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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Green Synthesis of Silver Nanoparticles with Black chokeberry [*Aronia melanocarpa* (Michx.) Elliot] Leaf Extract and Evaluation of Insecticidal Activity on the Greater Wax Moth [*Galleria mellonella* (Lepidoptera:Pyralidae)]

Aronya [*Aronia melanocarpa* (Michx.) Elliot] Yaprak Ekstresi ile Gümüş Nanopartiküllerin Yeşil Sentezi ve Büyük Balmumu Güvesi [*Galleria mellonella* (Lepidoptera:Pyralidae)] Üzerindeki Böcek Öldürücü Aktivitenin Değerlendirilmesi

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Abstract

The greater wax moth, *Galleria mellonella* (Lepidoptera:Pyralidae), is a harmful species that settles on honeycombs in beehives and causes a decrease in productivity. In the control of harmful insects, the discovery of alternative methods to chemical control, which are environmentally friendly and not effective on non-target organisms has been studied for many years. - Plant-derived substances are widely used among these alternative methods. Black chokeberry [*Aronia melanocarpa* (Michx.) Elliot] is a shrub-like plant belonging to Family Rosaceae and is cultivated for fruit production. While there are many studies on the medical importance of this plant, known as the "super/miracle plant", studies on its effects on insects are limited. Nanotechnology is one of the topics that has attracted a lot of attention in recent years. The use of nanotechnology is increasing in the agricultural sector as in other disciplines. An agrochemical system with superior properties such as targeted pesticide, controlled release of active substances, and biodegradable materials can be created from modified nanomaterials. With these features, nanotechnology gives hope to solve the environmental problems caused by traditional chemical pesticides. In this study, the leaf extract of *A. melanocarpa* was green synthesized with silver nanoparticles. The green synthesized nanoparticles were tested on late stage larvae of *G. mellonella* and their insecticidal effect was demonstrated. In addition, the binding ability of the main components in the leaf extract (n-hexadecanoic acid, oleic acid and octadecanoic acid) to the antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) of the insect has been demonstrated *in silico*. Thus, it was aimed to reveal a plant-derived extract, an effective bioinsecticide alternative to chemical control, by *in vivo* and *in silico* methods. LC_{50} and LC_{99} values of *G. mellanolla* larvae applied with silver nanoparticle leaf extract were determined as 5.51 and 11.58µl, respectively. Moreover, the effect of main components of *A. melanocarpa* on SOD and CAT enzymes, which are the main elements of the enzymatic defense system of *G. mellonella*, was demonstrated *in silico*.

Keywords: *Galleria mellonella, Aronia melanocarpa, in silico*, Nanoparticle, Leaf extract

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Öz

Büyük balmumu güvesi *Galleria mellonella*, arı kovanlarındaki peteklere yerleşen ve verimin azalmasına neden olan zararlı bir türdür. Zararlı böceklerin kontrolünde, kimyasal mücadeleye alternatif, çevre dostu ve hedef dışı organizmalar üzerinde etkili olmayan yöntemlerin keşfi uzun yıllardır çalışılmaktadır. Bu alternatif yöntemler arasında bitki kaynaklı maddelerin kullanımı geniş bir yer tutmaktadır. Aronya [*Aronia melanocarpa* (Michx.) Elliot], Rosaceae familyasına ait çalı benzeri bir bitki olup, meyve üretimi amacıyla yetiştirilmektedir. "Süper/mucize bitki" olarak bilinen bu bitkinin tıbbi önemi üzerine birçok çalışma bulunurken, böcekler üzerindeki etkilerine ilişkin çalışmalar sınırlıdır. Nanoteknoloji son yıllarda oldukça ilgi çeken konulardan biridir. Nanoteknolojinin kullanımı diğer disiplinlerde olduğu gibi tarım sektöründe de giderek artmaktadır. Modifiye edilmiş nano malzemelerden, hedef odaklı pestisit, aktif maddelerin kontrollü salınması, biyolojik olarak parçalanabilen malzemeler gibi daha üstün özellikli bir zirai kimyasal sistem oluşturulabilir. Bu özellikleri ile nanoteknoloji geleneksel kimyasal pestisitlerin yarattığı çevresel problemleri çözme ümidi vermektedir. Bu çalışmada *A. melanocarpa*'nın yaprak ekstraktı yeşil sentez yoluyla gümüş nanopartikül ile sentezlenmiştir. Sentezlenen yeşil nanopartiküller, *G. mellonella*'nın geç dönem larvaları üzerinde test edilmiş ve böcek öldürücü etkileri gösterilmiştir. Ayrıca yaprak ekstraktı içinde bulunan ana bileşenlerin (n-heksadekanoik asit, oleik asit ve oktadekanoik asit) böceğin antioksidan enzimlerine (süperoksit dismutaz (SOD) ve katalaz (CAT) bağlanma özelliği *in silico* olarak gösterilmiştir. Böylece kimyasal mücadeleye etkili bir biyoinsektisit alternatifi olan bitki kaynaklı bir ekstraktın *in vivo* ve *in silico* yöntemlerle ortaya çıkarılması amaçlanmıştır. Gümüş nanopartikül yaprak ekstraktı uygulanan *G. mellanolla* larvalarının LC_{50} ve LC_{99} değerleri sırasıyla 5.51 ve 11.58µl olarak belirlenmiştir. Ayrıca *A. melanocarpa*'nın ana bileşenlerinin *G. mellonella*'nın enzimatik savunma sisteminin ana elemanları olan SOD ve CAT enzimleri üzerindeki etkisi *in silico* olarak gösterilmiştir.

