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Structural characterization, antioxidant and cytotoxic effects of iron nanoparticles synthesized using *Asphodelus aestivus* Brot. aqueous extract

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Abstract: *ASP* was used to synthesize FeNP_A. They were characterized by UV-vis spectroscopy, FT-IR, TEM, SEM, XRD and ZP. The aim of this study was to evaluate in vitro cytotoxic activity and antioxidant activities of FeNP_A and *ASP*. The antioxidant properties were evaluated using DPPH, ABTS⁺ and H₂O₂ assays. FeNP_A had higher antioxidant activity comparing to *ASP* according to DPPH (IC₅₀: 3.48 µg/mL) and ABTS⁺ (60.52%) assays. Anti-cancer activities of FeNP_A and *ASP* were investigated in breast cancer, melanoma and control cell lines. FeNP_A was more cytotoxic than *ASP* in MCF-7, MeWo, CHL-1, and HEL 299 cells. FeNP_A had shown that mitochondria induce apoptosis through stress in MDA-MB-231, and cells MeWo. *ASP* also induced apoptosis 2.23-fold in MCF-7 cells. Progesterone receptor gene expression showed a 10-fold increase in a hormone-dependent MCF-7 cell line in *ASP*, and FeNP_A treatment. Expressions of BCL6, CXCL12, DNAJC15, RB1 and TPM1 in melanoma cancer cell lines were significantly increased in *ASP* and FeNP_A administration. It had been shown that FeNP_A regulates gene expressions that may be considered important in terms of prognosis in breast cancer and melanoma cell lines and it is suggested that gene expressions regulated by FeNP_A are also evaluated in animal models in vivo.

Keywords: *Asphodelus aestivus* Brot.; iron oxide nanoparticles; cancer cell line; gene

List of abbreviations

FeNP _A	Iron nanoparticles
FeNP _A	Iron oxide nanoparticles of <i>Asphodelus aestivus</i> aqueous extract
<i>ASP</i>	<i>Asphodelus aestivus</i> aqueous extract
UV-vis	Ultraviolet-vis spectroscopy
FT-IR	Fourier transform infrared spectroscopy
TEM	Transmission electron microscopy analysis
SEM/EDX	Scanning electron microscopy/energy disperse X-ray spectroscopy
TGA	Thermal gravimetric analysis
XRD	X-ray diffraction analysis
ABTS ⁺	2,2'-azino-bis (3-ethyl benzo-thiazoline-6-sulphonic acid)
DPPH	1-1-diphenyl-2-picryl-hydrazyl
H ₂ O ₂	Hydrogen peroxide
EMEM	Eagle's Minimum Essential Medium
DMEM	Dulbecco's Modified Eagle Medium
PI	Propidium iodide
ROS	Reactive oxygen species
AIF	Apoptosis inducing factor
KRT 5	Keratin 5
IGF	Insulin-like growth factor

1 Introduction

Nanotechnology allows to work scale in the 1-100 nm range and takes advantage of small structures for new properties and activities [1-3]. Since 1990s iron nanoparticles (FeNP_s) draw much attention in nanotechnology, because of their high intrinsic reactivity of surface sites [4], biocompatibility, surface modifiability and high surface to volume ratio [5]. Iron nanoparticles can be synthesized by different chemical and physical methods such as sol-gel reactions, hydrothermal methods, polyol methods, microemulsion process, radiolysis, aerosol pyrolysis and laser pyrolysis [6]. Recently synthesizing iron oxide nanoparticles using green chemistry attract scientists because of their non toxic and eco friendly properties [7].

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Iron oxide nanoparticles are widely used in various fields of science. Their usage in biomedical field such as anticancer, antimicrobial, larvicidal, antiplasmodial, targeted drug delivery and cancer therapy are remarkable. Besides, iron nanoparticles are used in lithium ion batteries, high performance CO gas sensors, biosensors and jet printing make use of iron nanoparticles [6,7]. However, in recent years the most prominent features of them are their antitumoral effects [8].

