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EPR-based study to monitor Free Radicals in Treated Silk Fibroin with Anthocyanins

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Abstract: Bioactive materials of natural origin have great demand in industry and medicine due to their versatility and useful properties. The main purpose of this work is to prepare biocomposites for the dual purpose of modified silk fibroin (*Bombyx mori* L.), which protects against the destructive effects of bioactive, antioxidant and ultraviolet rays. For this purpose, an aqueous extract of autumn leaves of the anthocyanin-rich smoke tree plant (*Cotinus coggygria* L.) was applied. 2% thiourea solution was used to increase the durability of the modified SF to external influences and for use in textiles. The intensity of free radicals in silk fibroin-anthocyanin (SFA) and silk fibroin-anthocyanin-thiourea (SFAT) biocomposites modified by the Electron Paramagnetic Resonance (EPR) method was studied. Maximum adsorption time was determined 20 minutes and the intensity of free radicals in SFA bio-composite was 80-85% and in SFAT biocomposite 50-55% in relation to silk fibroin untreated. For biomedical use of SFA, the radical scavenger activity kinetics were studied on a UV-2700 spectrophotometer and radical capture activity was calculated: $RSA\%_{(bioextract)} = 73.52 \pm 0.5$, $RSA\%_{(SF)} = 6.42 \pm 0.4$, $RSA\%_{(SFA)} = 45.23 \pm 0.8$

Keywords: EPR-spectroscopy, free radical, silk fibroin, autumn leaves, anthocyanins, antioxidant

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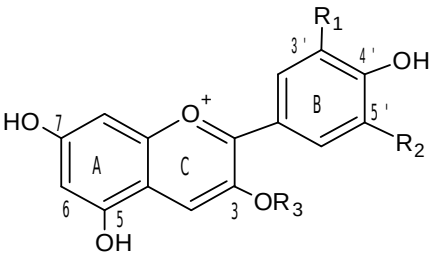
INTRODUCTION

Preparation of eco-friendly biomaterials is one of the important fields in the materials science, industry and technology, medicine as well as for to reduce occurring in the production process harmful garbage and poisonous gases (1). Silk fibroin (SF) obtained from mulberry silkworm are wide using in the weaving industry and medicine as biomaterial. This is related to its fibrillar structure. In addition, silk fibroin materials have disadvantage peculiarity as wrinkling, aging, deformation and degradation due to arising from biocompatibility properties. A lot of attempts have been made for modification and functional of the silk fibroin fibers and silk fibroin cloths arising from market demands for natural and smart materials basing on the supply of raw materials sufficient raw materials in the world. Modification of the silk in form successful may be increase use quality in addition to eliminating internal deficiencies. Hong Liu and other researchers summarized the latest ideas, methodologies, and processing technologies for the surface modification and functionality of silk fibroin, taking into account the application of improved properties of silk fibroin. Based on the results of experimental studies, the researchers hoped that such success would be perspective in the field of application of both textiles and biomaterials. The largely application of silk is related to its luster properties, effective mechanical indicators, biocompatibility, and controllable degradation. SF fibers have superior mechanical properties with a breaking elongation ~ 15%, a specific tensile strength ~ 0.5 GPa, and a breaking energy of ~ 62.104 J kg^{-1}. There are 3 main kinds of secondary structures, namely: α-helix, β-sheet, and random coil. These structures can move from one structure to another as a result of external influences, such as temperature changes, pressure and chemical effects (2-5).

One of the most widespread and important representatives of fibrillar proteins is silk fibroin. Silk

