Advances in Biology & Earth Sciences
Vol.3, No.3, 2018, pp.167-175

DETECTION OF MANGANESE-CONTAINING ENZYMES AND MAGNETIC NANOPARTICLES IN *JUNIPERUS COMMUNIS* AND RELATED BIOMATERIALS BY ESR SPECTROSCOPY

**Rovshan I. Khalilov[1,2,3], Taras S. Kavetskyy[3,4,5], Vladimir A. Serezhenkov[6]
Aygun N. Nasibova[2,3], Abolfazl Akbarzadeh[3,7,8], Soodabeh Davaran[3,7]
Mohammad Pourhassan Moghaddam[3,9], Siamak Saghfi[3,10], Nikolay A. Tkachev[6]
Morteza Milani[3,7], Mohammad Kouhi[3,11], Ondrej Šauša[12]
Svitlana Ya. Voloshanska[3,4]**

[1]Department of Biophysics and Molecular Biology, Faculty of Biology, Baku State University, Baku, Azerbaijan
[2]Institute of Radiation Problems, National Academy of Sciences of Azerbaijan, Baku, Azerbaijan
[3]Joint Ukraine-Azerbaijan International Research and Education Center of Nanobiotechnology and Functional Nanosystems, Drohobych, Ukraine & Baku, Azerbaijan
[4]Department of Biology and Chemistry, Faculty of Biology and Natural Sciences, Drohobych Ivan Franko State Pedagogical University, Drohobych, Ukraine
[5]Department of Applied Physics, The John Paul II Catholic University of Lublin, Lublin, Poland
[6]Semenov Institute of Chemical Physics, Russian Academy of Sciences, Moscow, Russian Federation
[7]Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[8]Universal Scientific Education and Research Network (USERN), Tabriz, Iran
[9]Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[10]Department of Plant Physiology, Faculty of Biology, Baku State University, Baku, Azerbaijan
[11]Islamic Azad University, Tabriz Branch, Iran
[12]Institute of Physics, Slovak Academy of Sciences, Bratislava, Slovak Republic

Abstract. An innovative approach based on the effect of bio-mineralization as a response reaction of cells to decrease their damage upon stress was applied for *Juniperus communis* and related biomaterials. The electron spin resonance (ESR) method was used as the main experimental tool for detecting the paramagnetic species as a result of existence of antioxidant activity system, represented by superoxide dismutase with manganese, catalase etc., and formation of superparamagnetic iron oxide nanoparticles (SPIONs). The ESR signals for the *Juniperus communis* shell and seeds and their mixture were recorded and analyzed. The SPIONs were found in the *Juniperus communis* shell, while the antioxidant activity due to manganese-containing enzymes was detected in the *Juniperus communis* seeds. For comparative analysis, the *Juniperus communis* based biomaterials, such as Nefrovil and Immunostan drugs were also investigated by ESR spectroscopy. As a result, the same features were obtained for Nefrovil (manganese-containing enzymes, prevailing superoxide dismutase with manganese) and Immunostan (prevailing SPIONs), when the choice of Nefrovil and Immunostan drugs has been made to increase the antioxidant activity and to improve the immune system of human organism respectively. The influence of temperature on the intensity of ESR signals from manganese-containing enzymes and SPIONs has also been examined. The detected bio-functionality of the *Juniperus communis* shell and seeds as well as biomaterials on its basis could be recommended for pharmaceutical and biomedical applications.

