Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2018, volume 103, livre 4, pp. 43-52
Youth Scientific Conference "Kliment's Days", Sofia 2017

EFFECTS OF [(DIMETHYLPHOSPHINYLMETHYL)AMINO] (CHLOROPHENYL)-METHYLPHOSPHONIC ACID AND N-(PHOSPHONOMETHYL) GLYCINE ON ATPase ACTIVITY OF RAT LIVER MITOCHONDRIA

MILENA SHKODROVA[1]*, MARIELA CHICHOVA[1],
VESELIN DOBRINOV[1,] DILYANA DONCHEVA-STOIMENOVA[1],
DIMITAR GANCHEV[1], YULIAN ZAGRANIARSKY[2],
TSVETANKA CHOLAKOVA[2], SABI VARBANOV[3]

1 Department of Animal and Human Physiology, Faculty of Biology, University of Sofia, Dragan Tzankov Blvd. 8, 1164 Sofia, Bulgaria
2 Department of Organic Chemistry, Faculty of Chemistry and Pharmacy, University of Sofia, James Bourchier Blvd. 1, 1164 Sofia, Bulgaria
3 Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, Akad. Georgi Bonchev Str. 9, 1113 Sofia, Bulgaria
**Corresponding author: e-mail: milshk@abv.bg*

Keywords: aminophosphonic acids, dimethylphosphinyl-substituted phosphonates, glyphosate, mitochondria, mitochondrial ATPase

Abstract: During the last decades, aminophosphonic acids and theirs derivatives are widely used as antimicrobial, antiviral, antitumor agents, herbicides, plant growth regulators. However, data were presented that the aminophosphonates applied as herbicides in agriculture exhibit toxic effects on animal and human organisms. A detailed study of the commercial aminophosphonates effects on cells and cell organelles would provide a better insight into the mechanisms of their biological action.

The purpose of the current work was to study the effects of two aminophosphonates –a newly synthesized [(Dimethylphosphinylmethyl)amino](chlorophenyl)-methylphosphonic acid (DMCPPA) and a commercially used N-(phosphonomethyl) glycine (glyphosate), on ATPase activity of rat liver mitochondria. Two different mitochondrial preparations were used: intact mitochondria and mitochondria uncoupled by freezing/thawing.

DMCPPA did not influence ATPase activity of intact mitochondria and insignificantly increased the activity of 2,4-dinitrophenol (DNP)-uncoupled mitochondria. This suggests that DMCPPA does not possess an uncoupling effect on intact liver mitochondria and is not able to pass the inner mitochondrial membrane. DMCPPA insignificantly reduced ATPase activities of freeze/thawed mitochondria. The herbicide Cosmic® with an active

ingredient glyphosate stimulated ATP hydrolysis in intact mitochondria and ATPase activity of DNP-uncoupled mitochondria, which suggests that the compound possesses uncoupling effect on intact mitochondria and is able to pass the inner mitochondrial membrane. Cosmic® increased ATPase activity of freeze/thawed mitochondria.

The differences in the effects of the newly synthesized DMCPPA and herbicide Cosmic® may be due to supplements in the composition of Cosmic® (anionic surfactant) and/or to different chemical structures of the glyphosate and DMCPPA, which determine different biological activity.

Our results provide an insight into the mechanisms of the biological action of the aminophosphonates on the living systems, in particular on mitochondria.

INTRODUCTION

During the last decades α-aminophosphonic acids and their biological activity was extensively studied because of their structural analogy with α-aminocarboxylic acids (Hudson, 2000; Mastalerz and Kafarski, 2000; Oleksyzsyn, 2000). Synthesis of α-aminophosphonic acid analogues resulted in a new class of bioactive compounds with a great variety of commercial applications, such as enzyme inhibitors, antifungal agents, herbicides, plant growth regulators and pesticides, immune system activators, neuroactive, antitumor, antiviral, and antibacterial compounds (Kafarski and Lejczak, 2000; Kafarski and Lejczak, 2001; Naydenova et al., 2007; Naydenova et al., 2010; Orsini et al., 2010).

The development of aminophosphonates as agrochemicals is one of the most significant discoveries in the agriculture. Such aminophosphonate is the commercially used glyphosate (N-(phosphonomethyl) glycine, Fig. 1A) which is the fundamental substance of some pesticides such as RoundupTM, etc.