Asphodelus aestivus is a species from Liliaceae family. *A. aestivus* shows a wide spread in Turkey. In the previous study, our research group obtained, characterized and investigated antioxidant activity of silver nanoparticles from *A. aestivus* [9,10].

Cancer is one of the most important fatal disease. It is specified as the second most common disease which causes death. Millions of people are reported to die because of cancer [11-13]. Cancer treatment has emerged as a separate issue in recent years due to the side effects [14]. Nano-technology may take care and compense some of the problems, so scientist are trying to use less toxic and useful natural compounds in nano dimensions [15-17].

In the whole world lung, breast, colorectal, stomach and liver cancers are most common ones. Breast cancer is the most common malignancy in women and is one of the three most prevalent cancers worldwide with lung and colon cancers [18]. A variety of methods and drugs have been discovered and used for their treatment [19-21]. MDA-MB-231 and MCF-7 are breast cancer cell lines derived from mammary gland tissue derived from the pleural effusion metastatic region (ATCC, 2016). MDA-MB-231 represents a basal-like subtype with a triple negative feature [22-25].

Melanoma is caused by malignant transformation of melanocyte cells responsible for the formation of melanin pigment [26]. MeWo is a human tissue-derived melanoma cell line of the skin tissue initiated by primer culture from the lymph node of the metastatic region. CHL-1, RPMI 7932 is a human malignant melanoma cell line derived from the skin tissue created by transefection of the human melanoma cell line. While MeWo cells morphologically have displayed fibroblast structure, CHL-1 cells have showed epithelial structure (ATCC, 2016). In the present study iron oxide nanoparticles of *A. aestivus* were prepared and FeNP_A were characterized. The aim of the present research was to evaluate the in vitro cytotoxic and antioxidant activities both FeNP_A and aqueous extract of *A. aestivus* and compare the differences caused by nanoparticles.

2 Materials and methods

2.1 Plant material

A. aestivus Brot. (Asphodelaceae) was collected from Kusadası (Aydın) on January 2014. The plant was identified by Prof. Dr. Bijen KIVCAK from Ege University Faculty of Pharmacy, Department of Pharmacognosy, Izmir, Turkey and a voucher specimen (No. 1520) is deposited in Herbarium of the Ege University, Faculty of Pharmacy.

2.2 Preparation of the plant extract

The aerial plant extract was prepared by infusion in the ratio of 5%. It was then filtered and the filtrate was concentrated using vacuum evaporator. *A. aestivus* aqueous extract (ASP) was obtained.

2.3 Synthesis of FeNP_A

5 mL of *A. aestivus* water extract was mixed with 5 mL of 0.001 M aqueous FeCl₃ solution in a 1 : 1 ratio. The mixture was incubated at 50°C-60°C for 20 min with shaking. The color development, which turns black from orange, had shown the formation of iron oxide nanoparticles. The UV spectrum of Fe particles solution was drawn. Therefore, mixture centrifuged at 5000 rpm for 30 min. The material was then stored at -80°C for 24 h and then lyophilized.

2.4 Characterization of nanoparticles

2.4.1 Ultraviolet-vis (UV-vis) spectroscopy

UV-vis spectra was recorded using Thermo Evaluation Array UV-vis spectrophotometer (Evolution Array; Thermo Scientific, Waltham, MA, USA) in the range of 200-800 nm at room temperature. In order to carry out the experiment quartz cuvettes were used. The purpose of UV-vis spectra was to observe the formation of iron nanoparticles [7].

