system, myocardium (atrial and ventricular), blood vessels, smooth gastrointestinal muscles (stomach, small intestine, colon), gallbladder, urinary bladder, prostate, skeletal muscles. Non-clinical trials have demonstrated the major implication of beta3-ARs in glucose metabolism, implicitly, in insulin release, and also in obesity. Therefore, new compounds were synthesized starting from beta-phenylethylamine nucleus and substituted in various positions, for possible antidiabetic and/or antiobesity action. **Materials and Methods:** In the present research, the antidiabetic action of newly synthesized compounds was investigated on an experimental model of alloxan-induced diabetes, administered in dose of 130 mg/kg body weight (bw), intraperitoneally (i.p.). After 14 days of treatment, glycemia and enzymes involved in homeostasis of glucose metabolism, glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphatase (G6Pase) and hexokinase were determined. Animals were then euthanized and histopathology examinations were performed on harvested liver, kidney, spleen and brain in order to document pathological changes induced by alloxan-induced diabetes and/or by tested compounds. **Results and Conclusions:** Glycemia in animals treated with the tested compounds decreased statistically significant for groups C2 and C3 (-42.13% and -37.2%, respectively), compared to diabetic control group. C2 was also the compound to favorably modify the dynamics of determined enzymes, together with the display of very good safety profile supported by minor, non-significant, histopathological changes.

**Keywords:** beta3-adrenergic receptors, beta-phenylethylamine derivatives, alloxan-induced diabetes, histopathological changes.

## Introduction

Diabetes describes a group of metabolic diseases associated with a high morbidity and mortality. In a *World Health Organization* (WHO) statistic, the number of people with diabetes increased from 108 million in 1980 to 422 million in 2014, and a projection shows that diabetes will be the 7<sup>th</sup> leading cause of death in 2030 [1, 2].

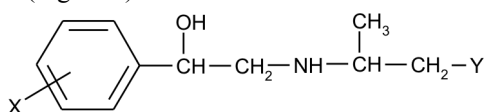
There are several approved drugs which can contribute to the reduction of high blood glucose in diabetic patients, none of them having a sympathomimetic mechanism of action. Several preclinical studies regarding compounds acting as agonists on beta-adrenergic receptors (beta3-ARs) proved some important metabolic effects, especially in the brown adipose tissue. This thermogenic tissue has the role of keeping constant body core temperature of small animals at cold ambient temperatures. Stimulation of beta3-ARs not only activates brown adipose tissue

thermogenesis in the short term, but also increases mitochondrial biogenesis and the expression of thermogenin, now known as uncoupling protein 1 (UCP1) [3–5]. This protein mediates transport across the internal mitochondrial membrane and interrupts oxidative phosphorylation of the beta-oxidation of fatty acids, increasing the use of energy [6]. The effects of beta3-adrenergic agonists on thermogenin have been investigated. The results have shown that beta3-adrenergic agonists activated thermogenin and also other uncoupling proteins (UCPs), such as UCP2, that is found in many tissues and UCP3 that is found in skeletal muscle and has an important role in basal thermogenesis [7].

The stimulation of thermogenesis by beta3-adrenergic agonists resulted in a number of experimental studies, which have shown that in animals, these substances lead to weight loss, a selective fat decrease, without reducing food intake [8].

Unfortunately, the human trials offered low confirmation for these preclinical results, the effects on lipolysis, metabolic rate and insulin sensitivity being modest. Many compounds proved to be non-selective beta-receptor agonists, causing unacceptable side effects such as tachycardia or tremor. It is the reason that new possible active compounds need to be full and highly selective beta<sub>3</sub>-adrenergic agonists, knowing now that the lipolysis in adipose tissue and thermogenesis are regulated by stimulation of these adrenergic receptors [9, 10].

Based on all these preclinical and clinical considerations, new chemical entities with beta-phenylethylamine nucleus, substituted in various positions on the nucleus or side chain, with potential action on diabetes and/or obesity were synthesized [11, 12]. Chemists led synthesis in order to obtain derivatives with increased β<sub>3</sub>-receptor selectivity. The compounds were conventionally named C1–C6 (Figure 1).



X: H; 3-chloro; 4-chloro; 2,3-dichloro; 3-methoxy.

Y: 4-carbopropoxy-phenoxy; 4-carbomethoxymethylene-phenoxy; 4-carbomethoxyethylene-phenoxy.

**Figure 1 – General structure of the newly synthesized compounds.**

## Materials and Methods

All experiments were carried out on male albino rats Wistar strain, 258±24.5 g average weight (*n*=200), obtained from the Animal Facility of “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania. They were housed eight per cage in a ventilated cage system, with a bedding of wood sawdust, under controlled light/dark cycle conditions (12 h light/12 h dark; lights on at 6:00 AM), with free access to water and food pellets (food was given twice daily: 8:00 AM, 5:00 PM). The animals were left to acclimate for three days, before starting the experimental procedures. The temperature ranged between 20–22°C and the relative humidity was maintained at 35–45%.

### Alloxan-induced diabetes

Type 2 diabetes (T2D) was induced by intraperitoneal (i.p.) administration of a single dose of Alloxan 130 mg/kg. Alloxan (Alloxan monohydrate, Sigma Aldrich, Germany) was dissolved in saline (0.9% sodium chloride, pH 7). We used a 13% solution, so each animal received 0.1 mL/100 g volume. Forty-eight hours following Alloxan administration, blood glucose was determined using Accu-Chek Active kit (Roche Diagnostics GmbH, Mannheim, Germany). Blood was obtained from tail vein through puncture.

### Study design

Animals with a level of blood glucose over 200 mg/dL were distributed in seven groups (eight animals/group). In addition, a control group (*n*=8) with normal levels of blood glucose (non-diabetic), was formed.

The groups received for 14 days:

- Group 1 – C1 20 mg/kg body weight (bw), 0.2% solution;

- Group 2 – C2 50 mg/kg bw, 0.5% suspension;
- Group 3 – C3 100 mg/kg bw, 1% suspension;
- Group 4 – C4 100 mg/kg bw, 1% suspension;
- Group 5 – C5 100 mg/kg bw, 1% suspension;
- Group 6 – C6 100 mg/kg bw, 1% suspension;
- Group 7 (diabetic control) – distilled water (1 mL/100 g bw);
- Group 8 (non-diabetic control) – distilled water (1 mL/100 g bw).