Anahtar Kelimeler: *Galleria mellonella, Aronia melanocarpa, in silico*, Nanopartikül, Yaprak ekstraktı

1. Introduction

Nanotechnology is one of the topics that has attracted a lot of attention in recent years. With this technology, nanoparticles appear as systems prepared in natural or synthetic structure, with sizes varying between 10-1000 nm, called nanospheres or nano capsules depending on the preparation method, and in which the active substance is dissolved in the particle, trapped or absorbed or bound to the surface. Nanoparticles can be obtained in nano sizes by certain physical or chemical means. Nanotechnology; It includes scientific fields such as organic chemistry, molecular biology, and semiconductor physics and is used in many fields such as environment, food, medicine, automotive, space, and agriculture (Wagner et al., 2007). With nanotechnology, the basic physical and chemical properties of materials can be changed, thus reducing the size of the materials to nanometers, creating new materials with unique electrical, optical and mechanical properties (Farre et al., 2009).

The agricultural sector and other disciplines are increasingly using nanotechnology. The use of nanotechnological products is gaining importance as a solution to the problems encountered in agriculture, which is one of the most important sources of income all over the world and in our country. Increasing the efficiency and reducing the use of agricultural inputs such as pesticides, and fertilizers by using nano carriers and accelerating product development are prominent nanotechnological applications in the agricultural industry. It offers hope of solving environmental problems created by traditional chemical pesticides. This technology aims to increase the solubility of the active ingredient of biopesticides, control/targeted release of active ingredients, and prevent the deterioration of active ingredients against environmental influences (Nuruzzaman et al., 2016).

Nano agrochemicals may have some outstanding properties such as high solubility, good stability, high efficacy, triggering release, etc. Thus, an agrochemical system with superior properties such as targeted pesticide, controlled release of active substances, and biodegradable materials can be created from modified nanomaterials. In this way, the environment and human health will be secured, while pesticides and fertilizers will protect and encourage the growth of plants. Due to the small size of nanoparticles, the surface area covered will be much larger than that of microparticles at the same dose. Therefore, it is thought that the effectiveness will gain a geometric increase. It seems that this approach is very important in terms of the policy of using less pesticides. Different methods are used in the synthesis of nanoparticles. One of these methods is biological methods, which enable the preparation of nanoparticles without using any toxic chemicals. In nanotechnology, biosynthesis occurs in plants, bacteria, fungi, yeast, algae, viruses, etc. Describes synthesizing nanoparticles using chemicals with living organisms.

Chemical pesticides have been widely used to manage agricultural pests for many years. The use of these chemicals can cause significant side effects on organisms and the environment, such as reduction in natural enemy populations, development of resistance in pest populations, and toxicity to humans. All of these problems have led researchers to develop natural and safe alternative methods - For this reason, plant essential oils and herbal extracts have begun to be investigated and their potential for use as botanical insecticides to eliminate the problem of insect resistance development and chemical residues - Plants are considered good sources of natural bioactive compounds with a wide range of nutritional and pharmacological properties.

Aronia melanocarpa is a shrub-like plant belonging to Family Rosaceae and is cultivated for fruit production. It grows naturally in the eastern region of North America and Canada. The plant known as "chokeberry" has two identified species: *A. melanocarpa* (black chokeberry) and *A. arbutifolia* (red chokeberry) (Kulling and Rawel 2008). It is known that *Aronia* is grown in a total of 78 hectares of land in our country, according to 2019 data. In cultivation, the provinces of Kırklareli (240 da), Bursa (141 da), Manisa (90 da), Kırşehir (48 da) and Yalova (48 da) are in the top five (Poyraz and Boz, 2019). *Aronia* is a plant with high adaptability to different soil and climate conditions. This feature allows it to be grown easily in different regions. Due to its high antioxidant activity, it is used in the prevention and treatment of many diseases, and therefore its production and use is becoming widespread around the world (Oszmiański and Lachowicz 2016).

