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Vitex Agnus-Castus L. Nanoparticles: Preparation, Characterization and Assessment of Antimicrobial and Anticancer Activity

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This study was aimed to synthesise antimicrobial and therapeutic silver nanoparticles (AgNPs) by green synthesis method using *Vitex agnus-castus* L. *(VAC)* seed extract. The synthesised *VAC* seed extract coated AgNPs were characterised by UV-Vis spectrometry, Zeta analysis and FT-IR spectrometry. The antifungal, antimicrobial, cytotoxicity by MTT assay, and anticarcinogenic activities on HeLa cells line were investigated. *VAC*-AgNPs exhibited high antimicrobial activity against *S. aureus* with a zone diameter of 11.3 ± 0.6 mm. In addition, the cytotoxicity of *VAC*-AgNPs was evaluated using the L929 fibroblast cell line and the HeLa cells line, with AgNPs applied at six different concentrations. At a concentration of 5.0 mM, viability was found to be 47.6% ± 4.2% in L929 fibroblast cells and 39.6% ± 7.9% in HeLa cells, suggesting a potential antiproliferative effect of *VAC*-AgNPs on HeLa cancer cells. The IC50 values of *VAC*-AgNPs against the HeLa cell line were calculated as 2.94 μg/ml.

Introduction

Today, many plant varieties are widely used in the cosmetics, pharmaceutical, and food industries. With the developing technology, instead of using plants directly, extracts of plants, specially synthesized with metal nanoparticles (NPs), are used in green chemistry processes. The obtained green metal synthesis has many advantages such as mild synthesis conditions, simplicity, low cost, and not harming the environment due to the absence of toxic reagents.[1,2] There have been many recent studies on the use of silver nanoparticles (AgNPs) in various industries, biomedicine, and environmental applications.[2–4] The potential of plants to reduce metal ions, both at the surface and in various organs and tissues far from the site of ion penetration, has been recognized for a long time. Plant-based synthesis of AgNPs is an important branch of biosynthesis processes. AgNPs have garnered considerable attention and applications in medical technology due to their excellent antimicrobial and anticancer properties. Organic acids such as amino acids, enzymes, vitamins, proteins, polysaccharides, and citrates, which are bioactive molecules found in plants, have the potential to reduce metal ions.[5–6]

It has been determined in many studies that AgNPs have broad-spectrum antimicrobial and antifungal properties against fungi, bacteria. Antimicrobial activity of AgNPs; Surface properties of AgNPs depend on the size, dose, and shape. Studies have shown that reducing the size of AgNPs can enhance their stability and biocompatibility, and the shape of nanoparticles is one of the properties that can affect other physicochemical properties. AgNPs have been found to interact with fungi and bacteria in a manner that is dependent on their shape.[7] In comparison with rod-shaped or spherical AgNPs, truncated triangular-shaped AgNPs are known to exert greater antibacterial activity.[8] The adhesion of AgNPs to microbial cells, penetration into cells, reactive oxygen species (ROS) and free radical formation, and modulation of microbial signal transduction pathways have been considered as the most prominent modes of antimicrobial action. Based on the research conducted to date, it is known that AgNPs can be designed to increase their efficacy, specificity, stability, biosafety and biocompatibility. To date, AgNPs have been used in combination or alone with antibiotics.[9] AgNPs were found to have synergistic and additive antimicrobial effects when used with antibiotics.[10–12] The tendency of AgNPs to bind to sulfur and phosphorus in gram-positive bacteria cell wall is the reason for their antibacterial activity.[13]

The antibacterial effect of AgNPs is much more effective on gram-negative bacteria and the reason for this is the water channels called purines on the outer surface of gram-negative bacteria. NPs that enter for bacteria bind to DNA, lipids and,

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proteins, as a result of the reaction of AgNPs with DNA, cell growth in bacteria stops and bacterial denaturation occurs.[14–16] It has been determined in many studies that nanotechnological particles can be applied against microorganisms that have gained multiple antimicrobial resistance against antibiotics, which is an important public health problem today.[17] Wei *et al.*[16] obtained with sea buckthorn berry extract and they determined the antimicrobial activities of AgNPs on *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). The results of the study show that NPs prepared against these pathogens can be used. Shaik *et al.*[17] Gram-positive bacteria (*S. aureus, Staphylococcus epidermidis* (*S. epidermidis*), *Micrococcus luteus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA)) and gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Shigella sonei*, *Salmonella typhimurium*) of AgNPs obtained using *Origanum vulgare* L. and pathogenic fungi (*Aspergillus flavus*, *Alternaria alternat*, *Phialophora alba*, *Paecilomyces variotii*) were investigated and the antimicrobial activity of NPs against all strains was determined. Carson *et al.*[18] examined the antibacterial effect of AgNPs obtained using Phyla dulcis extract on gram-positive bacteria (*Listeria monocytogenes* and *S. aureus*) and two gram-negative bacteria (*E. coli* and *Salmonella typhimurium*). In the study, it was determined that the plant did not have any antibacterial effect on these bacteria, but the synthesized Ag NPs had antimicrobial properties on all bacteria. Gomathi *et al.*[19] reported that AgNPs obtained by using *Gymnema Sylvestre* leaf extract had antibacterial activity on *E. coli* and *S. aureus*.