2.4.2 Fourier transform infrared spectroscopy (FT-IR)

To perform FTIR spectroscopy potassium bromide pellets were prepared for each experiment. This method was used to determine the stability and the attachment of

biomolecules to the iron oxide ions. A Perkin-Elmer, Spectrum 100 spectrometer was used for the analysis in the range of $4000\text{--}280\text{ cm}^{-1}$ (Perkin Elmer Inc., Wellesley, MA, USA). According to FT-IR analysis of FeNP_A and ASP, the bonds responsible from formation of iron oxide nanoparticles are detected. Among these analyses, we would determine the bonds responsible from formation of nanoparticles by comparison FT-IR results.

2.4.3 Transmission electron microscopy analysis (TEM)

Transmission electron microscopy (TEM) analysis was performed to get a preliminary idea about particle size and take a microscope image of nanoparticles [29]. The FeNP_A images were taken by a Libra 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). Electronic radiation was used to take images and it detects the electrons transported through the sample. 80-120 kV acceleration volt and in column OMEGA filter was used for the device.

2.4.4 Scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX)

This characterization method shows the size and shape of the nanoparticles. The morphology was determined by SEM. A SEM 250 FEG Quanta instrument was used for the determination. SEM 250 FEG Quanta instrument was used with an EDX detector (Oxford Azteck) to identify the composition of nanoparticles.

2.4.5 Thermal gravimetric analysis (TGA)

TGA experiments indicated the thermal stability of iron oxide nanoparticles [30]. A Perkin-Elmer Diamond TG/DTA (Thermogravimetric and Differential Thermal Analysis) was used for the experiments. Device was operated with a heating rate of $20^\circ\text{C}/\text{min}$ and achieved between 20°C – 500°C temperature rate.

2.4.6 X-ray diffraction analysis (XRD)

The analyses were done at room temperature by the device Rigaku Ultima IV X-ray diffractometer (Rigaku Corp., Tokyo, Japan) to examine the diffractions of nanoparticles. The device was operated at a voltage of 45 kV and copper potassium alpha radiation (40 mA) was used.

2.4.7 Particle size distribution and zeta potential

Nano-zs Zetasizer (Zetasizer-Nano-ZS, Malvern, UK) was used for particle size distribution and zeta potential. Particle size distribution and zeta potential are important to understand the homogeneity, stability and functionality of particles. High zeta potential shows how stable nanoparticle is [5]. Samples were diluted in distilled water. Particle size distribution and polydispersity index were examined at room temperature. A polydispersity index higher than 0.5 showed that the formation was not homogenous. Zeta potential was also evaluated at room 25°C using the angle 17° , with 78.5 dielectric constant. Electrical field was operated to 15 v/cm.

2.5 Determination of antioxidant activities

2.5.1 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺) assay

FeNP_A and ASP were determined for their ABTS cation radical scavenging activity. 245 mM potassium persulphate solution and 7 mM ABTS were mixed. The mixture was incubated for 16 h. After incubation the solution was diluted until the absorbance at 743 nm becomes 0.7. The prepared solution and 100 μL sample were stirred and incubated at room temperature for 6 min. The absorbance was measured at 734 nm using UNIQCAM 8625 UV/Vis spectrophotometer [31]. Ethanol was used as blank whereas α -tocopherol was used as a standard.

2.5.2 1-1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity was determined using the 1-1-diphenyl-2-picryl-hydrazyl (DPPH) 0.1 mL of 0.1 mM solution of DPPH was added to 3 mL of ASP and FeNP_A of different concentration. The mixture was incubated at room temperature for 30 min and absorbance was measured at 517 nm using UNIQCAM 8625 UV/Vis spectrophotometer [31]. The percentages of scavenging activity values was calculated using the following formula:

$$\text{Percentage of scavenging} = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

where:

A_0 = Absorbance of control

A_1 = Absorbance of sample.

α -Tocopherol was used as a positive control.

