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Evaluation of the toxicity and hepatoprotective properties of new s-substituted pteridins

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ABSTRACT

Liver damage is a common problem around the world, and pharmacocorrection of such disease is carried out by administration of various drugs. Natural and synthetic thio-containing compounds are important in this respect. Most of these, however, have side effects and do not always meet the criteria of evidence-based medicine. Therefore, the search for new drugs with hepatoprotective properties, characterized by high efficiency and low toxicity, is an urgent problem of current pharmacology and biochemistry. The purpose of this study was to assess the acute toxicity of new potentially bioactive S-substituted pteridinones, to select the least toxic substance, to improve the pharmaco-technological characteristics, and to study the hepatoprotective properties in an experimental model of tetrachloromethane hepatitis in rats. Herein, comparison of the hepatoprotective properties of compound 4.1 and the reference drug "Thiotriazoline" is based on biochemical studies. The research results showed that sub-stance 4.1 had a positive effect on biochemical processes by increasing the compensatory mechanisms of antioxidant systems, while reducing the infiltrative, destructive and inflammatory process in the liver, evoking decreases in the cytolytic process, restoring the structure of the components of the membrane of hepatocytes, stabilizing and enhancing the functional activity of the liver, restoring the liver's protein-synthesizing function and increasing the ability to restore metabolic disorders in the liver. As a result of the biochemical study of the hepatoprotective effect of compound 4.1, it was found that the studied substance is a non-toxic compound with antioxidant properties.

INTRODUCTION

Liver damage is one of the leading diseases affecting humans in today's world [1], and is associated with the wide presence of hepatotoxic agents in the environment [2,3]. Thus, the search for new drugs with hepatoprotective properties that have high therapeutic efficacy and low toxicity is an urgent task of molecular pharmacology and biochemistry. Pharmacocorrection of these diseases is carried out with drugs of various nature: of plant and animal origin, essential phospholipids, amino acids, vitamins and vitamin-like substances, antioxidants and synthetic drugs. Natural and synthetic thio-containing analogs (methionine, homocysteine, L-glutathione, S-adenosylmethionine, acetylcysteine, etc.) are important drugs in this respect. They are characterized by expressed antioxidant and nucleophilic properties, and are detoxifiers (L-glutathione) of endogenous metabolites and xenobiotics in the liver. S-adenosylmethionine, for example, takes part in the methionine cycle in the presence of folates (THF, 5-MTHF, 5,10-MTHF) [4-6]. Therefore, an interesting line of research is the modification of a natural heterocyclic matrix, namely pteridine, since this class of substances is characterized by high biological activity

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[7-14]. Moreover, the folates themselves, as noted above, are essential nutrients and are involved in various metabolic processes, including the formation of methionine, which in turn is used for the synthesis of S-adenosylmethionine, as a universal donor of the methyl group [6]. As, our previous studies have shown, S-substituted 3-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropteridin-6-yl)propanoic acids are free radical scavengers and dihydrofolate reductase inhibitors [15].

The aim of this work was to study the acute toxicity of new potentially bioactive S-substituted pteridine derivatives, to select the least toxic substance, improve the pharmaco-technological characteristics and to study the hepatoprotective activity.

MATERIALS AND METHODS

For screening of acute toxicity and hepatoprotective activity 3-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropteridin-6-yl)propanoic acid (2.1), 3-(2-((carboxymethyl)thio)-4,7-dioxo-3,4,7,8-tetrahydropteridin-6-yl) propanoic acid (3.1), 2-((6-(2-carboxyethyl)-4,7-dioxo-3,4,7,8-tetrahydropteridin-2-yl)thio)propanoic acid (3.2), 2-((6-(2-carboxyethyl)-4,7-dioxo-3,4,7,8-tetrahydropteridin-2-yl)thio)-3-methylbutanoic acid (3.3) and the reference drug, namely 2.5% solution of thiotriazoline (Arterium Ukraine, Series: 0006604, No: LSR-002170/10 dated 17.03.10) were used. Synthesis and physical-chemical data similar to pteridine (2.1, 3.1-3.3) were described earlier and shown in Figure 1 [15].