Vitex agnus-castus L. (*VAC*), a member of the Lamiaceae family, is a perennial shrub with a strong aromatic scent.[20] Although the homeland of the species is the Mediterranean region, it grows as an ornamental plant in many parts of the world such as subtropical regions.[21,22] In addition, it has been determined in studies that the medicinal plant *VAC* contains a large number of bioactive metabolites (flavonoids, ketosteroids, iridoids, essential oils, etc.) with high reducing properties and great activity in the green synthesis of AgNPs amount diversity varies according to habitat conditions.[23] Located in *VAC* the most well-known flavonoid is casticin, an important phytochemical. It is found in the seed of the casticin *VAC*.[24–25] Casticin has anti-cancer properties on the HeLa cell lines with Cell arrest by downregulation of cyclin B and activation of p21 and activation of c-Jun N-terminal kinase (JNK).[26–27] According to the European Pharmacopoeia, dried medicine must contain at least 0.08% casticin.[28] Therefore, whether as a medicine or as a food supplement, *VAC* extracts are safe for many diseases such as premenstrual syndrome (PMS), It can be used in the treatment and is very popular today. The *VAC* plant and its seeds have been used for decades in the treatment of certain diseases, especially gynecological disorders of women.[29–33] Cervical cancer is one of the most prevalent gynecological cancers affecting women of reproductive age.[34] According to the World Health Organization (WHO), cervical cancer is the fourth most common cancer in women, with over one million cases worldwide. In 2018, an estimated 570,000 women were diagnosed with cervical cancer, and approximately 311,000 women died from the disease.[35] In Turkey, the prevalence of cervical cancer is about 4 per 100 thousand. It is estimated to be 5 and it is stated to be the tenth most common cancer type.[36] Although these cancers are preventable, they are among the serious causes of death in underdeveloped and developing countries. Especially in developing countries, deaths from cervical cancer are at the level of 80–85%, and it is stated that it is about four times higher than in industrialized countries.[37]

Several studies in the literature have reported on the antifungal, antibacterial, anticancer, and cytotoxic properties of AgNPs synthesized by *VAC*.[38–39] Our study is the first to investigate the anticancer effects of *VAC*-AgNPs on cervical cancer cells. In this study, *VAC* seeds were used for the green synthesis of AgNPs, which were then characterized by UV-Vis, FT-IR, and Zeta analysis. The antifungal, antimicrobial, and cytotoxic activities of the synthesized *VAC*-AgNPs were examined. Furthermore, the anticancer properties of biogenic *VAC*-AgNPs were evaluated against both the human cervical cancer cell line (HeLa) and the healthy L929-Murine fibroblast cell line.

Results and discussion

GC-MS Results

The content of the ethanol extract of *Vitex agnus castus* L. seeds by GC-MS has given in Figure 1 and Table 1.

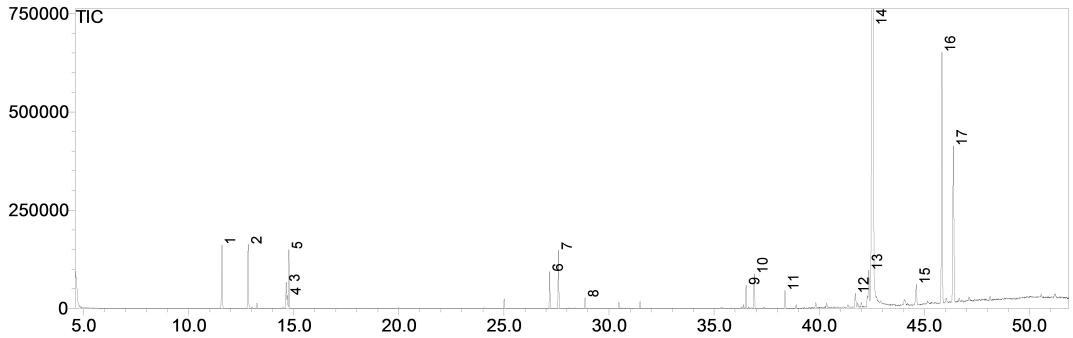

Figure 1. GC-MS chromatogram of *VAC*-seeds.

Table 1. Content of the ethanol extract of *VAC*-seeds by GC-MS.

Peak	R.Timto	Peak Report TIC Name	Area(%)	CAS	m/z	Similarity(%)
1	11.577	.ALPHA.-PINENE, (−)-	1.03	Formula:C10H16 CAS:80-56-8	93, 136	96
2	12.826	Sabineto	0.88	Formula:C10H16 CAS:3387-41-5	93, 136	96
3	14.638	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-	0.33	Formula:C10H16 CAS:5989-54-8	68, 93, 136	92
4	14.694	.beta.-Phellandrento	0.15	Formula:C10H16 CAS:555-10-2	93, 77, 136	92
5	14.766	1,8-Cineolto	0.65	Formula:C10H18O CAS:470-82-6	81, 108, 154	94
6	27.173	trans-Caryophyllento	0.41	Formula:C15H24 CAS:87-44-5	69, 93, 133, 147	94
7	27.593	(E)-.beta.-Famesento	0.64	Formula:C15H24 CAS:18794-84-8	69, 93, 133, 161	95
8	28.862	1,5-Heptadiene, 2,5-dimethyl-3-methylene-	0.12	Formula:C10H16 CAS:74663-83-5	93, 121	86
9	36.524	(E)-.beta.-Famesento	0.24	Formula:C15H24 CAS:18794-84-8	69, 81,93,121	85
10	36.913		0.42		80, 119, 135, 191	
11	38.378	Globulol	0.25	Formula:C15H26O CAS:489-41-8	55, 71, 95, 107, 135	81
12	41.725	1-Undecyne (CAS)	0.20	Formula:C11H20 CAS:2243-98-3	81, 95, 135	80
13	42.363	2-Nitro-2-(3-oxobutyl)cycloheptanone	0.58	Formula:C11H17NO4 CAS:86572-27-2	69, 181, 291	84
14	42.566	hexanedioic acid, dioctyl ester (CAS)	86.80	Formula:C22H42O4 CAS:123-79-5	57, 129, 341	93
15	44.624		0.36		69, 109, 207	
16	45.843	6.11-Undecadiene, 1-acetoxy-3,7-dimethyl-	4.01	Formula:C16H28O2 CAS:0-00-0	81, 302	82
17	46.388		2.94		55, 69, 318	
			100.00			

