

# Toxicological Assessment of P-9801091 Plant Mixture Extract after Chronic Administration in CBA/HZg Mice – A Biochemical and Histological Study

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## ABSTRACT

Acute, subchronic and chronic effects of the P-9801091 plant mixture extract at a dose of 20 mg/kg body mass were assessed in serum of healthy CBA/HZg mice at 24 hours, 7 days, 3 months and 6 months of treatment (experimental group), and compared with the values obtained in the control group of untreated healthy CBA/HZg mice. The P-9801091 plant mixture extract is an antihyperglycemic preparation containing *Myrtilli folium* (*Vaccinium myrtillus* L.), *Taraxaci radix* (*Taraxacum officinale* Web.), *Cichorii radix* (*Cichorium intybus* L.), *Juniperi fructus* (*Juniperus communis* L.), *Centaurii herba* (*Centaurium umbellatum* Gilib.), *Phaseoli fructus sine semine* (*Phaseolus vulgaris* L.), *Millefolii herba* (*Achillea millefolium* L.), *Mori folium* (*Morus nigra* L.), *Valerianae radix* (*Valeriana officinalis* L.) and *Urticae herba et radix* (*Urtica dioica* L.). Toxic effect of the P-9801091 plant mixture extract was assessed by the following biochemical parameters: urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and cholesterol. Also, histopathological examination of the kidneys, liver, spleen, pancreas, testes and lungs was performed. Results of biochemical testing performed at specified time points generally showed no statistically significant differences from control values, with the only exception of the catalytic concentration of AST in the experimental group measured on day 7, which was significantly increased as compared with the control group ( $p < 0.05$ ). Pathohistological examination including characteristic organ and tissue structure, and parenchyma relationship to the adjacent blood vessels and connective tissue in the examined organs revealed no major pathologic changes.

**Key words:** CBA mice, chronic toxicity, aminotransferase, urea and creatinine, histopathology, plant extract

## Introduction

Type 2 diabetes mellitus is the most common form of diabetes with specific diagnostic and therapeutic implications. Normoglycemia is necessary to achieve for the prevention of late diabetes complications<sup>1</sup>.

Several medicinal plants and their products have been used to control diabetes in the traditional medicine worldwide<sup>2</sup>. According to literature data, hypoglycemic effect has to date been scientifically assessed in 295 plants used in traditional medicine, 238 (81%) of them yielding positive results. Many studies investigated the hypoglycemic properties of medicinal plants<sup>3–5</sup>. Such a high percentage of hypoglycemically active plants points

to the great variety of active principles and mechanisms of action, not all of which may prove therapeutically useful<sup>6</sup>.

The aim of the present study was to assess acute, subchronic and chronic toxicity of the P-9801091 plant mixture extract containing *Myrtilli folium* (*Vaccinium myrtillus* L.), *Taraxaci radix* (*Taraxacum officinale* Web.), *Cichorii radix* (*Cichorium intybus* L.), *Juniperi fructus* (*Juniperus communis* L.), *Centaurii herba* (*Centaurium umbellatum* Gilib.), *Phaseoli fructus sine semine* (*Phaseolus vulgaris* L.), *Millefolii herba* (*Achillea millefolium* L.), *Mori folium* (*Morus nigra* L.), *Valerianae radix* (*Vale-*

*riana officinalis* L.) and *Urticae herba et radix* (*Urtica dioica* L.). The antihyperglycemic and antioxidative effects of the preparation, available on the Croatian market, have been previously described<sup>7,8</sup>. In order to assess the acute, subchronic and chronic toxic effects of a dried standardized extract of P-9801091, biochemical and histologic studies were performed in healthy CBA/H Zg mice over a 6-month period. Similar toxicological studies of *Momordica charantia* L. was performed from author El Batran *et. al.*<sup>9</sup>. The evaluation our study included determination of serum urea, creatinine, cholesterol, and catalytic concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and histology of the kidneys, liver, spleen, pancreas, testes and lungs.

## Material and Methods

### Plant material

In the P-9801091 plant mixture extract, different plants are present in the following proportion: *Centaurii herba* 12.3 % (m/m), *Cichorii radix* 17.7 % (m/m), *Juniperi fructus* 6.2 % (m/m) *Millefolii herba* 3.5% (m/m), *Myrtilli folium* 6.6 % (m/m), *Phaseoli fructus sine semine* 14% (m/m), *Taraxaci radix* 9.7 % (m/m), *Urticae herba* 7.4 % (m/m), *Urticae radix* 7.0 % (m/m), *Valerianae radix* 7.8 % (m/m), and *Mori folium* 7.4 % (m/m). Species were identified at the Ruder Bošković Institute, Department of Molecular Medicine, Zagreb, by testing of the external matter of plants<sup>10</sup>.