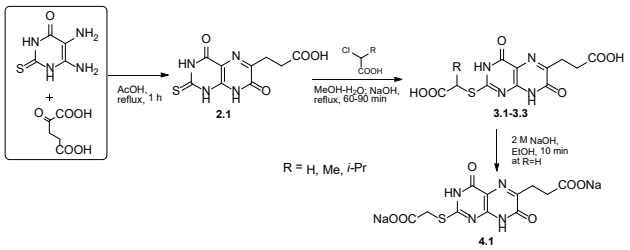

Figure 1. Methods of synthesis of S-substituted 3-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropteridin-6-yl)propanoic acid

The synthesis of disodium 3-(2-((carboxylatomethyl) thio)-4,7-dioxo-3,4,7,8-tetrahydropteridin-6-yl) propanoate (4.1)

Herein, 0.80 g (0.02 M) of sodium hydroxide was added to 3.26 g (0.01 M) of compound 3.1 in 20 ml of ethanol. The resulting suspension was heated to boiling, then activated carbon was added and refluxed for 5 minutes. The mixture was subsequently filtered, and the solvent was removed under vacuum. Afterwards, 10 ml of acetone was added and the precipitate was filtered. A light yellow crystalline substance was obtained after drying, soluble in water that had low solubility in organic solvents. Yield: 96.3%; m.p. >300°C; Calculated for: $C_{11}H_8N_4NaO_6S$: C, 35.68; H, 2.18; N, 15.13; S, 8.66; Found: C, 35.72; H, 2.23; N, 15.16; S, 8.71.

The prediction of acute toxicity of the studied compounds was carried out in silico using software packages (services) ProTox-II [16].

In vivo experimental studies were carried out on 48 outbred male mice weighing 16-24 g. The animals were kept on a standard vivarium diet [17]. The tested substances were administered intraperitoneally in the form of an aqueous suspension stabilized by Tween 80 in a volume of not more than 1 ml. The control group of animals was injected with saline and Tween 80 in the same volume as the studied group. Each group consisted of 8 animals (dose selection was carried out considering virtual data (Table 1)). The animals were observed for 2 days after a single injection of substances. During this time, their behavior, skin condition and mucous membranes, and the number of dead animals in each group were recorded. Mean lethal doses (LD_{50}) were determined by the Prozorovsky method [18].

The compounds hepatoprotective effect research was carried out according to the model of acute tetrachloromethane (TCM) hepatitis in adult male rats (6-8 months) of the Wistar line weighing 220-350 grams, which were kept under standard vivarium conditions (temperature 20±5°C, humidity 65±5%) on a standard diet with free access to water and food, in conditions of a natural change of day and night [17]. Animal care and experimental protocols were carried out in accordance with the requirements of the Directive of the European Council of November 24, 1986 for the care and use of laboratory animals (86/609/EEC), and the ethical principles of animal experiments adopted by the First National Congress of Ukraine on Bioethics (2001). International agreements and legislation of Ukraine in this area, were approved by the ethics committee, as well as in accordance with Directive 2010/63/EU of the European Parliament [19].

To assess hepatoprotective activity, the rats were divided into the following groups of 6 animals each:

- Group I: intact animals, which received by intraperitoneally injection, an appropriate volume of 0.9% sodium chloride solution for 14 days.
  In rats of groups II-IV, experimental hepatitis was reproduced by subcutaneous injection of TCM at a dose of 0.8 ml/100 g of weight in the form of a 50% oil solution once a day for 2 days [20].
- Group II: control (toxic hepatitis), animals received by injection, carbon tetrachloride, to reproduce experimental hepatitis according to the above scheme [21].
- Group III: from 1st till 14th day after TCM administration, rats received by intraperitoneally injection, 2.5% aqueous solution of thiotriazoline at the rate of 200 mg/kg (Arterium Ukraine, Series: 0006604, No: LSR-002170/10 dated 17.03.10) once per day [22,23].
- Group IV: from days 1st till 14th after TCM administration, rats receive by intraperitoneally injection, once a day, an aqueous solution of compound 4.1 at the rate of 1/50 LD_{50} [24].