fibroin consists of two phases, crystalline and amorphous. About 60% of content of the silk fibroin form the crystalline phase (Gly-Ala-Gly-Ala-Gly-Ser)n , and the rest amorphous phase. The formation of the crystalline and amorphous phases depends on the amino acid sequences. The fact that the crystalline phase is hydrophobic and the amorphous phase is hydrophilic also depends on the amino acid sequences. The content of *Bombyx mori* silk fibroin (in moles %) is mainly characterized by the predominance of five amino acids: Gly (42.9%), Ala (30.0%), Ser (12.2%), Tyr (4.8%) and Val (2.5) %) (6). *Bombyx mori* fibroin consists of two heavy-H chain and light-L chain proteins bound by disulfide bonds, as well as a glycoprotein called P25. The molecular weight of the H-chain is about 350 kDa, which is higher than that of the L-chains (26 kDa) and P25 (30 kDa). The H-heavy, L-light and P25 chains have a 6: 6: 1 molar mass ratio (7, 8). Anti-parallel β-sheets bound by hydrogen bonds form the second structure of fibroin. The predominance of β-structure in fibroin gives high mechanical strength to the materials obtained from it, and the amorphous part of the protein gives elasticity to fibroin. Silk fibers are superior to Kevlar (para-aramid), one of the best synthetic materials, in terms of their strength. Silk fibroin with a tensile strength of 740 MPa is widely used in the manufacture of hydrogels, various transparent layers, fibrous sponges, pipes, thin layers, and microlayers. Another advantage of fibroin-derived materials is that their mechanical strength is different from that of chemically-derived materials due to their low disintegration rate. They also indicate very good biocompatibility, even when implanted in living tissues (9).

Smoke tree is not only decorative, but also rich in anthocyanins (10). Anthocyanins occur naturally in plants in the form of glycosides, in the position bound glucose, galactose, rhamnose, xylose, or arabinose with the aglycone nucleus. Anthocyanins carry a positive charge in an acidic solution unlike other flavonoids. It dissolves well in water and is usually colored blue, purple, or red, depending on the pH and the presence of chelate-forming metal ions. Deglycosylation or aglycone forms of anthocyanins are known as anthocyanidins. The sugar components of anthocyanins are generally bound to the anthocyanin ring by the hydroxyl group in position 3 of the C-ring. The radical scavenging (antioxidant) activity of anthocyanins are mainly due to the presence of hydroxyl groups in the position 3 of the C ring and at the same time in the positions 3, 4 and 5 of the B ring of the molecule (Figure 1). The activity of anthocyanins (aglycones) is superior to the corresponding anthocyanins (glycosides) (11). The degree and position of hydroxylation and alkylation in the B ring, effects their stability and reaction activity, and in thus the antioxidant activity (12).



Anthocyanidin (aglycon)	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	H
Malvidin	OCH_3	OCH_3

R_3=H or Glycoside

Figure 1: General structure of anthocyanins.

Free radicals are formed in living systems by various biochemical ways, serious oxidative effect to biomolecules and cause diseases. Indian researchers have confirmed the use of fenugreek-silk fibroin biocomposites nano-fibers as antioxidant scaffolds in wound healing applications. Fibroin scaffolds indicate the best porosity and high biocompatibility and can support better cell adhesion and proliferation. In vivo wound healing researches have indicated that they increase the rate of wound healing and collagen formation (13). Oguz Bayraktar and other researchers have proved that silk fibroin is a promising adsorbent for polyphenols based on its hydrophobic. The researchers confirmed the adsorption of antioxidant polyphenols in olive leaf on silk fibroin by FTIR. Modified silk fibroin has been shown to have antioxidant and antimicrobial activity (14).

The above-mentioned Turkish researchers made a comparative analysis of the adsorption process of silk fibroin from water and 70% ethanol extract of rutin and oleuropein in olive leaves. Achieved higher results in aqueous extract (15).

Antioxidant and antimicrobial active pads have been developed from the interaction of pomegranate peel powder and SF solution (16). Ethyl acetate extract of smoke tree leaves has been used in both biomedical research and the development of anti-cancer therapeutic strategies and has yielded high results (17). Researchers Halil Aksoy and others have used diabetic wounds on rats using from bioextract of smoke tree leaves extracted with 96% ethanol and have proven effective (18).

The main goal of this study was to improve the bioactive properties of SF with aqueous bioextract of autumn leaves with rich in anthocyanins. Because anthocyanins accumulate in the vacuoles of leaf cells, they dissolve well in water (19) and are better

extracted with water (15). The ecological usefulness and economic efficiency of water make it possible to use water as a solvent. The presented article determined the optimal time for joint processing of SF and anthocyanin pigments, had been studied the radical scavenger activity and kinetics of the obtained bioextract and biocomposite.