There are many studies on the anticancer, antimutagenic and antibacterial activities of Aronia (Jurikova et al. 2017). It is known that it is very rich in phenolic compounds, especially the anthocyanins it contains. The positive effects of this important plant in the treatment of various diseases have been determined. It is predicted that it can be used against many diseases, especially cancer. Because of these properties, it is called "super/miracle plant". However, limited information is available regarding the insecticidal properties of these plant-derived products.

The greater wax moth, *Galleria mellonella*, is a harmful species that settles on honeycombs in beehives and causes a decrease in productivity. *G. mellonella* is a preferred species in entomological research with its nutritional needs, ecological adaptation and development characteristics. The negative problems caused by chemical control used against economically harmful insects have caused biological control studies to gain importance (Alkaş, 2007). For this purpose, in addition to the use of biological control agents, studies involving environmentally friendly techniques to determine the lethal and repellent effects of plant extracts and essential oils on harmful insects have been ongoing for many years. Recent studies show that essential oils and plant extracts produced by aromatic plants are used successfully in control of stored product pests (Erler, 2005; Negahban et al., 2007; Ayvaz et al., 2009; Ercan et al., 2013). At the same time, these products may have a repellent effect against the target pest and have a negative impact on various characteristics such as longevity and reproductive potential.

Since *G. mellonella* larvae can be produced in large quantities in cheap artificial foods under laboratory conditions, they are used as a model organism in physiology, biochemistry and molecular biology studies. Its importance is increasing due to its use as natural host insects in the breeding of parasitoid insects used in biological control, insecticide efficacy trials, and even its widespread use in determining the pathogenicity of microorganisms that cause diseases in humans and other mammals. They are also agriculturally important as many species in the family that this insect belongs to are pests of stored products (Sefer and Büyükgüzel, 2018).

In this study, *A. melanocarpa* leaf extract was green synthesized with silver nanoparticles, characterized (FTIR, SEM, EDX), and tested on *G. mellonella* late stage larvae and its insecticidal effect was demonstrated. In addition, the content of the leaf extract was analyzed by GC-MS and the primary substances in the content were used as ligands and tested with insect antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) binding properties were demonstrated in silico. Thus, the potential of a plant-derived extract to be an effective biopesticide alternative to chemical control was investigated, supported by nanotechnology and *in silico* data.

2. Materials and Methods

2.1. Plant Material 1

A. melanocarpa used in the study was purchased in an identified form from the Aronia Sante company, which produces it in the Kırklareli region (Türkiye). The leaves of the plant materials were dried and stored at 4 ^{o}C after grinding. Solid-liquid extraction method was used to prepare the leaf extract. Water was used as the solvent for extraction (Tosun, 2009).

2.2. Biosynthesis of Silver Nanoparticles from leaf extract of Aronia melanocarpa

In the study, after preparing the water extract of *A. melanocarpa*, 1mM silver nitrate was added to the extract (Ndikau et al., 2017). After mixing in a conical flask, it was kept at room temperature for 24 hours to monitor the color change, which is an indicator of nanoparticle formation. FTIR and SEM analyzes were performed to determine whether biosynthesis was successful.

2.3 Production of Galleria mellonella under laboratory conditions and application of leaf extract with silver nanoparticles

The eggs of *G. mellonella* used as a model insect were taken from the stock culture grown in the Entomology Laboratory of Kırşehir Ahi Evran University Faculty of Agriculture, Department of Plant Protection. Corn flour, water, bran, milk powder, honey, glycerol, yeast and honey nutrients were used in the cultivation of *G. mellonella*. To do this, wax moth eggs were first placed in 1-liter glass jars filled with 1/3 food, paper was placed around the mouth of the glass jar to allow them to lay eggs, and it was covered with fly screen and metal clamps. The last stage larvae of *G. mellonella* cultured in this way were reserved for use in experiments. The temperature of the incubator used was set to be 28± 2°C, the average proportional humidity was 65±5%, and it was in the dark.

The concentration of the plant product administered to the larvae via microinjection was determined during the applications (Gökmen et al., 2022). To apply the product at the specified concentration (0, 2, 4, 6, 8 µl.) to the larvae, the larvae were wiped with 70% alcohol using a sterile swab and a direct injection was performed with a microinjector into the last left leg (proleg) of the larvae (Alvandial et al., 2016). Solvent injection (dH_2O) was applied to the control group, and both control and treated larvae were monitored in the incubator at 28±2°C, 65±5%

relative humidity and in the dark all day long. For each dose, 6 replicates and 10 late-stage insect larvae were used in each replicate. After insect larvae were exposed to the plant product at determined doses for 24 h, mortality rates were recorded and probit analysis was performed to determine LC_{50} and LC_{99} doses (Abbott, 1925).