Dry plant material of the P-9801091 preparation was extracted with 60% ethanol. After 28 days, the macerate was filtered and ethanol was evaporated on a rotatory evaporator at a temperature of 45 °C, and lyophilized.

### Animals

Forty-eight male CBA/HZg mice divided in two groups (control and experimental), three-month-old, body mass 27–36 g, were used in the study. The mice were bred at the Laboratory of Molecular Medicine, Ruder Bošković Institute, Zagreb. The mice were cultivated in the Animal department of the Laboratory of Molecular Medicine. This Laboratory has a certificate of ethical committee from Institute Ruder Bošković with signature and stamp. During the study, animals were kept at 12-h light/dark cycle at a temperature of 22–24 °C and fed standardized laboratory chow (Pliva, Zagreb) with the addition of lyophilized P-9801091 plant mixture extract at a dose of 20 mg/kg b.m./day (experimental group) or standardized laboratory chow (Pliva, Zagreb) without the addition of P-9801091 plant mixture extract (control group).

For biochemical analysis, animals were sacrificed humanly in ether narcosis at 24 h, 7 days, 3 months and 6 months (each group consisted of six mice), and for histologic analysis at 6 months of treatment.

### Blood samples and serum chemistry

The animals were bled to death from orbital plexus under light ether anesthesia. Samples of approximately 0.1 mL (13–15 drops) were collected individually into plastic vials (Microtainer Brand Tube 0.5m, Becton Dickinson Cat No. 365973). Serum urea, creatinine, AST, ALT, and total cholesterol were determined on an Olympus AU-600 autoanalyzer. Urea was measured by urease-UV method<sup>11</sup>, creatinine by Jaffe kinetic method<sup>12</sup>, AST and ALT by enzymatic kinetic method of Boehringer Mannheim<sup>13</sup>. The reading was done at 340 nm at 30C<sup>14</sup>. Results were expressed in U/L. Total cholesterol was determined by enzymatic method<sup>15</sup>.

### Histologic analysis

For histologic evaluation, six mice from each group were chosen. The tissues examined included the kidneys, liver, spleen, pancreas and testes. The material obtained was fixed in 4% formalin solution. For preparation of paraffin sections, samples were stained with hematoxylin and eosin. Thus, permanent histologic slides for microscopy were obtained.

### Statistical analysis

The results are expressed as arithmetic mean and standard error of the mean. Non-parametric tests, Kruskal-Wallis analysis of variance, and Mann-Whitney comparison of two independent samples were used for statistical analysis. The level of significance was set at  $p < 0.05$  and  $p < 0.01$ .

## Results

### Body mass of the mice

In each individual mouse, body mass was determined once a week for 91 days, by weighing on a SAC 62 1,200 0.1 g Scaltec electric balance. Significant body mass differences between the control and experimental animals were recorded on days 7, 14 and 21 of the study ( $p < 0.01$ ) (Figure 1).

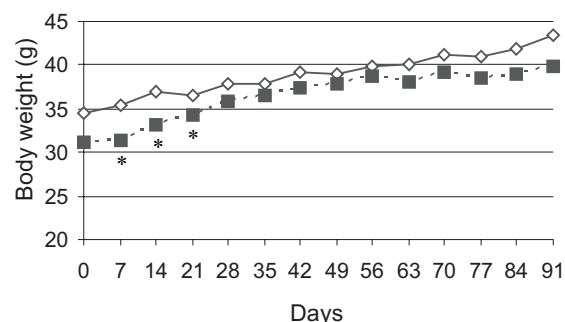


Fig. 1. Effect of P-9801091 plant mixture extract on body weight over a 90-day period.  $\diamond$ - - -  $\diamond$  control CBA/H Zg mice,  $\blacksquare$ - - -  $\blacksquare$  CBA/H Zg mice treated with P-9801091 plant mixture extract at a dose of 20 mg/kg b.m. \* $p < 0.01$  compared with control (meant  $\pm$  SEM;  $n = 10$ ).

