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Combined and alone apoptotic effects of Ankaferd hemostat and silver nanoparticles on chronic myeloid leukemia (CML) cell line K562

Ankaferd kanama durdurucu ve gümüş nanopartiküllerin kronik miyeloid lösemi (KML) hücre hattı K562 üzerindeki kombine ve tek başına apoptotik etkileri

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Abstract

Purpose: This study aimed to evaluate the effects of Ankaferd hemostat (ABS; Ankaferd Blood Stopper®) and silver nanoparticles (AgNPs), alone or in combination, on human chronic myeloid leukemia (CML) cells.

Materials and methods: The cytotoxicity of ABS and AgNPs on K562 CML cells was assessed using the XTT assay, measuring cell viability over time and across different doses. The half maximal inhibitory concentration (IC_{50}) was determined at 72 hours. Apoptosis-related gene expression was analyzed by Real-Time PCR, and oxidative stress was assessed by total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI).

Results: AgNPs reduced cell viability at higher doses, with the IC_{50} for 40 nm AgNPs being 107.8854 ppm at 72 hours. ABS reduced cell viability by 75% even at maximum dose. Significant changes (p<0.05) were observed in bcl-2, caspase-8 and CDK4 in the AgNPs group. In the ABS group, bcl-2, and CDK4 expressions were significantly elevated. The combined treatment increased caspase-8 and caspase-9 expressions, promoting apoptosis. No significant differences were found in TAS-TOS, but all groups showed higher oxidant activity compared to the control, with the combination group exhibiting the highest antioxidant effect.

Conclusion: ABS, a herbal treatment with minimal side effects, and AgNPs, a promising therapeutic agent, both showed potential in inhibiting tumor cells. Their combination enhanced apoptotic effects, warranting further investigation.

Keywords: Chronic myeloid leukemia, Ankaferd hemostat, silver nanoparticles, apoptosis, oxidative stress.

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Öz

Amaç: Bu çalışma, Ankaferd hemostat (ABS; Ankaferd Blood Stopper®) ve gümüş nanopartiküllerinin (AgNP'ler), tek başına veya kombinasyon halinde, insan kronik miyeloid lösemi (KML) hücreleri üzerindeki etkilerini değerlendirmeyi amaçlamıştır.

Gereç ve yöntem: ABS ve AgNP'lerin K562 KML hücreleri üzerindeki sitotoksik etkisi, XTT testi ile değerlendirilmiş ve farklı dozlar ve zaman dilimlerinde hücre canlılığı ölçülmüştür. Yarı maksimal inhibitör konsantrasyonu (IC_{50}) değeri 72 saat sonunda belirlenmiştir. Apoptoz ile ilişkili gen ekspresyonları Real-Time PCR ile analiz edilmiş, oksidatif stres ise total antioksidan durumu (TAS), total oksidan durumu (TOS) ve oksidatif stres indeksi (OSI) kullanılarak değerlendirilmiştir.

Bulgular: AgNP'ler, doz arttıkça hücre canlılığını düşürmüş ve 40 nm AgNP'ler için IC_{50} değeri 72 saat sonunda 107.8854 ppm olarak bulunmuştur. ABS, maksimum dozda bile hücre canlılığını %75 oranında düşürmüştür. AgNP grubunda bcl-2, kaspaz-8 ve CDK4 ekspresyonlarında anlamlı değişiklikler (p<0,05) gözlemlenmiştir. ABS grubunda ise bcl-2 ve CDK4 ekspresyonları anlamlı şekilde artmıştır. Kombine tedavi, kaspaz-8 ve kaspaz-9 ekspresyonlarını artırarak apoptozu teşvik etmiştir. TAS-TOS arasında anlamlı bir fark bulunmamış, ancak tüm gruplarda kontrol grubuna kıyasla daha yüksek oksidan aktivite gözlemlenmiş, kombinasyon grubunda ise en yüksek antioksidan etki saptanmıştır.

Sonuç: Yan etkileri minimal olan bir bitkisel tedavi olan ABS ve umut verici bir tedavi ajanı olan AgNP'ler, tümör hücrelerini inhibe etme potansiyeli göstermiştir. Kombinasyonları apoptoz üzerindeki etkilerini artırmış ve daha fazla araştırma yapılmasını gerektirmiştir.

Anahtar kelimeler: Kronik miyeloid lösemi, Ankaferd hemostat, gümüş nanopartiküller, apoptoz, oksidatif stress.