2.4 Molecular Docking and Simulation Studies

Content analysis of the leaf extract has been done in our previous study (Torun et al., 2023). For molecular docking studies, firstly ligand preparation was made. For ligand preparation; 3D drawings of the main substances determined by the content analysis obtained as a result of GC-MS of the plant leaf extract have been completed in BIODRAW and GAUSSIAN programs. Insects, like vertebrates, have enzymatic and non-enzymatic defense systems. In the study, superoxide dismutase (SOD) and catalase (CAT) enzymes, which are among the main elements of the enzymatic system, were selected as target proteins. Sequences of the enzymes were taken from UniProt (https://www.uniprot.org/) (Catalase: LOC113521268; Superoxide dismutase: LOC113520545).

Protein structure models were built using Phyre2 and Itasser online databases (Kelly et al. 2015; Yang et al., 2015; Yang & Zhang 2015; Zheng et al., 2021). Stereochemical analyzes of the homology model were performed using the Ramachandran plot obtained from https://zlab.umassmed.edu/32. Finally, detailed analysis of the 3D structure of the enzymes was performed using the CASTp 3.0 server (http://sts.bioe.uic.edu/castp/index.html?j_61c5a569932c0) to analyze the active sites of the enzymes. Autodock Vina program was used for docking studies (Trott and Olson, 2010).

To detect protein-ligand interactions, the protein-ligand file (.pdbqt), which is the output of the Autodock Vina program, was selected separately for ligand and protein with the VMD and Maestro (Academic free) program, and then 2- and 3-dimensional protein-ligand interactions were observed at the amino acid level (Humphrey et al., 1996). Simulation studies were carried out using the NAMD program (James et al., 2005).

3. Results and Discussion

3.1. SEM Analysis and EDX Results

Energy Dispersive Spectrum (EDX) is used to perform quantitative chemical analysis by taking advantage of the energies of the elements by adding them to SEM and/or TEM analysis. When high-energy electrons hit the surface of the sample, some electrons are detached from the sample surface due to these collisions. If these electrons are removed from the inner (closer to the nucleus) orbitals, the atoms lose their stability. In order to become stable again, electrons in the outer orbitals fill the gaps in the inner orbitals. Since the energies of the electrons in the outer orbitals are higher than the energies of the electrons in the inner orbitals, the outer orbital electrons have to lose a certain amount of energy while filling the inner orbitals. This lost energy appears in the form of X-rays. The X-rays detected by the detector are converted into signals and converted into an X-ray energy histogram consisting of peaks with certain intensities. With this X-ray histogram, the type and amount of each element in the material can be determined. The percentages of elements in the sample are proportional to the areas under the elements' peaks. EDX and SEM analyzes of nanoparticles were performed in the project (*Table 1*, *Figure 1-3*).

SEM Spectra of the synthesized Ag nanoparticles are presented in *Figure 1* and *2*. The morphology of nanoparticles synthesized through a green method was analyzed using SEM. The prepared nanoparticles exhibited spherical in shape and ellipsoidal, and displaying aggregation, as illustrated in *Figure 1* and *2*. Most of the Ag nanoparticles were observed to be with a moderate variation in particle sizes. According to the size distribution, the size of the nanoparticles is on average in the range of 15-50 nm. The properties of silver nanoparticles were also observed by energy dispersive microanalysis (EDX). Energy dispersive X-ray analysis (EDX) revealed a strong signal confirming the presence of silver (*Figure 3*, *Table 1*). The EDX results for elemental analysis indicated the presence of a silver absorption peak at 3 keV, signifying that silver is the predominant constituent.

The spectrum also revealed the presence of carbon and oxygen, attributed to the organic compounds from the leaf extract coating the nanoparticle surface. These elements play a crucial role in the reduction and stability of nanoparticles synthesized through the green method (Jagtap and Bapat, 2013).

