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PRELİMİNARY STUDİES ON GENERATİNG METAL NANOPARTİCLES İN POMEGRANATES (PUNICA GRANATUM) UNDER STRESS

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ABSTRACT

The submitted article was dedicated to research of biosynthesis and generation of metal nanoparticles in *Punica Granatum.* Researches carried out with dried leaves, bark and extracts made from the leaves and fruits. Our study was carried out in two directions: biosynthesis of nanoparticles was carried out by using plant extracts and metallic salts. At the same time in living systems studied biogeneration of nanoparticles and determined mechanism of this event.

INTRODUCTION

Nanotechnology can be applied to lots of spheres of human activity. In the twenty-first century nanotechnology has achieved a lot of success in the field of science and technology. The development of nanotechnology paves the way for the solution of many problems that were not known until recently. Nanotechnology and manipulation at the atomic-molecular level in particular allows us to solve problems in the medical field and variety of environmental issues. Primarily this is due to clean and safe biosynthesis of nanoparticles. Thus, the application of nanoparticles in medicine is wide spread in treatment as well as diagnostics (http://www.den-za-dnem.ru). Overall, some nanoparticles now have a wide range of practical applications due to their unique properties. Physical and chemical methods are being used for the synthesis of nanoparticles with specific properties. However, these methods are dangerous to the environment and living organisms, they are also economically inefficient, labor and time consuming. There is a great need for economically efficient as well as environmentally clean and safe methods for synthesizing nanoparticles, that is why, over the past few years, scientists have begun to deal with the synthesis of biogenic nanoparticles (Basha *et al*., 2010; Gubin et.al., 2005; Senthil and Ramesh 2012). It is possible to synthesize nanoparticles in different concentrations, sizes and forms by adding metal salts to various plant extracts. This method makes synthesis of nanoparticles more time efficient. In addition, this approach makes it possible to control the shape and size of these nanoparticles (eg; controlling the PH of the environment and temperature of the reaction). In addition to the biosynthesis of nanoparticles, their bioinformation, biogeneration and the effects of some stress factors on this event is of great interest for study.

The generation of nanoparticles in plants using method of electron paramagnetic resonance (EPR) revealed by us for the first time (Khalilov and Nasibova, 2010). We observed wide EPR signal ($\Delta H \approx 320$ Э, g = 2, 38) (Fig.1) in some dominant plant species in Absheron peninsula (Azerbaijan) and determined that it belonged to the presence of ironoxide magnetic nanoparticles. These signals were identical with the synthetic nanoparticles of magnetite (Fe_3O_4 polyacrylamide matrix) because of their characteristic parameters (Khalilov *et al*., 2011; Nasibova *et al*., 2013).

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The presented case studies were carried out in two directions

1) Biosynthesis of nanoparticles using metal salts from plant extracts;
2) Biogeneration of nanoparticles in living systems and clarification of the mechanism of this event.

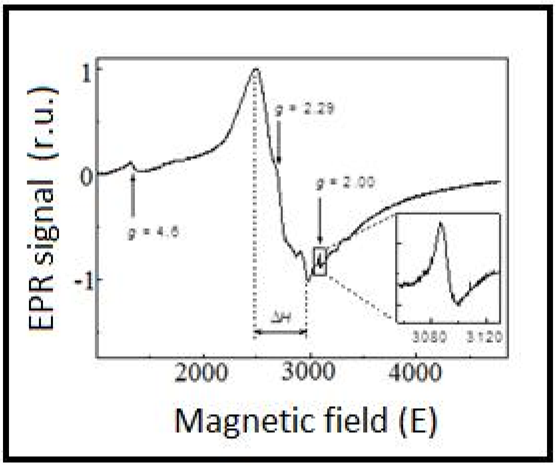


Fig.1. EPR spectrum, characteristic of magnetic nanoparticles (plant samples)

MATERIALS AND METHODS

Research subject pomegranate (*Punica Granatum*) were: dried leaves, peel, and extracts obtained from pomegranate fruit and leaves. The pomegranate is rich in (iron, iodine, calcium, potassium, magnesium) minerals and (A, B1, B2, B6, B12, C, E) vitamins. Research shows extreme value and benefits of pomegranate. All parts of pomegranate are useful due to its chemical composition: the peel, leaves and fruits. This plant is rich in vitamins, minerals and amino acids. Pomegranate juice and corns of pomegranate containing seeds are the most beneficial parts (http://www.vseprozdorovje.ru/zdorove.; Kim *et al*., 2002; Kohno *et al*., 2004). Its juice has variety of vitamins and minerals that is why pomegranate juice is being used for the rehabilitation after surgery and infectious diseases. Since it contains natural antioxidants, pomegranate is being used as anti-bacterial, anti-viral and a way to deal with inflammation. Antioxidants are barriers against the effects of free radicals that are generated as a result of negative impact of environmental pollution factors. Free radicals may cause development of cancer tumors, angina, heart disease and Alzheimer disease. Therefore, pomegranate is of a great importance in the prevention of these diseases. In alternative medicine Pomegranate is used to treat disorders caused by oxidative stresses (Toi *et al*., 2003).