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Introduction

Chronic myeloid leukemia (CML) is a disease characterized by aberrant clonal growth of myeloid precursor cells. The annual global incidence rate of CML is 0.87/100 000, and it can reach 1.52 in people over the age of 70. The average age of diagnosis is 56 [1-3]. CML is caused by the Philadelphia chromosome, which causes the bcr-abl fusion gene and its end result, the bcr-abl protein. In the early 2000s, the initiation of a tyrosine kinase inhibitor (TKI) by targeting this protein dramatically changed the treatment of CML.

Prior to TKI, allogeneic hematopoietic stem cell transplantation was attempted as soon as a suitable donor was found for the patients. Imatinib, dasatinib, nilotinib, and bosutinib are some of the most common treatment choices now available. Allogeneic hematopoietic stem cell transplantation is now only used on a very limited basis [4]. CML has been transformed from a fatal disease to a chronic disease because of TKI. However, due to the prevalence of TKI resistance, treatment-free remission and deep molecular response are difficult to achieve.

TKI resistance can be dependent on bcr-abl or independent of bcr-abl. Dose escalation or switching to another TKI is preferred in bcr-abl-dependent resistance. Nevertheless, this approach has limitations, particularly when higher doses are not tolerated. On the other hand, bcr-abl independent resistance is more complex and difficult to manage. Cancer cells exhibit negative effect adaptation behaviors in addition to pharmacogenetics in bcr-abl-independent resistance and its effects on patients. These cells are attempting to survive, which causes changes in the expression of a variety of proteins (such as bcl-2 and MCL1, MDR1, transcription factor STAT5, and imatinib uptake transporter OCT1). These cancer stem cells are strong, resistant, and incurable; they form tumors, renew themselves, and frequently cause disease progression and relapse [5].

Ankaferd hemostat (ABS; Ankaferd Blood Stopper®) is an herbal mixture utilized for many years in Anatolia for hemostasis. ABS contains Glycyrrhiza glabra, Alpinia officinalis, Thymus vulgaris, Vitis vinifera, and Urtica dioica, among others [6]. It also contains anti-inflammatory, antioxidative, anti-infective, and lastly, antineoplastic properties in addition to hemostasis. ABS inhibits cell proliferation by influencing cell metabolism and cell cycle mechanisms. KPNA2, SND1, and PARK7 are potential cancer therapy targets. It also increases the expression of the tumor suppressor proteins RPL5 and UCHL1. RPL5 causes apoptosis by directly activating the p53 apoptotic pathway [6, 7]. ABS toxicology studies have also revealed that it is a very safe agent [8].

Silver nanoparticles (AgNPs) are structures ranging in size from 1 to 100 nm. They have low electrical and thermal resistance and have been used in a variety of consumables, electronics, and healthcare applications [9]. It possesses potent antimicrobial properties [10]. Recent research on lymphoma, breast cancer cells, and liver cancer cells has also revealed that these molecules have anti-tumor properties [11].

CML is one of the most well-controlled cancers, yet it remains an important issue for research due to resistance development, complications induced by patients' extra co-morbidities, and TKI side effects. Additionally, although ABS and AgNPs have shown promising antitumor effects in various cancer models, there is still a notable gap in research on their combined effects on chronic CML cells. This study hypothesizes that the combination of ABS and AgNPs will synergistically enhance apoptosis in CML cells, providing a more effective therapeutic approach than either agent alone. By investigating these effects on the K562 CML cell line, we aim to assess the potential of ABS and AgNPs to induce apoptosis via both extrinsic and intrinsic pathways, which may offer an additional therapeutic mechanism compared to traditional treatments. This study's findings could establish a basis for new adjunctive strategies in CML treatment, addressing drug resistance and enhancing antitumor efficacy through a multi-mechanistic approach.

Materials and methods

Cell culture

In this study, K562 human CML cells were cultured in RPMI 1640 (Gibco™) enriched with 10% heat-inactivated fetal bovine serum (FBS; Capricorn Scientific), 20 units/mL penicillin and 20 µg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM amino acid solution (Biological Industries) and cultured at 37°C in 5% CO_2.

AgNPs (Sigma-Aldrich, 40 nm) were applied to the K562 cells with concentrations including 5, 10, 25, 50, 100, and 200 ppm, and Ankaferd hemostat (ABS; Ankaferd Blood Stopper®, Istanbul, Türkiye) was decided to apply to the K562 cells with various concentrations including 2.5, 5, 10, 25, 50, and 100 ppm to evaluate the antiproliferative effect at 24, 48, and 72 hours.