Synthesis of VAC-AgNPs

Characterization

Using UV-Vis spectroscopy is a common method to confirm the formation of silver nanoparticles in solution. This technique measures the absorbance of light by the nanoparticles at a specific wavelength, which is indicative of their size and concentration. The formation of a peak in the UV-Vis spectrum at around 400–420 nm is a characteristic feature of silver nanoparticles, which confirms their presence in the solution. As a result of UV-Vis analysis of *VAC*-AgNPs solutions, it was observed that the absorbance value increased with increasing $AgNO_3$ concentrations, proving that silver nanoparticles were formed at approximately 403 nm (Figure 2).[40,41]

Hydrodynamic diameters and zeta potentials of *VAC*-AgNPs synthesized at different concentrations were analyzed with Zeta sizer. It was determined that all of the synthesized AgNPs had negative zeta potential. For example, the average zeta potential of 1.0 mM *VAC*-AgNPs was determined as -22.1 ± 5.18 mV. This value shows that the synthesized particles are both covered with acidic groups and are stable in solution.[42] In addition, the formation of a single peak in the Zeta potential distribution graph shown in Figure 3a. proves that the solutions remain stable and are not subject to precipitation and aggregation. In addition, the average hydrodynamic diameter of the synthesized *VAC*-AgNPs was found to be less than 100 nm (Figure 3b). It has been reported in the literature that as AgNPs decrease in size, their surface area increases, and accordingly, their antimicrobial activities increase.[43]

FT-IR spectroscopy is a widely used analytical technique that can provide valuable information about the chemical structure and composition of a sample. It works by measuring the absorption of infrared radiation by the sample, which can reveal the types of chemical bonds and functional groups present in the molecule. Since *VAC*- seeds extract has various components such as iridoid glycosides, flavonoids, progestins, alkaloids, essential oil and essential fatty acids. And *VAC*-seeds extract is likely to contain a significant amount of carbonyl, carboxyl and hydroxyl groups in its chemical.[44] The presence of plant functional groups was investigated in both *VAC*-seeds extract and VAC-AgNPs synthesized at different $AgNO_3$ concentrations by FT-IR analysis, and the results are shown in Figure 4. The broad absorption band observed at 3252 cm^{-1} is due to –OH (hydroxyl) stretching vibrations. The absorption peak at 2922 cm^{-1} corresponds to the methylene C–H stretch. The peaks at 1732 cm^{-1} can be attributed to stretching vibrations of the ester groups. The strong vibration peaks at 1386 cm^{-1} and 1586 cm^{-1} are due to the carbonyl (C=O) groups of carboxylic acids and phenols. Peaks between 1260 cm^{-1} and 560 cm^{-1} can be attributed to vibrational frequencies of aromatic ethers, aromatic esters, alcohols, amines, amides, alkyl halides and sulfides.[45,46] When the FT-IR spectra of the synthesized 10 mM *VAC*-AgNPs were examined, the peak around 1260 cm^{-1} disappeared with AgNPs formation, while an increase in the peak density at 1386 cm^{-1} was observed. It was also observed that the intensity of the O–H vibration frequency at 3250 cm^{-1} decreased. The decrease in the intensity of the O–H stretching peaks indicates that the OH