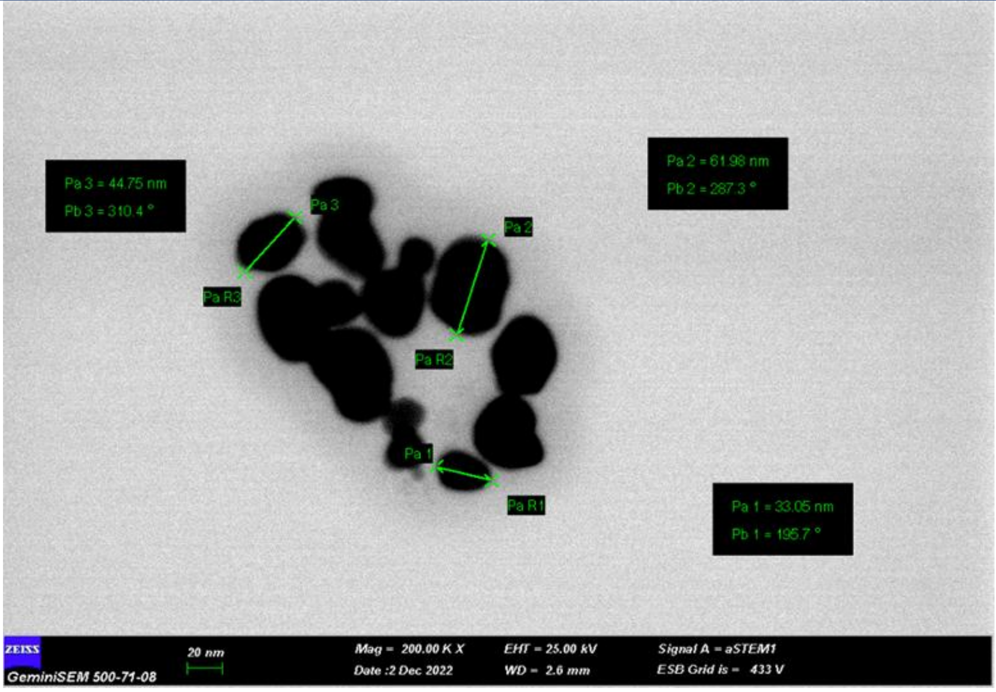


Figure 1. SEM analysis result (liquid)

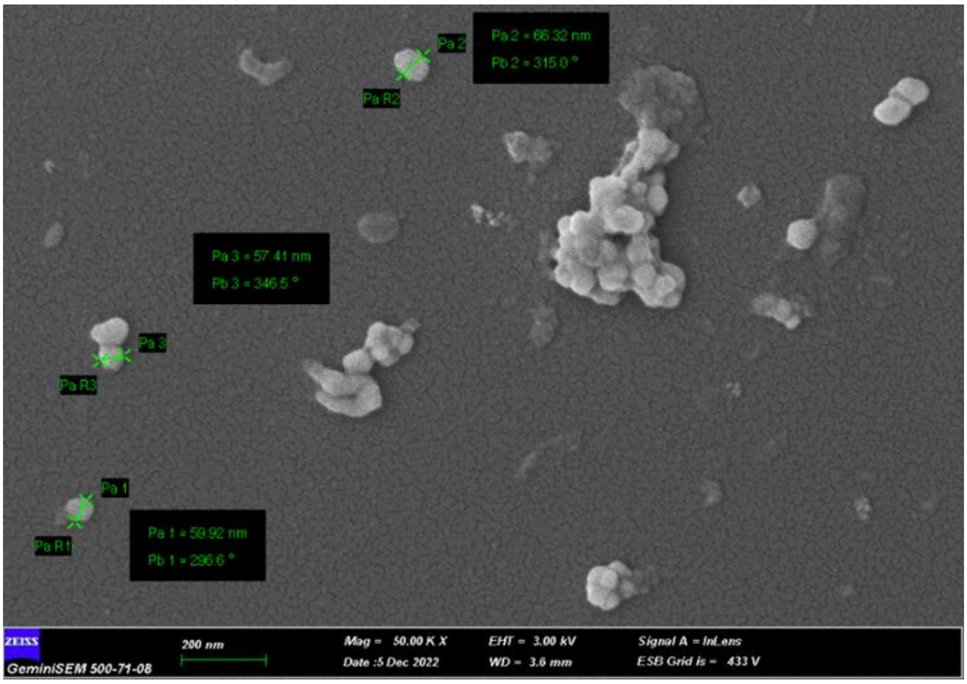


Figure 2. SEM analysis result

Table 1. Results of EDX spectrum

Element	Weight %	Atomic %	Net Int.
C K	44.59	55.38	1628.79
O K	45.69	42.6	1161.78
AgL	6.83	0.94	388.09
CaK	2.89	1.07	244.73

