Investigation of the biocompatibility and in vivo wound healing effect of *Cotinus coggygria* extracts

Esra BOZKAYA[1,2,a,✉], Mustafa TÜRK[3,b], Hüsamettin EKİCİ[4,c], Siyami KARAHAN[5,d]

[1]Kırıkkale University, Institute of Science, Department of Biology, Kırıkkale, Türkiye; [2]Kırıkkale University, Scientific and Technological Research Application and Research Center, Kırıkkale, Türkiye; [3]Kırıkkale University, Faculty of Engineering, Department of Bioengineering, Kırıkkale, Türkiye; [4]Kırıkkale University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Kırıkkale, Türkiye; [5]Kırıkkale University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Kırıkkale, Türkiye

[a]ORCID: 0000-0002-9259-2538; [b]ORCID: 0000-0003-4242-4445; [c]ORCID: 0000-0001-6403-737X; [d]ORCID: 0000-0002-2744-1717

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✉Corresponding author
esraarat@kku.edu.tr

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ABSTRACT

Cotinus coggygria is widely recognized its antiseptic, anti-inflammatory, antimicrobial, antihemorrhagic, and wound-healing properties. In this, aimed to evaluate the phenolic contents, cytotoxicity/proliferation, hemolytic, antimicrobial, genotoxic, apoptotic, necrotic activities, and *in vivo* wound healing effects of *C. coggygria*, a plant species known to have beneficial effects on wound healing. TOF-LC/MS analyzes revealed that the methanol extract of *C. coggygria* leaves contained flavonoids and phenolic compounds such as gallic acid (18.5 mg/kg), catechin (4.6 mg/kg), protocatesic acid (0.6 mg/kg), vanillic acid (8.4 mg/kg), ellagic acid (0.1 mg/kg), rosmarinic acid (0.1 mg/kg), quercetin (15 ppb) and *C. coggygria* stems contained such as gallic acid (24.6 mg/kg), catechin (155.1 mg/kg), chlorogenic acid (1.9 mg/kg), 4-hydroxybenzoic acid (383.3 mg/kg), rutin (2.5 mg/kg), ellagic acid (15.1 mg/kg), apigenin 7-glycoside (10.5 mg/kg), rosmarinic acid (0,4 mg/kg), quercetin (15.2 mg/kg), naringenin (279.1 mg/kg). Consequently, C. coggyria has a positive effect on wound healing with antibacterial properties, particularly against E. coli, and without cytotoxic, genotoxic, or hemolytic effects at test concentrations. In the *in vivo* burn model, wounds treated with leaf and stem extracts healed faster than the control group. Thus, *C. coggygria* is an effective plant for wound healing with antibacterial properties, particularly against E. coli, and without cytotoxic, genotoxic, and hemolytic effects.

Introduction

The skin is the human body's largest organ, providing protection to internal organs from microbial infection, mechanical damage, radiation, and extreme heat (34). The breakdown of the anatomical and physiological integrity of the skin due physical damage is called a “wound” (7). There are wound types determined according to different characteristics such as acute wound, chronic wound, burn wound and necrotic wound (3, 7, 38). Acute wounds are characterized by a healing process that can be completely healed by the body and not longer than 8 weeks while chronic wounds take a minimum of 12 weeks to heal. A burn is an injury to the skin caused by thermal, electrical, chemical, or radiation. Dry or wet sources account for 80% of all burns (19, 35). Burn wounds are classified based on the layer of skin affected as first, second, third and fourth degree burns. First-degree burns affect only the epidermis while second-degree burns are partial thickness and involve the epidermis and dermis. In third-degree burns, the reticular part of the dermis is affected and in fourth-degree burns the skin and tissues such as muscle and bone are damaged (31). Wound healing is a complex process that involves the systematic work of many cells, in which a series of cellular, physiological and biochemical events enabling the damaged tissue to regain its structure and function (18, 23, 33). The inflammation phase is the first phase of the wound healing process beginning after the injury and lasting 4-6 days during which a vascular and cellular response develops. The first event that occurs when bleeding is stopped is hemostasis.