Keywords: *Electron spin resonance spectroscopy, Biomaterials, Juniperus communis, Superoxide dismutase with manganese, Superparamagnetic iron oxide nanoparticles.*

Corresponding Authors: *Rovshan I. Khalilov, Prof. Dr. Sci., Department of Biophysics and Molecular Biology, Faculty of Biology, Baku State University, 23 Z. Khalilov Str., Baku, Azerbaijan, e-mail: hrovshan@hotmail.com; Taras S. Kavetskyy, Ph.D., Department of Biology and Chemistry, Faculty of Biology and Natural Sciences, Drohobych Ivan Franko State Pedagogical University, 24 I. Franko Str., Drohobych, Ukraine, e-mail: kavetskyy@yahoo.com*

Received*: 23 October 2018;* ***Accepted****: 22 November 2018;* ***Published****: 11 December 2018.*

1. Introduction

The natural biologically active dietary supplements (BADS) are very useable now for the eco-biomedicine and eco-human health applications. As a rule, BADS are important for improving physiological, healthy and prophylactic properties with aim to stabilize a normal functionality of human organism. There are a lot of companies in all over the world producing the bioactive dietary supplements. Having a huge choice to use BADS, physicians can introduce them into the ration of diet or rational food for optimization of exchange processes and functions of human organism, taking into account of its health state. However, as a practice experience shows, in many cases usefulness and prophylactic functions of BADS are determined by physicians through a chemical compound only, that is not enough to be as a basis for estimation of biological action of natural dietary supplements.

The influence of physical (stress) factors (e.g., temperature, humidity, strong UV and/or lower level of illumination, radiation, soil salinity, etc.) on such plant as *Juniperus communis* has formed an antioxidant system with a larger activity than for other plants. The presence of such antioxidant enzymes as catalase and superoxide dismutase (SOD) – iron SOD (FeSOD), manganese SOD (MnSOD), copper-zinc (CuZnSOD), and nickel SOD (NiSOD) (Abreu *et al*., 2010) – in *Juniperus communis*, makes it as a model biomaterial object with potential biomedical applications. As an example, many pharmacological effects of *Juniperus communis* are antitumor properties, antioxidant, antitoxic and anticarious activities.

Pharmacological activity of *Juniperus communis* could be significantly changed in dependence as one using a shell and/or seeds. Also, by adding the *Juniperus communis* into composition of other BADS, it is possible to distinguish the bio-functionality of the drug for medical purposes. For instance, the screening of *Juniperus communis* based drugs Nefrovil and Immunostan has been made (Roslyna Karpat Co., Ltd, Ukraine, http://roslynakarpat.com.ua) to increase the antioxidant activity and to improve the immune system of human organism respectively. But, if stress factors are affected on the *Juniperus communis* bioactivity (that is expectedly happened in different seasons), the properties of Nefrovil and Immunostan could be uncontrolled. The present research will focus on the resolving this problem by using a new approach based on the effect of bio-mineralization as a response reaction of stress-damaged cells (Khalilov *et al*., 2010; Khalilov *et al*., 2011a; Khalilov *et al*., 2011b; Khalilov *et al*., 2015). This effect is coupled with electron-transport chain of photosynthesis that leads to a decrease the Fenton's reaction due to the transformation of iron ions (Fe^{2+}, Fe^{3+}) to maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) superparamagnetic iron oxide nanoparticles (SPIONs). Among all analytical tools, electron spin resonance (ESR) spectroscopy is the only technique that directly detects free radicals and paramagnetic species. Thus, ESR method is used in the research as the main experimental tool for detecting the

paramagnetic species due to the presence of antioxidant enzymes and SPIONs as a result of bio-mineralization effect.