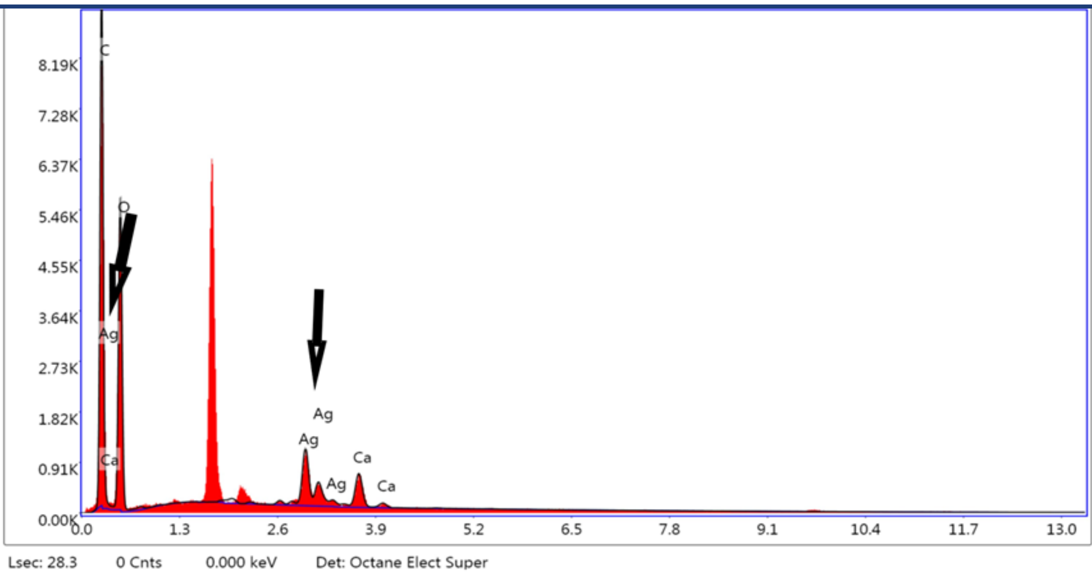


Figure 3. EDX spectrum of nanoparticles

3.2 FT-IR Analysis

FT-IR spectra of the molecules were taken using the Perkin-Elmer Spectrum One FT-IR spectrometer in the 4000-400 cm-1 region at room temperature with the KBr pellet method. FTIR spectra of these structures were analyzed using the solid-state KBr disk method. These spectra were recorded in the range of 4000–400 cm-1 at room temperature with the Thermo Scientific Nicolet 6700 FT-IR device at Kırşehir Ahi Evran University Central Research and Application Laboratory. Experimental FTIR spectra of the structure of plant extract and plant extract-loaded nanoparticles were plotted (*Figure 4* and *5*). As a result of the interpretation of these spectra, the presence of bands between 1231, 1315,1374, 1444, and 1742 cm-1 in Figure 4 and 5 shows that the synthesis of green nanoparticles has occurred.

As a result of the GC-MS analysis in our previous study, three substances found to be in the highest content were used as ligands in the study (Oleic Acid, Octadecanoic acid, n-Hexadecanoic acid) and their binding properties with insect antioxidant enzymes SOD and CAT were demonstrated molecular docking studies.