First step of the study: Pomegranate plant leaves were boiled in water and precipitated in centrifuge. silver nanoparticles were synthesized after adding silver nitrate (AgNO3) to leaf extract. The mixture was blended at a temperature of 30º C and incubated for 7 minutes. UV-Vis spectroscopy measurements were carried out by means of spectroscopy SPECORD 250 plus. Second step study has been carried out by Electron Paramagnetic Resonance (EPR) method (Varian E-4 - US; Bruker German).

Samples of research pomegranate leaves (yellow and green) and 20 ml of pure pomegranate extract (control) and appropriate concentrations of FeCl2, FeCl3 were added to the samples. After drying the leaves in room temperature they were ground to powder and their EPR spectra registered at room temperature (293 K). The results have been confirmed once again by Mossbauer spectroscopy methods. To observe Mossbauer spectrum, yellow and green leaves of pomegranate plants collected from both relatively clean and contaminated sites were used. Samples were placed in a standard container - a plastic tube. During the course of study, the weight of the sample was gradually increased, it was ground and pressed. Then Mossbauer measurements were carried out. Green synthesis and biogeneration of various nanoparticles (silver and iron oxide) have been studied in pomegranates plant.

RESULTS AND DISCUSSION

Currently, many studies have proved that it is possible to carry out the synthesis of Fe, Cd, Ag, Au and other nanoparticles using plant extracts. For this purpose, various plant extracts and salt solutions are used (Basha *et al*., 2010; Thirumurugan *et al*., 2013). We have done our studies using extract from the pomegranate leaves.

Fig.2. Extract from the Pomegranate leaves: on the right– 1.0 ml $AgNO_3$ solution of 10^{-2} M concentration is added to the extract

Pomegranate plant extract was incubated with 1.0 ml $AgNO_3$ solution of 10^{-2} M concentration and color changes were observed in the solution from yellow to reddish-brown (Fig.2). Samples from the extract were taken and they were placed in quartz test tubes and then their UV-VIS spectrums were measured (Fig.3). As shown in Fig.3 from the control sample, extract made only from the leaves of the pomegranate no peak was recorded in absorption spectrum.

However, absorption spectrum of pomegranate plant extract incubated with 1.0 ml $AgNO_3$ solution of 10^{-2} M concentration has shown maximum peak in the range of 410- 450 nm. It is clear that the peak intensity depends on the concentration of silver salt and the degree of agglomeration of nanoparticles in extract. According to acquired curve, we can say, synthesized silver nanoparticles could be the sizes of 50-80 nm and more.