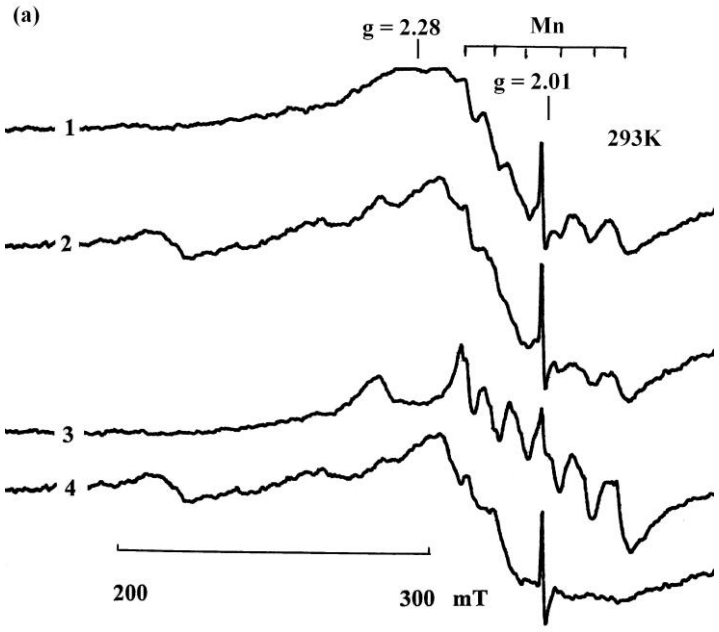
2. Experimental

The methodology of the present work is based on the application of the above mentioned effect of bio-mineralization for the *Juniperus communis* samples of Carpathian region of Ukraine. The *Juniperus communis* based Nefrovil and Immunostan drugs were used as provided by Roslyna Karpat Co., Ltd, Ukraine (http://roslynakarpat.com.ua). The ESR spectra of the biomaterials studied were registered with the aid of an X-range ESR spectrometer ECS-106 (Bruker, Germany) at the following conditions of registration: magnetic field HF-modulation amplitude 0.5 mT, field center 260 mT, field scanning 290 mT, temperatures 293 and 77 K, and gain 10^4 and 5×10^5, respectively.

3. Results and discussion

Juniperus communis

Figure 1 shows the ESR spectra of the *Juniperus communis* shell and seeds and their mixture along with differential spectrum between shell and seeds at room temperature (Fig. 1a). We may emphasize on the two ESR signals registered in the biomaterials. Namely, the ESR signal at g = 2.01 is attributed to the paramagnetic species of Mn (Khalilov *et al*., 2011a), characterized by six-component hyperfine structure, in the Mn-containing enzymes that is found only in the *Juniperus communis* seeds. At the same time, the broad ESR signal at g = 2.28 is attributed to the paramagnetic species of SPIONs (Khalilov *et al*., 2011b) that is found only in the *Juniperus communis* shell.



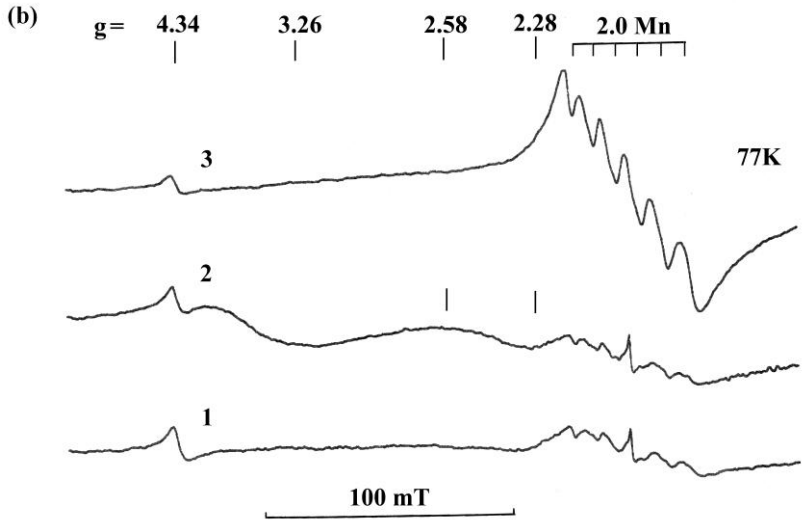


Fig. 1. ESR spectra of the *Juniperus communis* shell, seeds, and mixture of shell and seeds at temperatures (a) 293 K (1 - mixture of shell and seeds, 2 - shell, 3 - seeds, and 4 - differential spectrum (curve 2 -curve 3)) and (b) 77 K (1 - mixture of shell and seeds, 2 - shell, and 3 - seeds)