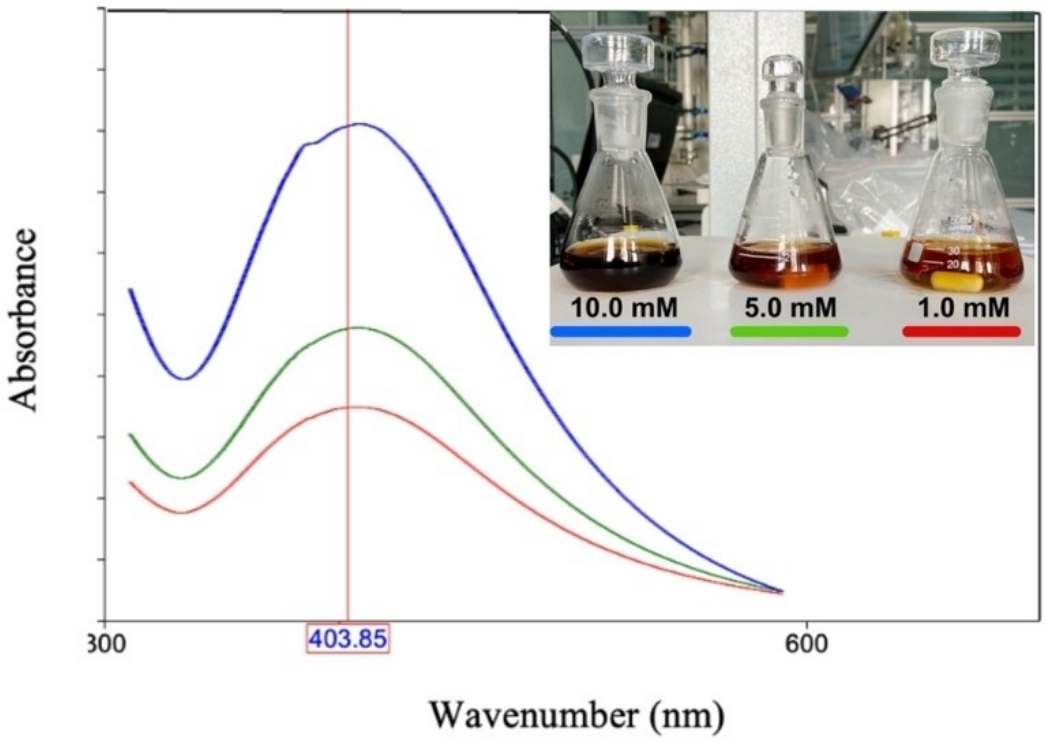

Figure 2. UV–Vis spectrum of *VAC*-AgNPs.

groups of the plant extract are involved in the reduction and stabilization of AgNPs. The high similarity between the *VAC*-AgNPs and the FT-IR spectra of the extract is strong evidence that AgNPs are coated with plant functional groups.[40]

Antimicrobial and Antifungal Activity

For antimicrobial activity studies, primarily disc diffusion studies were carried out. Disc diffusion results of *VAC*-AgNPs are given in Table 2.

Table 2. Zone diameters obtained as a result of disc diffusion experiment.

	Zone diameters (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
VAC-AgNPs	–	11.3 ± 0.6	–

Table 3. Minimum inhibitory concentration (MIC) values (mM) of the *VAC*-AgNPs extracts of the plant against selected microorganisms.

	MIC values (mM)		
	Escherichia coli	Staphylococcus aureus	Candida albicans
1 mM	0.13	> 0.50	0.13
5 mM	0.16	0.63	0.31
10 mM	0.16	0.16	0.16

According to the data obtained as a result of disc diffusion studies, the process of coating the sample seeds extract with silver nanoparticles was carried out. Antimicrobial activity studies of *VAC*-AgNPs were performed with minimal inhibition concentration determination study. Results are given in Table 3.

AgNPs are synthesized by physical, chemical, and biological/green synthesis methods.[46] Synthesis of AgNPs by green synthesis methods; It continues to attract the attention of researchers because it is a low-cost, environmentally friendly method without high temperature and pressure.[40] Ghani *et al.*[38] conducted a study to evaluate the antimicrobial activity of AgNPs that were synthesized using *VAC*-seeds extract. The study aimed to determine the MIC of AgNPs against two bacterial species, *E. coli* and *Bacillus cereus.* They found that the AgNPs synthesized from *VAC*-seeds extract exhibited antimicrobial activity against both pathogens, and the MIC values were measured to determine the concentration required to inhibit the growth of these bacterial species. Overall, this study suggests that AgNPs synthesized from *VAC*-seeds extract have potential as a natural antimicrobial agent against certain bacterial pathogens. And Al-Mudhafar *et al.*[39] conducted a study to investigate the antibacterial activity of *VAC*-AgNPs against several pathogenic bacteria, including *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa.* They found that *VAC*-AgNPs exhibited antibacterial effects against all tested pathogens, with the strongest activity observed against Proteus mirabilis, as indicated by the zone of inhibition diameter (24 mm). However, They also found that the antibacterial activity of *VAC*-AgNPs against *Klebsiella pneumoniae* was relatively weak compared to

