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Comprehensive Study of the Hemostatic Properties of *Achillea millefolium* and Silver Nanoparticles

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ABSTRACT

The Achillea millefolium plant is used in traditional medicine for various purposes, such as wound healing, disinfection, etc., due to its bioactive compounds, such as sesquiterpenes and flavonoids. This study aimed to investigate the hemostatic properties of silver nanoparticles, *Achillea millefolium* extract, as well as the effect of combining these two substances and the antibacterial effect of yarrow. To conduct this study, first, the *Achillea millefolium* plant was extracted using ethanol, and then GC-MS was used to examine the components of the extract. Also, coagulation properties were evaluated using PT, PTT, and CT tests. In order to investigate the antibacterial properties of *Achillea millefolium* extract, two species of *Staphylococcus aureus* and *Escherichia coli* were used. The results showed that silver nanoparticles and *Achillea millefolium* extract have hemostatic properties and are able to reduce blood clotting time, and *Achillea millefolium* also has antibacterial properties against *Staphylococcus aureus*. Given the hemostatic and antibacterial properties of *Achillea millefolium*, this plant can be used to stop bleeding from wounds.
Keywords: *Achillea millefolium*, Antibacterial, Blood coagulant, Silver nanoparticles

INTRODUCTION

Uncontrolled bleeding is the leading cause of death among the injured, and trauma kills more than four million people worldwide each year [1]. The important point is that many of these deaths can be prevented by covering the wound and stopping the bleeding [2]. Several methods are used to reduce and prevent bleeding, including direct pressure on the wound and the use of tourniquets. However, the use of tourniquets can cause pain and also thrombosis in the limb [3]. Of course, the use of hemostatic agents such as tranexamic acid, desmopressin, recombinant coagulation protein, etc. can improve hemostasis by stimulating fibrin formation or inhibiting fibrinolysis. Unfortunately, the side effects associated with them and the small number of available drugs mean that only a small number of people can use these drugs. Hence, there is a need for a cheaper, safer, and more effective treatment. Medicinal plants have been used in many parts of the world since ancient times to reduce bleeding [4].

Achillea millefolium (Achillea), known in Persian as *bumadaran*, is a perennial plant belonging to the Asteraceae family. The common name *Achillea* refers to Achilles, an ancient hero in the Trojan War, who used *Achillea* to treat his and his soldiers' wounds [5]. Because *Achillea* grows in temperate regions, there is a great deal of diversity among the species, with about 130 species and varieties native to Europe and Asia, and a few in North America. The plant can grow to a height of about 0.2 to 1 m, and its leaves are pointed and have distinct hairs. The flowers of *Achillea* are in a flat raceme and vary in color from white to pink depending on the species [6]. Different parts of the *Achillea* plant (flowers, leaves, and stems) are used for medicinal purposes due to the presence of compounds such as alkaloids, coumarins, flavonoids, sesquiterpene lactones (achillin, achillicin), triterpenes, tannins, etc [7]. The medicinal effects of the *Achillea* plant include antidiabetic, anti-inflammatory, antiseptic, antitumor, antioxidant, antifungal, etc [8]. This plant also has wound-healing properties and an anti-spermatogenic effect [5].

Silver nanoparticles are more effective against bacteria than conventional antibiotics and have shown broad antimicrobial properties due to their high surface-to-volume ratio [9]. Silver nanoparticles are utilized in various applications, including disinfectants and water purifiers [10], and are also employed as coatings on storage bags to prevent the growth of microorganisms and facilitate the long-term preservation of food [11]. They are also used as coatings in surgical instruments, wound and burn dressings, drug delivery systems, etc [12]. Additionally, silver nanoparticles have demonstrated anti-inflammatory properties [13].

Due to the properties of *Achillea* and silver nanoparticles, research on the effectiveness of these compounds and their synergistic effect on blood coagulation is limited. This study aimed to investigate the efficacy of silver nanoparticles and the ethanolic extract of *Achillea*, as well as the synergistic effect of these two substances on the blood coagulation process, and to determine the effective concentrations.