XTT Assay

The antiproliferative activities of AgNPs (40 nm), ABS, and their combination on K562 cells were evaluated using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay. The test was conducted on 1×10^4 cells per well in 96-well plates, the provided instructions (Cell Proliferation Kit; Biological Industries Cat No: 20-300-1000). Following the completion of the dosing procedures, the XTT combination was administered corresponding to the recommended dose and time. The synthesis of farmazon was assessed spectrophotometrically (using a Multiskan GO microplate spectrophotometer, Termo) and chromatically at 450 nm (reference wavelength: 630 nm). The viability percentage is calculated by dividing the absorption of the experimental well by the absorption of the control well, and then multiplying the ratio by 100.

The AAT Bioquest online web page was utilized to calculate the IC_{50} dose of AgNPs (40 nm) and ABS on K562 cells (https://www.aatbio.com/tools/ic50-calculator). The IC_{50} dosage was utilized as the dose group in the remaining molecular tests.

RT- PCR assay

Trizol (Invirogen, USA) was applied to obtain total RNA from cells, in accordance with the directions provided by the manufacturer. The A.B.T. synthesis kit with RNase Inh. was used to synthesize cDNA (ABT, Türkiye). RT-PCR was performed to measure changes in mRNA expression of bcl-2, caspase-3, bax, caspase-9, CDK4, caspase-8, CDK6, URG4, p21, BID, and CCND1 (Applied Biosystem, StepOne Plus). Beta-actin was utilized to normalize the results. The identical primer sequences as in Dodurga et al. [12] 2015 and Alur et al. [13] 2016 were applied. Real-time RT-PCR was utilized for PCR testing with the SYBR Green qPCR Master Mix ABTTM 2X qPCR SYBR-Green MasterMix (Türkiye) methodology.

TAS- TOS and OSI measurement

Rel Assay kits (Rel Assay Kit Diagnostics, Gaziantep, Türkiye) were applied to assess the total antioxidant status (TAS) and total oxidant status (TOS) in control cells as well as cells treated with AgNPs, ABS, and their IC_{50} value combinations. TAS and TOS values were obtained by a microplate reader (Biotek). The oxidative stress index (OSI) was calculated by converting the mmol unit of TAS to the µmol unit of TOS. The oxidative stress index was determined by the OSI= TOS / TAS* 1/10 technique.

Statistical analysis

The statistical analysis was carried out using SPSS 23.0 (IBM SPSS Statistics 23 software (Armonk, NY: IBM Corp.)). Continuous variables were defined by mean ± standard deviation. One-way ANOVA test was used for comparisons of multi-group parameters (TOS (µmol H_2O_2 equiv./L) and TAS (mMOL Trolox Equiv./L). The $2^{-\Delta\Delta CT}$ method in the RT² ProfilerTM PCR Array Data Analysis program was used to quantify RT-PCR data. Data were expressed as mean + standard deviation and for group comparisons. Independent samples t test was used with cRT² ProfilerTM PCR Array Data Analysis program. Statistical significance was determined as $p\leq0.05$.

Results

The results of this study provide a comprehensive assessment of the effects of ABS and AgNPs on K562 CML cells, demonstrating distinct responses across cell viability, gene expression, and oxidative stress. The XTT assay showed that AgNPs, both alone and in combination with ABS, significantly reduced cell viability in a dose- and time-dependent manner,

achieving an IC_{50} of 107.8854 ppm at 72 hours, whereas ABS alone had a comparatively limited effect. Gene expression analysis via RT-PCR further revealed that combination treatment increased apoptotic markers, particularly in caspase-8 and caspase-9 expressions. The antioxidant activity was most pronounced in the combined treatment. These findings collectively underscore the apoptotic and oxidative impacts of ABS and AgNPs on CML cells, with combination therapy showing potential for more substantial therapeutic effects. The results obtained from this study are given below in detail.

XTT assay

The viability of K562 cells was evaluated by the XTT assay following treatment with ABS, AgNPs (40 nm), and their combination. K562 cell viability decreased in a time- and dose-dependent way. At the 24- and 48-hour marks, the vitality of the cells treated with AgNPs decreased somewhat as the dose rose; however, at the 72-hour mark, a more significant effect was observed. The IC_{50} dose of AgNPs (40 nm) was found to be 107.8854 ppm at 72 hours in the K562 cell line (Figure 1a, Figure 2). The influence of ABS on K562 cell viability is illustrated in Figure 1b. A comparable reduction in cell viability was not found with increasing ABS dosage. Even at the highest dose rate, ABS could only reduce cell viability up to 75% within 72 hours.