During the proliferative phase, there is the development of granulation tissue to fill the wound cavity, along with the formation of epithelium to line the wound site. The formation of granulation tissue includes the proliferation of fibroblasts, the deposition of collagens and other extracellular matrixes, and the development of new blood vessels. When new tissue is formed in the wound area, the matrix formation and remodeling phase begins by restoring the structural integrity and functionality of the tissue (25, 32, 41). Depending on the condition of the wound, the selection of methods for wound healing is crucial to the healing process as it can reduce the risk of complications, sped up the wound healing process or minimize scar formation after complete healing. Skin grafts, wound dressings, cell therapy, hyperbaric oxygen therapy, ozone therapy and medicinal plants are used in wound healing treatment (30, 39). Using medicinal plants or herbal products in the treatment of various skin problems has become widespread, especially in recent years (17). Many herbs are of great importance in the wound healing process as they support natural repair mechanisms. It is used as a wound healing agent thanks to bioactive molecules such as flavonoids, tannins, terpenoids, saponins and phenolic compounds in medicinal plants. The purpose of using medicinal plants in wound healing is to contribute to the wound healing process by facilitating blood clotting, fighting infection, and speeding up wound healing (8, 15, 22). The genus *Cotinus* and *Cotinus coggygria* species are widely distributed in southern Europe, the Himalayas, Southwest China, and Southwest America. *C. coggygria Scop*. (Anacardiaceae), as is growing common in some parts of Turkey, in some parts of Balkans, in the Himalayas, is a plant that grows in the southwest of China and the United States. In traditional medicine, the plant is used for its anti-inflammatory, antimicrobial, antiseptic, antipyretic, antidiarrheal, antihemorrhagic and wound-healing properties. According to the researches, some phenolic compounds isolated from the *C. coggygria* plant have been determined to be effective in wound healing (1, 2, 10, 26, 27). The study aimed to investigate the *in vitro* biocompatibility and antimicrobial activity of extracts of *C. coggygria* (leaves and stem parts). In addition, the study aimed to demonstrate the proteins that play a critical role in wound healing by immunocytochemical techniques, to determine gylcosaminoglycans (GAGs) expressions and to investigate the *in vivo* wound healing properties.

Materials and Methods

Materials: *Cotinus coggygria* collected from Kümeevler locality in Akyurt district of Ankara in June 2013. Voucher number: M.Türk 6089 (ADO: Kırıkkale University, Faculty of Arts and Sciences Department of Biology Anatolian Herbarium). DMEM, FBS, Tyrpsin/EDTA solution and Penicillin streptomycin used in cytotoxicity and genotoxicity testing were obtained from Biological Industries (USA). For antibacterial analyses, the cultures of *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231) were taken from Kırıkkale University Scientific and Technological Research Laboratories (KÜBTUAM). The solid and liquid broth used in antibacterial tests and bacterial culture were obtained from Sigma-Aldrich (Germany). For cell proliferation assay e-plate was obtained from Elips (Roche). Hemolysis test standards-chemicals and DMMB assay chemicals were obtained from Sigma-Aldrich (Germany).

Cell Culture: L929 fibroblast cells obtained from the cell culture collection of the ŞAP Institute in Turkey were used. L929 cells were cultivated in DMEM (Biological Industries, USA) medium supplemented 10% Fetal bovine Serum (Biological Industries, USA) and 1% penicillin and streptomycin (Biological Industries, USA) in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Preparation of Extracts: Plant extracts were prepared by two different methods. The first of the methods used was to prepare the water extract by boiling the leaves and stems of the *C. coggygria* at 6 g/L for 15 minutes. The second method is the preparation of methanol extract by approximately 10 g of the leaves and stems of the *C. coggygria* were taken and extracting in 100 ml of methanol with an automatic extraction system (BÜCHİ, B-811) for 2 hours. After extraction, solid extract was obtained by removing methanol with a rotary evaporator (BÜCHİ, R210) system (24).

TOF-LC / MS Analysis: Quantitative analysis was performed by TOF/LC-MS to determine the phenolic compounds and flavonoids contained in *C. coggygria*. Separation was performed on the ZORBAX SB column. After the analysis protocol was determined as mobile phase and gradient program, it was carried out in positive ion mode in 30 minutes (13).

In vitro WST-1 Assay for Cytotoxicity: L929 fibroblast cells were placed per well ($10x10^3$ cells) in 96 well plates. After the cells were incubated for 24 hours (37 °C in 5% CO^2), *C. coggygria* extracts were applied at concentrations (1.2 and 0.6 mg/ml). After the cells were incubated with the extracts for 24 hours, 15 µL of WST-1 (water-soluble tetrazolium salt) reagent was added to the wells. It was measured on an Elisa Microplate Reader (BioTek, USA) at 440 nm after 4 hours of incubation (9, 21).