MATERIALS AND METHODS

Collection of *Achillea*

Aerial parts of *Achillea* were collected in autumn from northeastern regions of Alborz Province and identified by the Botany Department, Faculty of Biological Sciences, Al-Zahra University.

Extraction

Extraction was performed using the method of Banai *et al*., [14] with slight modifications. The aerial parts of *Achillea millefolium* were dried in the shade and ground. Then, 30 g of *Achillea* powder was mixed with 500 ml of ethanol and placed on a shaker for 24 hours at

room temperature. The alcoholic extract was then filtered using filter paper. The alcoholic extract was then placed in an oven to remove the solvent and concentrate. Finally, the resulting extract was dried and weighed [14]. The yield for the ethanolic extract of the *Achillea* plant was 10.16%.

GC-MS Analysis

Chromatographic analysis was performed on a 6890N GC-MS system with a 5973 mass selective detector (GC-MS). The capillary column was a HP 5 MS column (30 m × 0.25 mm × 0.25 µm). The temperature was set at 40 °C and maintained for 3 min. The sample was heated from 40 to 280 °C at a rate of 5 °C/min and held at 280 °C for 5 min.

Preparation of Silver Nanoparticles

Silver nanoparticles were purchased as gray powder, with dimensions of 20 nm, a density of 10.49 g/cm^3, a molecular mass of 107.87 g/mol, and a purity of 99.99% from Fine Nano Company.

Evaluation of Coagulation Indices

To investigate the effect of *Achillea* plant extract on blood coagulation, concentrations of 0.025, 0.05, 0.075, and 0.1 g/ml were prepared in normal saline solution. Also, the main stock of silver nanoparticles was prepared at a rate of 10 mg/ml in distilled water, and then concentrations of 10, 50, and 250 µl were prepared from it. PT, APTT, and CT tests evaluated the plant extract and silver nanoparticles on selected indicators.

Prothrombin Time (PT) Test

This test measures the clotting time of blood plasma in the presence of an optimal tissue factor concentration (tissue thromboplastin) and indicates the efficiency of the extrinsic pathway of blood coagulation. First, to examine the control sample, 200 µl of PT solution and 100 µl of warm normal plasma (maintained at 37 °C) were mixed, and after 10 seconds, the blood clotting time (formation of the first white fibrin strands) was recorded using a reading lamp. To examine the coagulation effects of different concentrations of *Achillea* extract and silver nanoparticles, 10 µl of the desired concentration were mixed with 190 µl of warm normal plasma in a glass tube, and after 5 minutes in a 37 °C incubator, 100 µl of warm PT solution was added to it, and about 10 seconds later, the time of coagulation was recorded [15].

Partial Thromboplastin Time (APTT) Test

This test measures the clotting time of blood plasma after activation of coagulation factors (without the addition of tissue thromboplastin), thus indicating the efficiency of the intrinsic pathway of blood coagulation. To examine the control sample, 100 µl of warm APTT solution was mixed with 100 µl of warm normal plasma solution and placed in a water bath for 5 minutes at 37 °C. Then, 100 µl of warm calcium chloride solution was added to the above mixture (APTT and warm normal plasma) and shaken slightly for 20 seconds in a water bath, and then the clotting time was recorded using the aforementioned method. To investigate the coagulation effect of different concentrations of *Achillea* extract and silver nanoparticles, 10 µl of the desired concentration was mixed with 190 µl of normal plasma and 100 µl of APTT solution in a tube. After 5 minutes in a 37 °C incubator, 100 µl of calcium chloride solution was added, and the coagulation time was recorded 20 seconds later [15].

Clotting Time (CT) Test

This test measures the ability of the intrinsic pathway to initiate clot formation by activating factor XII, and also plays an important role in the common blood coagulation pathway [16]. The Lee and White method was used to measure this index [17]. In this way, first, 150 µl of different concentrations of *Achillea* extract and silver nanoparticles were poured into test tubes and made up to a final volume of 1 ml with blood. The tubes were placed in a water bath at 37 °C and examined every 30 seconds for clot formation.
To investigate the synergistic effect of *Achillea* extract and silver nanoparticles, two concentrations of 0.075 g/mL and 50 µL, respectively, which had better effects, were selected and combined, and PT, PTT, and CT tests were performed again using the method described above.