Administration was made using a single dose, at 8:30 AM, before feeding. At the end of the experimental period, rats were fasted for 12 hours, and then sacrificed after one hour and 30 minutes following administration, by cervical decapitation. Fasting blood samples were collected from the sacrificed animals in tubes with heparin as well as several organs (liver, kidney, brain, spleen).

The blood was used to determine blood glucose levels. Samples of liver tissue were used for assessing glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphatase (G6Pase) and hexokinase activity, three enzymes involved in glucose metabolism.

The rest of liver samples, the kidneys, brain and spleen were submitted to histopathological examination.

The selection of doses was based on the LD<sub>50</sub> (lethal dose, 50%) determined in mice and taking into consideration a value of the therapeutic index over 10 [13]. These doses were also used in previous stages of our pharmacological studies regarding the effect of the above-mentioned substances on the baseline blood glucose level [13].

### Blood glucose determination

Blood glucose was determined using an enzymatic assay based on the glucose oxidase activity. The glucose is oxidized to gluconic acid and H<sub>2</sub>O<sub>2</sub> is generated. In the presence of 4-aminophenazone and phenol, H<sub>2</sub>O<sub>2</sub> forms a complex, which can be determined colorimetrically, using the semi-automatic chemistry analyzer Cormay Multi Biochemical [14].

### Enzyme assessment

#### Liver homogenate preparation

Liver samples were minced and homogenized (10%, w/v), separately, in ice-cold 0.9% sodium chloride (NaCl) (1 g tissue/3 mL solution), sodium phosphate buffer (pH 7.4) in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 12 000 rotations for 20 minutes, at 4°C, and the resultant supernatant was used for different enzyme assays.

#### Assessment of G6PD activity

G6PD catalyzes the conversion of glucose 6-phosphate to 6-phosphogluconolactone. Briefly, the conversion of nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) to nicotinamide adenine dinucleotide phosphate reduced form (NADPH), catalyzed by the dehydrogenase enzyme was measured by the increase of absorbance at 340 nm. Data are expressed in arbitrary absorption units [14].

#### Assessment of G6Pase activity

To determine the activity of glucose-6-phosphatase, inorganic phosphate formed after the enzymatic reaction

is determined by reaction with ammonium molybdate; phosphomolybdate ammonium treated with a solution of reducing agent (1-amino-2-naphthol-4-sulphonic acid or eikonogen, in the presence of  $\text{NaHSO}_3$  and  $\text{Na}_2\text{SO}_3$ ), it is converted to molybdenum blue (combination of  $\text{Mo}_2\text{O}_5$  with  $\text{MoO}_3$ ). The color intensity is directly proportional to the concentration of phosphate and reflects the catalytic activity of the enzyme [14].

#### Assessment of hexokinase activity

To determine the activity of the liver hexokinase, we assessed  $\text{NAD}^+$  reduction to  $\text{NADH} + \text{H}^+$ . This leads to a change in optical density ( $\Delta \text{OD}$ ), which is a measure of the enzymatic activity. The activity of G6PD, G6Pase and that of the hexokinase within liver homogenate was expressed per mg of soluble protein (as determined by the Lowry method). The degree of dilution of the homogenate of the liver is 4 [15]:

$$\text{Enzymatic activity} = (\Delta \text{OD} \times \text{homogenate degree of dilution}) / \text{protein concentration [mg/mL]}$$

#### Histological technique

Tissues were fixed using 10% formalin solution and embedded in paraffin. Staining was carried out using Hematoxylin and Eosin (HE). Blades analysis was performed at the Department of Pathology, University of Agronomic Sciences and Veterinary Medicine, Bucharest, Romania.

#### Ethics rules

The experiment was conducted in accordance with Directive 86/609/EEC of November 24, 1986 and with the Order No. 143/April 1, 2002 (Ministry of Agriculture, Food and Forestry), Order No. 400/May 20, 2002 (Ministry of Water and Environmental Protection) and Order No. 84/August 30, 2005 (National Sanitary Veterinary and Food Safety Authority – ANSVSA), on the protection of animals for research purposes.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). The type of distribution of the animal response was established with D'Agostino & Pearson test. Data are reported as means  $\pm$  SEM (standard error of the mean) and were analyzed statistically using ANOVA (analysis of variance), followed by Dunnett's *post-hoc* test (normal distribution). Comparison was made vs. diabetic and non-diabetic control groups for all determined parameters (blood glucose and enzymes). *P*-values of 0.05 or less were considered to be significant.

### Results

#### Diabetes induction

T2D was induced in 54.5% of the animals, following Alloxan administration in dose of 130 mg/kg (Figure 2). The average blood glucose level of the collectivity was  $395.872 \pm 186.812$  mg/dL. 13.5% animals died after 48 hours after Alloxan administration. 32% of the animals

presented a blood glucose level  $<200$  mg/dL and were not included into the experimental groups.

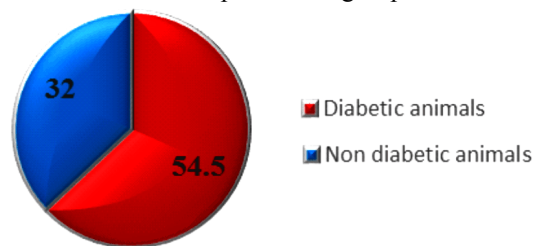


Figure 2 – Diabetic vs. non-diabetic animals, following Alloxan administration (130 mg/kg bw, i.p.).

Alloxan toxicity led to a progressive decrease in body weight for the administered rats as it follows: 4.84%, 24 hours after administration, respectively 7.5% after 48 hours from Alloxan administration.

#### Blood glucose assessment

Mean blood glucose levels for all experimental groups are presented in Figure 3. The blood glucose level of the animals treated with the experimental compounds (Figure 3) decreased in a statistically significant manner for groups receiving C2 and C3 (-42.13%, respectively -37.2%) vs. diabetic control group. For groups treated with C1, C4, C5 or C6, the decrease in blood glucose level ranges 8.21–19.72%. Groups receiving C2 or C3 have a blood glucose level similar to that of non-diabetic control group (Figure 4).