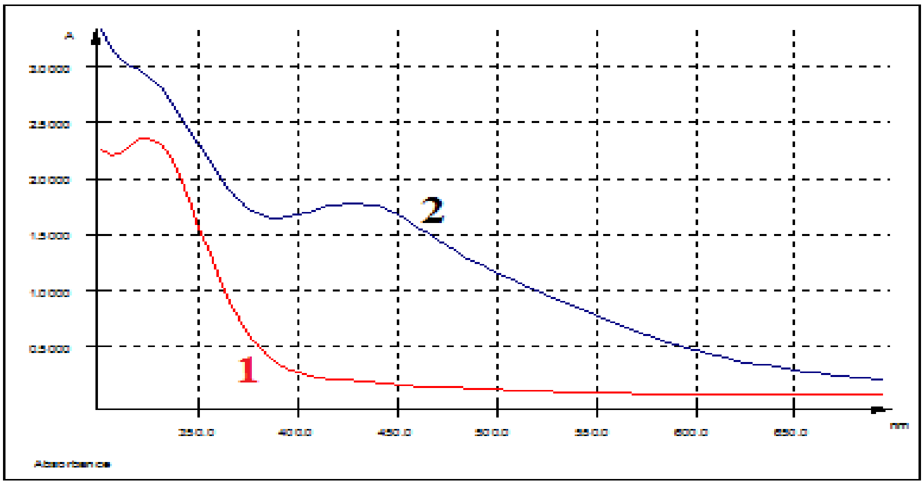
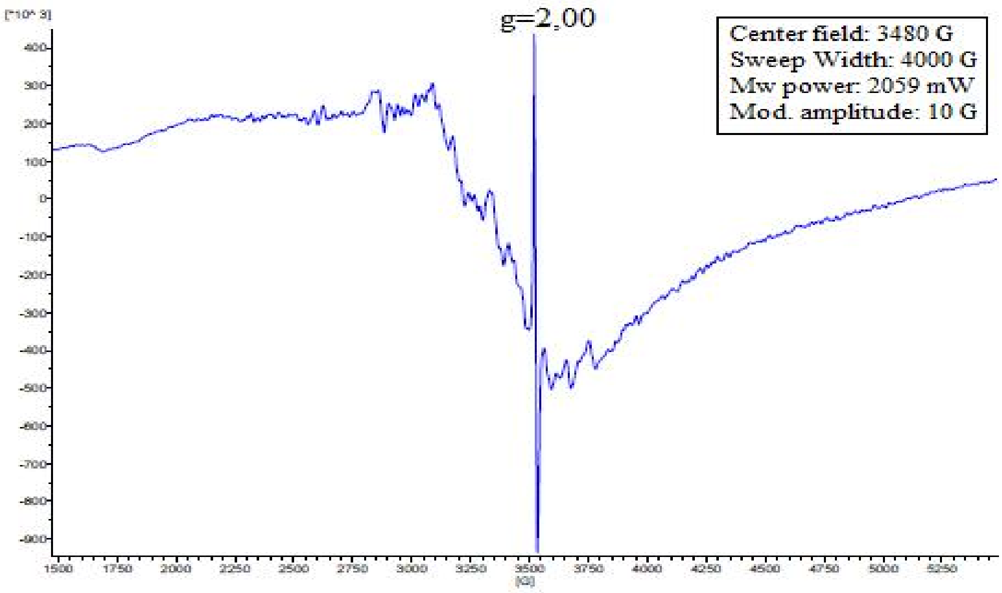


Fig. 3. UV-Vis spectrum of pomegranate leaves extract. 1-pure extract; 2-extract that was incubated with silver salt solution



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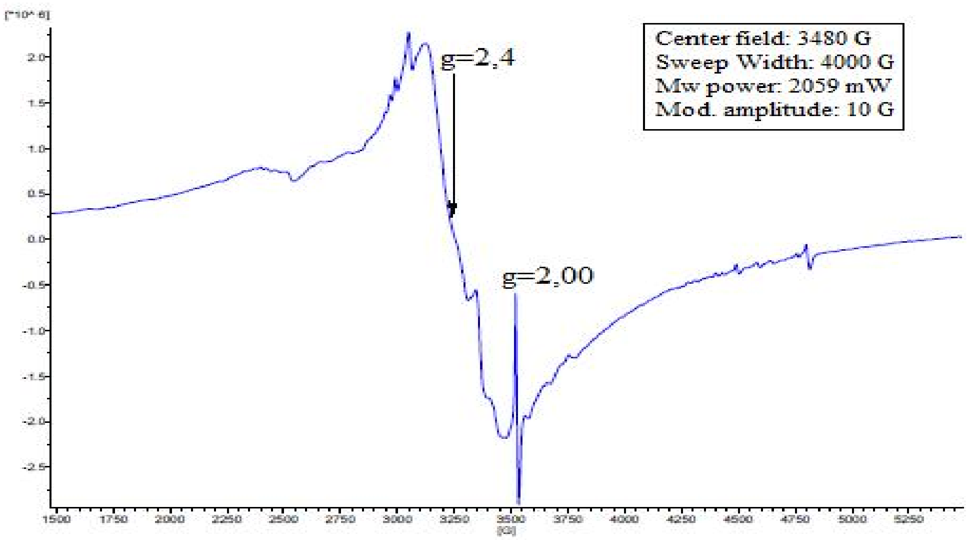