2.5.3 Hydrogen peroxide scavenging assay (H₂O₂)

0.1 M, pH 7.4 phosphate buffered saline was added in hydrogen peroxide (40 mM) solution. 600 μ L of hydrogen peroxide solution and 1 mL of the ASP and FeNP_A containing samples of different concentration were rapidly mixed. The mixture was incubated at room temperature for 10 min. The absorbance was measured at 230 nm by UNIQCAM 8625 UV/Vis spectrophotometer against a blank (without hydrogen peroxide). The percentage of scavenging of hydrogen peroxide was calculated using the following formula. Quercetin was used as a positive control [32].

$$\text{Percentage scavenging (H}_2\text{O}_2) = (A_0 - A_1)/A_0 \times 100 \quad (2)$$

where:

A₀ = Absorbance of control

A₁ = Absorbance of sample

2.6 Cell lines

Cancer cell models were selected as breast cancer models MCF-7 and MDA-MB-231 and melanoma cancer models MeWo and CHL-1 and fibroblast cell models WI-38 and HEL 299 (control cell models). Cell lines were cultured and maintained in mediums (EMEM for MCF-7, MeWo, HEL-299, WI-38 cell lines, DMEM for CHL-1 cell line and L15 for MDA-MB-231 cell line) containing 1% L-glutamine supplemented with 10% inactivated FBS and 1% penicillin/streptomycin and were maintained in a humidified incubator at 37°C with 5% CO₂. Analysis of cell viability was carried with trypan blue test.

2.7 Cytotoxicity assay

To determine the cytotoxic effect of FeNP_A and ASP on the MCF-7, MDA-MB-231, MeWo, CHL-1, WI-38 ve HEL 299 cell lines, cells were seeded in 96-well E-plate and treated with variable concentrations (3.13-100.00 μ g/mL) of FeNP_A and ASP. Cytotoxicity activity was achieved by normalize cell index using xCELLIGENCE software and IC₅₀ values and analysis were determined by sigmoidal dose-response analysis.

2.8 Analysis of apoptosis

Apoptotic effects of the ASP and FeNP_A on the cells were evaluated by AnnexinV-EGFP Apoptosis Detection Kit

Biovision. Initially, 5 x 10⁵ cells were treated with their IC₅₀ doses of each for 72 h. 72 h later, cells were collected. Then washed and resuspended. AnnexinV-EGFP and propidium iodide (PI) were added, and the cells were incubated. After incubation, cells were examined by flow cytometry named BD Accuri C6 Flow Cytometer.

2.9 Gene expression analyzes

In breast cancer and melanoma cell models, effects of the FeNP_A and ASP on gene expressions were assessed by real time online RT-PCR (LightCycler 480 II) by using RT² Profiler PCR Array Human Breast Cancer (Table 2) and Human Melanoma (Table 3) PCR Array, Qiagen Kits. Total RNA extraction and cDNA were performed according to the kit manual, by using RNeasy Mini Kit (Qiagen) and RT2 First Strand Kit cDNA Synthesis kit (Qiagen), respectively.

3 Results and discussion

Green synthesized FeNP_A had been characterized by various methods. These methods are: ultraviolet-vis (UV-vis) spectroscopy, Fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy analysis (TEM), scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX), thermal gravimetric analysis (TGA), X-ray diffraction analysis (XRD) and particle size distribution and zeta potential.

Basically, the characteristic color change during process from yellow to reddish brown showed the formation of FeNP_A. In the next step UV spectrums for FeNP_A and ASP were evaluated [33]. After the color change a peak occurred at 250 nm and 450 nm which proves iron oxide nanoparticles presence (Figure 1) [30,34,35]. The FTIR spectrum was performed by A Perkin-Elmer Spectrum 100 Spectrometer. ASP and FeNP_A spectrums were compared in Figure 2. ASP spectrum gave absorbance bands at 3397 cm⁻¹, 2917.24 cm⁻¹, 1618.74 cm⁻¹, 1384.47 cm⁻¹, 1076.66 cm⁻¹ and 824.97 cm⁻¹. Subsequent to formation

Table 1: Determination of antioxidant activity, total phenolic and flavonoid content of FeNP_A and ASP.