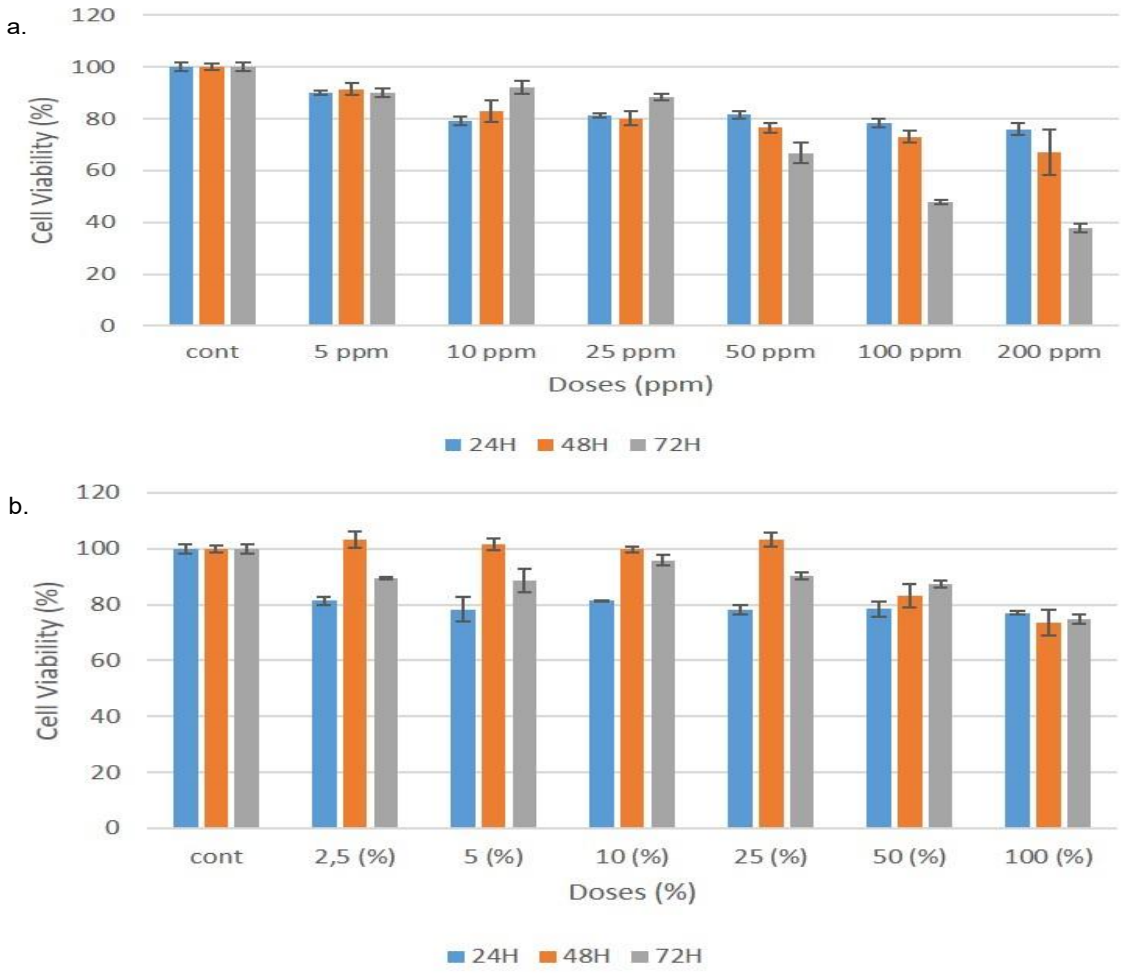


Figure 1. Cell viability of groups

a. K562 cells were treated with AgNPs at different concentrations and time intervals and their viability was assessed by XTT assay. Data shows the average results of three independent experiments. IC_{50} doses of AgNPs in K562 cells were detected as 107.8854 ppm at the 72 hours

b. K562 cells were treated with ABS at different concentrations and time intervals and their viability was assessed by XTT assay. Data shows the average results of three independent experiments