Antimicrobial Activity Assay

The antimicrobial activity of Achillea extract was determined using an agar diffusion assay. First, a concentration equivalent to 0.5 McFarland standard was obtained using bacteria and Mueller-Hinton broth medium, then 100 µl of it was spread evenly on the surface of Mueller-Hinton agar. 70 µl of the extracts was added to 9 mm diameter wells. The diameter of the zones of no growth was measured in mm, including the well's diameter. Each antimicrobial assay was performed at least three times. The following reference bacterial strains were used as test cultures: *Staphylococcus aureus (ATCC29213)* and *Escherichia coli (ATCC25912)*. As a positive control, erythromycin (15 mg per disk) was used for gram-positive bacteria, ciprofloxacin (5 mg per disk) for gram-negative bacteria, and Mueller-Hinton Broth medium was used as a negative control [18].

Statistical Analyses

Data were analyzed using one-way analysis of variance (ANOVA) using Prism software version 8. All experiments were performed in triplicate. The results were calculated and expressed as mean and standard deviation.

RESULTS

GC-MS Analysis Results

The results of GC-MS analysis showed that the most abundant compound in the ethanolic extract of *Achillea* plant is part of the sesquiterpene lactone group (Achillicin). This extract also contains compounds from the monoterpene family (Camphor and 1,8-Cineol) (Figure 1 and Table 1).

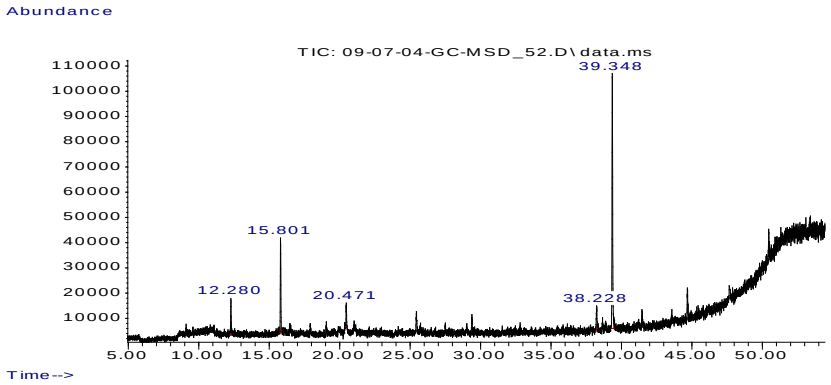

Fig. 1 GC-MS analysis results of the ethanolic extract of the *Achillea* plant

Table 1 GC-MS analysis results of the ethanolic extract of the *Achillea* plant

Components identified	Retention times (R_t , min)	Molecular Formula	Molecular Mass	Similarities	Area %
1,8-Cineole	12.28	$C_{10}H_{18}O$	154	82	6.76
Camphor	15.80	$C_{10}H_{16}O$	152	93	18.70
Methoxyacetic acid, 2-(1-adamantyl) ethyl ester	20.47	$C_{15}H_{24}O_3$	252	80	4.13
Arsenous acid, tris(trimethylsilyl) ester	38.22	$C_9H_{27}AsO_3Si_3$	342	82	5.44
Achillicin	39.34	$C_{15}H_{18}O_3$	246	98	64.95

Analyses Related to Silver Nanoparticles

The results of scanning electron microscopy (SEM) of silver nanoparticles are presented in Figure 2.

Fig. 2 Scanning electron microscope (SEM) images of silver nanoparticles. a: 100 nm magnification, b: 25 nm magnification

The Effect of Different Concentrations of Ethanolic Extract of *Achillea* Plant and Silver Nanoparticles on Coagulation Indices

As can be seen in Figure 3a, *Achillea* extract at concentrations of 0.025, 0.05, 0.075 g/ml had no significant effect on the PT coagulation index compared to the control group and no effect was observed on the reduction of coagulation time, but at a concentration of 0.1 g/ml, blood coagulation time increased compared to the control group and a significant effect was shown compared to the control. However, silver nanoparticles at concentrations of 10, 50, and 250 µl increased the blood coagulation time compared to the control group, and a significant effect was shown compared to the control (Fig. 3b).