MATERIALS AND METHODS

Sample Preparation

The cocoon of SZEM-4 mulberry silkworm breed which it is belong *Bombyx mory* family, was removed from sericin according to accepted protocol (20). The degummed silk fibroin fibers have washed initially with tap water and in the end distilled water, and have dried at 25-30 °C. Eight silk fibroin samples weighing 0.4 g and one control sample for comparison have prepared for dyeing with smoke tree autumn leaf extract.

Getting of dye, adsorption process

The red leaves collected to extract the dye from autumn leaves of the smoke tree were dried until constant weight. The extracts were obtained by brew of ground leaves and distilled water in volume ratio of 2:1 at a temperature of 60 °C for 4 hours. From this extract, anthocyanins have treated with silk fibroin fibers at different times (15,21), and after the treatment process, the samples have been dried at room temperature. 4 samples have been taken from the obtained biocomposites and in 2% aqueous solution of thiourea ($CS(NH_2)_2$) for 30 minutes have been fixed.

Measurement in EPR

One of the most important aspects of EPR spectroscopy is to determine the concentration of radical species in special biological systems (22). All EPR spectra have been measured at room temperature using an ELEXSYS E580 spectrometer manufactured by Bruker. The G factors of free radicals have determined using the software provided with the device. The value of the G-factor is related not only to the electronic condition, but also to anisotropy (23).

Preparation of SF and SFA solution

SF and SFA were dissolved in LiBr (Sigma-Aldrich, USA) solution (9.3 M) at 60 °C for 4-5 h. The mixture solutions were dialyzed for 24 hours and centrifuged in Centrifuge 4500 rpm at 4 °C, 20 minutes, the concentration of both solutions was adjusted to 15 µM by using a UV-Vis spectrophotometric method (24).

Radical Scavenging Activity (RSA)

The free radical scavenging activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) assay. In methanol, 60 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution were prepared and noted absorbance at 515 nm. The ability of the bioextract, SF and SFA biocomposite to act as free radical scavengers against DPPH radical was tested spectrophotometrically with UV-2700 spectrophotometer (Shimadzu) by measuring. The DPPH radical scavenging activity was calculated using the following equation: $RSA\%=(A_0-A_s)/A_0$ (12).

RESULTS AND DISCUSSION

The adsorption process of SF samples in the bioextract was carried out at different times in pH = 4.5, T = 80 ± 5 °C and in the same concentration condition, in separately test tubes. Four samples dried at 20 ° C were fixed in 2% thiourea solution for 30 min and were again dried. Finally, all samples were thoroughly washed in distillated water, dried and were prepared for EPR measurements. The spectra of the samples were monitored and the parameters were analyzed.

The frequency and the intersection of the magnetic field (3555 Gauss) that causes absorption are highly dependent on the nature of the radical and the concentration of the radicals (25). The values of the G-factors in the measured spectra have shown in Table 1. The value of the G-factor has determined on the basis of a program provided by Bruker.

Some researchers have determined with EPR using that free radicals in sik fibroin are stable state. Differential signals in EPR are caused by tyrosyl radicals. Tyrosyl radicals are present in the hydrophobic part and are functional for modification (26). In Figures 2 and 3, the amplitude of the EPR spectra of both treated with anthocyanin-rich extract silk fibroins, treated and $CS(NH_2)_2$ fixed fibroins show that, concentrations of free radicals characterized by the highest signal intensity in treated silk fibroin for 20 minutes and the weakest in treated silk fibroin for 40 minutes. Compared to the intensity of free radicals of untreated SF, the intensity of free radicals in SF treated with anthocyanins rich extract increased by 80-85% for 20 minutes (Figure 2). But in treated and fixed silk fibroins, this value is 50-55% (Figure 3). Experiments have shown that the intensity of EPR signals increases when silk modifies fibroin fibers. This helps to understand the function and application of free radicals in silk fibroids for biocomposites.

Table 1: G-factor of the samples.