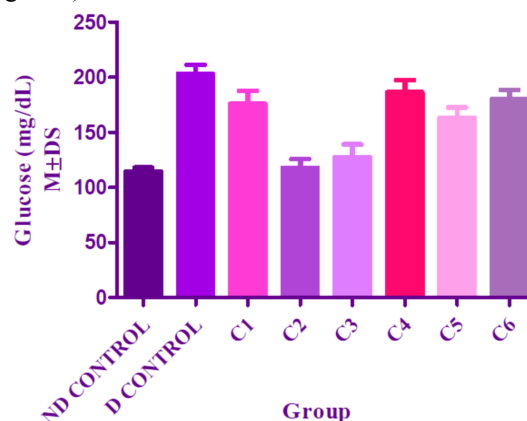


Figure 3 – Mean blood glucose levels for all experimental groups. ND control: Non-diabetic control group; D control: Diabetic control group; C1–C6: Groups treated with tested compounds C1, C2, C3, C4, C5 or C6.

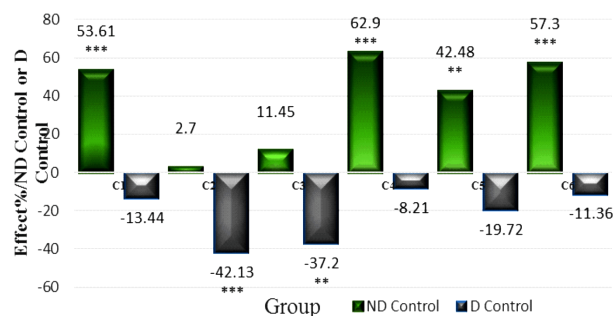
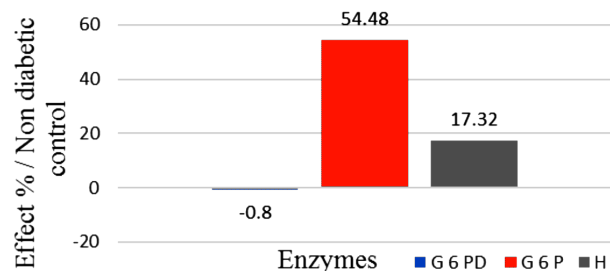


Figure 4 – Change in blood glucose levels for groups treated with tested compounds vs. non-diabetic (ND) control group and diabetic (D) control group. \**p*<0.05, \*\**p*<0.001, \*\*\**p*<0.0001.

### Enzymatic activity assessment

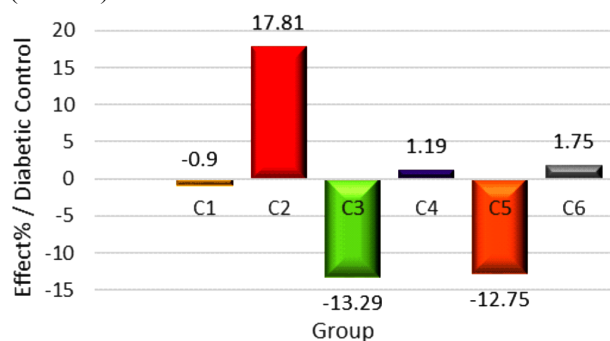
Alloxan-induced diabetes led to changes (Figure 5) in the activity of the three enzymes we assessed, enzymes involved in regulating carbohydrate metabolism:

- A reduction in the activity of G6PD activity, enzyme involved in metabolizing glucose on pentose phosphate pathway;
- An increase in the activity of G6Pase, the enzyme which catalyzes the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate;
- A reduction in the activity of hexokinase, the enzyme involved in glucose phosphorylation.



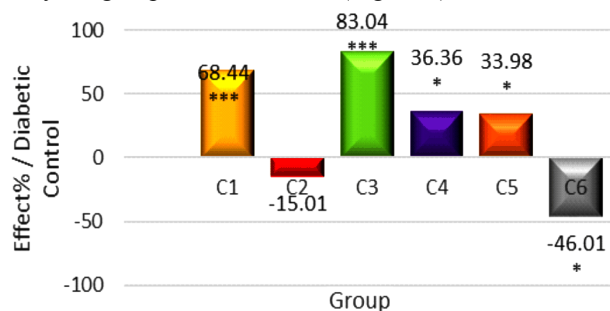
**Figure 5 – Changes in enzymatic activity for G6PD, G6Pase and hexokinase for diabetic animals vs. non-diabetic control group.**

According to data from literature [16], the activity of G6PD is reduced in diabetes. Only compounds C2, C4 and C6 determined an increase in this enzyme activity (Figure 6), the maximal effect being noticed for C2 (17.81%).



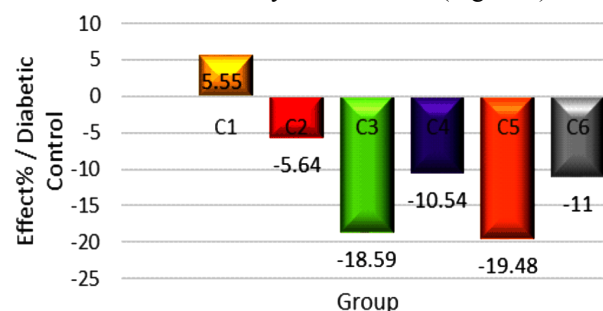
**Figure 6 – Change in the activity of G6PD for groups receiving tested compounds vs. diabetic control group.**

Same literature data support the hypothesis that the activity of G6Pase increases in diabetes [16]. Both compounds C2 and C6 reduce the activity of this enzyme vs. diabetic control group, but this effect is significant only for group treated with C6 (Figure 7).



**Figure 7 – Change in the activity of G6Pase for groups receiving tested compounds vs. diabetic control group.**  
\* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ .

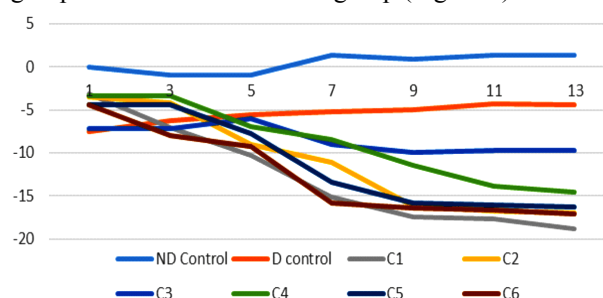
Ansari *et al.* (1993) proved that the reduction of blood glucose level leads to the decrease of hexokinase activity [16]. This effect (a reduction of hexokinase activity) is noticed for compounds C2, C3, C4, C5, C6, with a maximum efficacy of C3 and C5 (Figure 8).