On the 15th day of the experiment, from 9:00 to 11:00, the animals were decapitated under ether anesthesia. Immediately thereafter, blood was collected in glass tubes without anticoagulant. The serum was separated from the blood clot, centrifuged for 15-20 min at 1000xg, and then stored at (-20)°C until the biochemical parameters were studied [25].

Measurement of biochemical parameters in blood serum was carried out on a semi-automatic open-type biochemical analyzer BTS 330 (Bio Systems, Spain) with reagent

kits manufactured by Bio Systems (Spain). The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) [26,27], the concentration of urea in blood serum [28], total protein [29], and the concentration of albumin [30] were studied.

Liver samples were homogenized with cooled trichloroacetic acid. The homogenates were centrifuged at 3000xg for 10 minutes at 4°C. The concentration of substances reacting with 2-thiobarbituric acid (malonic dialdehyde) was determined in protein-free supernatants (extracts) [31].

Subcellular fractionation of liver homogenates was performed to obtain a mitochondrial fraction for subsequent determination of catalase activity in it (EC 1.11.1.6) [32]. To do this, the extracted liver samples were washed from blood, carefully minced using scissors, and homogenized in a Potter-Elveheim glass homogenizer in a 10-fold volume of 0.25 M sucrose (pH 7.4). The homogenates were filtered through 2 layers of gauze and centrifuged at 1000xg for 10 minutes. The supernatant was then centrifuged at 10.000xg for 20 minutes. Subsequently, the precipitate was suspended in 5 ml of 0.25 M sucrose (pH 7.4) and washed twice at 10.000xg for 20 minutes. All procedures were performed at 4°C [33]. The precipitate of purified mitochondria was suspended in 1 ml of 0.25 M sucrose (pH 7.4) and stored in a frozen state at -20°C until the study.

For histopathological examination, liver tissue samples were fixed in 10% formalin and stained according to the Best method [34].

Statistical data processing was carried out using the non-parametric Wilcoxon-Mann-Whitney method.

RESULTS AND DISCUSSION

An important stage in the creation of new drugs is the establishment of a threshold of toxicity and harmlessness, the rationale being to establish safe levels of exposure of promising compounds exposure on the body. Since this kind of experimental research is quite expensive, in silico methods for determining toxicity are now widely used [16]. Taking this into account, for a preliminary assessment of the safety parameters of the compounds we researched, we carried out a prediction of toxicity using the ProTox-II software. The results of acute toxicity calculations using these services showed that S-substituted pteridines are low-toxic, practically non-toxic or non-toxic substances (Table 1). The prediction data allowed the calculation of a safe dose for the experimental determination of acute toxicity.

The results of the experimental study of the acute toxicity of substances 2.1, 3.1-3.3 themselves confirmed the prediction data (Table 1). Our work established that their LD_{50} after intraperitoneal administration is in the range of 714-2830 mg/kg. An analysis of the data on the "structure-acute toxicity" relationship showed that the modification of the thio-group of 2nd position of pteridine (2.1) with fragments of ethanoic (3.1), α-propionic (3.2), and α-(3-methyl)butanoic (3.3) acids leads to a decrease in indicators of acute toxicity by 2-4 times. Acute toxicity in this series is located as follows: 2.1>3.3>3.2>3.1. The preparation of the water-soluble salt of acid 3.1 (substance 4.1) led to an even more significant increasing of LD_{50} (6240 mg/kg). The data obtained made it possible to classify them as low-toxic, practically non-toxic or non-toxic compounds (IV-VI class) [35]. For further research, we selected substance 4.1, which, like the reference drug "Thiotriazoline", is a non-toxic substance.