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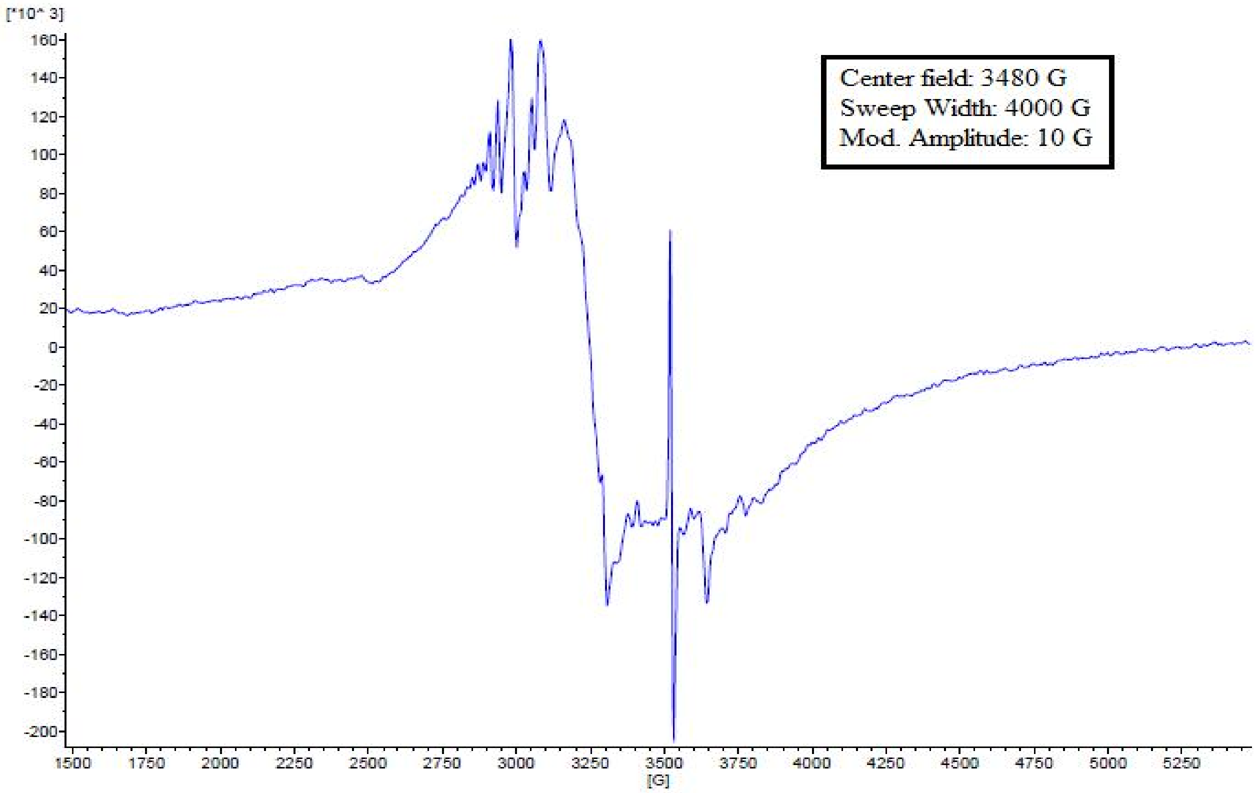
Fig. 4. Green leaves: A-from the garden area; B-from the industrial territory