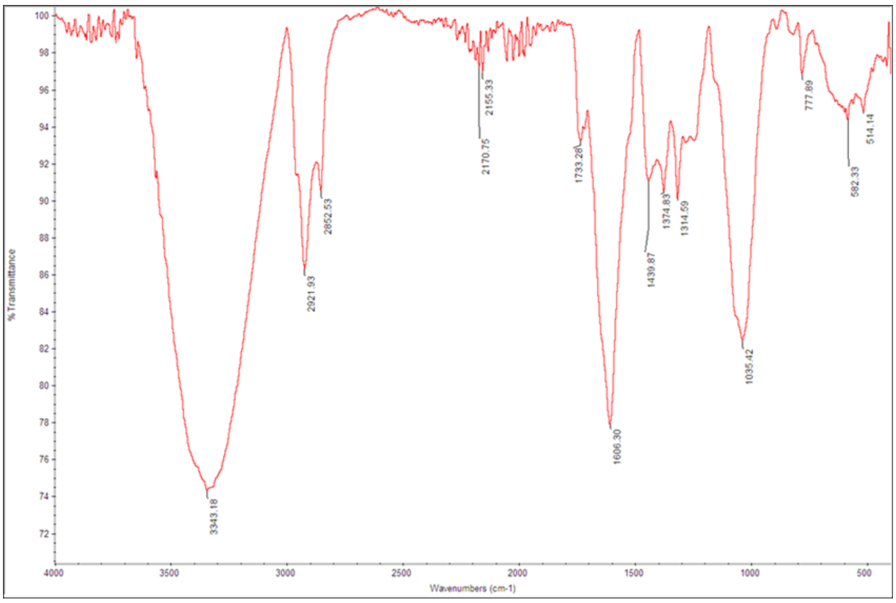


Figure 4. FTIR spectrum of plant extract

Docking results were obtained using Autodock Vina, VMD and Maestro. Binding free energy can include electrostatics, hydrogen bonding, and van der Waals interactions. The highest binding value represents tight binding between the protein and the ligand. In our study, the highest binding values were obtained between the CAT protein and Octadecanoic acid and n-Hexadecanoic acid molecules (-7.1 kcal/mol) (*Table 2*). All three ligands bound to SOD protein with relatively lower energies. LC_{50} and LC_{99} values were calculated after the determined doses of leaf extract with silver nanoparticles were applied to the last instar larvae of the insect (*Table 3*).

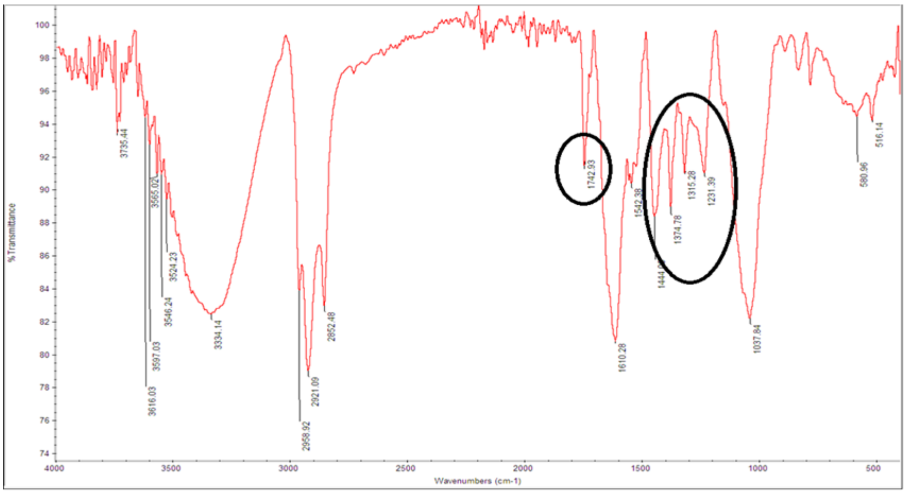


Figure 5. FTIR spectrum of plant extracted silver nanoparticles

Table 2. Molecular docking results between insect antioxidant enzymes and selected ligands

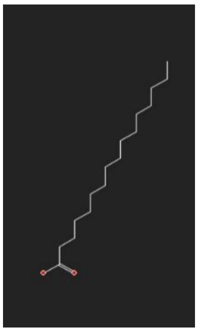
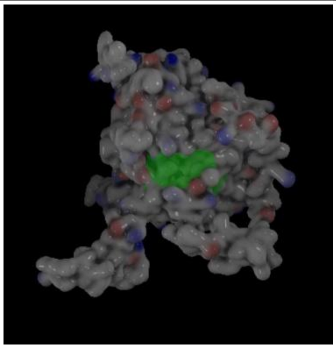
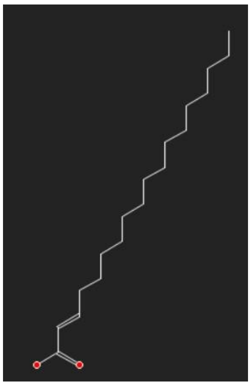
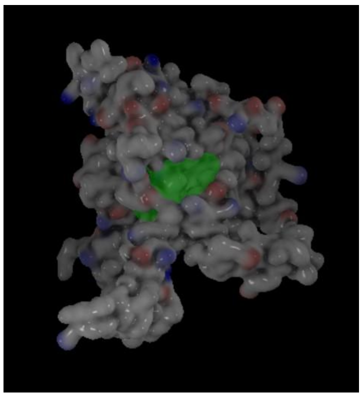
PROTEIN	LIGAND	ENERGY VALUES	HYDROGEN BOND	LIGAND	PROTEIN+LIGAND
CAT	n-Hexadecanoic acid	-7.1 kcal/mol	1		
CAT	Octadecanoic acid	-7.1 kcal/mol	1		