**Figure 8 – Change in the activity of hexokinase for groups receiving tested compounds vs. diabetic control group.**

### Effect of tested compounds on body weight

Data from literature reveals the reduction of body weight in obese rats, after treatment with compounds having a  $\beta$ -phenylethylamine ring-containing structure, possible agonists of the beta3-ARs. Our research has shown that all tested compounds reduced progressively the body weight of treated groups vs. non-diabetic control group and vs. diabetic control group (Figure 9).



**Figure 9 – Body weight evolution for animals from non-diabetic control, diabetic control and for the groups treated with the tested compounds C1, C2, C3, C4, C5, C6.**

### Histopathological examination

For non-diabetic control group, no significant changes were detected in the liver (Figure 10A). A discrete glomerular and interstitial stasis accompanied by the partial degeneration of epithelial tubules and focal epithelial desquamation was observed for the kidneys (Figure 10B). No obvious changes were seen in the brain, although there were noticed a discrete inflammatory meningeal process and oxyphilia for some isolated neurons (Figure 10C). No changes were seen in the spleen.

For diabetic control group, degeneration of hepatocytes was noticed in some regions (cellular edema) as well as mononuclear cell infiltration in one microregion (Figure 11A). Focal hyalinosis of the epithelium could mean a glomerular filtration defect (no obvious morphological changes in structure glomerulus were noticed). Microregions with mononuclear cell infiltration were observed in the parenchyma and within the medulla, in



the tubule lumen, hyaline and cellular cylinders were formed (Figure 11B). The brain had no obvious changes, presenting rare contracted neurons, oxyphilic or basophilic, and neuronal necrosis (Figure 11C). No changes were observed for the spleen.

For the group treated with C1, no significant changes were noticed. A slight mononuclear cell infiltration was seen in portobiliary space (Figure 12A). No changes were detected in the brain, but several meningeal microregions with inflammation and hemorrhage were noticed (Figure 12B). In the kidneys, glomerular and interstitial stasis as well as some regions of oxyphilia were seen in the epithelium of renal tubules (degeneration/incipient necrosis) (Figure 12, C and D). No pathological changes were observed for the spleen.

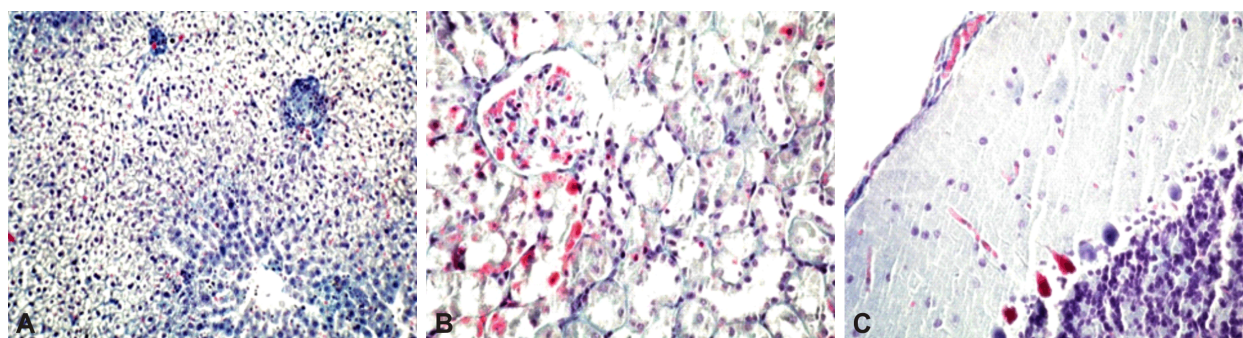
For the animals receiving C2, the liver presented a general normal appearance (Figure 13A), and in the kidney a reduced glomerular and interstitial stasis were noticed (Figure 13B). The brain presented no changes (Figure 13C) with basophilic neurons and an appearance of neuronal necrosis (although no typical necrosis reactions were seen, which suggest a transient phenomenon). Moderate stasis was noticed in the spleen (Figure 13D).

For the group receiving C3, focal, discrete stasis was noticed in liver tissue (Figure 14A). Discrete glomerular stasis (Figure 14B) and degeneration and desquamation of the renal tubules epithelium (aspect of toxic tubular necrosis) was noticed. No changes were observed in the brain tissue, but isolated microregions with hemorrhage were noticed (Figure 14C). Stasis was observed in the spleen (Figure 14D).

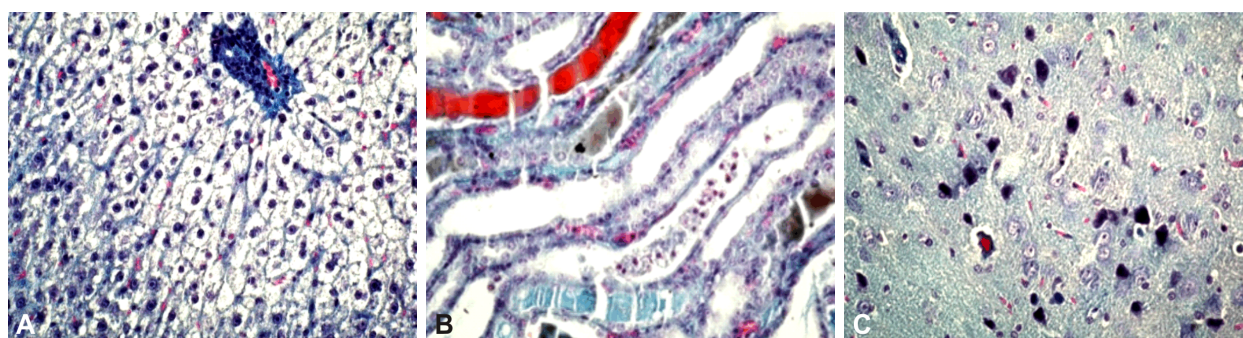
No pathological changes in the liver were noticed for the group treated with C4. C4 has renal toxicity, pathological changes such as: severe glomerular stasis, oxyphilia, necrosis, desquamation of tubules epithelium, interstitial mononuclear cell infiltration, being noticed (Figure 15, A–C). Tubular lesions indicate acute toxic tubular necrosis. The overall appearance of the brain was unchanged (Figure 15D). One region of neuronal necrosis, with glial nodules, activated pericytes and perivascular cuffs was identified (non-suppurative focal encephalitis appearance). The spleen presented no pathological changes.