In vitro Cell Proliferation Assay: The proliferation of L929 fibroblast cells applied to *C. coggygria* (leaves and

stem extract) was determined with Real Time Analyzer (RTCA) SP (Roche, Germany). Cell growth was followed by seeding L929 fibroblast cells ($5x10^3$ cells per well) with e-plate 96 (Roche). Leaves and stem extracts (1.2 - 0.6 - 0.3 mg/mL concentrations) were applied at 18th and 40th hours of incubation. The logarithmic increase of L929 fibroblast cells incubated in 5% CO_2 at 37 °C for 65 hours was measured (9).

In vitro Genotoxicity Assay: The micronucleus test was performed according to the ISO 10993-3 OECD guidelines 487. The micronucleus test, in which the genotoxic effect of *C. coggygria* water extracts was investigated. CHO cells-Chinese hamster ovary cells (20 x 10^3 cells per well) were seeded in 48-well plates with DMEM medium containing 10% fetal calf serum and 1% penicillin-streptomycin. L929 cells were treated at two concentrations of extracts (1.2 and 0.6 mg/ml) for 24 h. Mitomycin C, known to have genotoxic effects, was used as a positive control in the experiment. 3 µg/ml Cytochalazine-B (Santa Cruz) was added to the culture media prepared for the micronucleus test in order to obtain cells with binucleus at the 44th hour of incubation. At the 72nd h, the media in the wells were discarded and incubated with cold 0.075 M KCI (Ambresco) hypotonic solution at room temperature for 4 min in order to allow the cells to swell and disintegrate and chromosomes to separate from each other. At the end of the incubation, methanol: glacial acetic acid (Merck, Germany) in a ratio of 3:1 was fixed with a freshly prepared fixative in order to detect the opened metaphase plates. The fixation process was repeated 3 times. Propodium iodide (Life Technologies, USA) was dripped and incubated in the dark for 15 minutes to visualize binucleus cells with micronuclei formation at the final stage. Binucleated cells with micronuclei were examined under a fluorescent microscope (Leica, DMI6000B, Germany) (37).

In vitro Antimicrobial Activity: To determine the antimicrobial activity of water and methanol extracts of *C. coggygria*, agar applied to *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *C. albicans* (ATCC 10231) strains by disk diffusion method. The 0.5 Macfarland turbidity standard prepared from 24 h fresh cultures of bacteria. Homogenized bacterial suspensions were inoculated into petri dishes with Mueller Hinton Agar. Blank discs impregnated with 20 µl of water and methanol extracts (1.2 mg/ml and 0.6 mg/ml) of *C. coggygria* were placed on agar. Penicillin (Bioanalyse P10) for *S. aureus*, Nystatin (Bioanalyse NY100) for *C. albicans*, tetracycline (Bioanalyse TE10) for *E. coli*, antibiotic discs were used as controls. Petri dishes were incubated for 18-24 h at 37 °C. The zone diameters around the disc were then measured (42).

In vitro Hemolysis Assay: The hemolysis test was performed according to the TS EN ISO 10993-4 standard. Hemoglobin standard was prepared according to ASTM F756-00 standard. In order to determine the amount of hemoglobin in plasma in blood samples taken from rabbits, 3 ml of blood was transferred to falcon tubes. 500 µl of the plasma part of the blood samples centrifuged at 700 G-force was taken and transferred to the eppendorf tube. 500 µl of Drabkin's reagent was added to tubes and incubated for 15 minutes at room temperature. After incubation, blood samples transferred to well plates were measured at 540 nm wavelength in Elisa reader. The amount of hemoglobin in the plasma should be less than 2 mg/ml. 20 µl of blood was added to 5 ml of Drabkin's reagent and incubated for 15 minutes. The amount of hemoglobin in whole blood was determined by measuring at a wavelength of 540 nm. Blood samples were diluted to 10 mg/ml of hemoglobin. Blood samples diluted with PBS were incubated with *C. coggygria* water extract in falcon tubes for 3.5 hours at 37 °C. Control groups were also treated with blood for 3.5 hours. Water was used as positive control and PBS was used as negative control. At the end of the incubation, blood samples interacted with plant extracts in falcon tubes were centrifuged at 750 G-force for 15 minutes. Plant extracts were transferred to Eppendorf tubes by taking 500 µl from the supernatant part of the tubes that were interacted with the positive and negative control. 500 µl of Drabkin's reagent was added to it. It was incubated for 15 minutes at room temperature. Measurements were made at 540 nm by placing 100 µl on 96 well plate. Percent hemolysis rates were calculated with the determined absorbance values (5). According to ASTM F756-00 standard directed by ISO 10993-4 standard: <2% not hemolytic, 2-5% mild hemolytic and> 5% hemolytic.