Table 2. continiued

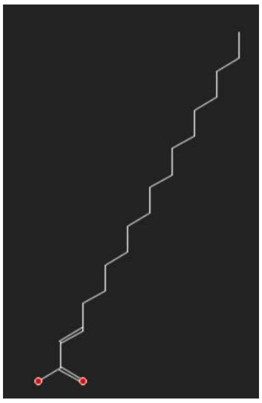
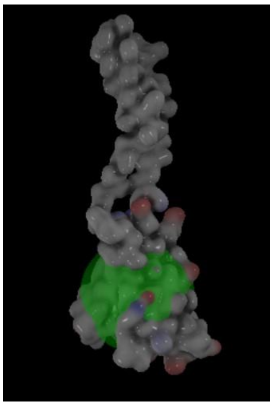
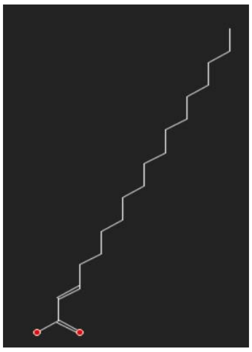
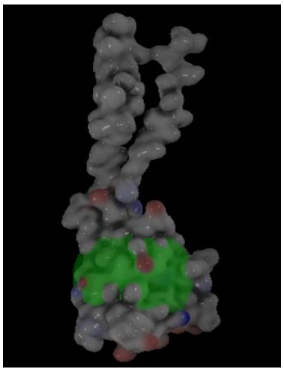
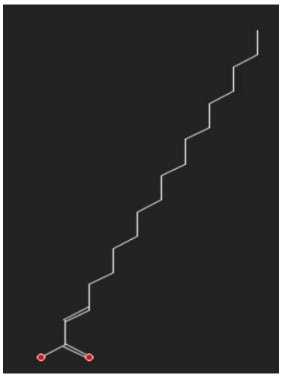
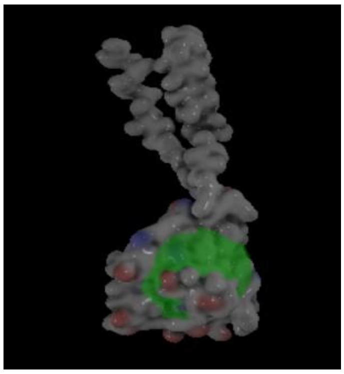
SOD	n-Hexadecanoic acid	-4.8 kcal/mol	3		
SOD	Octadecanoic acid	-5.7 kcal/mol	-		
SOD	Oleic Acid	-5.4 kcal/mol	1		

Table 3. LC_{50} and LC_{99} values of G. mellanolla larvae applied with silver nanoparticle leaf extract

Süre (24 h)	N	LC_{50} (µl)	LC_{99} (µl)	DF	Sig.
	60	5,51	11,58	3	0,960[a]

3.2 Discussion

Since plants constitute very rich bioactive chemical sources, they appear as potential alternatives to currently used chemical-based insect control products. In this way, they can be a resource for the development of safer insect control. There are many plant-originated products that are being researched for their biological activity. *A. melanocarpa*'s leaf extracts is one of them. Although it has become popular in recent years, there has been little research done on the effectiveness of this plant against insect pests.

Among the studies conducted with insects, there is a study in which the in vivo part of this plant's ability to reduce the negative effects of human diseases was conducted and *Drosophila melanogaster* was used as a model organism (Savıć et al. 2019). Živković et al. (2020) created polyphenol-based microencapsulated extracts from *A. melanocarpa* fruits as insecticides and tested its effect on *Halyomorpha halys* and concluded that the microencapsulated extract was promising in the sustainable fight against the pest. Gökmen et al. (2022) investigated the insecticidal and anti-fungal effects of leaf, stem, fruit extracts and hydrosol obtained from Aronia. It was revealed that especially the leaf extract of Aronia showed insecticidal effect on *G. mellonella* and inhibitory effect on *Fusarium oxysporum f.* sp. *lycopersici*.

4. Conclusions

Considering the damage caused by chemical control in agricultural production to human health and the environment, the development of plant products and their use to control pests is an important step in terms of human health and the environment. Examining plants with high biological activity and using them to suppress insects will bring more positive results for the environment and human health over the years. Phenolic compounds such as anthocyanins, cyanidins, phenolic acids, proanthocyanidins, triterpenoids and their analogues have been identified as the major active components of *Aronia.* It is obvious that these substances in its content enable *Aronia* to reveal its properties such as anti-cancer, antioxidant, anti-infective. In addition, there are few studies on the insecticidal activity of products obtained from *Aronia.* This study will form the basis for further studies in this aspect.

In the study, the lethal effect of leaf extract of *A. melanocarpa p*lant with silver nanoparticles, known to be of medical importance, on *G. mellonella* larvae as a model insect was evaluated for the first time. Thus, information was obtained about the biopesticide potential of the *Aronia* plant, whose cultivation is increasing day by day in our country, and at the same time, the content analysis of *A. melanocarpa* leaf extract was made. By *insilico* analysis of some substances found in the plant, their binding properties with the antioxidant enzymes of the insect were investigated.

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Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

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