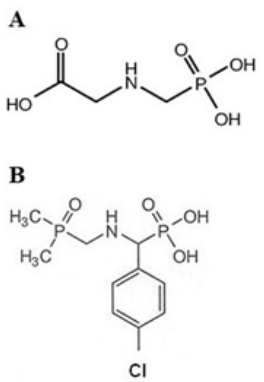

Fig. 1 Structures of N-(phosphonomethyl) glycine (glyphosate, A) and [(Dimethylphosphinylmethyl)amino] (chlorophenyl)-methylphosphonic acid (DMCPPA, B)

Original derivatives of α-aminophosphonic acid with dimethylphosphinyl-substituent in the molecule are synthesized from imine of aminomethyl-dimethylphosphine oxide (Zagraniarsky et al., 2008). One of them is [(dimethylphosphinylmethyl)amino](chlorophenyl)-methylphosphonic acid (DMCPPA). Its molecular structure (Fig. 1B) includes two different phosphorus-containing groups – phosphonyl and dimethylphosphinyl, which might determine potential biological activity. The presence of a free phosphonyl group and/or a

lipophilic cyclic part in the molecule of this compound creates the possibility of interaction with enzymes using adenosine triphosphate (ATP) as a substrate.

Mitochondrial ATP synthase/ATPase (ATP hydrolase: EC 3.6.1.34) is an enzyme complex, responsible for ATP synthesis in the cell. The complex catalyzes the synthesis of ATP from adenosine diphosphate and inorganic phosphate (Pi) by utilizing the transmembrane proton gradient and membrane potential generated during substrate oxidation. This reaction can be reversed by pumping protons in the opposite direction resulting in ATP hydrolysis (Boyer, 1997). Although effects of α-aminophosphonic acid derivatives on some living systems was observed (Szarek et al., 2000, Peixoto, 2005, Lushack et al., 2009), there are no reports on direct influence on the mitochondrial ATPase.

Along with the wide range of agricultural and medical applications of α-aminophosphonic acid derivatives, the necessity of profound research on possible adverse effects on human health and the environment is growing.

The present work was undertaken to evaluate the in vitro effects of two aminophosphonates – a newly synthesized DMCPPA and a commercially used glyphosate on ATPase activity of rat liver mitochondria. More detailed research on the effects of the α-aminophosphonic acids on living systems will clarify the mechanisms of their biological activity and potential toxicity.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Fresh stock solution of ATP was prepared prior to each experiment and kept on ice to preserve it from hydrolysis.

DMCPPA was synthesized in the Department of Organic Chemistry, Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski", and the Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences.

Cosmic® (aquatic solution of the active ingredient glyphosate, isopropylamine salt, 360 g/l) was purchased from Arysta LifeScience, France.

Double distilled water was used for preparation of all reagent solutions.

Animals and isolation of rat liver mitochondria

Liver mitochondria were isolated from male albino rats (Wistar strain, 50-60 days of age; 120-150 g), supplied by the vivarium of the Faculty of Biology, Sofia University "St. Kliment Ohridski". The animals received a standard laboratory diet (commercial rat chow, TopMix Ltd, Bulgaria) and water ad libitum and were fasted prior to use. All studies were performed in accordance with the European ethical guidelines.

Intact mitochondria were isolated as previously described (Shkodrova et al., 2013).

Assay of ATPase activity

ATPase activity was determined by measurement of Pi released from ATP. The reaction was carried out in reaction medium consisting of 200 mM sucrose, 10 mM KCl, 50 mM Tris-HCl, and 100 μM EDTA-KOH, pH 7.5.

In part of the experiments, ATPase activity was studied in course of the reaction using intact mitochondria. In these experiments ATPase activity was assayed at room temperature and continuous stirring with 1 min pre-incubation of the mitochondria with the studied substances in final concentrations of 10 and 100 μM. ATP in 1 mM final concentration was added to the reaction medium, as well as 2,4-dinitrophenol (DNP) was included in concentration of 50 μM wherever indicated. The reaction was started by adding mitochondrial protein. Samples of 0.5 ml were taken after 15, 30, 60, 120, 180 and 300 s incubation and added to 0.2 ml of 3M perchloric acid for termination of the reaction.

In second group of experiments, freeze/thawed mitochondria were pre-incubated for 5 min at 37°C with DMCPPA or Cosmic®, added in the assay medium in final concentrations of 10, 25, 50 and 100 μM respectively. The reaction was initiated by the addition of ATP in final concentration of 1 mM, continued for 5 min at 37°C and terminated by adding 0.4 ml of 3M perchloric acid.