**TABLE 1**  
SERUM CONCENTRATIONS OF UREA, CREATININE, AST, ALT AND CHOLESTEROL IN HEALTHY CBA/HZg MICE

Biochemical parameter	Group of animals	24 hours	7 days	3 months	6 months
Urea ( $\mu\text{mol/L}$ )	A	6.6 $\pm$ 0.6	8.5 $\pm$ 0.8	7.6 $\pm$ 0.3	7.0 $\pm$ 0.8
	B	7.7 $\pm$ 0.7	7.1 $\pm$ 0.8	6.8 $\pm$ 1.3	6.8 $\pm$ 0.3
Creatinine (mol/L)	A	44.5 $\pm$ 1.0	51.2 $\pm$ 4.1	46.5 $\pm$ 2.4	40.4 $\pm$ 2.3
	B	47.0 $\pm$ 1.8	50.3 $\pm$ 1.6	48.5 $\pm$ 2.9	40.8 $\pm$ 2.2
AST (U/L)	A	53.9 $\pm$ 17.5	78.5 $\pm$ 11.9	58.9 $\pm$ 8.9	54.2 $\pm$ 12.7
	B	70.6 $\pm$ 11.9	122.6 $\pm$ 10.3*	76.4 $\pm$ 11.4	53.3 $\pm$ 11.6
ALT (U/L)	A	27.2 $\pm$ 2.4	28.0 $\pm$ 1.9	27.6 $\pm$ 3.1	28.4 $\pm$ 4.5
	B	26.6 $\pm$ 2.7	25.7 $\pm$ 3.1	29.3 $\pm$ 4.2	27.8 $\pm$ 6.5
Cholesterol (mmol/L)	A	2.5 $\pm$ 0.3	3.0 $\pm$ 0.7	2.7 $\pm$ 0.6	3.2 $\pm$ 0.4
	B	2.3 $\pm$ 0.3	3.1 $\pm$ 0.4	3.4 $\pm$ 0.5	3.2 $\pm$ 0.3

A – control group: untreated, healthy CBA/HZg mice, B – experimental group, healthy CBA/HZg mice daily treated with the P-9801091 plant mixture at a dose of 20 mg/kg b.m., \* p < 0.05 B vs. A, each group containing of six animals

### Biochemical analysis

Serum concentrations of urea, creatinine, catalytic concentration of AST, ALT and cholesterol in the experimental and control mice are shown in Table 1.

Urea and creatinine were determined as parameters of the renal function<sup>16,17</sup>. During the study period, serum concentrations of urea and creatinine underwent no statistically significant changes either within or between the experimental and control group of animals. In the control group, no significant changes were recorded for the catalytic concentration of AST, however, in the experimental group of mice a significant increase in the serum catalytic concentration of AST was observed on day 7 as compared to the values recorded at 24 h of treatment (p<0.05). Comparison of the catalytic concentration of AST between the control and experimental group of animals on day 7 revealed a statistically significant increase in the experimental group (p<0.05). The catalytic concentration of ALT and the concentration of cholesterol showed no significant changes during the study period in either group of animals (Table 1).

### Histologic analysis

Histologic analysis was performed on the kidneys, liver, spleen, pancreas, testes and lungs (Figure 2.).The weight of these organs was determined. No changes were recorded in the weight of the spleen, kidneys, testicles and liver of either experimental or control group animals

(Table 2). None of the organ sections revealed any histologic or macroscopic alterations.

### Discussion

Although numerous plants with a hypoglycemic effect have been described in the literature<sup>18–21</sup>, there are others reporting on the toxicity of some plant extracts<sup>22,23</sup>. Study of Stickel et. al. 2005<sup>24</sup>, summarizes the current evidence on the potential hepatotoxicity from herbal drugs, and outlines the problems that need to be addressed to correctly assess their risk-benefit ratio. In this study are described that certain herbals have been identified as a cause of acute and chronic hepatitis, cholestasis, drug-induced autoimmunity, vascular lesions and even hepatic failure and cirrhosis. Conclusion were that only rigorous pharmacological testing of herbals can prove their safety and efficacy. Therefore, the P-9801091 plant mixture extract was given to healthy male CBA/HZg mice in a daily dose of 20 mg/kg b.m. to assess its acute, subchronic and chronic toxicity by analysis of relevant biochemical parameters and organ histology. AST and ALT are hepatospecific enzymes<sup>25</sup>. An increase in the enzymatic activity of AST and ALT in serum is known to directly reflect cell rupture<sup>26</sup>, thus these enzymes are frequently used in the evaluation of liver function<sup>27,28</sup>.