Sample	Adsorption time, min.	G-factor
Silk fibroin		2.0049
Silk fibroin+anthocyanins (SFA)	10	2.0053
	20	2.0053
	30	2.0052
	40	2.00504
Silk fibroin+anthocyanins+$CS(NH_2)_2$ (SFAT)	10	2.00513
	20	2.00505
	30	2.00491
	40	2.00473

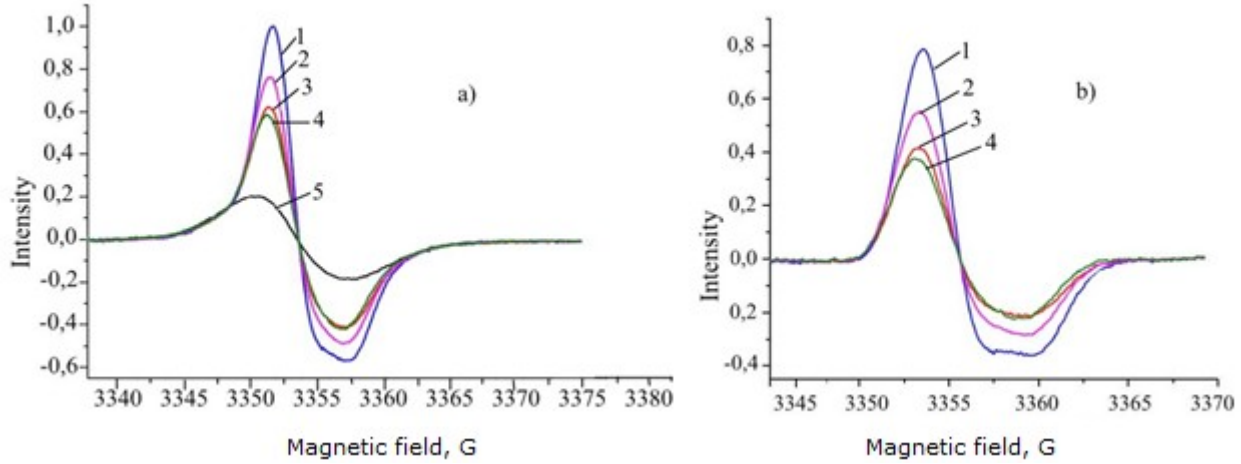


Figure 2: a) EPR spectra of silk fibroin treated with extract in various times: 1: 20 minutes, 2: 30 minutes, 3:10 minutes, 4: 40 minutes treated silk fibroin, 5: untreated silk fibroin. **b)** EPR spectra of anthocyanins obtained after subtracting untreated silk fibroin data from treated silk fibroin data in various times: 1: 20 minutes, 2: 30 minutes, 3: 10 minutes, 4: 40 minutes.