For the group treated with C5, the liver did not show any significant changes, but in the kidneys, we found: interstitial and glomerular stasis, degeneration, focal necrosis of the epithelium of the renal tubules, mononuclear cell infiltration region – aspect of tubulointerstitial acute nephropathy (Figure 16, A and B). In the brain tissue, we found perivascular cuffing, satellitosis, neuronal necrosis and non-suppurative focal encephalitis (Figure 16, C and D). No changes were seen in the spleen.

For the group receiving C6, generalized hepatic steatosis with centroacinar localization was observed, as well as a slight stasis within the centrolobular vein and in the portobiliary space (Figure 17A). Hyperemia and glomerular stasis, degeneration of the epithelium of renal tubules and regions of steatosis were observed in the kidneys (Figure 17B). No changes were detected in the brain, but there appeared several microregions with hemorrhage (Figure 17C). Stasis was seen in the spleen, but the general appearance was unchanged (Figure 17D).

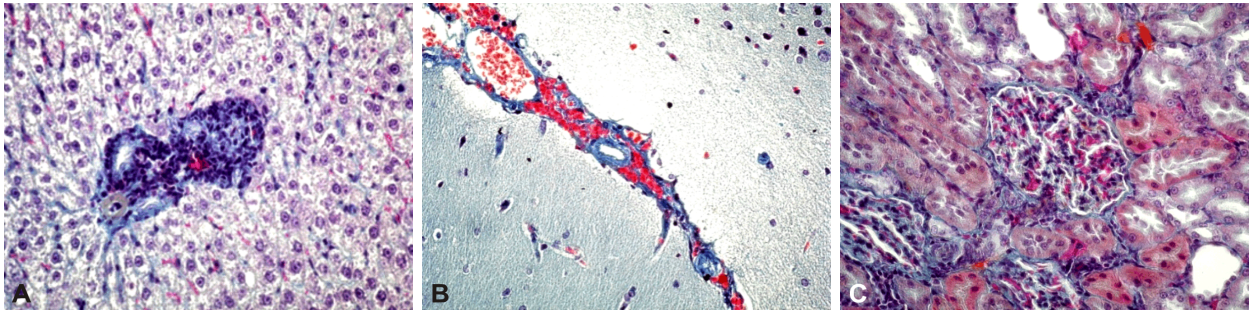


**Figure 10 – (A) Liver – hepatocytes hypertrophy and cytoplasmic vacuolation; (B) Kidneys, glomerular hypertrophy – partial desquamation of the renal tubules; (C) Brain tissue – discrete inflammatory meningeal process, isolated necrotic neurons. HE staining: (A)  $\times 200$ ; (B and C)  $\times 40$ .**

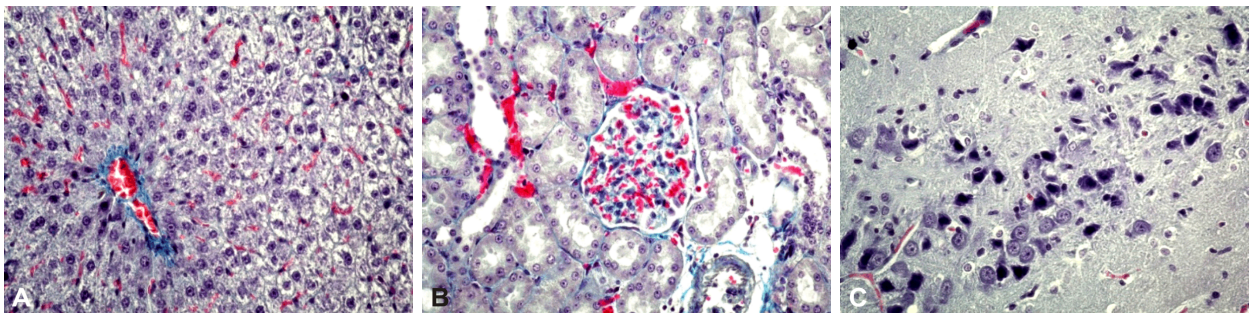


**Figure 11 – (A) Liver – mononuclear cell infiltration in portobiliary space; (B) Kidneys – cellular desquamation and hyalinosis within the medulla, in the tubules; (C) Brain – rare neuronal necrosis. HE staining: (A and C)  $\times 400$ ; (B)  $\times 40$ .**

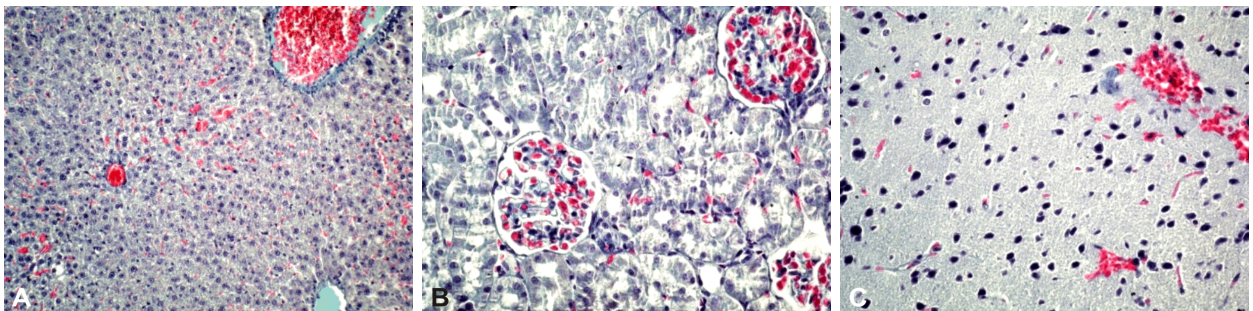




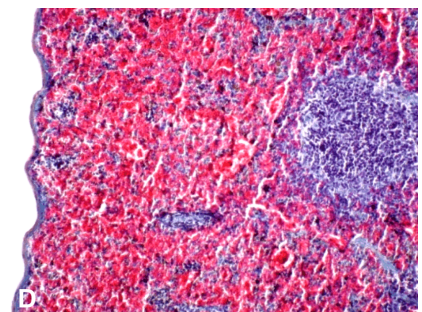
**Figure 12 – (A) Liver – slight mononuclear cell infiltration in portobiliary space; (B) Brain – discrete hemorrhage; (C) Kidneys – focal tubules nephrosis, glomerular stasis; (D) Kidneys – interstitial stasis. HE staining,  $\times 400$ .**