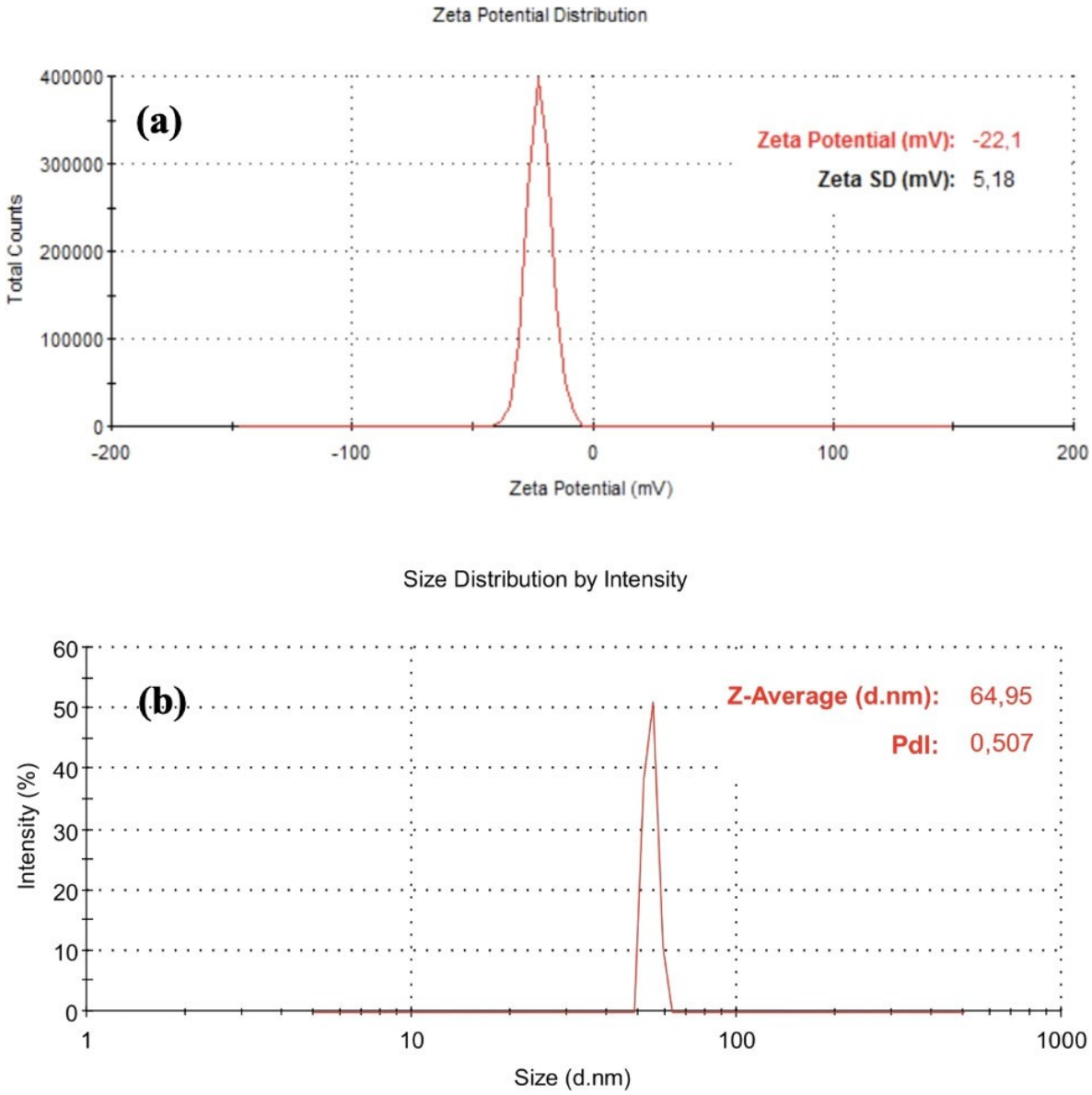

Figure 3. Zeta potential distribution spectrum of *VAC*-AgNPs (a), hydrodynamic diameter distribution spectrum (b) (VAC-AgNPs: 1.0 mM).

the other tested pathogens. These findings could be useful for the development of novel antibacterial agents or treatments targeting specific pathogens. Study results are similar to our study. In our study, we aimed to determine the antimicrobial activity of *VAC*-AgNPs extract against a different group of microorganisms, including *S. aureus, E. coli* and *C. albicans*. and we determined that the zone diameter was 11.3 ± 0.6 mm only against *S. aureus* strain. We found that *VAC*-AgNPs obtained from of our region have antibacterial and antifungal properties, and we believe that these findings may have potential implications for the development of natural antimicrobial agents to combat certain bacterial and fungal infections. Long term as significant contributors to higher plants' resistance to certain therapeutic microorganisms, antibacterial compounds found in plants. Thus, scientists have long recognized that screening plants for potential therapeutic properties could aid in the discovery of countless new drugs by pharmacologists and phytochemists.[47] Additionally, further research is required to evaluate the potential of bioactive chemicals' antimicrobial effectiveness expressed in *VAC*.[48]

Cytotoxicity and Anticancer Activity with the MTT Assay

MTT test specified in TS EN ISO 10993–5 Standard was used to determine the cytotoxicity of ethanol extracts of *VAC*-AgNPs. L929 fibroblast cells specified in the standard were used to determine cytotoxicity by MTT assay. *VAC*-AgNPs were applied in 6 different concentrations. Viability values results of *VAC*-AgNPs are given in Table 4. and Figure 5. At 5 mM concentration, the viability was calculated as 47.6% ± 4.2% in L929 fibroblast cells, and 39.6 ± 7.9% in HeLa cells. No cytotoxic