It must be noted here that the SPIONs detected in the investigated material have an important role in development of pathological conditions and are frequently used for biomedical applications, where their toxic potential is still a major concern (Ito *et al*., 2005; Huber, 2005; Gupta & Gupta, 2005; Liu *et al*., 2006; Hautot *et al*., 2007; Roca *et al*., 2009; Berry, 2009; Singh *et al*., 2010; Soenen & De Cuyper, 2010; Ankamwar *et al*., 2010; Mahmoudi *et al*., 2011; Kandasamy & Maity, 2015). The SPIONs have also a promising application in food science (Miller, 2010; Hilty *et al*., 2010; Zimmermann & Hilty, 2011). These magnetic nanoparticles lead to appearance of the magnetic properties in plant systems and emergence of ESR signals (Khomutov & Koksharov, 2009). Magnetic nanoparticles are present in various natural systems including living organisms of all levels and types of organization. Biogenic generation of nanophase magnetic oxides is associated with physiological processes including a number of pathologies, in particularly, human neurodegenerative diseases, Type 2 diabetes, premature ovarian insufficiency (POI), and hemochromatosis. As a result, the investigation of mechanisms of formation iron oxide nanoparticles in living systems is important from fundamental viewpoint and also is necessary for development of novel efficient methods in pharmacy. The appearance of ESR signal characteristic for magnetic nanoparticles has been found (Khalilov *et al*., 2011b; Nasibova & Khalilov, 2016) in various plants as a result of effects of external stress factors.

In the present study, the stress factors affecting on the *Juniperus communis* shell are also directly confirmed by ESR detected SPIONs as a result of bio-mineralization effect under stress. In other words, the *Juniperus communis* shell with externally-attained functionality, resulting in formation of SPIONs, plays a role as protection for the *Juniperus communis* seeds. On the other hand, Mn-containing enzymes, representing the antioxidant activity, are normally identified to be in the *Juniperus communis* seeds. Therefore, the SPIONs in the drugs based on the *Juniperus communis* shell and the antioxidant activity detected by enzymes in the case of the *Juniperus communis* seeds could be applied for pharmaceutical and biomedical purposes.

Figure 1 also shows the ESR spectra of the *Juniperus communis* shell and seeds and their mixture at low temperature (Fig. 1b). As it was expected, the measurement performed at 77 K showed the enhancement of the ESR signal from enzymes (g = 2.0) for the *Juniperus communis* seeds. At the same time, the intensity of the ESR signal from SPIONs (g = 2.28) for the *Juniperus communis* shell is reduced at 77 K. Also, the intensity of the broad ESR signal at g = 3.26 for the *Juniperus communis* shell is found to be decreased at low temperature. Note, that in all dried soil samples at room temperature the broad ESR signal with half-width 150 mT and the position of maximum of the low-field component at g = 3.3 has been observed (Khalilov *et al*., 2011b), while upon lowering the temperature from room to 80 K the intensity of this broad signal was reduced. Registration of the broad ESR signal at g = 3.3 in soil samples has been interpreted (Khalilov *et al*., 2011b) due to a high content of iron aggregates, among which there could be magnetic nanoparticles (SPIONs) in the soil on which plants grew. As seen in our case for the *Juniperus communis* shell, the behavior of the ESR signals at g = 2.28 and g = 3.26 at 77 K is quite similar, supporting the finding of the authors (Khalilov *et al*., 2011b) that these both signals are connected with SPIONs and iron aggregates (containing SPIONs). Besides, a weak signal of Fe^{3+} at g = 4.3 (recording at 80 K in the works of Khalilov *et al*., 2011b) is also detected for the *Juniperus communis* samples at 77 K (Fig. 1b). A broad ESR signal with the position of maximum of the low-field component at g = 2.58 is assigned to the SPIONs too, as that signal is the most close to that at g = 2.69 detected for the samples with magnetic nanoparticles (Khalilov *et al*., 2011b).