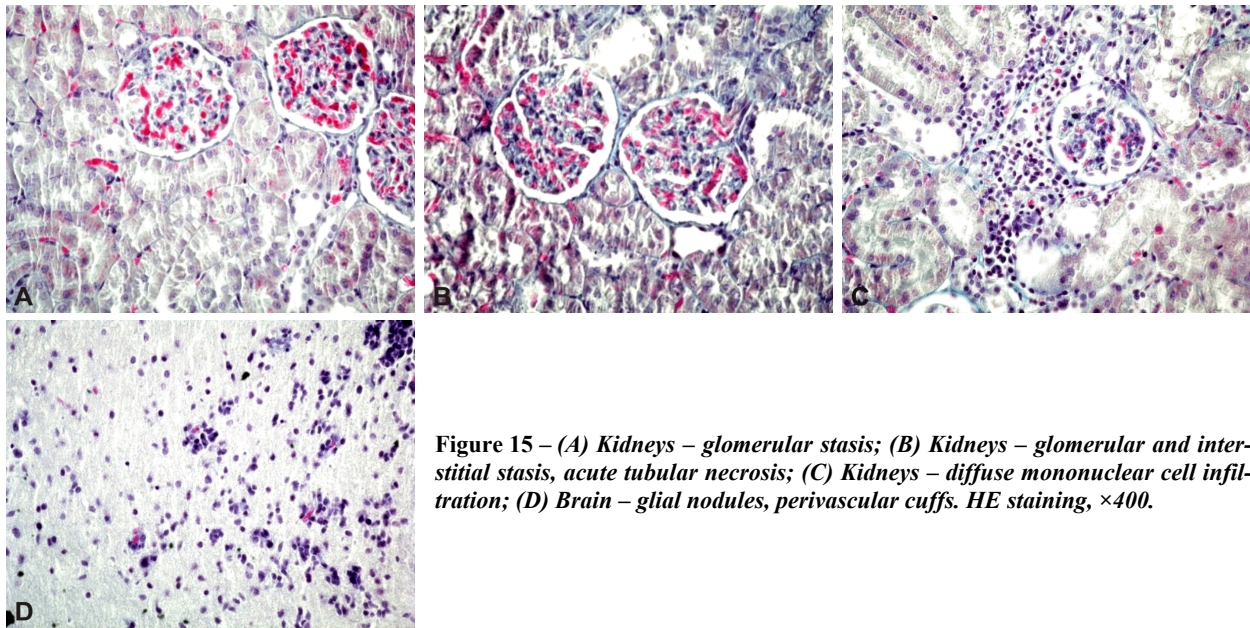
**Figure 13 – (A) Liver – reduced regions with degenerated hepatocytes; (B) Kidneys – slight stasis; (C) Brain – basophilic neurons; (D) Spleen – stasis. HE staining: (A and D)  $\times 200$ ; (B and C)  $\times 400$ .**



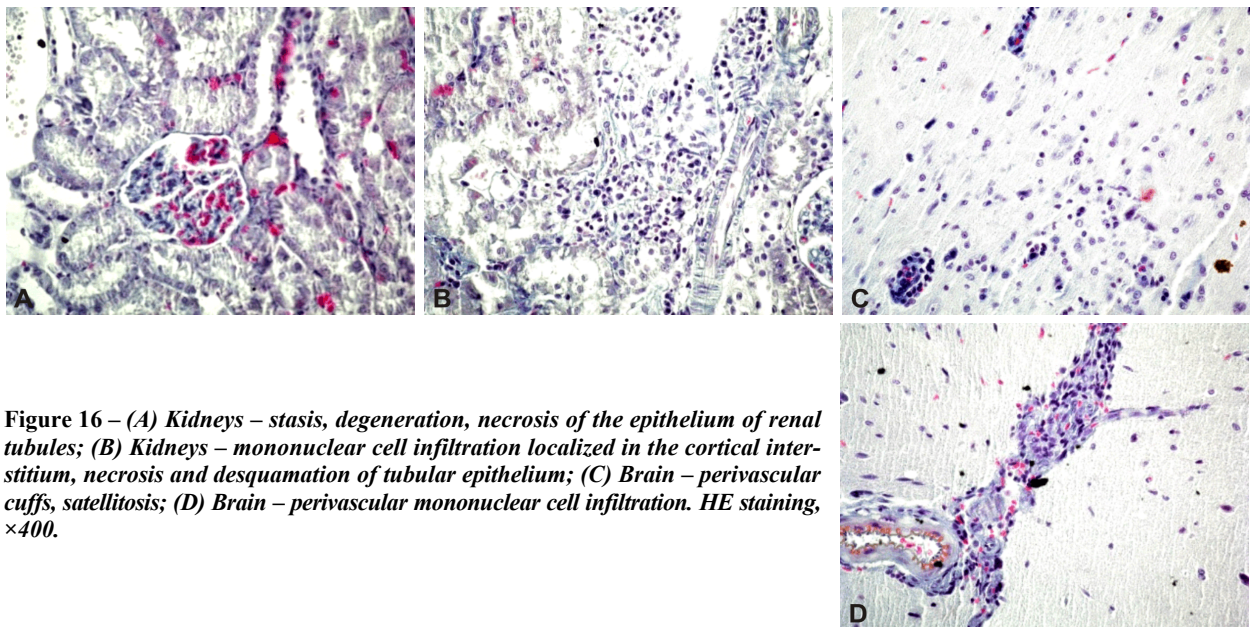
**Figure 14 – (A) Liver – focal discrete stasis; (B) Kidneys – necrosis and desquamation of the renal epithelium; (C) Brain – small region with hemorrhage; (D) Spleen, stasis. HE staining: (A and D)  $\times 200$ ; (B and C)  $\times 400$ .**