The hepatoprotective activity of compound 4.1 was evaluated by limiting liver damage by tetrachloromethane in experiments on rats [36]. At the same time, their effects on the value of biochemical markers in blood serum, liver homogenates and the state of infiltrative-destructive processes in the liver were analyzed. Changes in the activity of ALT, AST, as well as total protein, albumin and urea in the blood indicate the occurrence of toxic liver damage when carbon tetrachloride was administered to animals (group II) (Table 2). In rats of this group, the activity of ALT was increased by 3 times and AST by 2 times, compared with intact animals (group 1; $p \leq 0.05$). Such shifts indicate the development of hepatocyte cytolysis [37]. At the same time, AST activity in rats of the 3rd and 4th groups was significantly lower by 25% and 46%, respectively, than in animals of the control group (group II). Moreover, simultaneously, the effect of lowering AST activity with the introduction of compound 4.1 was 20.9% higher than with the administration of the reference drug thiotriazoline (group III). With the administration of compound 4.1 (group IV),

Table 1. Results of predicted and experimental acute toxicity of the investigated compounds

Compd.	Molweight	ProTox-II		Experimental data (intraperitoneally)	
		LD_{50}, mg/kg	Toxicity Class*	LD_{50} mouse, mg/kg	Toxicity Class**
2.1	268.25	19	(II)	714±56	IV
3.1	326.28	300	(III)	2830±230	V
3.2	340.31	300	(III)	1420±110	V
3.3	368.36	1000	(IV)	1131±89	V
4.1	370.25	300	VI	6240±830	VI
Thiotriazoline	288.29	2000	(IV)	5890*	VI

Notes:
* – Class I: fatal if swallowed ($LD_{50} \leq 5$); Class II: fatal if swallowed ($5 < LD_{50} \leq 50$); Class III: toxic if swallowed ($50 < LD_{50} \leq 300$); Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$); Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$); Class VI: non-toxic ($LD_{50} > 5000$);
** – Class I: extremely toxic ($LD_{50} \leq .2$); Class II: highly toxic (LD_{50}=0.3-10.0); Class III: moderately toxic (LD_{50}=11-100); Class IV: low toxicity (LD_{50}=101-1000); Class V: practically non-toxic (LD_{50}=1001-3000); Class VI: relatively rocky ($LD_{50} > 3000$)

Table 2. Influence of the compound 3.1 on the biochemical parameters of the blood serum of rats with experimental hepatitis (M±m)

Parameters	Experimental groups			
	group I (intact)	group II (control)	group III (TCM)	group IV (compound 4.1)
Number of tested subjects	6	4	6	6
ALT, mmoL/(g×L)	41.8 ±2.8	138.9 ±28.0*	61.5 ±9.1**	60.8 ±6.1**
AST, mmoL/(×L)	77.8 ±5.2	175.5 ±26.6*	132.1 ±11.1**	95.5 ±11.1**/***
Total protein, g/l	54.85 ±5.33	47.86 ±1.58*	48.70 ±0.98	50.78 ±2.01
Albumins, g/l	28.38 ±1.80	24.80 ±0.82*	26.52 ±1.28	27.83 ±0.94**
Urea, mmol/l	5.41 ±0.83	3.87 ±0.42*	4.66 ±0.53	6.05 ±0.41**/***

Notes:
* - significant difference (P<0.05) from intact (group I)
** - significant difference (P<0.05) from control (group II)
*** - significant difference (P<0.05) from group III (administration of the reference drug Thiotriazoline against the background of the administration of carbon tetrachloride)

the activity of ALT in the blood significantly decreased by 56%, compared with its value in rats of the control group (group II) and at the same time corresponded to that in animals of the 3rd group. Therefore, the results of the study indicate that with the administration of compound 4.1, there is a decrease in the severity of aminotransferase hyperenzymemia. This demonstrates its protective effect on the liver during the development of toxic hepatitis. The magnitude of the hepatoprotective effect of the drug, according to results of the study of ALT activity, is commensurate with the manifestation of a similar effect in the reference drug (thiotriazoline), and even exceeds it in relation to AST.