In all cases, the protein precipitates were removed by centrifugation at 8800 g for 10 min. The concentration of Pi hydrolyzed from ATP was measured by the method of Fiscke and Subbarow (1925) with modifications. Blanks in which the reaction was blocked by addition of perchloric acid before ATP addition were carried out in parallel in order to determine the background Pi amount as a result of non-enzymatic hydrolysis. ATPase activity was expressed as μmol Pi.mg protein^{-1}.min^{-1} or μmol Pi.mg protein^{-1}.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments performed in duplicate unless otherwise indicated. ATPase activity values were calculated as percentages of the activity measured under control conditions (DMCPPA- or Cosmic®-free assay medium). The difference between DMCPPA- or Cosmic®-treated samples and the untreated control was tested by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Influence of DMCPPA on ATPase activity of intact mitochondria

To test the possible uncoupling action of DMCPPA a set of experiments with intact mitochondria was carried out. Figs. 2A and 2B show the results from two representative experiments with intact and DNP-uncoupled mitochondria, respectively. ATPase activity remained low until the end of the registration under control conditions, indicating the low permeability of the mitochondrial membrane. Addition of the uncoupler DNP in final concentration of 50 μM powerfully stimulated ATP hydrolysis. DMCPPA in concentrations of 10 μM and 100 μM did not influence ATPase activity of intact mitochondria (Fig. 2A) and insignificantly increased the activity of DNP-uncoupled mitochondria (Fig. 2B). This suggests that DMCPPA does not possess an uncoupling effect on intact liver mitochondria and is not able to pass the inner mitochondrial membrane.

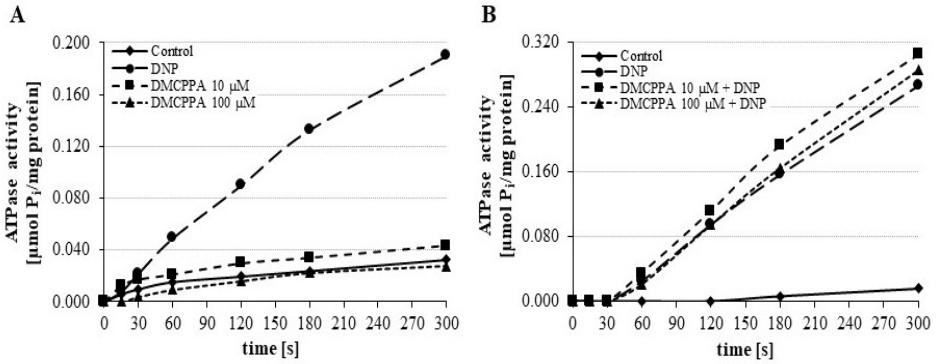

Fig. 2. Effects of DMCPPA on ATPase activity of intact (A) and 2,4-dinitrophenol (DNP)-uncoupled mitochondria (B). DNP was included in the reaction medium in concentration of 50 µM. The reactions were started by adding 80 µl of mitochondrial suspension (protein 9.38 mg/sample and 5.17 mg/sample for A and B, respectively) and carried out for 5 min as described in Materials and Methods. Data present curves registered during two experiments. The abbreviations are the same as in Fig. 1

Influence of DMCPPA on ATPase activity of freeze/thawed mitochondria

Freezing/thawing disrupts the mitochondrial inner membrane leading to uncoupling and stimulation of initial ATPase activity. In parallel with this, mitochondria preserve their structure and the membrane spanning F0 sector of the ATPase.

ATPase activity values were calculated as percentages of the activity measured under control conditions (DMCPPA-free assay medium). DMCPPA in concentrations of 10, 25, 50, and 100 µM reduced ATPase activity of freeze/thawed mitochondria to 83,54±6,58% of the control (in concentration of 50 µM), but the differences between the groups were not statistically significant (Fig. 3).