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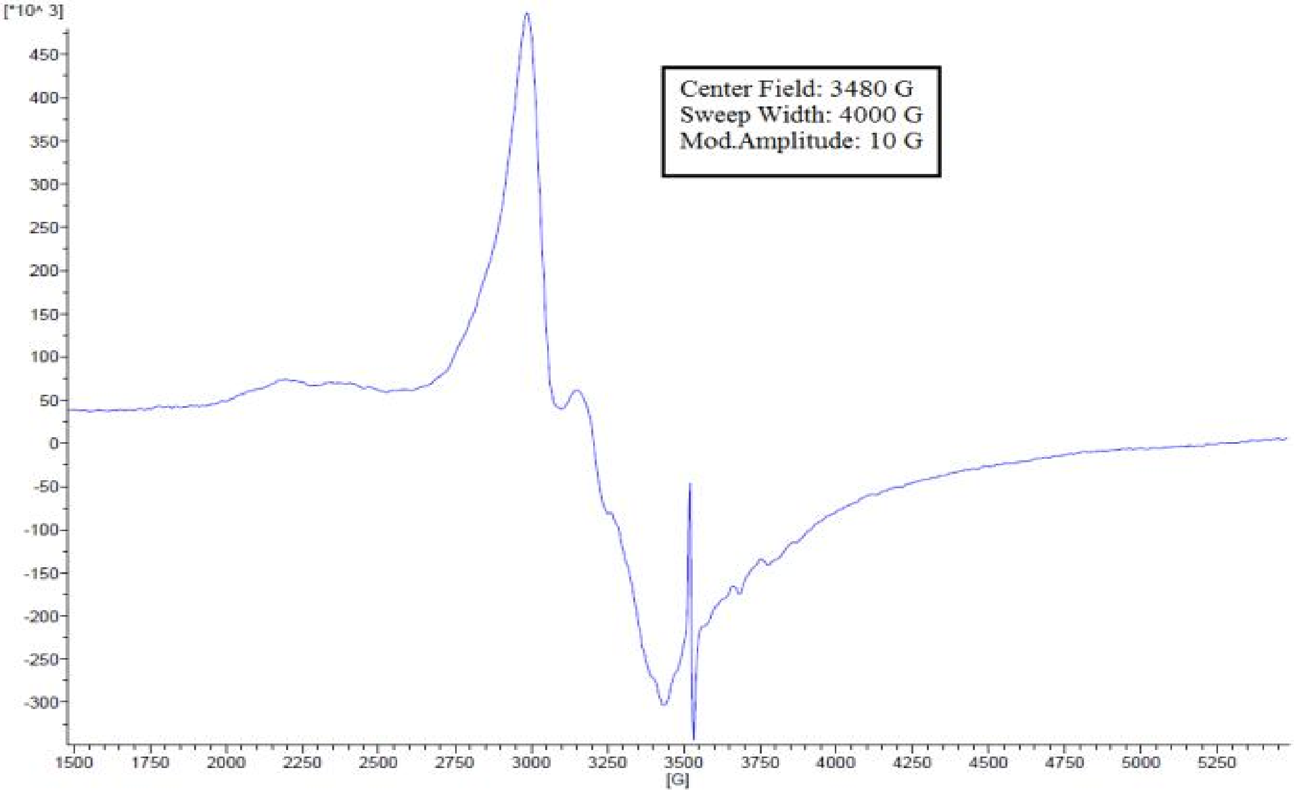


B

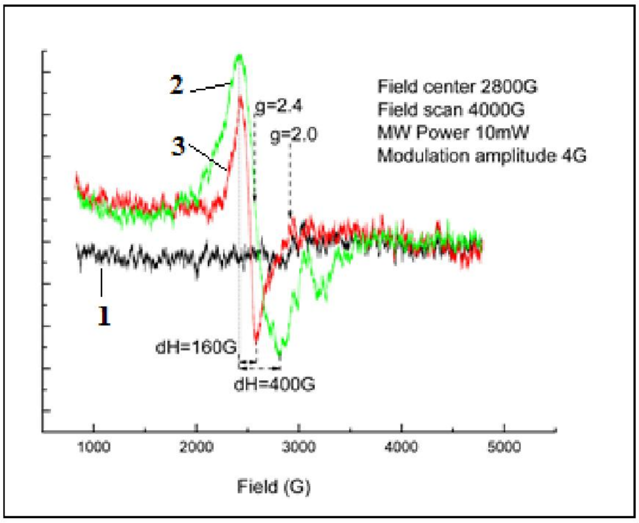
Fig. 5. Yellow leaves: A-from the garden area; B-from the industrial territory

UV-Vis spectrum was re-examined for monitoring the stability of the colloidal solution of silver nanoparticles after 4 weeks. It was determined that the spectrum of UV-VIS absorption peak during this time remained unchanged. It is known that from bacteria to the human, as a result of natural processes of biomineralization process in organisms, naturally happening iron oxide is in nanoparticle form (Khomutov *et al*., 2011; Mann *et al*., 2001). In animate and inanimate nature the most common magnetic nanoparticles are magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3). They play an important role in functionalizing living systems, as well as the development of pathological conditions. These nanoparticles cause formation of magnetic properties in biosystems and the emergence of a wide EPR signal in plants and that was demonstrated by us for the first time (Fig.1).

Therefore, with the help of plant extracts, green synthesis of nanoparticles along with the study of the mechanisms of nanoparticles synthesis in natural systems and their biogenic origin is of a great interest. Thus, in our next experiment, (2-nd direction), biogeneration of iron-based magnetic nanoparticles in pomegranate plant and the effects of stress factors associated with this event have been studied. We collected green and yellow leaves of pomegranate plants from both ecologically clean, garden area (control), and from industrial territory which is considered relatively polluted area (experimental) and studied their paramagnetic centers. As can be seen from Fig. 4. EPR signals of free radicals (g = 2.0023) in wide interval of magnetic field and manganese ions (g=2,00) were observed in leaf samples collected from the garden area. In the (experimental) sample (green leaves), beside EPR signal of free radicals, emergence of the iron oxide characterizing signal, (g=2,32; ΔH=400 Qs) was observed. This shows that, with increasing stress factors, biomineralization intensity increases in pomegranate leaves and amplitude of characteristic EPR signal is growing as a result (Khomutov *et al*., 2011).