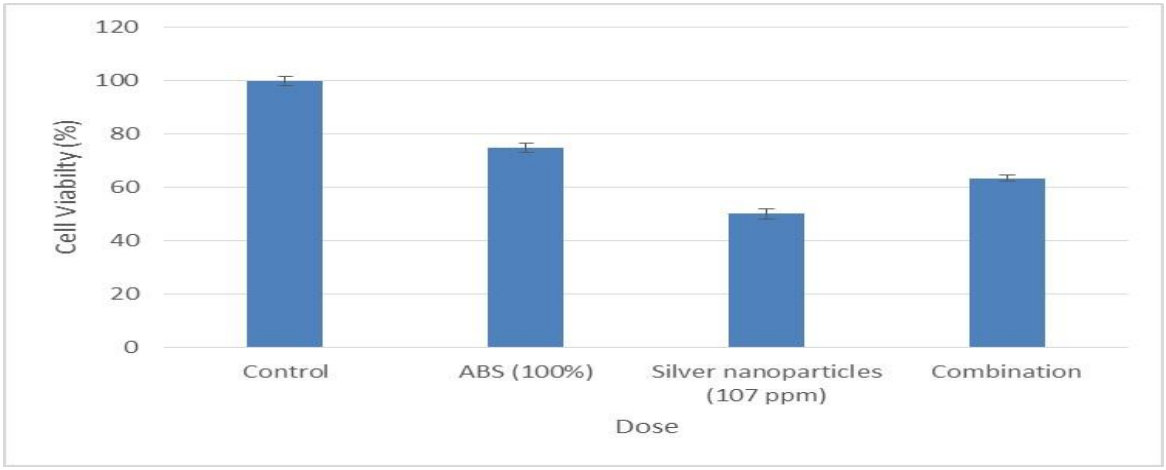


Figure 2. Cell viability of control and dose groups including IC_{50} value of AgNPs, ABS (100%) and their combination in K562 cells

Real Time-PCR

Following total RNA removal from the cells, cDNA synthesis was carried out. RT-PCR was used to examine the gene expression of bcl-2, caspase-8, caspase-9, bax, caspase-3, CDK6, CDK4, URG4, p21, BID, and CCND1. The SYBR Green qPCR Master Mix technique was followed. RT- PCR was utilized to assess mRNA expression alterations in genes implicated in the triggering of apoptosis and cell cycle arrest. In comparison to the control group, the expression of key genes related to apoptosis, such as caspase-8 and caspase-9, increased in the combination group. Significant alterations (p<0.05) were seen in bcl-2, caspase-8, and CDK4 expressions in the AgNPs group. Statistically significant (p<0.05) increases in bcl-2 and CDK4 expressions were found in the ABS group. In the combined group, the increase in both caspase-8 and caspase-9 expressions was found to be statistically significant in inducing apoptosis. Table 1 summarizes all gene phase changes and associated p-values (Table 1).

Table 1. Fold regulation and p values in dose groups with comparing to control group

		Group 1*		Group 2*		Group 3*	
		Fold Regulation	*p*-value	Fold Regulation	*p*-value	Fold Regulation	*p*-value
1	Beta-actin	1.00	non	1.00	non	1.00	non
2	Caspase-3	-10.96	0.343256	9.44	0.555935	14.21	0.353469
3	Caspase-8	4.14	0.313246	28.27	0.005590	12.06	0.004980
4	Caspase-9	1.88	0.213277	1.39	0.537163	5.89	0.011216
5	bax	2.89	0.201103	2.56	0.100986	1.87	0.083722
6	bcl-2	8.81	0.012183	5.48	0.006919	37.03	0.019985
7	CDK4	5.64	0.003648	2.14	0.019540	7.18	0.000171
8	CDK6	9.39	0.050577	20.24	0.306986	451.47	0.003172
9	CCND1	4.55	0.272031	5.89	0.000166	6.27	0.015260
10	URG4	1.74	0.048724	1.78	0.178441	2.09	0.320371
11	p21	2.20	0.055106	1.55	0.026678	1.49	0.374144
12	BID	1.80	0.410042	1.16	0.935613	9.65	0.081735