Nefrovil and Immunostan

Similar ESR study as for the *Juniperus communis* shell and seeds was performed in the case of the *Juniperus communis* based Nefrovil and Immunostan drugs, where the preparation of Nefrovil and Immunostan has been made (http://roslynakarpat.com.ua) to increase the antioxidant activity and to improve the immune system of human organism respectively.

Figure 2 shows the ESR spectra of the Nefrovil at temperatures 293 and 77 K. It is clearly seen that the dominant contribution into the ESR spectra demonstrates the Mn-containing compounds (g = 2.0, characterized by six-component hyperfine structure) compared to the contribution of SPIONs (g = 2.30). The presence of both signals from Mn-containing compounds and SPIONs in the Nefrovil drug is due to the mixture of *Juniperus communis* shell and seeds used during the drugs preparation. Thus, the obtained results testify that the purpose of preparation of Nefrovil to increase the antioxidant activity is strongly confirmed using the approach developed in the present work.

In the case of Immunostan drug, we also confirmed its bioactivity to improve the immune system of human organism as shown in Figure 3. Indeed, the dominant contribution into the ESR spectra demonstrates the SPIONs (g = 2.28) and iron aggregates (g = 2.58 and 3.26) compared to the contribution of Mn-containing compounds (g = 2.0). Moreover, the behaviour of the ESR signals at room and low temperatures for the Immunostan is in consistent with the results obtained for the *Juniperus communis* materials.