Same experiment with yellow leaves yields the same results and proves the above mentioned results. Fig. 5 EPR signals of yellow leaf samples that were collected from same areas at the end of the autumn. In both cases, we see wide EPR signals characterizing iron-based magnetic nanoparticles. This can be explained by considering exposure of yellow leaves to UV radiation as a stress factor (Aygun Nasibova *et al*., 2016; Nasibova *et al*., 2013; Rovshan Khalilov *et al*., 2015). L.A. Blumenfeld and colleagues have shown in their previous work (Samoilova *et al*., 1995) that they record wide EPR signals with yeast cells. As mentioned above, it has been shown in our studies conducted in recent years by the EPR method (Khalilov *et al*., 2011; Nasibova *et al*., 2013) that these signals (g=2,38; ΔH=320 Qs) belong to iron-containing magnetic nanoparticles. In our studies we have obtained wide EPR signals from plant samples that are compatible with EPR signals recorded from synthetic magnetic nanoparticles (Khalilov *et al*., 2011). Our experiments and measurements show that as environmental pollution level increases, the generation of magnetic nanoparticles is accelerating in the leaves of the plants growing in that areas (Rovshan Khalilov *et al*., 2015). Therefore, we believe that these signals can be used as bioindicators for investigation and monitor of ecological status of environment.

The spectra from control pomegranate extract were recorded by the electron paramagnetic resonance method after adding the $FeCl_3$ and $FeCl_2$ (Fig. 6 A, B). Signals characterizing iron-oxide nanoparticles were noted for $FeCl_3$ iron oxide-added samples. But, we didn't get this signal from the control sample (Fig. 6 A).



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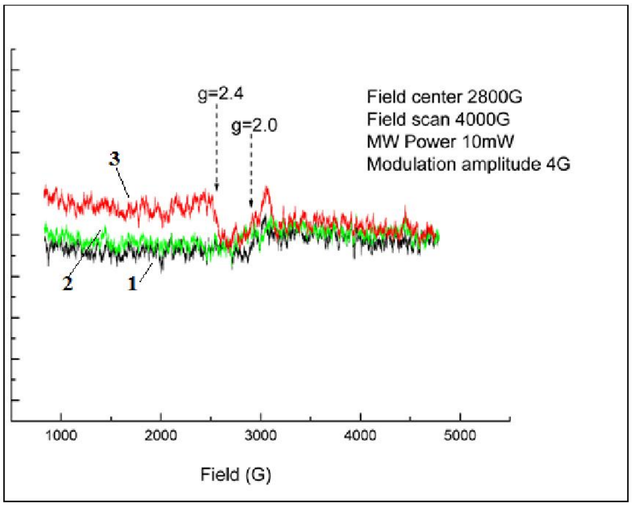


B

Fig.6. EPR signals from pomegranate extract. A)1-control; 2,3 $FeCl_3$ has been added to B) 1-control; 2,3 $FeCl_2$ has been added to

Adding $FeCl_2$ to pomegranate juice does not give rise to a wide EPR signal. This shows that in order for characteristic EPR signals to form the reaction environment (pomegranate juice) have to have reductive properties. Adding $FeCl_3$ to pomegranate juice can cause partial reduction of Fe^{3+} ions to Fe_3O_4 (Senthil and Ramesh 2012; Shengtai *et al*. 2001).

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad ....(1)$$

$$Fe(OH)_3 + R\text{-}CHO \rightarrow Fe_3O_4 + R\text{-}COOH \quad ....(2)$$

At the same time EPR signals were observed in pomegranate leaves (yellow and green) (Fig.7) and peel (Fig.8) dried at room temperature.