*Group 1: ABS, Group 2: AgNPs, Group 3: Combination

TAS-TOS and OSI determination

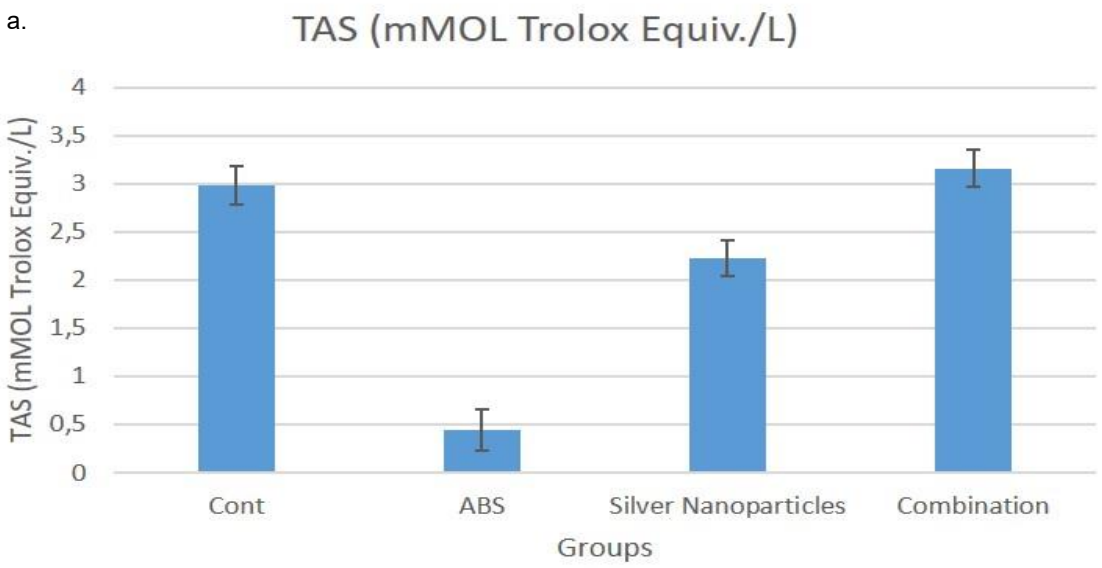
Table 2 shows that there was no statistically significant difference (p>0.05) in the TAS-TOS between the groups. ABS, AgNPs, and their combination showed increased total oxidant activity than the control group (p>0.05), despite the lack of a statistically significant difference (Table 2). After assessing each group's total antioxidant capacity, we discovered that, while there was no statistically significant difference, the combination group had the greatest antioxidant impact (Table 2). OSI values are shown in the Figures 3a-c.

Table 2. Comparison of TAS and TOS values

	Control	Group1*	Group 2*	Group 3*	*p*-value
TAS	2.97±0.08	0.43±0.09	2.22±0.15	3.15±0.04	0.417 (F=0.325)
TOS	25.31±0.11	103.8±0.22	61.12±0.16	36.97±0.13	0.561 (F=0.733)

*Group 1: ABS, Group 2: AgNPs, Group 3: Combination

a.



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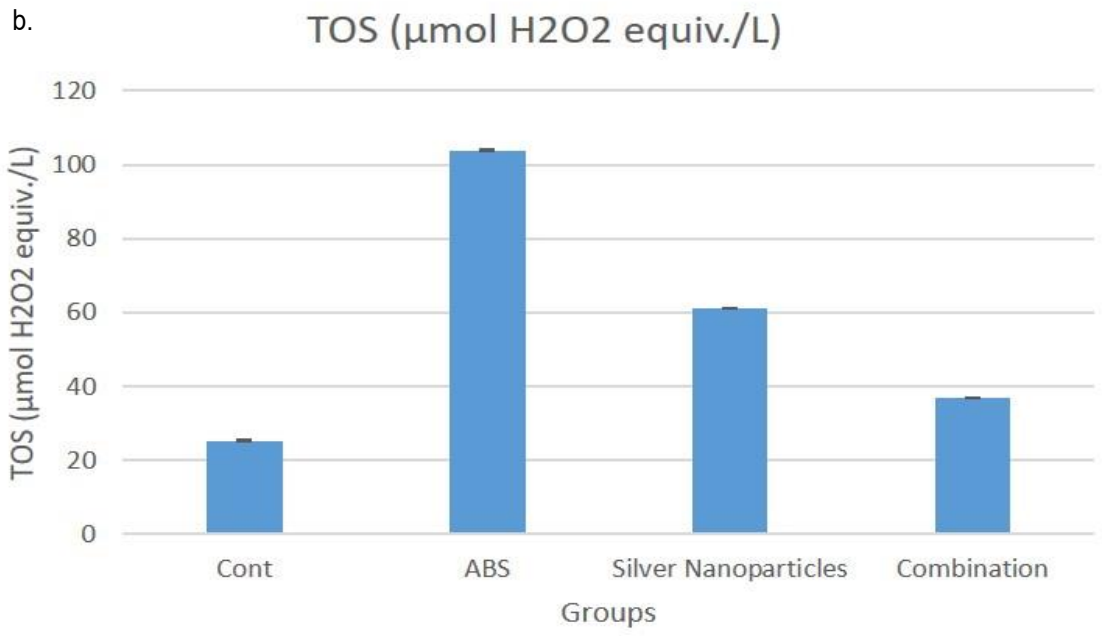