	DPPH(IC ₅₀) (μ g/mL)	ABTS*(%) (μ g/mL)	H ₂ O ₂ (%) (μ g/mL)
ASP	31.65 $\pm$ 0.01 ^a	39.62 $\pm$ 0.02	55.86 $\pm$ 0.14
FeNP _A	3.48 $\pm$ 0.01	60.52 $\pm$ 0.01	16.83 $\pm$ 0.06

^a Results are mean $\pm$ SD of three replicate analysis

Table 2: “PCR Array Human Breast Cancer” 96-well microplate gene design.

ABCB1	ABCG2	ADAM23	AKT1	APC	AR	ATM	BAD	BCL2	BIRC5	BRCA1	BRCA2
A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
CCNA1	CCND1	CCND2	CCNE1	CDH1	CDH13	CDK2	CDKN1A	CDKN1C	CDKN2A	CSF1	CST6
B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
CTNNB1	CTSD	EGF	EGFR	ERBB2	ESR1	ESR2	FOXA1	GATA3	GLI1	GRB7	GSTP1
C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
HIC1	ID1	IGF1	IGF1R	IGFBP3	IL6	JUN	KRT18	KRT19	KRT5	KRT8	MAPK1
D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
MAPK3	MAPK8	MGMT	MKI67	MLH1	MMP2	MMP9	MUC1	MYC	NME1	NOTCH1	NR3C1
E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
PGR	PLAU	PRDM2	PTEN	PTGS2	PYCARD	RARB	RASSF1	RB1	SERPINE1	SFN	SFRP1
F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
SLC39A6	SLIT2	SNAI2	SRC	TFF3	TGFB1	THBS1	TP53	TP73	TWIST1	VEGFA	XBP1
G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Table 3: Melanoma PCR Array 48 well microplate gene design.

ADAMTS18	CLEC3B	LRP2	NRP1	RB1	HPRT1
A01	A02	A03	A04	A05	A06
AKAP12	CXCL12	LRRC2	PCNA	SOCS2	ACTB
B01	B02	B03	B04	B05	B06
ARPC2	DNAJC15	MME	PPP1R3C	SOCS3	GAPDH
C01	C02	C03	C04	C05	C06
BCL6	DPP4	MMP1	PPP2R4	THBD	B2M
D01	D02	D03	D04	D05	D06
BIRC5	ENC1	MMP12	PRDX2	TNFRSF10A	HGDC
E01	E02	E03	E04	E05	E06
BRAF	GDF15	MMP14	PTEN	TP53INP1	RTC
F01	F02	F03	F04	F05	F06
CDH3	IRF8	MMP2	QPCT	TPM1	RTC
G01	G02	G03	G04	G05	G06
CDH8	LAMC2	MMP9	RASEF	RPL13A	PPC
H01	H02	H03	H04	H05	H06

Figure 2: FT-IR spectra of *A. aestivus* aqueous extract (ASP) and FeNP, synthesized from ASP.

Figure 1: UV–visible spectra of the iron nanoparticles synthesized from *Asphodelus aestivus* extract and the aqueous extract of *Asphodelus aestivus* (FeNP_A:Fe nanoparticles prepared by *Asphodelus aestivus* aqueous extract; ASP: the aqueous extract of the aerial part of *Asphodelus aestivus*).