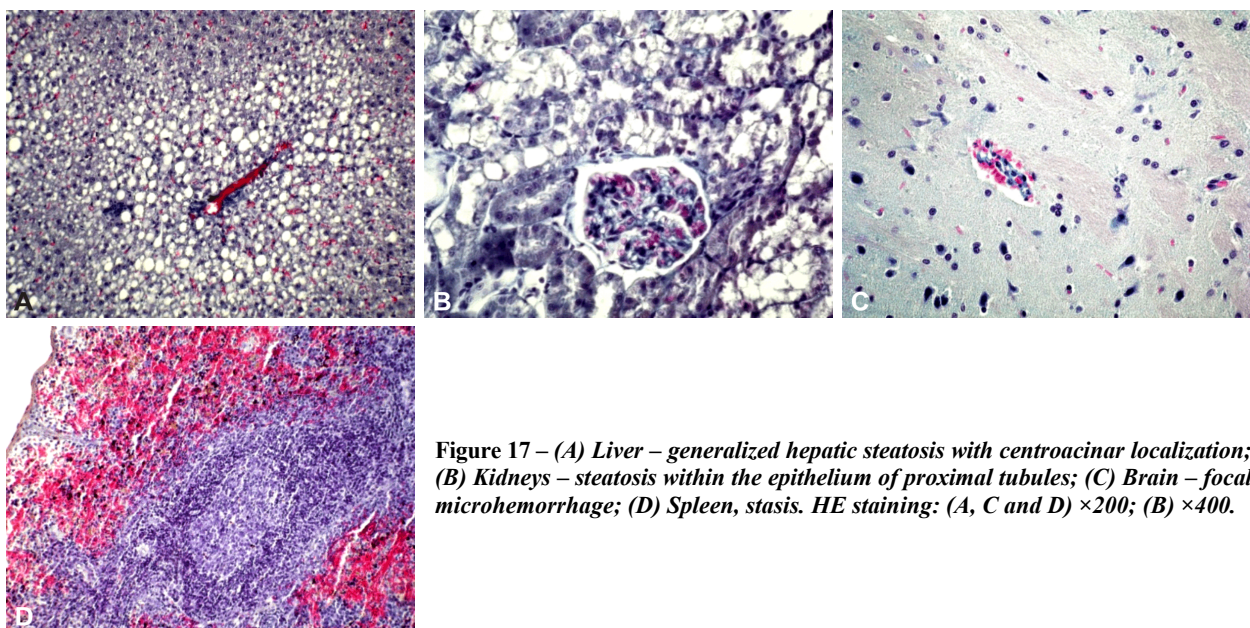




**Figure 15 – (A) Kidneys – glomerular stasis; (B) Kidneys – glomerular and interstitial stasis, acute tubular necrosis; (C) Kidneys – diffuse mononuclear cell infiltration; (D) Brain – glial nodules, perivascular cuffs. HE staining, ×400.**



**Figure 16 – (A) Kidneys – stasis, degeneration, necrosis of the epithelium of renal tubules; (B) Kidneys – mononuclear cell infiltration localized in the cortical interstitium, necrosis and desquamation of tubular epithelium; (C) Brain – perivascular cuffs, satellitosis; (D) Brain – perivascular mononuclear cell infiltration. HE staining, ×400.**



**Figure 17 – (A) Liver – generalized hepatic steatosis with centroacinar localization; (B) Kidneys – steatosis within the epithelium of proximal tubules; (C) Brain – focal microhemorrhage; (D) Spleen, stasis. HE staining: (A, C and D) ×200; (B) ×400.**

## Discussion

### Diabetes induction

Alloxan is a diabetogenic agent currently used in animal models of diabetes mellitus [17]. The first tests regarding the mechanism of action of Alloxan were performed *in vitro* on isolated islet cells [18] and perfused rat pancreas [19]. *In vivo* studies demonstrated that Alloxan is readily absorbed by beta pancreatic cells, effect contributing to diabetogenic action. This action determined by the formation of oxygen reactive species [20]. The formation of reactive oxygen species is preceded by Alloxan reduction, the substance manifesting an increased affinity for substrates that include –SH groups, like reduced glutathione, cysteine, proteins with sulphide groups [21].

One of the targets for oxygen reactive species is the deoxyribonucleic acid (DNA) of the pancreatic islet cells, in which fragmentation of DNA occurs when beta cells are exposed to Alloxan [22, 23]. The DNA alteration stimulates poly(DNA-ribose), enzyme which contributes to affected DNA repair. Some studies suggest that glucose administration help counteracting the cytotoxicity of Alloxan, probably by a dual mechanism: glucokinase protection and interaction with glucose transporter GLUT2 resulting in reduced absorption of alloxan [24].

At the dose that used in our experiment (130 mg/kg bw i.p.), the pancreatic toxic effect for Alloxan was manifest in 54.5% of the treated animals [25], phenomenon signaled also in other literature data, which states that diabetes does not occur in all of the animals following administration of the toxic [26, 27]. These trials also highlight the decrease in body weight for the animals following toxic administration, which is in concordance with our findings [27].

### Blood glucose and enzymatic activity

Of all tested compounds, only C2 and C3 resulted in a statistically significant reduction of glycemia for the diabetic animals, after 14 days of treatment. The blood glucose level approached the values noticed in the animals from control non-diabetic group.

The results obtained for these substances are concordant with those from other non-clinical trials in the literature which showed that beta3-agonist's administration have reduced plasma concentrations of glucose and lipids in diabetic mice of genetically-modified strains (kk, C57BL/KsJ-db/db) or in rats with experimentally induced diabetes [28, 29].

Several mechanisms [30] were suggested for the anti-diabetic effect of these compounds:

- reduced insulin resistance;
- increased tissue response to hormone;
- increased release of insulin in beta pancreatic cells [31];
- reduced glucose liver release;
- increased non-insulin-dependent re-uptake of glucose in white and brown adipose tissues and in skeletal muscles [32];
- increased glucose tolerance [33].

Antidiabetic activity was low and statistically non-significant for the compounds C1, C4, C5 and C6, probably because of the affinity of these compounds for other adrenergic receptors.

The administration of Alloxan has altered the activity of the enzymes, which ensure the balance of glucose metabolism: G6PD, G6Pase and hexokinase. The common point for these enzymes is glucose-6-phosphate. Glucose-5-phosphate is a biomolecule with central role in glucose metabolism. Its biosynthesis and transformation is catalyzed by hexokinase, G6Pase, respectively, G6PD, the metabolic balance being hormonally controlled by insulin.