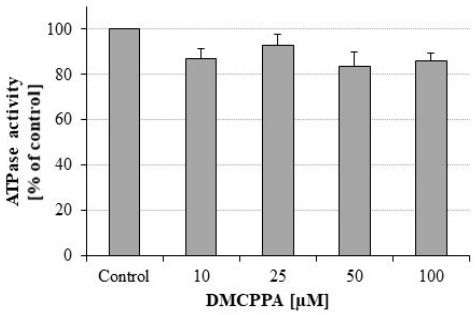

Fig. 3. Effect of DMCPPA on ATPase activity of freeze/thawed mitochondria. ATPase reaction carried out for 5 min at 37°C. ATPase activity values were calculated as percentages of the activity measured under control conditions (DMCPPA-free assay media). Data are plotted as mean ± SEM of six independent experiments (two parallel samples per group per experiment). The difference between DMCPPA-treated samples and the untreated control was tested by one-way analysis of variance (ANOVA). The abbreviations are the same as in Fig. 1

Influence of Cosmic® on ATPase activity of intact mitochondria

In order to compare the effects of the newly synthesized DMCPPA on ATPase activity with those of the commercially used glyphosate, a similar set of experiments was carried out with the widely used in Bulgarian agriculture pesticide Cosmic® (aquatic solution of the active ingredient glyphosate, isopropylamine salt, 360 g/l).

The effects of Cosmic® on ATPase activity of intact mitochondria are presented in Fig. 4. Addition of the compound in concentrations of 10 μM и 100 μM led to stimulation of ATP hydrolysis similarly to the effect of DNP in concentration of 50 μM (Fig. 4A). These results suppose an uncoupling action of Cosmic® on mitochondria.

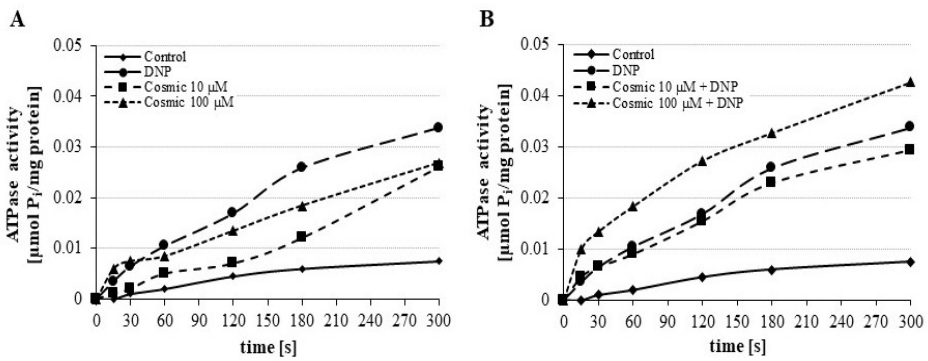

Fig. 4. Effects of Cosmic® on ATPase activity of intact (A) and DNP-uncoupled mitochondria (B). DNP was included in the reaction medium in concentration of 50 μM. The reactions were started by adding 80 μl of mitochondrial suspension (protein 7.8 mg/sample) and carried out for 5 min as described in Materials and Methods. Data present curves registered during one experiment. The abbreviations are the same as in Fig. 2

Cosmic® in concentration of 10 μM did not significantly influence DNP-stimulated ATPase activity, but in concentration of 100 μM stimulated the activity (Fig. 4B). In comparison with the data for DMCPPA, Cosmic® exerted stronger effects on DNP-stimulated ATPase activity, which suggests that the pesticide is able to pass the mitochondrial membrane with subsequent stimulation of ATPase activity.

Influence of Cosmic® on ATPase activity of freeze/thawed mitochondria

Cosmic® in concentrations of 10, 25, 50, and 100 μM enhanced ATPase activity of freeze-thawed mitochondria to 132,30±18,09% of the control (in concentration of 100 μM) (Fig. 5).

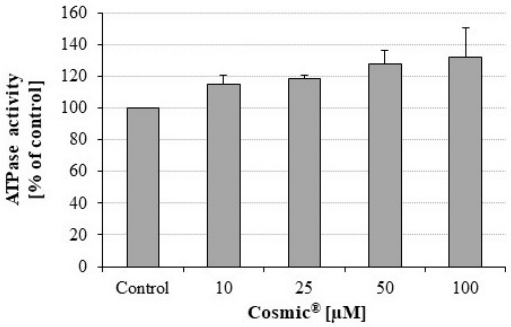

Fig. 5. Effect of Cosmic® on ATPase activity of freeze/thawed mitochondria. ATPase reaction carried out for 5 min at 37°C. ATPase activity values were calculated as percentages of the activity measured under control conditions (Cosmic®-free assay media). Data are plotted as mean ± SEM of three independent experiments (two parallel samples per group per experiment). The difference between Cosmic®-treated samples and the untreated control was tested by ANOVA.