spectrum had changed and shifts occurred. The absorbance band at 1618.74 cm^{-1} which is responsible from C=C aromatic chain stretching vibration of phenolic compounds shifted to 1611.75 cm^{-1} . The bending vibration of phenols of -OH which gave band at 1384.47 cm^{-1} shifted to 1366.54 cm^{-1} . C-O or C=O stretching vibration formed by polysaccharides which gave band at 1076.66 cm^{-1} shifted to 1041.78 cm^{-1} . All of these shifts were due to a new formation: FeNP_A. In addition to the shifts new absorption bands around $1000\text{-}1400\text{ cm}^{-1}$ (1449.67 cm^{-1} , 1212.24 cm^{-1} , 1041.78 cm^{-1}) occurred because of C=C, OH, C-O, C=O or C-O stretching vibration [36]. One of the most important band was at 612.98 cm^{-1} occurred because of the Fe-O stretching proved FeNP_A transformation from ASP. [37]. In the next step TEM analysis was evaluated. TEM analysis provided information about general morphology. According to results, the particle size of FeNP_A were about 20-25 nm (Figure 3). In order to acquire

more detailed information about morphology, surface properties and elemental composition SEM/EDX analysis were used. Results indicated FeNP_A had rough surfaces. They were cavity like structures as shown in Figure 4. As it was occurred in Figure 5 analysis confirmed the presence of Fe and O [7]. An other characterization method TGA analysis was presented in Figure 6. The initial weight loss till 193°C was due to removal of moisture. Second step between 193.12°C-383.26°C may due to combustion of organic constituents, elimination of carbon groups. Last weight loss was because of the crystal structure of

Figure 3: Transmission electron microscopy (TEM) images of FeNP_A synthesized by ASP.

Figure 4: Scanning electron microscopy (SEM) images of FeNP_A synthesized by ASP.

FeNP_A [30,38,39]. The XRD pattern of FeNP_A were shown in Figure 7. The 2θ peaks at 18.106° , 35.4° and 40.48° were attributed to crystal planes at (111), (220) and (311) respectively. The position and the relative intensity of the diffraction peaks match well with the standard XRD data for iron oxide nanoparticles (JCPDS file No. 19-0629) [3,30]. Finally, zeta potential analysis was carried out to prove the stability of FeNP_A

Figure 5: EDX spectrum of synthesized FeNP_A using ASP.

Figure 6: TGA analysis of synthesized FeNP_A using ASP.

Figure 7: X-ray diffraction (XRD) patterns of the FeNP_A synthesized by ASP.

(Figure 8) The value (-21.2 mV) guides the stability [40,41]. ABTS⁺, DPPH and H₂O₂ scavenging activities were performed for both ASP and FeNP_A. According to results FeNP_A was more active than ASP by DPPH and ABTS⁺ methods. On the other hand results of H₂O₂ scavenging assay showed that ASP was more active than FeNP_A (Table 1). Mostly the FeNP_A showed more activity than ASP which gave rise to thought that FeNPs could be used in many diseases caused by oxidative stress.

3.1 Cytotoxicity of FeNP_A and ASP

Breast cancer models MCF-7 and MDA-MB-231 and melanoma cancer models MeWo and CHL-1 and fibroblast cell models WI-38 and HEL 299 (control cell models) were treated with FeNP_A and ASP 72 h to analyze the cytotoxic effects. The IC₅₀ values of FeNP_A were determined respectively as 139.40 µg/mL, 47.00 µg/mL, 38.48 µg/mL, 5.26 µg/mL, 179.12 µg/mL, 32.70 µg/mL and ASP were 4.39 mg/mL, 25.56 µg/mL, 74.18 µg/mL, 29.44 µg/mL, 98.98 µg/mL, and 146.10 µg/mL, respectively (Table 4). FeNP_A were more cytotoxic than ASP in MCF-7, MeWo, CHL-1, and HEL 299 cells (>31-, >1.9-, >5-, and >4-fold change in IC₅₀ values, respectively). When nanoparticle cytotoxicity

was assessed, FeNP_A showed cytotoxicity in all cell lines. Aslanturk and Çelik used aqueous, diethylether, ethyl acetate and methanol extracts of *A. aestivus*. Aqueous extracts gave results 185.92 µg/mL at 24 h and 89.68 µg/mL for 72 h in MCF-7 cells (40). This difference in cytotoxicity is due to the use of different parts of the *A. aestivus* plant. In the present study, the upper parts of *A. aestivus* plant were used, however Aslanturk and Çelik were used ground tubers.