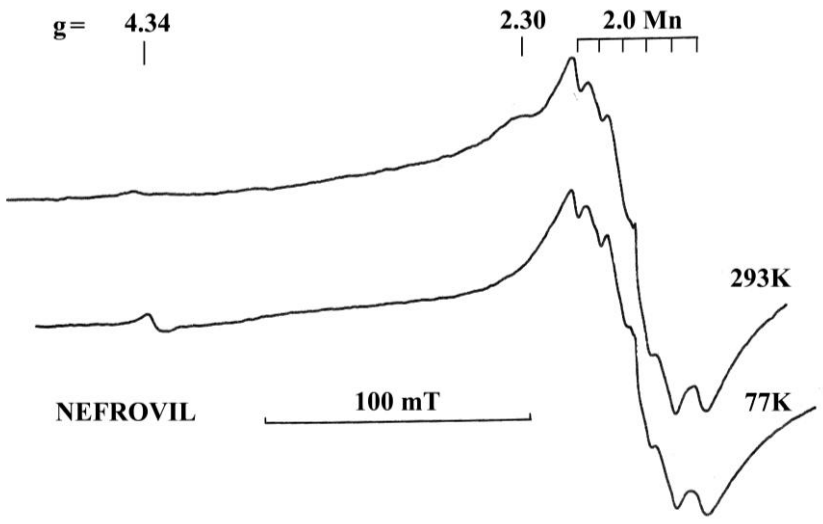


Fig. 2. ESR spectra of the Nefrovil at temperatures 293 and 77 K

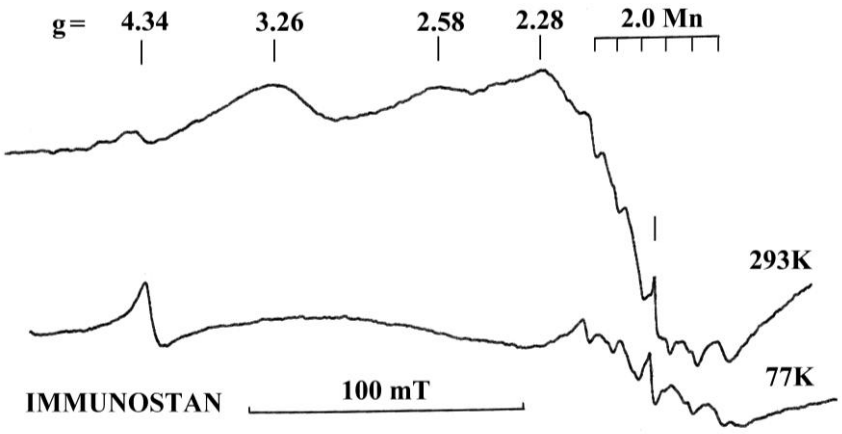


Fig. 3. ESR spectra of the Immunostan at temperatures 293 and 77 K

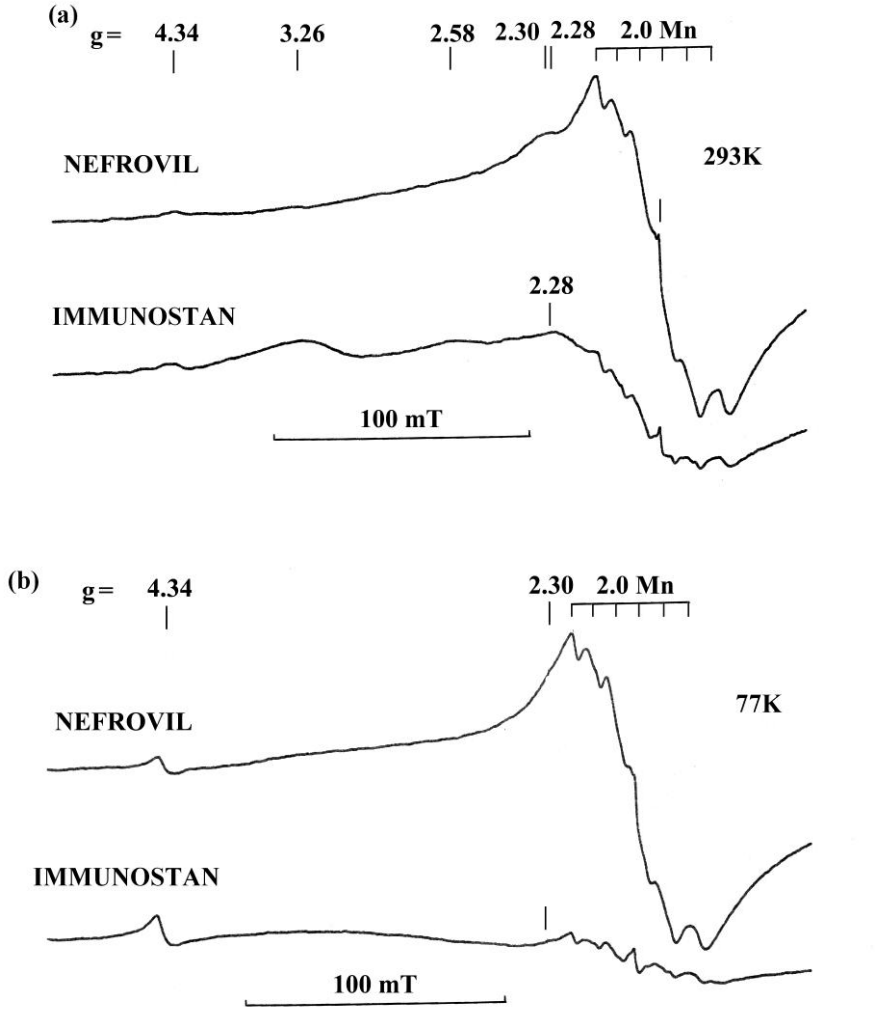


Fig. 4. Comparison of ESR spectra of the Nefrovil and Immunostan at temperatures (a) 293 K and (b) 77 K

Finally, for comparative analysis, Figure 4 shows the ESR spectra of the Nefrovil and Immunostan at temperatures 293 and 77 K. The dominant roles of MnSOD for the Nefrovil and SPIONs for the Immunostan are plausibly verified. Moreover, comparing the ESR signal from Fe^{3+} at g = 4.34, which is resolved better at low temperature, for the Immunostan and Nefrovil drugs, it is established that the prevailing role of SPIONs and Fe^{3+} ions in the Immunostan are fully correlated. It may be considered as an additional experimental evidence of the bio-mineralization effect under physical stress factors for the biomaterials studied, which is coupled with electron-transport chain of photosynthesis that leads to a decrease the Fenton's reaction due to the transformation of ferric ions (Fe^{3+}) to magnetite (Fe_3O_4) SPIONs.