As can be seen from the data presented in Table 2, toxic liver damage in rats of group II was accompanied by a significant decrease in blood levels of urea, albumin and total protein by 1.4, 1.1 and 1.1 times, respectively, compared with the value of these indicators in intact animals (group I). The reason for their occurrence is the inhibition of the protein-synthesizing function of hepatocytes and the violation of the process of urea formation in them, as a particular manifestation of the antitoxic function of the liver. The administration of compound 4.1 to rats of group IV was accompanied by an increase in the level of urea in the blood by 35.5%, compared with its value in animals of group II. At the same time, the value of this indicator was 23% higher than in group III (administration of the reference drug thiotriazoline).

The value of total protein in the blood of rats of the 4th group remained at the level of intact animals, as well as of rats that were injected with a reference drug, thiotriazoline, against the background of carbon tetrachloride. All this reflects the manifestation of the hepatotropic properties of the newly synthesized substance, the outcome of which is the restoration of the protein-synthesizing function of liver cells [38].

The level of albumin in rats of the IV group after the administration of compound 4.1 authentically increased by 10.8%, compared with that in rats of the control group II. This may indicate the restoration of the protein-synthesizing function of the liver in rats with experimental hepatitis [39].

According to existing concepts, liver damage by carbon tetrachloride is closely related to the occurrence of oxidative stress in hepatocytes, which results in free radical oxidation of cell membrane lipids, proteins and nucleic acids. As a result, the cell death of the liver parenchyma occurs, and all its functions are suppressed [40].

To assess the state of processes of free radical oxidation of lipids in the liver in protein-free extracts of its homogenates of all studied groups, the content of malondialdehyde was determined (Table 3). Behavioral studies revealed that the concentration of this metabolite in protein-free liver extracts of group II animals was 2.5 times higher than in intact animals (group I), which indicates an increase in free-radical lipid oxidation processes and the occurrence of oxidative stress in the liver of rats with experimental hepatitis [41]. With the injection of compound 4.1 (group IV), we observed a significant decrease in the content of malondialdehyde in protein-free liver extracts by 54.5%, compared with the control group (group II). A similar effect was observed in animals treated with the reference drug thiotriazoline (group III).

Table 3. Influence of compound 3.1 on the content of malondialdehyde in protein-free extracts and catalase activity in the mitochondrial fraction of rat liver with experimental hepatitis (M±m)

Parameters	Experimental groups			
	group I (intact)	group II (control)	group III (TCM)	group IV (compound 4.1)
Number of tested subjects	6	4	6	6
Malondialdehyde, μ*mol/g	5.7 ±0.4	14.8 ±0.2*	7.1 ±0.4**	6.7 ±0.9**
Catalase (EC 1.11.1.6), μmol/mg protein min	12.0 ±1.1	6.0 ±1.0*	8.2 ±1.8	11.0 ±0.8**/***

Notes:
* – significant difference (P<0.05) from intact (group I)
** – significant difference (P<0.05) from control (group II)
*** – significant difference (P<0.05) from group III (introduction of the reference drug Thiotriazoline against the background of the introduction of carbon tetrachloride)

The study of catalase activity in the mitochondrial fraction of the liver showed (Table 3) that the introduction of carbon tetrachloride led to a twofold decrease in the activity of the enzyme in group II, compared with its value in intact animals (group I). The occurrence of this shift contributed to the aggravation of oxidative stress in the liver, as a result of impaired functioning of the enzymatic system of the first line of antioxidant defense in hepatocytes [42]. The administration of compound 4.1 prevented the inhibition of catalase in the mitochondrial fraction of the liver in group IV. This is indicated by the fact that their enzyme activity is at the level of intact animals. Moreover, the introduction of the reference drug, thiotriazoline, was not accompanied by such shift. The obtained data indicate the existence of certain differences in the manifestation of the protective effect of compound 4.1 and thiotriazoline on hepatocytes under conditions of oxidative stress that occurs in the liver after the administration of TCM.