3.2 Apoptotic effect of FeNP_A and ASP

The apoptotic effect of FeNP_A IC₅₀ values was determined and apoptosis was increased 2.21 fold in MCF-7 cells, 2.31 fold in MDA-MB-231, 1.29 fold in CHL-1 cells, 1.72 fold in MeWo cells, 19.85 fold in WI-38 cells and 3.71 fold in HEL 299 cells compared to the control group and ASP induced 4.93 fold, 1.21 fold, -1.03 fold, -2 fold, 1.55 fold, and 31.85 fold, respectively. FeNP_A had shown that mitochondria induce apoptosis through stress in MDA-MB-231, and cells MeWo (>1.9-, and >1.7-fold). ASP also induced apoptosis 2.23-fold in MCF-7 cells (Table 4). Aslanturk and Çelik were observed similar apoptosis for ASP. They had suggested that the apoptotic effect of *A. aestivus* plant extract was due to oxidative DNA damage resulting from proximal flavonoids present in the extract [42]. In the present study, results indicated that FeNP_A was caused by oxidative DNA damage in apoptotic effect in MCF-7 and MDA-MB-231. However, it has been shown that FeNP_A did not have apoptotic effects on melanoma cell lines, CHL-1 and MeWo it was referred to the fact that the antioxidant systems worked well in these cell lines so that no oxidative DNA damage could occur. Austin et al. had suggested that synthesized nanoparticles increase reactive oxygen species (ROS) levels, damage mitochondrial membranes, decrease ATP levels, and induce caspase-independent apoptosis in the nucleus loci of p38 MAPK and apoptosis inducing factor (AIF) proteins [43]. In the course of these findings, it was also been considering the apoptosis induction through the caspase independent pathway of FeNP_A synthesized in the present research.

Figure 8: Zeta potential stabilization pattern analysis of synthesized FeNP_A using ASP.

Table 4: IC₅₀ values of FeNP_A and ASP in cell lines (µg/mL).

Cancer model	Cell lines	FeNP _A	Water extract	Fold change
Breast	MCF-7	139.40	4390.00	31.49
	MDA-MB-231	47.00	25.56	-1.84
Melanoma	MeWo	38.48	74.18	1.93
	CHL-1	5.26	29.44	5.60
Control	WI-38	179.12	98.98	-1.81
	HEL 299	32.70	146.10	4.47

3.3 Effect of FeNP_A on gene expression

FeNP_A reduced 2 to 6 times of the 14 gene's expression and increased the expression of 5 genes 2 to 9 times in MCF-7 cells, reduced 7 gene expression by 2 to 9 fold, and increased 3 gene expressions by 2 to 10 fold in MDA-MB-231 cells. ASP reduced the expression of 11 genes by 2 to 7 fold and increased the expression of 4 genes by

2.11 fold compared to the control group in MCF-7 cells and increased expression of 4 genes by 2-9 fold in MDA-MB-231 cells (Figure 9). In breast cancer cells, Keratin 5 (KRT5) gene expression was detected 4-fold increase and a 2-fold reduction in BIRC5, ESR1, IGF1 and TWIST1 gene expressions by FeNP_A and ASP treatment.

It had been shown that FeNP_A treatment in breast cancer cell lines (MCF-7 and MDA-MB-231) affects the expression of many genes in mRNA. Kabos et al. observed a decrease in ESR1 protein and an increase in KRT5 protein in the combined administration of 17 β -estradiol and anti-estrogen tamoxifen or fulvestrant in MCF-7 cell line [44]. The same effect was found in ER+ tumors of neoadjuvant endocrine therapy patients and they suggested that it may be related to resistance to standard endocrine and chemotherapeutic agents. In the present study, FeNP_A treatment showed an increase in KRT5 expression and a decrease in ESR1 expression in breast cancer cell lines. The similar response in two gene expressions without positive endocrine and/or chemotherapy was evaluated positively. Insulin-like growth factor (IGF) signaling was an important regulator of breast development and breast cancer. IGF1 and IGF2 were ligands of the IGF signaling pathway. It had been shown that IGF1 and IGF2 overexpression increase cell proliferation and tumor formation in mouse xenografts and do not alter resistance to chemotherapeutic drugs such as taxol. Overexpression