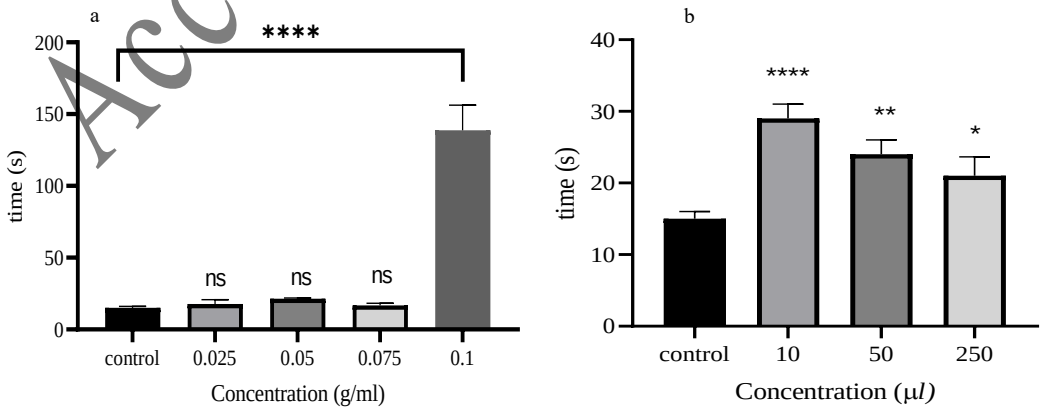

Fig. 3 Effect of different concentrations of ethanolic extract of *Achillea* plant and silver nanoparticles on coagulation index PT: (a) different concentrations of ethanolic extract of *Achillea* plant, (b) different concentrations of silver nanoparticles.

The results of combining the two ingredients, *Achillea* extract and silver nanoparticles, at concentrations of 0.075 g/ml and 50 µl, are shown in Figure 4. This combination increased the coagulation time by about 3 minutes and had a significant effect compared to the control.

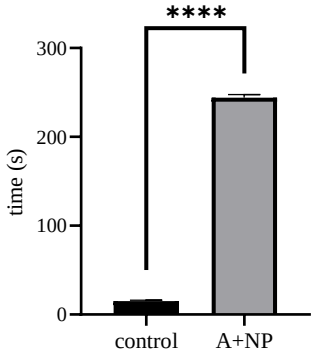

Fig. 4 Effect of concentrations of 0.075 g/ml and 50 µl of ethanolic extract of *Achillea* plant and silver nanoparticles on the PT coagulation index

As can be seen in Figure 5a, concentrations of 0.025 and 0.05 g/ml of ethanolic extract of *Achillea* increased the coagulation time, and a significant effect was observed on the PTT coagulation index compared to the control group. Still, at concentrations of 0.075 and 0.1 g/ml, no significant effect was observed compared to the control group. As shown in Figure 5b, a concentration of 10 µl of silver nanoparticles increased the coagulation time compared to the control group, with a significant effect observed. However, no significant effect was observed at concentrations of 50 and 100 µl compared to the control group.

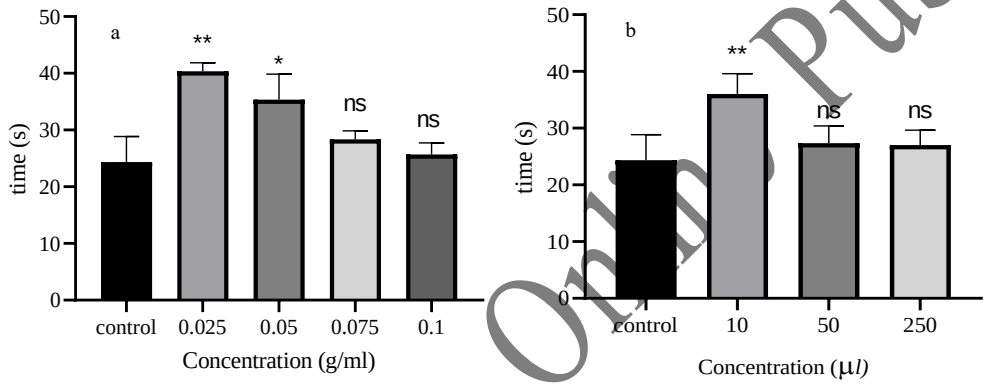

Fig. 5 Effect of different concentrations of ethanolic extract of *Achillea* plant and silver nanoparticles on the PTT coagulation index: (a) different concentrations of ethanolic extract of *Achillea* plant, (b) different concentrations of silver nanoparticles.