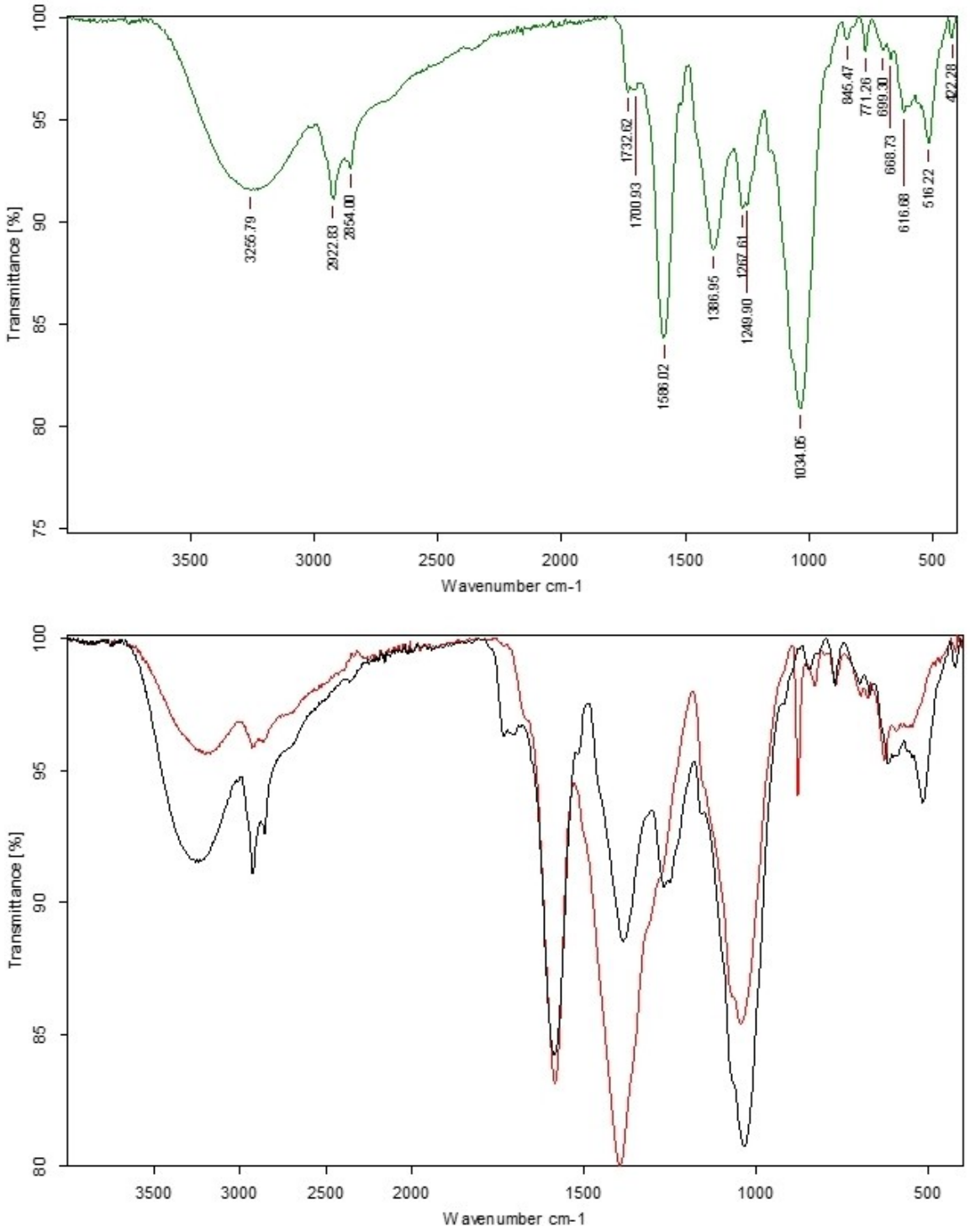

Figure 4. FT-IR spectra of *VAC*-AgNPs synthesized with 10 mM $AgNO_3$ (red) and *VAC*-seeds extract (green/black).

effects were observed in L929 fibroblast cells at 2.5–0.15 mM concentrations. The viability in HeLa cell at 2.5 mM concentration was calculated as 63.4%±7.6%. *VAC*-AgNPs show antiproliferative effect at 5 mM concentration in Hela cells, and it was determined that cell viability increased in a dose-dependent manner at lower concentrations.

Al-Mudhafar *et al.*[39], determined the anticancer activity of *VAC*-AgNPs on MCF7 s. In addition, in this study, the cytotoxicity of AgNPs in the normal cell line was evaluated similar to our study and it was determined that it was not very toxic. Kathiravan *et al.*[49] examined the anticancer activity of AgNPs obtained from the leaf extract of *Melia dubia* in the human breast cancer cell line, and they found that AgNPs had very

Table 4. *VAC*-AgNPs viability values

Concentration	HeLa	L929
control	100 ± 8.5	100 ± 7.5
0.15 mM	95.2 ± 9.2	91.4 ± 5.4
0.31 mm	80.5 ± 9.7	93.5 ± 5.1
0.62 mm	84.3 ± 3,5	89.1 ± 5.1
1.25 mm	81.4 ± 9.5	79.6 ± 5.6
2.5 mm	63.4 ± 7.6	79.4 ± 4.2
5 mm	39.6 ± 7.9	47.6 ± 4.2
IC50	3.27	2.94

good implantation abilities in cancer cells. According to Devi *et al.*[50] determined the cytotoxic effect of AgNPs extract synthesized from the plant *Gelidiella sp.* and its anticancer activity on Hep-2 (Human laryngeal) cell. In a study by Kelkawi *et al.*[51], the cytotoxic effect of AgNPs synthesized from Mentha plant and the anticancer effect of AgNPs against MCF-7 and HeLa cancer cells were examined[51]. In another study by Devaraj *et al.*[52], the anticancer activity of AgNPs synthesized from *Cleome viscosa L* seed extracts against ovarian cancer cells (PA1) and lung (A549) cancer cell lines was investigated. These studies support our study.

In our study, the cytotoxic effect of AgNPs obtained from *VAC*-seeds by green synthesis and their anticancer activity in HeLa cells were investigated. According to the data we obtained from our study, we believe that AgNPs of vagnus castus, which is a medical deposit, can be used reliably in cervical cancer.