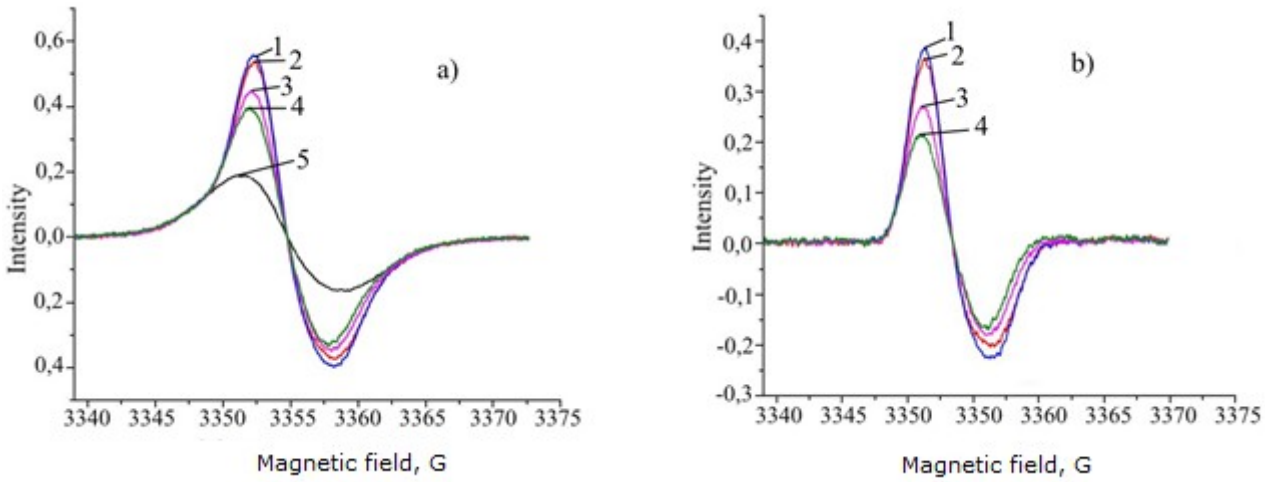


Figure 3: a) EPR spectra of silk fibroin treated with extract and fixed in various times:1: 20 minutes, 2: 30 minutes, 3: 10 minutes, 4: 40 minutes treated silk fibroin, 5: untreated silk fibroin **b)** EPR spectra of anthocyanins obtained after subtracting untreated silk fibroin data from treated and fixed silk fibroin data in various times: 1: 20 minutes, 2: 30 minutes, 3: 10 minutes, 4: 40 minutes.

The treatment process is based on the adsorption process. Since adsorption is a chemical (irreversible) and physical (reversible) process, van der Waals forces involve in physical adsorption, and chemical binding (covalent, hydrogen) occurs between fibroin and anthocyanins in chemical adsorption. In a range of 10-20 minutes, physical and chemical adsorption occur in parallel, in 30-40 minutes ranges only physical adsorption occur (27-29). The intermolecular H bonds and covalent bonds have been described in Figure 4 (30,31).