The results of combining two substances, *Achillea* extract and silver nanoparticles, at concentrations of 0.075 g/mL and 50 µL in the PTT test are shown in Figure 6. This combination did not show any effect on coagulation time, and no significant effect was observed compared to the control.

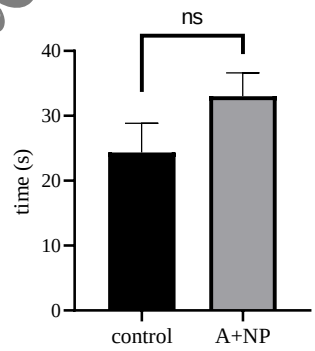

Fig. 6 Effect of concentrations of 0.075 g/mL and 50 µL of ethanolic extract of *Achillea* plant and silver nanoparticles on the PTT coagulation index

As can be seen in Figure 7a, *Achillea* extract at concentrations of 0.05 and 0.075 g/mL had no significant effect in the CT test compared to the control group. Still, at concentrations of 0.025 and 0.1 g/mL, it reduced the coagulation time and had a significant effect compared to the control group. To more accurately examine the effect of different concentrations in the CT test, we increased the tested concentrations until we reached a concentration that demonstrated good effectiveness. For this purpose, we examined two concentrations of 0.125 and 0.15. The concentration of 0.125 had no significant effect, but the concentration of 0.15 caused a significant increase in coagulation time and had a significant effect compared to the control group. A concentration of 10 µL of silver nanoparticles reduced the coagulation time and had a significant effect compared to the control group, but no significant effect was observed at concentrations of 50 and 250 µL (Figure 7b).

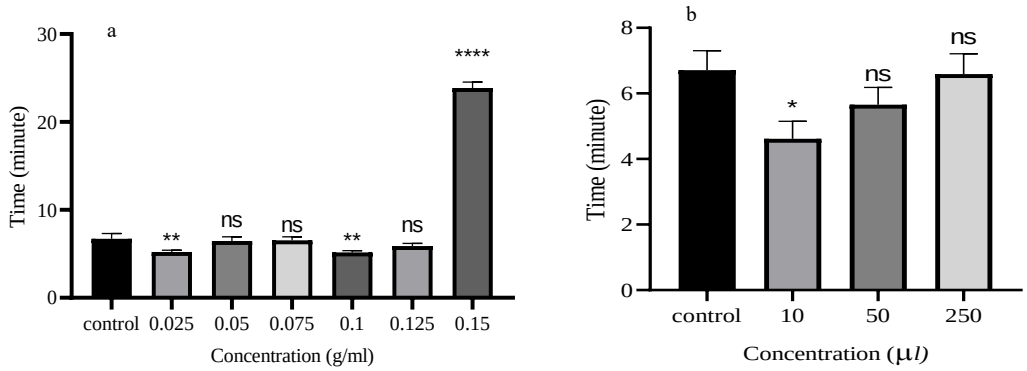

Fig. 7 Effect of different concentrations of *Achillea* ethanolic extract and silver nanoparticles on the CT coagulation index: (a) different concentrations of *Achillea* ethanolic extract, (b) different concentrations of silver nanoparticles.

The results of combining two substances, *Achillea* extract and silver nanoparticles, at concentrations of 0.075 g/mL and 50 µL, are shown in Figure 8. This combination increased the coagulation time by about 24 minutes and had a significant effect compared to the control.