4. Conclusion

An innovative identification method for bioactivity of natural BADS has been proposed on the example of *Juniperus communis* based biomaterials (*Juniperus communis* shell and seeds, Nefrovil and Immunostan drugs). Due to the effect of bio-

mineralization under stress factors, the presence of antioxidant activity system, represented by MnSOD and other enzymes, and formation of SPIONs in the biomaterials studied has been examined by means of ESR spectroscopy. The SPIONs in the *Juniperus communis* shell are only detected, while the Mn-containing enzymes are found only in the case of the *Juniperus communis* seeds. The same features have been discovered for the Nefrovil (prevailing MnSOD) and Immunostan (prevailing SPIONs), when the screening of Nefrovil and Immunostan drugs based on the *Juniperus communis*, both produced by Roslyna Karpat Co., Ltd (Ukraine), has been made to increase the antioxidant activity and to improve the immune system of human organism respectively. It has been shown that the intensities of the ESR signals from SPIONs and iron aggregates (containing SPIONs) as well as Mn-containing enzymes are sensitive to the influence of temperature. It has been established that the prevailing role of SPIONs and Fe^{3+} ions in the Immunostan are fully correlated, showing an additional experimental evidence of the bio-mineralization effect under stress (i.e., transformation of Fe^{3+} ions to Fe_3O_4 SPIONs) for the investigated biomaterials. The results obtained demonstrate the possibilities and perspectives for development and functionalization of innovative *Juniperus communis* based drugs with controlled properties for pharmaceutical and bio-medical applications, in particular, for goals where antioxidant enzymes and SPIONs should be involved.

Acknowledgments

This work was supported by the Science Development Foundation under the President of the Republic of Azerbaijan – Grant No. EIF/MQM/Elm-Tehsil-1-2016-1(26)-71/04/3. This work was supported in part by the Ministry of Education and Science of Ukraine (projects Nos. 0114U002617, 0116U004737 and 0118U000297 to T.S.K. and S.Ya.V.), State Program for ICP RAS 0082-20140-0001 (project No. AAAA-F17-117040610310-6 to V.A.S. and N.A.T.), Slovak Grant Agency VEGA (project No.2/0157/17 to O.Š.) and Slovak Research and Development Agency (project No.APVV-16-0369 to O.Š.).

The authors are grateful to Roslyna Karpat Co., Ltd (Ukraine) for providing Nefrovil and Immunostan drug samples.

References

Abreu, I.A., Cabelli, D.E. (2010). Superoxide dismutases – a review of the metal-associated mechanistic variations. *Biochimica et Biophysica Acta, 1804*, 263-274.

Ankamwar, B., Lai, T.C., Huang, J.H., Liu, R.S., Hsiao, M., Chen, C.H., Hwu, Y.K. (2010). Biocompatibility of Fe_3O_4 nanoparticles evaluated by in vitro cytotoxicity assays using normal, glia and breast cancer cells. *Nanotechnology*, *21*, 075102(1-9).

Berry, C.C. (2009). Progress in functionalization of magnetic nanoparticles for applications in biomedicine. *Journal of Physics D: Applied Physics*, *42*, 224003(1-9).

Gupta, A.K., Gupta, M. (2005). Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials*, *26*, 3995-4021.

Hautot, D., Pankhurst, Q.A., Morris, C.M., Curtis, A., Burn, J., Dobson, J. (2007). Preliminary observation of elevated levels of nanocrystalline iron oxide in the basal ganglia of neuroferritinopathy patients. *Biochimica et Biophysica Acta*, *1772*, 21-25.