AST is an enzyme that is present in a high concentration in the cytoplasm and mitochondria of the liver, and in lower concentrations in the heart, skeletal muscle,

**TABLE 2**  
STANDARDIZED ORGAN WEIGHT (ORGAN WEIGHT/BODY WEIGHT) IN CBA/HZg MICE, CONTROL AND TRATED WITH P-9801091 PLANT MIXTURE EXTRACT AT A DOSE OF 20 mg/kg b.m.

CBA/H Zg mice	Spleen	Kidneys	Gonads	Liver
Control	0.2802 (0.0231)	0.6325 (0.0424)	0.1933 (0.0213)	3.5023 (0.2132)
Treated with P-9801091	0.3089 (0.0272)	0.7196 (0.0592)	0.2410 (0.0431)	3.7997 (0.3422)

Mean (SEM); n=6; \*p<0.01

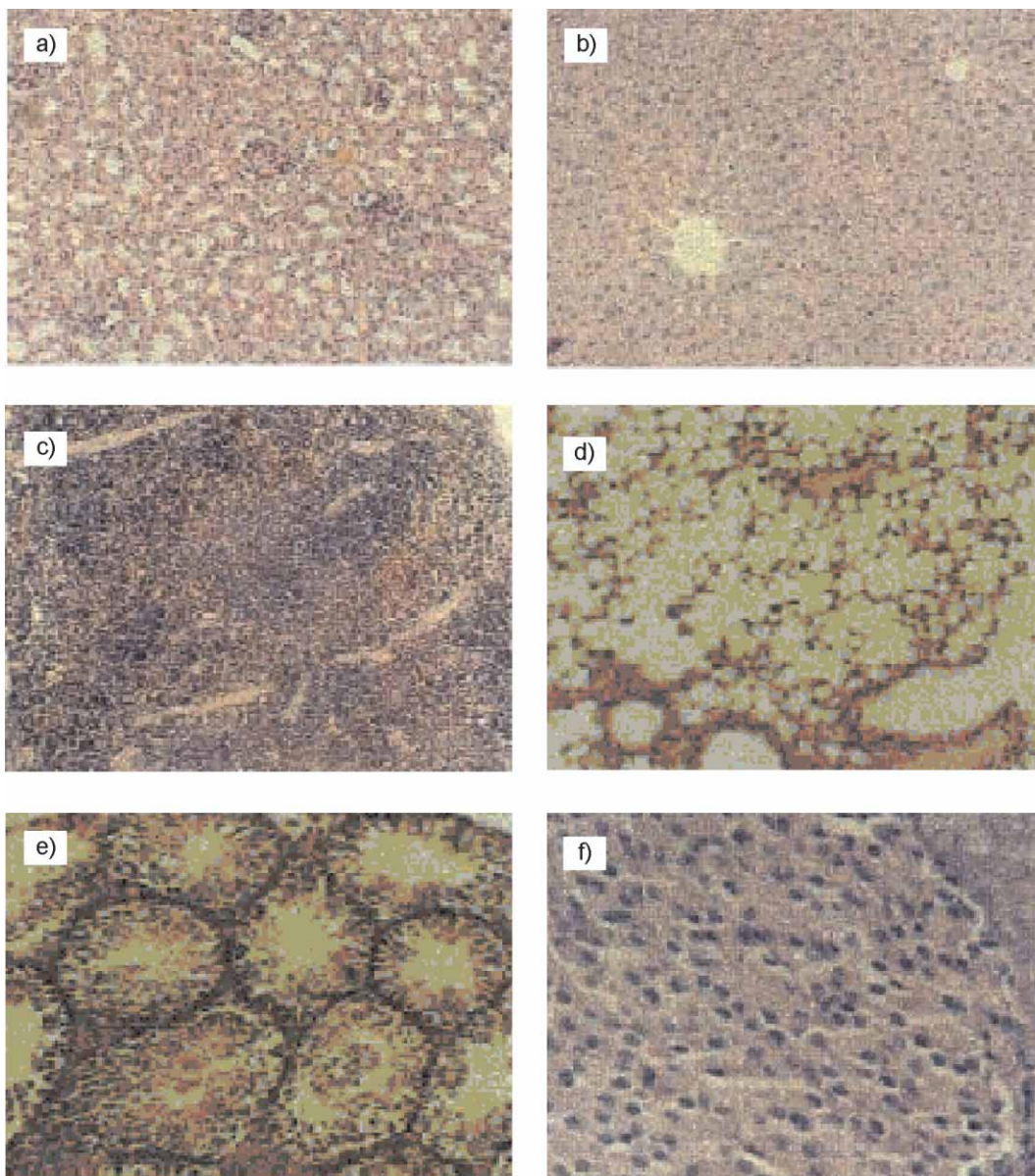


Fig. 2. Histologic section of (A) kidney, (B) liver, (C) spleen (D) pancreas, (E) testicles, and (F) lung from mice treated with P-9801091 plant mixture extract for 6 months (20 mg/kg b.m.) (hematoxylin – eosin, x40).