c.

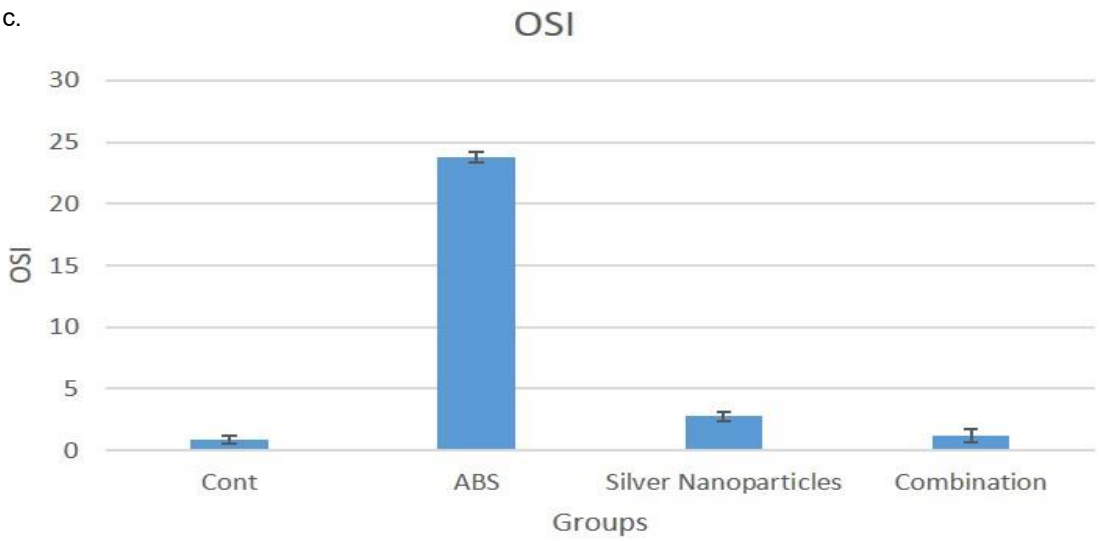

Figure 3. Total antioxidant (a), oxidant (b) and oxidative stress index (c) in K562 cells treated with AgNPs, ABS and their combination

Discussion

This study demonstrates that the combined treatment of ABS and AgNPs significantly enhances apoptotic activity in K562 CML cells. Key findings include a dose- and time-dependent decrease in cell viability with AgNPs alone, reaching optimal efficacy at 72 hours, while the combined treatment markedly increased expression of apoptotic markers, both caspase-8 and caspase-9. These results indicate that the ABS-AgNP combination amplifies apoptotic responses beyond the effects of either agent alone, which could offer a multi-faceted approach for targeting CML cells. This combined effect highlights the potential of ABS and AgNPs as adjunctive agents in CML therapy, addressing limitations of current treatments and potentially overcoming challenges related to drug resistance.

Apoptosis is a physiological condition required for a cell's normal growth and development. It is also necessary for a stable internal environment. The mitochondrial pathway is divided into segments, such as the death receptor pathway. Caspase-mediated apoptosis occurs because of mitochondrial cytochrome c release. When the mitochondrial permeability transition pores open in response to an external stimulus, the mitochondrial membrane potential decreases and apoptosis is induced [11, 14, 15]. In our study, we noticed a statistically significant increase in caspase 8 activities in the AgNPs group. This suggests that the inhibitory effect of these nanoparticles on malignant cells in CML in our study occurs via apoptosis. The increase in caspase-9 expression was also shown to be statistically significant in inducing apoptosis in the combined group. One of the expressions of genes implicated in inducing cell cycle arrest, known as Cyclin D1, increased in the AgNPs group.