Hilty, F.M., Arnold, M., Hilbe, M., Teleki, A., Knijnenburg, J.T.N., Ehrensperger, F., Hurrell, R.F., Pratsinis, S.E., Langhans, W., Zimmermann, M.B. (2010). Iron from

nanocompounds containing iron and zinc is highly bioavailable in rats without tissue accumulation. *Nature Nanotechnology 5*, 374-380.

Huber, D.L. (2005). Synthesis, properties, and applications of iron nanoparticles. *Small*, *1*, 482-501.

Ito, A., Shinkai, M., Honda, H., Kobayashi, T. (2005). Medical application of functionalized magnetic nanoparticles. *Journal of Bioscience and Bioengineering*, *100*, 1-11.

Kandasamy, G., Maity, D. (2015). Recent advances in superparamagnetic iron oxide nanoparticles (SPIONs) for *in vitro* and *in vivo* cancer nanotheranostics. *International Journal of Pharmaceutics*, *496*, 191-218.

Khalilov, R. I., Nasibova, A.N. (2010). The endogenous EPR-detectable iron nanoparticles in plants. *News Baku University*, *3*, 35-45.

Khalilov, R.I., Nasibova, A.N., Gasimov, R.J. (2011a). Magnetic nanoparticles in plants: EPR researches. *News Baku University*, *4*, 56-62.

Khalilov, R.I., Nasibova, A.N., Serezhenkov, V.A., Ramazanov, M.A., Kerimov, M.K., Garibov, A.A., Vanin, A.F. (2011b). Accumulation of magnetic nanoparticles in plants grown on soils of Apsheron peninsula. *Biophysics*, *56*, 316-322.

Khalilov, R.I., Nasibova, A.N., Youssef, N. (2015). The use of EPR signals of plants as bioindicative parameters in the study of environmental pollution. *International Journal of Pharmacy and Pharmaceutical Sciences*, *7*, 172-175.

Khomutov, G.B., Koksharov, Y.A. (2009). *Magnetic nanoparticles* (Edited by Gubin SP). WILEY-VCH Verlag GmbH & Co KGaA, 117-195.

Liu, G., Men, P., Harris, P.L., Rolston, R.K., Perry, G., Smith, M.A. (2006). Nanoparticle iron chelators: a new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance. *Neuroscience Letters*, *406*, 189-193.

Mahmoudi, M., Sant, S., Wang, B., Laurent, S., Sen, T. (2011). Superparamagnetic iron oxide nanoparticles (SPIONs): Development, surface modification and applications in chemotherapy. *Advanced Drug Delivery Reviews*, *63*, 24-46.

Miller, D.D. (2010). Food nanotechnology: New leverage against iron deficiency. *Nature Nanotechnology*, *5*, 318-319.

Nasibova, A.N., Khalilov, R.I. (2016). Preliminary studies on generating metal nanoparticles in pomegranates (Punica Granatum) under stress. *International Journal of Development Research 6*, 7071-7078.

Roca, A.G., Costo, R., Rebolledo, A.F., Veintemillas-Verdaguer, S., Tartaj, P., Gonzalez-Carreno, T., Morales, M.P., Serna, C.J. (2009). Progress in applications of magnetic nanoparticles in biomedicine. *Journal of Physics D: Applied Physics*, *42*, 224002(1-11).

Singh, N., Jenkins, G.J.S., Asadi, R., Doak, S.H. (2010). Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). *Nano Reviews*, *1*, 5358(1-15).

Soenen, S.J.H., De Cuyper, M. (2010). Assessing iron oxide nanoparticle toxicity in *vitro*: Current status and future prospects. *Nanomedicine*, *5*, 1261-1275.

Zimmermann, M.B., Hilty, F.M. (2011). Nanocompounds of iron and zinc: Their potential in nutrition. *Nanoscale*, *3*, 2390-2398.