Conclusions

In our study, *VAC*-AgNPs obtained from *VAC-seeds* by green synthesis method were used. AgNPs obtained from *VAC-seeds* were characterized by UV-Vis, FT-IR, zeta analysis obtained by various spectroscopic tools and *VAC*-AgNPs homogeneous distribution was found. When the antimicrobial and antifungal activity of *VAC*-AgNPs on *E.coli, S. aureus, C. albicans* was examined, MIC values were determined for all 3 pathogens with a zone diameter of 11.3 ± 0.6 mm against only *S. aureus* strain. It was determined that the synthesized *VAC*-AgNP showed high cytotoxic effects against HeLa cancer lines after 24 hours. In addition, when the results of the toxicity test were examined, it was determined that it was less toxic to the healthy cell line (L929-Murine Fibroblast cell line). Today, many studies are carried out for the effective use of NPs in the medical world. Alternative approaches to antibiotics are very important, especially due to the low number of new generation antibiotics and the rapid resistance of pathogens. Anti-microbial, antifungal and anti-cancer effects of NPs synthesized from many different plants in terms of green synthesis have been determined in studies. In addition, despite these effects, studies have been carried out in recent years on the anticancer effects of these Nps, which are inexpensive and environmentally friendly. In our study, the anticancer activity of *VAC*-AgNps was revealed. It has long been accepted by the scientific world that NPs obtained from plants by the green synthesis method can be an important step for new drug discoveries to be used in the treatment of many infectious diseases and cancers. In addition, more research is needed to evaluate the potential of antimicrobial, anticancer and other activities of NPs obtained from *VAC*, which is very common in the world and has been used in many ailments for many years.

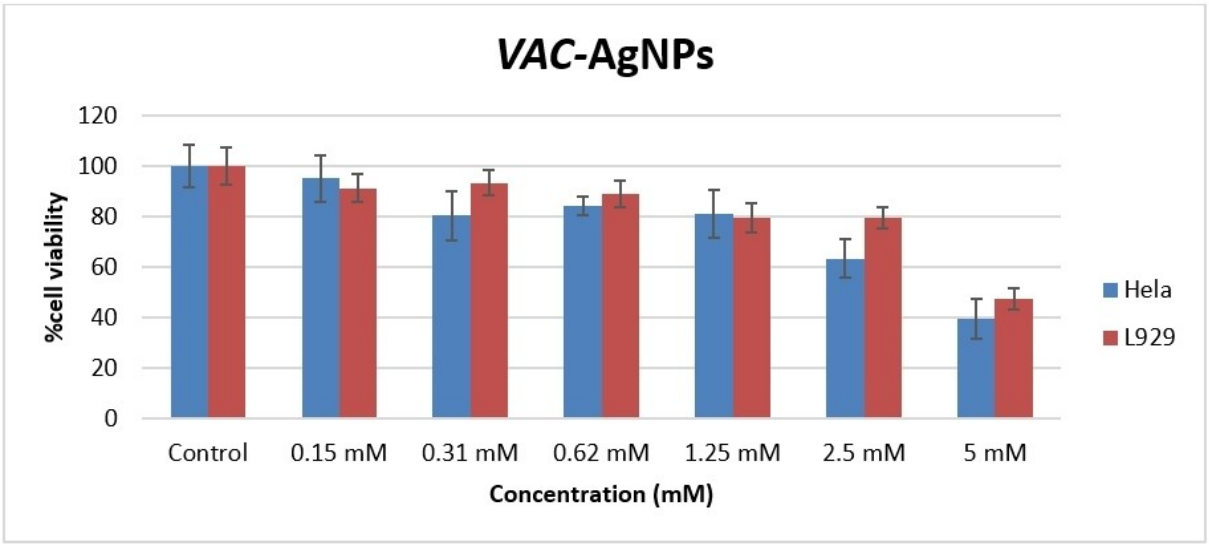

Figure 5. *VAC*-AgNPs viability values

Experimental Section

Identification of the plant

VAC L. plant was collected from İzmir Seferihisar in the autumn of 2020. GAZI2020 number was given to the plant by confirming that it is *Vitex agnus castus* L. in Gazi University Herbarium.

Extraction of VAC L.

For the extraction of *VAC* seeds, a low-pressure extraction system was used in order to minimise the loss of volatile components and prevent their decomposition at high temperature. Before extraction, approximately 10 grams of seeds were ground and placed in a glass flask containing 100 mL of ethyl alcohol. The glass flask was then immersed in a water bath at 40 °C and extracted for 1 hour at a rotational speed of 100 rpm and a pressure of 200 mbar. At the end of this time, the extract obtained was filtered through a 0.45 μm filter and stored at + 4 °C.

GC-MS analyses

Shimadzu QP-2010 GC-MS (Shimadzu, Kyoto, Japan). GC analysis was performed on a Shimadzu QP 2010 system with a fused silica capillary column (60 m, 0.25 mm id and film thickness of 0.25 mm with chemically bonded phase TRB-5MS). The oven temperature was hold at 60 °C for 2 min, programmed to rised at 5 °C/min to 150 °C, and held for 2 min. Then rised at 10 °C/ min to 250 °C and held for 2 min then rised at 5 °C/min to 300 °C and held for 1 min. Finally, the programmed to rised at 5 °C/min to 310 °C and held for 5 min A sample volume of 0.1–1 μL was injected in splitless mode (high pressure). The injector was 250 °C and the interface temperature was kept at 300 °C. Helium was used as the carrier gas.

Mass spectrometric parameters: the electron impact ionization energy was 70 eV, the detector voltage was 0.91 kV, the ion source temperature was 250 °C, and the solvent delay time was 4.5 min. MS detector was used in multiple ion monitoring mode (ions characteristic of *VAC* L in SIM mode were: m/z = 357 and 372).