Analyzing the results of the conducted studies, it could be concluded that the new derivative 4.1 has a pronounced hepatoprotective effect. Its administration inhibits damage to hepatocytes induced by carbon tetrachloride, and prevents the violation of their protein-synthesizing and antitoxic functions. One of the reasons for the observed effect of this compound is its pronounced antioxidant effect, which predetermined the inhibition of the process of lipid peroxidation in the liver with the injection of TCM.

The pronounced hepatoprotective effect of S-substituted pteridine (4.1) was also confirmed by the results of morphological studies of liver tissue in rats of the studied groups. In rats of group II, after the introduction of carbon tetrachloride, fatty degeneration of the liver proceeds (Fig. B), expressed violations of the cytoarchitectonics of the hepatic lobule, displacement of the nucleus, and a change in the shape of hepatocytes was observed. The content of glycogen in the composition of intracytoplasmic inclusions was also reduced compared to intact animals. In the liver of rats treated with compound 4.1, the relative density of distribution of glycogen granules in group IV (Fig. D) reached the values of the intact group (Fig. A) and restoration of the cytoarchitectonics of liver tissues was observed.

Thus, the expressed hepatoprotective properties and low toxicity make it possible to list the newly synthesized compound 4.1 from the group of S-pteridine derivatives

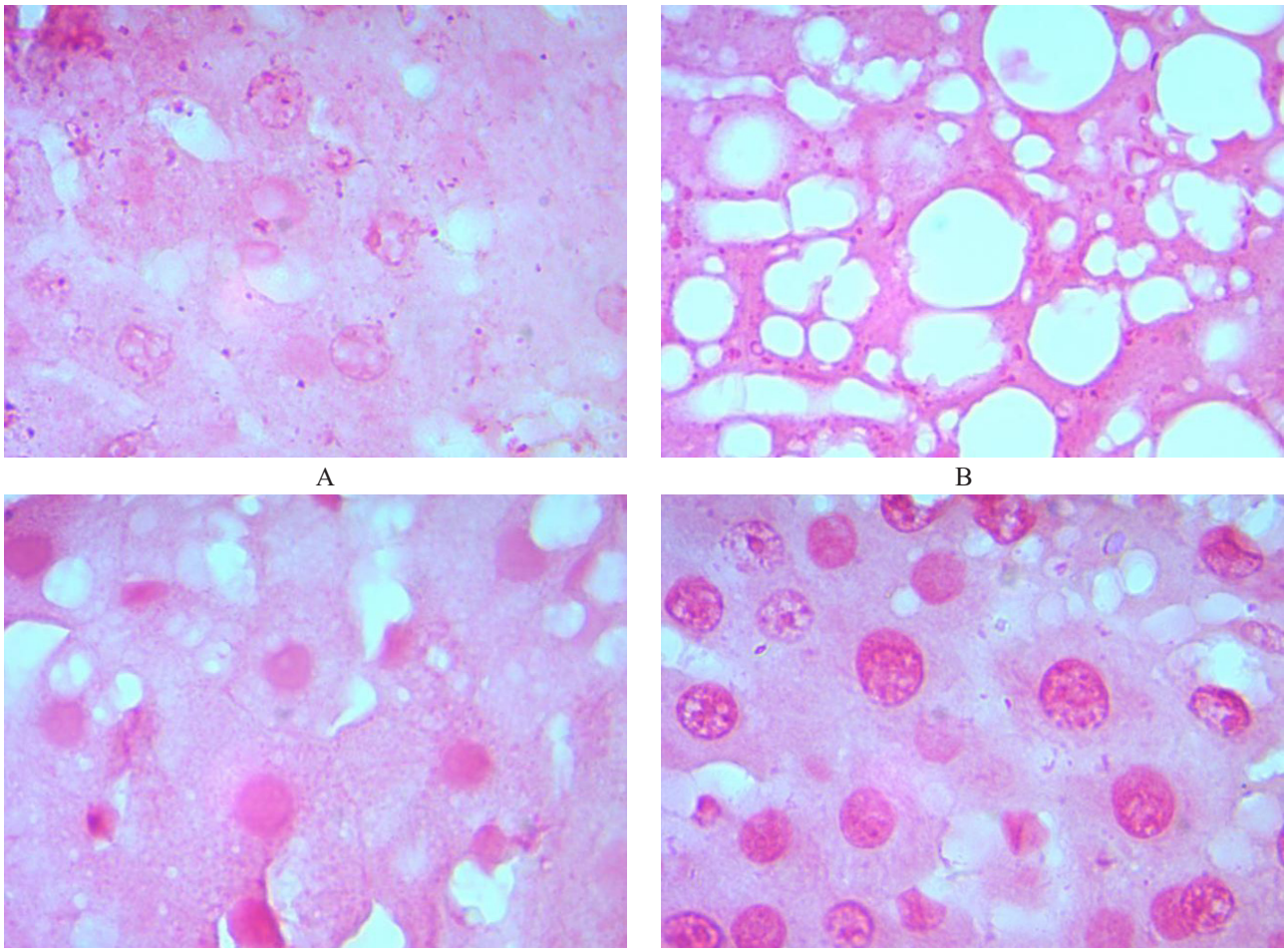

Figure 2. Liver of rats. A – intact (I group), B – rats with carbon tetrachloride hepatitis (II group), C – animals with carbon tetrachloride hepatitis and administration of 2.5% solution of thiotriazoline (III group), D – rats with carbon tetrachloride hepatitis and correction with an aqueous solution of compound 4.1 (group IV). Coloring according to best. ×400