These data were confirmed from the experiments in the course of the ATPase reaction (data are not shown) as the strongest effect was observed in the onset of the reaction (during the first 60 s of the reaction). In these experiments, two approaches were applied to examine the effects of the compounds on the mitochondrial ATPase activity. A set of experiments was conducted, in which the mitochondrial suspensions were added to the reaction medium and pre-incubated for 1 min in the presence of DMCPPA or Cosmic®, whereupon the reaction was started by addition of ATP. This approach allowed to investigate the influence of the compounds under conditions providing their interactions with the active site of the enzyme prior to its saturation with the substrate. The second approach was to enable conditions allowing simultaneous interactions of the substrate ATP and compounds studied with the active site of the enzyme. For that purpose, ATP and DMCPPA or Cosmic® in the same concentrations were preliminary introduced into the reaction medium, and the reaction was initiated by addition of the corresponding mitochondrial suspensions. The results from these experiments (data are not shown) were similar. This suggests a lack of competitive interactions between the compounds and the substrate with the active site of the enzyme.

According to the WHO Classification of active pesticide ingredients, glyphosate is class III – "slightly hazardous". Poisonings with glyphosate are documented, as the symptoms severity depends on the dose exposure (Roberts et al., 2010; Zouaoui et al., 2013).

Effects of RoundupTM on some living systems have been observed. It has been found that the pesticide suppressed the activity of superoxide dismutase, glutathione-S transferase, glutathione reductase and glucose-6-phosphate dehydrogenase in goldfish tissues (Lushack et al., 2009). Electron microscopy revealed that RoundupTM in concentrations of 40- to 20-fold lower than those

used in practice, caused appearance of myelin-like structures in carp hepatocytes, swelling of mitochondria and disappearance of internal membrane of mitochondria (Szarek et al., 2000).

Our results for the effects of Cosmic® on the mitochondrial ATPase activity are in agreement with data presented by other authors. It was observed that the herbicide RoundupTM (with an active ingredient glyphosate) stimulated succinate-supported respiration, with simultaneous collapse of transmembrane electrical potential in isolated rat liver mitochondria (Peixoto, 2005). The same study showed a partial inhibition of respiratory chain complexes II and III, as well as uncoupling of oxidative phosphorylation due to a non-specific membrane permeabilization induced by RoundupTM. In opposition to RoundupTM formulation products, the authors did not find any relevant effect of glyphosate alone on the mitochondrial bioenergetics. They suggest that the differences in the effects could be attributed either to some products of RoundupTM or to a synergic effect of glyphosate and to formulation products.

Besides glyphosate, the pesticides contain anionic surfactant. Along with its main application to enhance the penetration into the leaves, the surfactant might increase the compound toxicity.

The composition of the pesticide Cosmic® is similar to that of RoundupTM. The differences in the effects of the newly synthesized DMCPPA and herbicide Cosmic® may be due to supplements in the composition of Cosmic® (anionic surfactant) and/or to different chemical structures of the glyphosate and DMCPPA, which determine different biological activity.

It should be noted that in the present study we demonstrate the inhibitory effects of DMCPPA and Cosmic® on the ATPase activity of a membrane-bound enzyme. There is a possibility that the compounds could affect the enzyme surroundings rather than enzyme itself. Further studies on compounds' influence on the activity of isolated soluble ATPase (F1) could provide opportunity to explain the mechanisms of the potential biological activity of the α-aminophosphonic acids.

CONCLUSIONS

We found that DMCPPA did not influence ATPase activity of intact mitochondria and insignificantly increased the activity of DNP-uncoupled mitochondria. This suggests that DMCPPA does not possess an uncoupling effect on intact liver mitochondria and is not able to pass the inner mitochondrial membrane. DMCPPA insignificantly reduced ATPase activities of freeze/thawed mitochondria. The herbicide Cosmic® with an active ingredient glyphosate stimulated ATP hydrolysis in intact mitochondria and ATPase activity of DNP-uncoupled mitochondria, which suggests that the compound possesses uncoupling effect on intact mitochondria and is able to pass the inner mitochondrial membrane. Cosmic® increased ATPase activity of freeze/thawed mitochondria.

The differences in the effects of the newly synthesized DMCPPA and herbicide Cosmic® may be due to supplements in the composition of Cosmic®

(anionic surfactant) and/or to different chemical structures of the glyphosate and DMCPPA, which determine different biological activity.