of IGF1 hd also been shown to cause overexpression of 9 genes in the IGF signaling pathway. It had been suggested that upregulation of these genes has stimulating effects on the IGF signaling pathway on global protein synthesis [45]. In this study, FeNP_A administration reduced IGF1 expression about 3 fold in the MCF-7 cell line and about 9 fold in the MDA-MB-231 cell line. The present research supposed that the downregulation of IGF1 expression and the decrease of cell proliferation in FeNP_A cytotoxicity were effective and apoptosis was induced by a decrease in global protein synthesis (Figure 11).

In MeWo cells, FeNP_A reduced the expression of 14 genes by 2 to 7 folds and increased the expression of 19 genes by 2 to 13 times and in CHL-1 cells, increased the expression of 17 genes by 2 to 8 times and decreased the expression of 7 genes by 3 to 9 times compared to the control group. ASP reduced the expression of 12 genes by 2 to 13 fold and increased the expression of 21 genes by 2-14 fold compared to the control group in CHL-1 cells and increased expression of 28 genes by 3-15 fold and reduced the expression of 8 genes by 2 to 11 fold in MeWo cells (Figure 10). BCL6, CXCL12, DNAJC15, RB1 and TPM1 gene expressions were increased in melanoma cancer cell. Bai et al. demonstrated that overexpression of BCL6 induces apoptosis by downregulation of BCL2 and BCL-XL in the diffuse large B cell lymphoma [46]. In CHL-1 and MeWo cell lines, FeNP_A and ASP treatment had been shown to increase BCL6 gene expression and FeNP_A and ASP administration in apoptosis induction has been assessed as providing BCL2 and BCL-XL suppression (Figure 12).

Figure 9: IC₅₀ value of FeNP_A were determined. (a) IC₅₀ was 139.40 μ g/mL in MCF-7, (b) 47.00 μ g/mL in MDA-MB-231, (c) in MeWo 38.48 μ g/mL, (d) 5.26 μ g/mL in CHL-1, (e) 179.12 μ g/mL in WI-38 and (f) 32.70 μ g/mL in HEL 299.

Figure 10: Apoptotic effect of FeNP_A. Apoptosis was increased (b) 2.21 fold in MCF-7 cells, (d) 2.31 fold in MDA-MB-231, (f) 1.29 fold in CHL-1 cells, (h) 1.72 fold in MeWo cells, (j) 19.85 fold in WI-38 cells and (l) 3.71 fold in HEL 299 cells compared to the control group (a, c, e, g, i and k are untreated control groups of the cell lines, respectively).

Figure 11: Effect of FeNP_A and ASP on gene expression in breast cancer model.

Figure 12: Effect of FeNP_A and ASP on gene expression in melanoma model.

4 Conclusion

This is the first report on the antioxidant activity, cytotoxic activity and characterization of iron oxide nanoparticles synthesized from the aerial part of *A. aestivus*. Iron oxide nanoparticles were characterized by UV-visible, FT-IR, TEM, SEM/EDX, TGA, XRD and Zeta potential characterization methods. Biological activities of the extract and nanoparticles were compared. FeNP_A showed higher antioxidant and cytotoxic activities than ASP. FeNP_A was highly effective in breast cancer and melanoma cell lines, induced apoptosis and altered gene expression regulation that is important for prognosis. It was suggested that gene expressions regulated by FeNP_A should be evaluated in further in vivo studies.

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