among the promising drugs for the treatment of toxic liver damage. Its pronounced hepatoprotective properties show its promise for further studies of biochemical markers on this and other experimental models.

CONCLUSIONS

A set of studies on the acute toxicity of S-substituted pteridines showed that the modification of the thio-group in 3-(4,7-dioxo-2-thioxo-1,2,3,4,7,8-hexahydropteridin-6-yl)propanoic acid fragments of alkanecarboxylic acids, as well as the synthesis of a water-soluble salt, leads to a decrease in acute toxicity. The data obtained made it possible to attribute them to the set of low-toxic, practically non-toxic or non-toxic compounds (IV-VI class) and to select compounds 4.1 for further research in toxic liver damage. At the same time, we found that compound 4.1 has expressed hepatoprotective properties, which manifest themselves in limiting cytolysis and maintaining the protein-synthesizing and detoxifying functions of the liver at the initial level after the administration of carbon tetrachloride to animals. The hepatoprotective effect of the noted compound is based on its high antioxidant activity, which protects liver cells under conditions of pronounced oxidative stress that occurs when animals are injected with the hepatotropic poison – carbon tetrachloride. The severity of the hepatoprotective effect of compound 4.1 is commensurate with that of the reference drug thiotriazoline.

CONFLICT OF INTERESTS STATEMENT

We declare no conflict of interests.

FINANCIAL DISCLOSURE STATEMENT

The work was carried out on the budgetary theme of the Ministry of Health of Ukraine “Design and synthesis of condensed pyrimidines and elaboration of based on them potential medications with anti-inflammatory, neurotropic and metabotropic activity” (problem “Pharmacy”, № state registration 0122U000584; period of study 2022-2024).

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