kidneys and brain<sup>25</sup>. This enzyme showed significantly higher values in the experimental group ( $p < 0.05$ ) compared with control animals on day 7 (Table 1), pointing to the possible initial toxic lesion of the liver. However, this effect must have subsequently disappeared, since the catalytic concentration of AST in the experimental group did not differ significantly from the catalytic concentration found in the control group at 3 and 6 months of treatment. There were no significant changes in the catalytic concentration of ALT during the study period either.

Serum urea and creatinine are determined as renal lesion parameters<sup>16,17</sup>. As the P-9801091 plant mixture extract is administered *per os* and is composed of various plants, some metabolic components are likely to be elimi-

nated in the urine. Therefore we embarked upon the study of renal lesion markers. Throughout the experiment, there were no significant differences in the urea and creatinine concentrations between the experimental and control group of animals. As the lipid (including cholesterol) metabolism is frequently impaired in diabetes mellitus,<sup>29</sup> we considered it necessary to assess the effect of P-9801091 plant mixture extract on the blood cholesterol level. No significant differences in the cholesterol concentration were recorded between the experimental and control groups of mice throughout the 6-month experiment (Table 1). During the first 21 days of the study, body mass was significantly lower in the experimental group than in the control group of mice ( $p < 0.01$ ), how-

ever, no change in the weight curve was recorded from day 21 through day 91 of the experiment.

Histologic analysis of the kidneys, liver, spleen, pancreas, testes and lungs from the experimental group mice showed no major pathologic alterations (Figure 2).

It can be concluded that plant mixture extract P-9801091 given to healthy male CBA/HZg mice in a daily dose of 20 mg/kg b.m. during 6 mounts did not toxic effect on the liver, kidney, spleen, pancreas, testes and lungs.

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## TOKSIKOLOŠKA PROCJENA BILJNOG EKSTRAKTA P-9801091 NAKON KRONIČNE PRIMJENE NA CBA/HZg MIŠEVIMA-BIOKEMIJSKA I HISTOLOŠKA STUDIJA

### SAŽETAK

Akutni, kronični i subkronični učinak biljnog ekstrakta P-9801091 u dozi od 20 mg/kg tjelesne mase ispitan je na serumu zdravih CBA/HZg miševa nakon 24 sata, 7 dana, 3 mjeseca i 6 mjeseci od početka tretmana (pokusna skupina) i uspoređen s dobivenim vrijednostima u kontrolnoj skupini koju su činili netretirani, zdravi CBA/HZg miševi. Biljni ekstrakt P-9801091 je antihiperlipidemijski preparat koji sadrži: *Myrtilli folium* (*Vaccinium myrtillus* L.), *Taraxaci radix* (*Taraxacum officinale* Web.), *Cichorii radix* (*Cichorium intybus* L.), *Juniperi fructus* (*Juniperus communis* L.), *Centaurii herba* (*Centaurium umbellatum* Gilib.), *Phaseoli fructus sine semine* (*Phaseolus vulgaris* L.), *Millefolii herba* (*Achillea millefolium* L.), *Mori folium* (*Morus nigra* L.), *Valerianae radix* (*Valeriana officinalis* L.) and *Urticae herba et radix* (*Urtica dioica* L.). Toksični učinak biljnog preparata P-9801091 procijenjen je praćenjem slijedećih biokemijskih parametara: uree, kreatinina, aspartat aminotransferaze (AST), alanin aminotransferaze (ALT) i kolesterola. Također je provedeno i histopatološko ispitivanje bubrega, jetre, slezene, gušterače, testisa i pluća. Rezultati biokemijskih analiza pokusne skupine u navedenom vremenu pokazuju nepostojanje statistički značajnih razlika u odnosu na kontrolnu skupinu zdravih, netretiranih CBA/HZg miševa, s iznimkom katalitičke koncentracije AST u pokusnoj skupini mjereno sedmog dana ( $p < 0.05$ ). Patohistološko ispitivanje, koje je uključilo ispitivanje strukture karakterističnih organa također ne pokazuje patološke promjene u odnosu na zdravu, netretiranu skupinu.