Our results provide an insight into the mechanisms of the biological action of the aminophosphonates on the living systems, in particular on mitochondria.

Acknowledgements: This work was supported by Grant № 80-10-17/12.04.2017 from Sofia University "St. Kliment Ohridski".

REFFERENCES

1. Boyer, P. D. 1997. The ATP synthase – a splendid molecular machine. *Annual Reviews of Biochemistry,* 66: 717–749.
2. Fiske, C., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *Journal of Biological Chemistry*, 66: 375-400.
3. Hudson, H. R. 2000. Aminophosphonic and aminophosphonic acids and their derivatives. In: Kukhar, V. P. and Hudson, H. R. (Eds.). *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity.* John Wiley&Sons, Chichester-Toronto, pp. 443–482.
4. Kafarski, P., Lejczak, B. 2000. The biological activity of phosphono- and phosphinopeptides. In: Kukhar, V. P. and Hudson, H. R. (Eds.). A*minophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity.* John Wiley&Sons, Chichester-Toronto, pp. 407–442.
5. Kafarski, P., Lejczak B. 2001. Aminophosphonic acids of potential medical importance. *Current Medicinal Chemistry. Anticancer Agents,* 1: 301–312.
6. Lushchak, O., Kubrak, O., Storey, J., Storey, K., Lushchak, V. 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. *Chemosphere*, 76 (7): 932–937.
7. Mastalerz, P., Kafarski, P. 2000. Naturally occurring aminophosphonic and aminophosphonic acids. In: Kukhar, V. P. and Hudson, H. R. (Eds.). A*minophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity.* John Wiley&Sons, Chichester-Toronto, pp. 1–32.
8. Naydenova, E., Todorov, P., Troev, K. 2010. Recent synthesis of aminophosphonic acids as potential biological importance. *Amino Acids*, 38: 23–30.
9. Naydenova, E., Troev, K.; Topashka-Ancheva, M., Haegele, G., Ivanov, I., Kril, A. 2007. Synthesis, cytotoxicity and clastogenicity of novel a-aminophosphonic acids. *Amino Acids*, 33: 695–702.
10. Oleksyzsyn, J. 2000. Aminophosphonic and aminophosphonic acid derivatives in the design of transition state analogue inhibitors: biomedical opportunites and limitations. In: Kukhar, V. P. and Hudson, H. R. (Eds.). *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity.* John Wiley&Sons, Chichester-Toronto, pp. 537–558.
11. Orsini, F., Sello, G., Sisti, M. 2010. Aminophosphonic acids and derivatives. Synthesis and biological applications. *Current Medicinal Chemistry,* 17 (3): 264-289.
12. Peixoto, F. 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere*, 61(8): 1115–1122.

13. Roberts, D. M., Buckley, N. A., Mohamed, F., Eddleston, M., Goldstein D. A., Mehrsheikh. A., Bleeke M. S., Dawson A. H. 2010. A prospective observational study of the clinical toxicology of glyphosate-containing herbicides in adults with acute self-poisoning. *Clininical Toxicology* (Phila), 48 (2): 129–136.
14. Shkodrova, M., Chichova, M., Ganchev, D., Sazdova, I., Minkov, I. 2013. Influence of sodium nitroprusside as a donor of nitric oxide on ATPase activity of rat liver mitochondria. *Bulgarian Journal of Agricultural Sciences,* 19: 167-170.
15. Szarek, J., Siwicki, A., Andrzejewska, A., Terech-Majewska, E., Banaszkiewicz T. 2000. Effects of the herbicide RoundupTM on the iltrastructural pattern of hepatocytes in carp (Cyprinus carpio). *Marine Environmental Research,* 50 (1-5): 263-266.
16. Zagraniarsky, Y., Ivanova, B., Nikolov, K., Varbanov, S., Cholakova, T. 2008. Synthesis of dimethylphosphinyl-substituted α-amino(aryl)methylphosphonic acids and their esters. *Zeitschrift für Naturforschung*, 63b: 1192–1198.
17. Zouaoui, K., Dulaurent, S., Gaulier, J., Moesch C., Lachâtre G. 2013. Determination of glyphosate and AMPA in blood and urine from humans: about 13 cases of acute intoxication. *Forensic Science International*, 226 (1-3): 20–25.