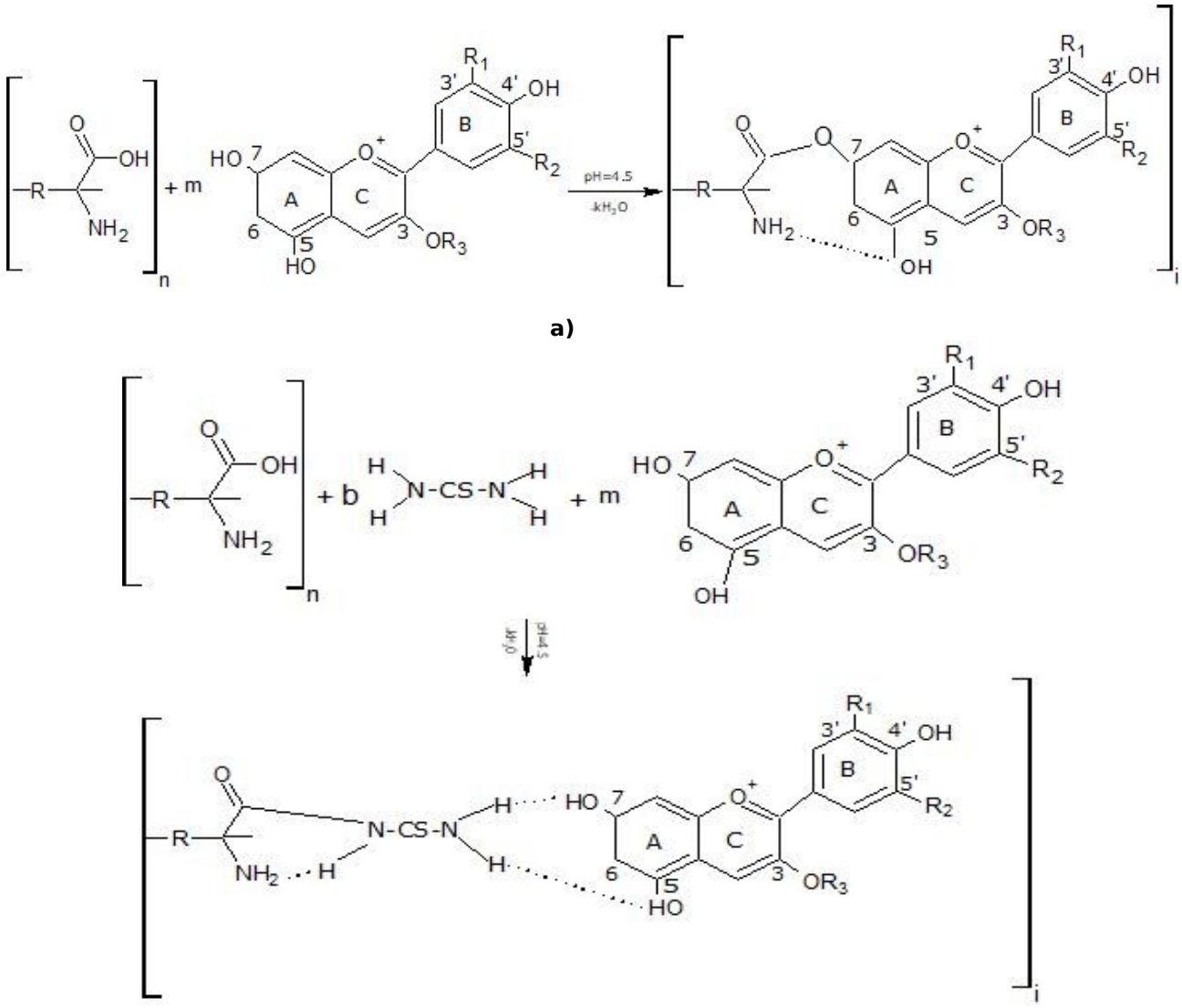


Figure 4: a) H-bond and covalent bond between anthocyanin and fibroin molecules, **b)** H- bond and covalent bond between anthocyanin, thiourea, and fibroin molecules.

The EPR analysis showed that the intensity of free radicals in the SFAT biocomposite was lower than that of the SFA. 20 minutes treated SFs were used to achieve the define goals. The resistance of SFAT biocomposite to detergents and sunlight, and SFA to 95% ethanol solution was tested for 24 months. These tests showed that the application of SFAT in textiles and SFA in biomedicine is considered appropriate. The SFA biocomposite can be stored in ethanol without any changes.

One of the goals of this study was to obtain a natural fibroin-based bioactive, antioxidant, antimicrobial biomaterial. For this purpose, the free radical capture activity of bioextract, SF and SFA solutions by DPPH was analyzed on a UV-2700 spectrophotometer. In a cuvette, 2950 µL of 60 µM methanolic DPPH solution was mixed with 50 µL of bioextract to start the reaction, kinetics was monitored for 30 minutes. The experiment was repeated for SF and SFA solutions with same way and in result: Bioextract > SFA > SF (Figure 5) (32). Then, absorbance of mixture solution was measured

at end of each process. The absorption data at 515 nm were recorded and the RSA (%) were calculated: $RSA\%_{(bioextract)}$=73.52±0.5, $RSA\%_{(SF)}$=6.42±0.4 and $RSA\%_{(SFA)}$=45.23±0.8

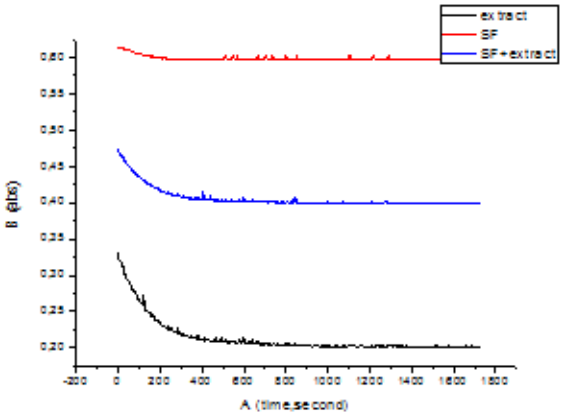


Figure 5: Kinetics of samples on UV-Vis spectophotometer.

CONCLUSION

It was determined in result of research that the free radical scavenger activity of silk fibroin increased from $RSA\%_{(SF)}$ = 6.42 ± 0.4 to $RSA\%_{(SFA)}$ = 45.23 ± 0.8 using an anthocyanin-rich bioextract obtained from the autumn leaves of the smoke tree.

The EPR method is determined that the intensity of free radicals and the amount of anthocyanins were higher in silk fibroins adsorbed for 20 minutes at a temperature of 80 ± 5 °C.

SFA can be used in biomedicine due to the intensity of free radicals, RSA and long-term (24 months) resistance in 95% ethanol and SFAT can be used in textiles due to resistance to detergents and sunlight.

EPR spectra showed that, according to the laws of the adsorption process, the combination between silk fibroin and anthocyanins in the autumn leaf extract of the smoke tree is obtained by hydrogen, covalent bonding and van-der-Waals forces.

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