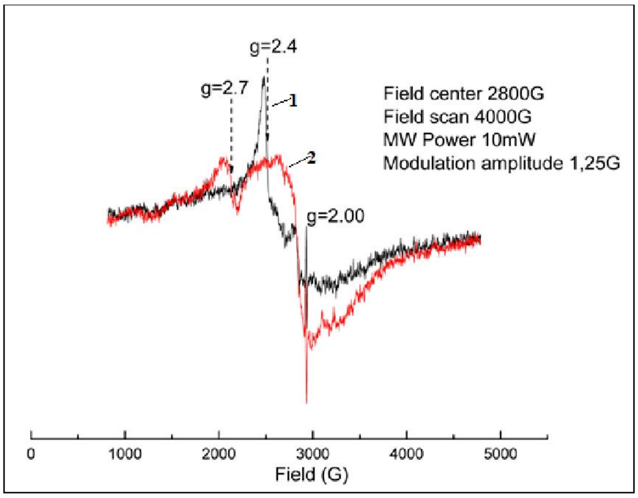


Fig.7. Pomegranate leaves EPR signals. 1- yellow; 2-green

We see from the Fig.7 that the amplitude of the signals of iron oxide magnetic nanoparticles in wide interval of magnetic field in yellow leaves is much greater than the amplitude of the signal from the green leaves.

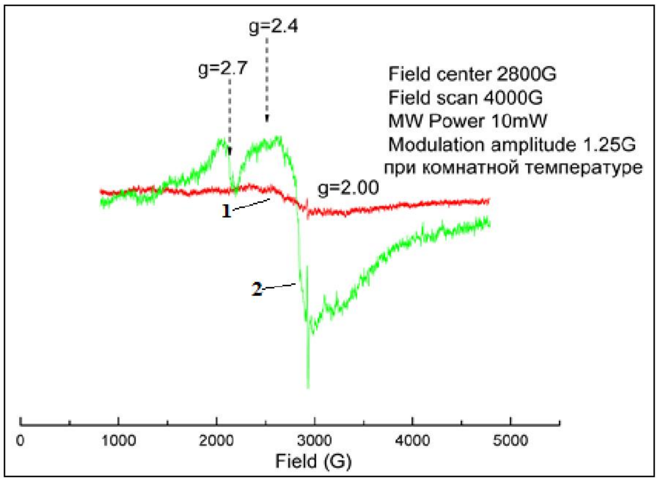
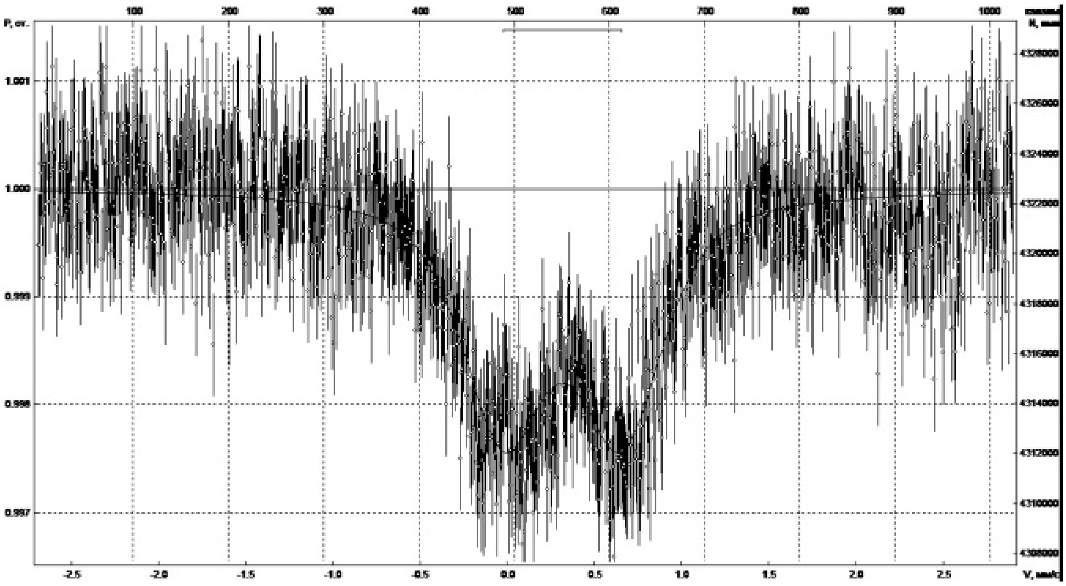


Fig.8. Green leaves of pomegranate (1) and EPR signals of pomegranate shells (2)