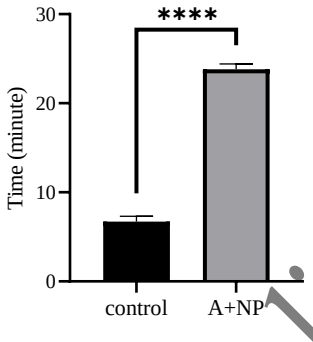

Fig. 8 Effect of concentrations of 0.075 g/mL and 50 µL of ethanolic extract of *Achillea* plant and silver nanoparticles on the coagulation index CT

Antibacterial Test

As can be seen in Table 2 and Figure 9, concentrations of 0.025 and 0.1 g/ml of *Achillea* extract did not have an inhibitory effect on the growth of *Staphylococcus aureus*, but at concentrations of 0.05 and 0.075 g/ml, the presence of a growth inhibition zone indicates the presence of antibacterial compounds at these concentrations. *Escherichia coli* bacteria lack a growth inhibition zone and are therefore resistant to different concentrations of *Achillea* extract. According to Fine Nano, 0.1% silver nanoparticles can play an important role in suppressing and eliminating dozens of pathogenic microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, etc.

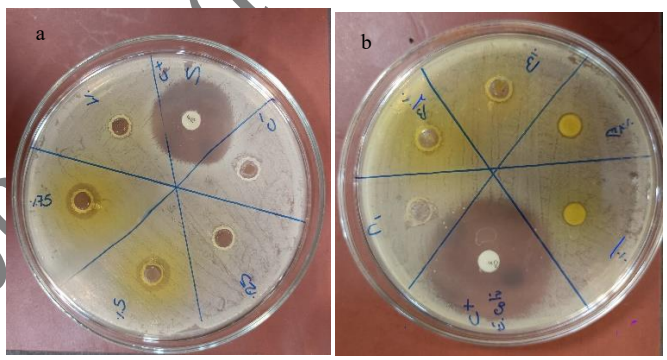

Fig. 9 Antimicrobial activity of different concentrations of *Achillea* extract, a: *Staphylococcus aureus* bacteria, b: *Escherichia coli* bacteria

Table 2 Antimicrobial activity of different concentrations of *Achillea* extract, zones of no growth in millimeters, including well diameter

Species name \ Concentration (g/ml)	0.025	0.05	0.075	0.1	Control +	Control -
Staphylococcus aureus	-	12.33 ± 0.57 *	13.33 ± 0.57 *	-	32.33 ± 0.57	0
Escherichia coli	-	-	-	-	33.33 ± 0.57	0

Note: Well diameter 9 mm; 0: no inhibition, -: no growth zone, *: data were statistically significant compared to control.

DISCUSSION

Whenever a blood vessel is torn or damaged, the process of clot formation (hemostasis) occurs in several stages in the following order: 1- Vessel contraction (reduction of blood flow at the site of injury) 2- Platelet plug formation (platelets adhere to the damaged vessel wall

and accumulate) 3- Blood clot formation and, as a result, blood coagulation. The platelet plug does not have sufficient strength to control the flow of bleeding, so a net-like network of fibrin strands forms around the platelets, creating a strong clot. Due to vascular injury, a cascade of coagulation factors is activated one after the other, and two internal and external coagulation pathways proceed to ultimately lead to the formation of a fibrin network in a common pathway [19-22].

According to the principles of blood coagulation, substances that can be used as blood coagulants can be divided into 5 categories, depending on their function and effect on the stages of hemostasis: 1- Coagulation and procoagulant factors (function as coagulation factors or stimulation of factors) 2- Concentration of coagulation factors (increasing the concentration of coagulation factors, increasing platelet density) 3- Substances that create a physical barrier to blood flow 4- Physiological coagulants (stimulating platelets, causing vasoconstriction) 5- Chemical coagulants (occluding the vascular pathway) [2].