Synthesis of VAC-AgNPs

A 1% solution was prepared from the extract prepared for the synthesis of *VAC*-AgNPs and negative charge groups such as COO^- were formed in the plant extract by adjusting the pH of the solution to 11 with 5 M NaOH. 5 mL of this solution was taken and added to the $AgNO_3$ solution (20 mL) prepared at different concentrations (1.0, 5.0 and 10.0 mM). The color of $AgNO_3$ solutions immediately changed to yellow-brown upon addition of the plant extract. The resulting mixtures were stirred at room temperature until this color change was stable (approximately 24 hours). The synthesized *VAC*-AgNPs were then filtered through a 0.45 μL filter and stored in falcon tubes at + 4 °C.

Characterization of VAC-AgNPs

The surface plasmon resonance (SPR) peaks of the synthesized *VAC*-AgNPs were measured at 350 and 600 nm with a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer, USA) to prove the formation of AgNPs. The average hydrodynamic diameters and zeta potential values of the *VAC*-AgNPs were determined using a Zeta-Sizer device (Nano ZS, Malvern In- struments, Malvern-UK). Chemical bond analysis of the synthesized *VAC*-AgNPs was performed using FT-IR with ATR attachment (Vertex 70 V, Bruker).

Antimicrobial and Antifungal Activity

The present study collected the plant extracts antimicrobial effects to discover the potential antimicrobial agents against the selected bacterial strains. The plant extracts were tested for their in vitro antibacterial activities against *S. aureus* (G(+), ATCC 25923), *E. coli* (G(−), ATCC 25922), and also for their antifungal activities against *C. albicans*, (ATCC 10231), using the agar-well diffusion method. Microorganisms were acquired from the collections of Kırıkkale University Scientific and Technological Research Application and Research Center Culture Collection, Turkey. *E. coli* and *S. aureus* were grown on Tryptic Soy Agar (TSA) plates and incubated at 37 °C for 24 h. Whilst, the yeast strains were grown in Sabouraud Dextrose Agar (SDA) medium and incubated at 30 °C for 48 h. After incubation, microbial suspensions were adjusted to a turbidity of 0.5 McFarland. Then microorganisms wered cultivated with a swab stick on Mueller Hinton Agar (Merck) for bacteria (*E. coli* and *S. aureus*), and with 0.5 μg/mL methylene blue (Merck), 2% glucose (Merck) enriched Mueller Hinton Agar for albicans Plant extracts were placed on petri dishes by absorbing 20 microliters into empty discs. After 24 hours of incubation, the zone diameters formed around the discs were measured and recorded.

The antimicrobial activity of the seed extracts coated silver nanoparticles was determined with the broth microdilution method. A preculture of bacteria and yeast were grown in Tryptic Soy Broth (TSB) and Sabourud Dextrose Broth overnight at 37 °C and 30 °C, respectively. The three microbial strains were evaluated for the concentration of extracts in Mueller Hinton Broth (MHB) ranging from 1–0.02 mM, 5–0.08 mM and 10–0.16 mM. The MIC value was determined as the lowest concentration of the plant extract that inhibited the visible growth of the test microorganism.

Cytotoxicity and Anticancer Activity with the MTT Assay

L929 fibroblast cells were seeded into 96-well plates at 1.0×10^4 cells per well. Cells were incubated for 24 hours. The extract was passed through a 0.45 micron filter before application. Then, the ethanol extracts were diluted 1/10 with DMEM and applied at 6 different concentrations and incubated for 24 hours at 37 °C 5% CO_2. Only the medium was used as a blind. After 24 hours, the media in the wells were discarded and 50 μl of MTT (1 mg/mL) solution was added to each well. After incubation at 37 °C for 2 hours, 100 μl of isopropanol was added to the wells and the absorbance values of the 96-well plate were read at 570 nm in a microplate reader to determine cell viability. Based on the control (Blind) groups, the % viability was calculated according to the following equation. HeLa cervical cancer cells were used to determine the anticancer activity of *VAC*-AgNP ethanol extracts. HeLa cells were seeded into 96-well plates at 1.0×10^4 cells per well. Cells were incubated for 24 hours. Extracts prepared as in the cytotoxicity test were applied at 6 different concentrations and incubated for 24 hours at 37 °C, 5% CO_2. Only the medium was used as a blind. After 24 hours, the media in the wells were discarded and 50 μl of MTT (1 mg/mL) solution was added to each well. After incubation at 37 °C for 2 hours, 100 μl of isopropanol was added to the wells and the absorbance values of the 96-well plate were read at 570 nm in a microplate reader to determine cell viability.

HeLa and L929 fibroblast cell line were used to investigate the anticancer and cytotoxic effect of *VAC*-AgNPs. *VAC*-AgNPs were applied at concentrations of 5–2.5–1.25–0.62–0.31–0.15 mM. The percentage of cell viability was determined using the following formula:

$$\text{Cell Viability} = \frac{100 \times \mathbf{OD}570\mathbf{e}}{\mathbf{OD}570\mathbf{b}} \quad (1)$$

OD570e: The value of the optical density of the sample

OD570b: Optical density of control (blind) group

Supporting information summary

The supplementary file contains the details of GC-MS chromatogram of *VAC*-seeds, tables as well as SI images of the results.

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Conflict of Interests

The authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Anticancer · green synthesis · HeLa cells · silver nanoparticles · vitex agnus-castus

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