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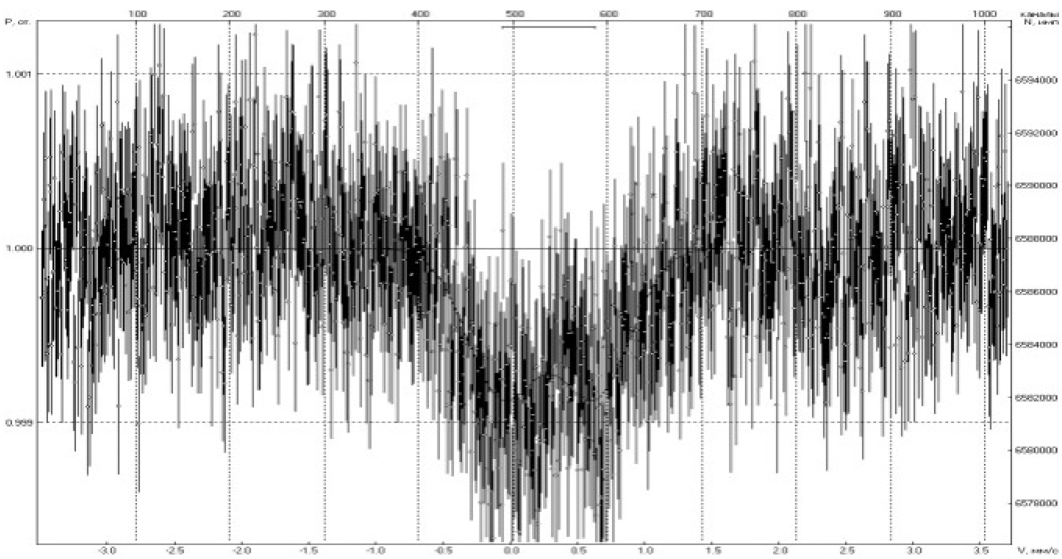

B

Fig.9. Mossbauer spectra of the pomegranate leaves. A-sampled from ecologically clean and B - ecologically polluted area

This confirms our hypothesis about the impact of stress factors as a stimulator in formation of magnetic nanoparticles (Aygun Nasibova *et al*., 2016; Khomutov *et al*., 2011). Obtained EPR results were approved by Mossbauer method (Fig. 9 A, B). Fig. 9 Mossbauer spectra of the pomegranate leaves sampled from ecologically clean (control) and ecologically polluted (experiment) area that are dried at room temperature (after processing by UNIVEM program). Spectra are calibrated to α-Fe. At room temperature spectrum have double transition, which means iron-oxides are in paramagnetic form. Spectrum is processed with one component. Values of isomeric shift (IS) and quadrupole splitting (QS) obtained by analysis. Value of isomeric shift 0.33 mm/sec at high spin (S=5/2) Fe^{3+} is characteristic for iron ions.

According to IS and QS parameters in components such as this we may come across superparamagnet spectrum in iron-oxide nanoparticle (Fe_3O_4, Fe_2O_3 and etc.) spectra. We came across the same effect in our control samples. Spectra of samples in standard test tubes were measured. The resonance absorption was 0.1%, which was less in experiment sample. The spectrum of control sample has paramagnetic appearance. In contrast to experiment samples, control samples did not have double transition appearance. Recently biomineralization processes have been topical issues of biological and ecological researches. It is clear that biominerals play an important role in life activity of many organisms. For example, calcium-containing biominerals form skeletons which are crucial for the normal life processes of the organism. But the biomineralization mechanisms are still not fully understood.

Photosynthetic systems in particular cyanobacteria play a key role in CO_2 cycle of nature, incorporating the carbon from CO_2 into organic compounds and mineral carbonates formation (Tverdislov *et al*., 2009; Kim *et al*., 2002). In this case the chemical composition of mineral deposits of photosynthetic origin significantly depends on the properties of the environment. Data from literature (Gubin *et al*., 2005; Khomutov *et al*., 2011) shows, Carbon-containing minerals are also generated within cells of some species of cyanobacteria. In this study, in addition to the green synthesis of silver nanoparticles in pomegranate extract and generation of iron-based nanophase particles in pomegranate plant, we have identified the mechanism of effect of stress factors on this event. As in our previous studies, the results of research conducted on pomegranate plant proves once again that stress factors has a stimulating effect and plays a role in the formation of the magnetic nanoparticles in plants.

And this effect can be applied to synthesis of functional iron-oxide nanoparticles used in different fields. Researches carried out by EPR spectroscopy method showed that this method is very promising for the detection of the formation of iron oxide magnetic nanoparticles in biological systems and can give new information for biomonitoring the degree of contamination of plants and environmental assessment. Detection of generation of magnetic nanoparticles in plants is a new tool for the study of biological processes. These results give us the possibility of deep understanding of the biogenic formation of nanoparticles and their role in living system.

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