According to the results obtained from Figures 3 and 5, different concentrations of *Achillea a*lcoholic extract did not have much effect on the intrinsic and extrinsic pathways of blood coagulation, and no decrease in blood clotting time was observed compared to the control group. However, according to Figure 7, at a concentration of 0.025 g/ml, the clotting time was reduced compared to the control group and reached 5 minutes. Considering that the PT and PTT tests were performed with plasma, which contained a small amount of platelets, and the CT test was performed on whole blood, which contained platelets, it can be concluded that the effect of *Achillea* extract on platelets could possibly lead to platelet stimulation and increased platelet aggregation, thereby reducing blood clotting time. At some concentrations, we observed an increase in blood clotting time, which probably indicates the presence of compounds with contradictory activities. A study has shown that a hot water extract of *Achillea (Achillea millefolium)* at a concentration of 5% m/v significantly reduced the recalcification time (blood clotting test) in human plasma to 43% of the time required for the reference substance. This flowering plant had the highest hemostatic activity, while its pressed juice significantly prolonged blood clotting. It was also shown that drying and storage conditions did not affect the test result [23]. A study was also conducted on the hemostatic effect of the methanolic extract of *Achillea (Achillea millefolium L.)* on rat liver. The results showed that *Achillea* extract at a concentration of 150 mg/kg significantly reduced the bleeding time from the liver, and no signs of toxicity or liver damage were observed [24]. In another study, it was shown that nanoparticles synthesized with *Achillea* extract, at concentrations of 500 and 1000 µg/mL, could effectively shorten blood clotting time compared to the control group. They also showed that the chemical composition or type of coating and the intrinsic properties, size, morphological features, and charge of silver nanoparticles synthesized with *Achillea* affect their hemostatic potential and blood clot formation [25]. A study has shown that *Achillea* plant and flowers have hemostatic properties due to their high alkaloids and are effective for external and internal bleeding due to their property of sealing blood vessels and preventing blood leakage into capillaries [26].

According to scientific reference, the medicinal effects of *Achillea* are mainly due to its essential oil, prazolenes, and other sesquiterpene lactones, phenolic compounds such as dicaffeoylquinic acids, and flavonoids [27]. Sesquiterpene compounds were also present in the highest amount in the alcoholic extract studied in this study (Table 1). Scientific sources also report that the main compounds – achillin and achillicin- are often cited as being responsible for stopping internal and external bleeding. The wound healing effect of this plant is also apparently due to the hemostatic activity, as well as the anti-inflammatory and antibacterial properties of *Achillea* drugs [28].

According to the results shown in Figures 3 and 5, the different concentrations of silver nanoparticles studied in this experiment did not have much effect on the intrinsic and extrinsic pathways of blood coagulation, and the clotting time did not decrease compared to the control group; in fact, in some concentrations, it even increased the clotting time. However, in the CT test, where a whole blood sample was examined, a concentration of 10 µL of silver nanoparticles significantly reduced the clotting time, and this time was reduced to 5 minutes (Figure 7). A study has shown that silver nanoparticles interact with blood components such as red blood cells, platelets, and the blood coagulation system. Their research results showed that silver nanoparticles have procoagulant effects and also increase platelet adhesion, and all of these effects were observed at concentrations higher than 5 µg/mL [29], Another study has also shown that silver nanoparticles accelerate thrombin formation and activate platelets [30].

Figures 4 and 8 show that the combination of two substances, *Achillea* extract and silver nanoparticles, increased blood clotting time and reduced or neutralized each other's effects, indicating the antagonistic effect of these two substances.

According to the results in Table 2, concentrations of 0.05 and 0.075 g/ml of *Achillea* extract prevented the growth of *Staphylococcus aureus* bacteria, indicating the antibacterial properties of *Achillea* extract. In a study, the antimicrobial properties of *Achillea* extract and essential oil were investigated. The results of this study showed that the *Staphylococcus aureus* strain showed the highest sensitivity to the extract and moderate sensitivity for *E. coli*. Also, the essential oil of this plant had no significant antimicrobial effect compared to the extracts [31]. Other studies have shown that *Achillea* extract exhibited concentration-dependent antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [32]. A study has shown that the ethanolic extract of *Achillea* (*Achillea wilhelmsii L.*) has antibacterial activity against clinically resistant *Staphylococcus aureus* [33]. The results of the aforementioned research are consistent with the present study.

CONCLUSION

Achillea, with its diverse bioactive compounds, has health-promoting effects, which, according to the findings of this study, exhibit hemostatic and antibacterial properties, making it suitable for use as a blood-clotting agent. However, in vivo studies can be used to further investigate its effectiveness.

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Conflict of Interests

The authors have not declared any conflicts of interest.

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