G6PD is involved in glucose transformation on pentose-phosphate pathway, which generates NADPH reduced equivalents, having the role of controlling the speed of the process. Literature mentions that hyperglycemia is an inhibiting factor for G6PD activity and that biguanides administration activates the enzyme in experimental models of insulin-resistance syndrome [34–36]. Experimental researches reported a reduction in G6PD activity after five weeks from diabetes induction, in laboratory animals with Streptozotocin-induced diabetes. Moreover, administration of antidiabetic sulphonamides determined the stimulation of the enzymatic activity, thus enforcing antioxidative defense mechanisms [36, 37].

The results of the present research concur with literature data that Alloxan-induced diabetes decreases the activity of this enzyme. Of all tested compounds, C2 has significantly increased, after two weeks of administration, the activity of the enzyme (17.81%), suggesting both pancreatic (in accordance with previous data regarding sulphonamides activity) and also extra-pancreatic (effects observed in previous non-clinical trials for biguanides) antidiabetic action mechanisms.

Hexokinase is the enzyme which catalyzes glucose phosphorylation, an irreversible reaction which consumes adenosine triphosphate (ATP) and initiates glucose transformation both by aerobic and anaerobic pathways in order to generate the necessary cellular energy. Insulin induces hexokinase activity, one of the mechanisms through which the pancreatic hormone manifests its hypoglycemic action.

The functioning of enzymatic system hexokinase–G6Pase is hormonally controlled. When blood glucose concentration increases, the activity of hexokinase (insulin induced) increases in parallel with activity of glycogen synthase activated by G6Pase, thus determining glycogen synthesis. Reduced blood glucose concentration determines the activation of G6Pase and reduces the activity of hexokinase, thus releasing glucose. Experimental work demonstrated that, in laboratory diabetic animals (Alloxan- or Streptozotocin-induced diabetes), significant reductions in activity of hexokinase and G6PD were observed [36, 38]. Results of our research highlighted that in Alloxan-induced diabetes group the activity of hexokinase was by 17.32% when compared to non-diabetic control group.

Several non-clinical trials [16] have underlined that the decrease of blood glucose concentration determines the reduction of hexokinase activity. The present research show a reduction in enzymatic activity compared to diabetic control group for the compounds C2, C3, C4, C5 and C6, the maximal effect of reducing hexokinase activity being registered for C3 (-18.59%) and C5 (-19.48%). A similar but smaller effect was observed for C2 (-5.64%).



In the liver, G6Pase releases glucose from its phosphorylated form, glucose passes in the blood stream and ensures the energy needs of the glucose-dependent tissues. Under insulin action, the activity of the enzyme is inhibited. Streptozotocin-induced diabetic animals have shown an increased activity of G6Pase activity in several non-clinical experiments. The same effect was noticed after Alloxan administration, the activity of the enzyme being increased by 54.48%. Other experimental trials, using Streptozotocin-induced diabetic animals, have shown that the administration of plant extracts with possible anti-diabetic effect have determined a reduction of blood glucose level by inhibiting G6Pase and stimulating G6PD activity. On the other hand, Umbelliferone and Glibenclamide administration decreases the activity of G6Pase while it increases the activity of hexokinase and G6PD in laboratory animals with Streptozotocin-induced diabetes [36, 39]. Out of the tested compounds, it was only C2 and C6, which decreased G6Pase activity by 15.01%, and 46.01%, respectively. These results demonstrate altogether the inhibition of glucose release into blood stream.

All the tested compounds have decreased the body weight of the treated animals, phenomenon quoted in literature and supported by beta3-ARs involvement in thermogenesis stimulation [40]. Antiobesity effect was proven in different obesity models, using rodent species [41, 42] of normal or genetically modified strains (with intended obesity predisposition). Ferrer-Lorente *et al.* (2005) [43] have demonstrated that in Wistar rat, the agonist of beta3-ARs have readily increased energy consumption by thermogenesis stimulation in deep adipose tissue. The same effect was reported by Souza *et al.* (1997) [26] in non-obese non-diabetic Sprague–Dawley rats with selective beta3-agonist CL 316 243, which induced increase in food consumption, metabolic rate and body temperature after seven days of treatment. The present trial also underlined the decrease in intra-abdominal and subepithelial fat tissue. It was shown using rat strains with different genetic predispositions to obesity [male eight weeks Osborne Mendel (OM) and S5B/P1 (S5B)] that beta3-agonist CL 316 243 significantly reduces the food intake in both animal strains.

Our results confirmed that the newly synthesized compounds with beta-phenylethylamine core, designed to have an affinity for beta3-ARs, possess an effect of body weight reduction for the animals with experimentally induced diabetes.

### Histopathological results

Histopathological examinations have revealed for the diabetic control group a toxic alteration of the liver, manifested as degeneration of the hepatocytes and small areas of mononuclear cell infiltration, explained in literature by local absorption of Alloxan, which determines toxic alterations [44]. It has also induced renal pathological changes by local toxic mechanism manifested as degeneration of the epithelium of renal tubes. These changes resemble to focal acute tubular necrosis. In the brain, no evident changes from Alloxan were noticed besides contracted neurons (oxyphilic or basophilic) and partial neuronal necrosis.

For all groups treated with the tested compounds, renal toxicity was recorded but it was concluded that it

is not exclusively the effect of the compounds' toxic activity, but also that of the Alloxan used to induce diabetes.

Compounds C2, C3, C4, and C5 have protected the animals against Alloxan-induced liver toxicity, the organ being generally unaltered. Exception was made for C6, which triggered a generalized hepatic steatosis with centroacinar localization.

No significant changes of the brain tissue were observed for the animals receiving the tested compounds, similar to the animals from the diabetic control group.

### Conclusions

By correlating C1–C6 modified enzymatic activities with decreased glycemia (indicative of antidiabetic effect), it was concluded that the most active compound was C2 (20 mg/kg), with the following effects when compared to diabetic control: decreased glycemia by 42.13% ( $p < 0.0001$ ); increased activity of G6PD by 17.81% ( $p > 0.05$ ); decreased activity for G6Pase by 15.01% ( $p > 0.05$ ); decreased activity for hexokinase by 5.64%. Together with low systemic toxicity demonstrated by the histopathological exam, C2 compound should be submitted to a future detailed research in order to pinpoint the mechanisms for the strongly documented antidiabetic actions.

### Conflict of interests

The authors declare that they have no conflict of interests.

### Author contribution

Simona Negreş and Anca Zanzfirescu equally contributed to the manuscript.

### Acknowledgments

The paper was supported by Project Excellence Research (CEEX) 51/2005.

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