Mitochondria produce reactive oxygen radicals and provide energy to cells. These radicals are a type of molecule that causes apoptosis. AgNPs have been shown in some studies to cause oxidative damage to tumor cells by causing the production of reactive oxygen radicals. This impact was not only detected in our study, but it was also observed more frequently in ABS [11, 16, 17].

CML is one of the most prevalent hematological cancers. TKI in CML affects not only bcr-abl1, but also a wide range of other targets. This situation has a slew of unintended consequences. Furthermore, despite its mildness, it interferes with humoral and cellular immunity. Side effects make life difficult for both the doctor and the patient, especially in patients with co-morbidities and the elderly [18]. Immunity issues are addressed, particularly in pediatric patients with CML. Concerns are also raised by the fact that these children will need

to use TKIs for an extended period of time. These children, for example, have immunization programs, which must be carefully managed due to the use of TKIs. In these two difficult patient groups, research is being conducted on various treatment options. Future investigations into the potential use of ABS and/or AgNPs in the treatment of these adult or pediatric patients may be guided by our findings.

Numerous cellular processes, including angiogenesis, apoptosis, cell cycle regulation, inflammation, signal synthesis, metabolism, and immunological pathways, have been shown to be subject to the pleiotropic effects of ABS [19, 20]. ABS has been demonstrated to prevent the B-CLL cell line from transforming into an aggressive, blastic lymphoid form [21]. Mumcuoglu et al. [19] investigated the effects of ABS in two different human leukemia cell lines, K562 and Jurkat cells, and discovered that it induced apoptosis by regulating the expression of PAR1 and EPCR (Endothelial cell protein C receptor) in these cells. ABS has been shown to have a dose- and time-dependent effect on EPCR expression in both cell lines, causing cycles of decreasing and increasing EPCR expression. However, in high-dose ABS application, the effect of decreased EPCR expression was observed to be statistically more stable and continuous in Jurkat cells compared to K562 cells. Given the results of this study in the literature, we investigated the effects of ABS and AgNPs on the CML cell line. Tumor cell viability decreased as the dose and period of therapy increased. ABS and/or AgNPs may be able to help these patients, as demonstrated in our study, because of their good efficacy and low side-effect profile.

We can see that AgNPs have hosted some cancer studies in recent years. In one study, AgNPs were modified with folic acid to actively and selectively recognize tumor cells, and it was demonstrated that these particles inhibit lymphoma cells by increasing the apoptosis of stem cells in vitro and in vivo, with no significant side effects [11]. Guo et al. [22] found that AgNPs may enter K562 cells, a CML cell line, and reside in endosomes. In our study, the K562 cell line was also selected. According to Guo et al. [22], the effect of AgNPs on these cells could be cytotoxicity and apoptosis via reactive oxygen radicals. These effects have been shown to be reversible with vitamin C, an antioxidant. Another study found that AgNPs increase the intracellular accumulation of daunorubicin, a common chemotherapeutic agent, in K562 leukemia cells, activating the killing effect of these cells [23].

While this study provides valuable insights, several limitations should be addressed in future research. The scope of gene analysis was limited to key apoptotic markers, and additional gene targets and pathways could be explored to better understand the molecular mechanisms at play. Additionally, potential off-target effects of AgNPs, especially in a clinical setting, require further investigation. The lack of in vivo data restricts the ability to fully translate these findings to therapeutic applications. Future studies should include in vivo experiments to evaluate the safety and efficacy of the ABS-AgNP combination in a living organism, alongside pathway-specific analyses to confirm and expand these initial findings. Exploring the pharmacokinetics, biodistribution, and long-term effects of these agents in vivo would be essential for assessing their therapeutic potential in CML treatment.

In conclusion, due to the development of drug resistance in CML, additional co-morbidities in patients, and TKI side effects, research into different treatment options is still ongoing. We tested two different options on CML cell lines, ABS, a herbal medicine with very low potential for side effects, and AgNPs, which is a very effective and promising agent in this regard, and found that they inhibited tumor cells. Furthermore, it has been demonstrated that when these two agents are used together, the apoptotic anti-tumor effects increase. This study, we believe, is a groundbreaking study for future research.

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Authors contributions: B.U.K. and Y.D. constructed the hypothesis of the study. B.U.K. and M.S. arranged the material and method section. M.S., B.U.K. and Y.D. evaluated the data of the Results section. Discussion section of the article was written by B.U.K. Also B.U.K. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

Conflict of interest: No conflict of interest was declared by the authors.

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