In vivo Burn Wound Healing: In vivo burn studies were performed at Kırıkkale University Hüseyin Aytemiz Experimental Research and Application Center. A total of 9 male Sprague-Dawley rats (250 - 300 g weight), 3 each (n=3) for the leaves, stem and control groups, were used to evaluate the efficacy of the extracts in wound healing. 50 µl of 0.6 mg/ml *C. coggygria* leaves and stem water extracts were applied to the burned areas.

After intraperitoneal ketamine-xylazine anesthesia was applied to the rats, their back regions were shaved. A cylindrical disc with a diameter of 1 cm was kept in 100 ◦C water and contacted with the back of the rats for 20 s to create a burn wound. Wound healing rates were calculated by measuring the wound areas at regular intervals for 27 days. The percentage wound contraction was determined by the following formula (36).

$$\%\ wound\ closure = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \text{x100}$$

n is the number of days (3rd, 7th, 14th, 21st and 27th).

Statistical Analysis: The data was first analyzed if it met the parametric test assumptions. For this Shapiro Wilk and Levene tests were performed. The results revealed that the data did not normally distributed. Therefore, Kruskal Wallis test was performed to see if there are group differences. Mann-Whitney U test was performed when group difference was existed for pairwise comparison. The Bonferroni correction was applied, and new significance levels was calculated as P=0.017 (for three groups comparison).

Results

TOF-LC/MS Analysis: Phenolic compounds, chemical compounds and amino acids in the leaves and stem methanol extract of *C. coggygria* were determined by TOF-LC/MS analysis. According to TOF-LC/MS phenolic compounds and flavonoids found in methanol extract of *C. coggygria* leaves and stem parts; gallic acid, catechin, protocatechic acid, vanillic acid, ellagic acid, rosmarinic acid, quercetin, 4-hydroxybenzoic acid, chlorogenic acid, routine and apigenin 7-glycoside (Table 1).

In vitro Cytotoxicity Assay: WST-1 test was performed to determine the toxicity of *C. coggygria* leaves and stem extracts (methanol-water). The % viability values of L929 fibroblast cells are given in Table 2 and Figure 1. The extracts were applied at two different concentrations, 0.6 mg/ml and 1.2 mg/ml. Cell viability was calculated as 117±3.5% and 114±4.6%, respectively, in cells treated with 0.6 mg/ml water and methanol extracts of leaves. While it was determined that the viability of L929 fibroblast cells, in which 1.2 mg/ml *C. coggygria* leaves water and methanol extracts were applied, increased viability, the % viability was calculated as 125±2.8% and 118±4.3%, respectively. L929 fibroblast cells treated with 0.6 mg/ml water and methanol extract of stem the % viability was calculated as 118±5.1 and 117±5 respectively. In cells treated with 1.2 mg/ml water and methanol extracts of stem, the % viability rates were calculated as 128±3.6 and 123±4.7, respectively.

Table 1. Total phenolic and flavonoid contents various parts of *Cotinus coggygria.*

Plant part used	
Leaves methanol extract Main compounds (mg/kg)	**Stem metanol extract Main compounds (mg/kg)**
Gallic acid (18.5), Catechin (4.6), Protokatesic acid (0.6), Vanilic acid (8.4), Ellagic acid (0.1), Rosmarinic acid (0.1), Quercetin (15 ppb)	Gallic acid (24.6), Catechin (155.1), Chlorogenic acid (1.9), 4-hidroksibenzoic acid (385.3), Rutin (2.5), Ellagic acid (15.1), Apigenin-7-glycoside (10.5), Rosmarinic acid (0.4), Quercetin (15.2), Naringenin (279.1)

Table 2. % viability values of L929 fibroblast cells to which *C. coggygria* leaves-stem water and methanol extracts were applied.

Concentration mg/mL	Leaves water extract	Stem water extract	Leaves methanol extract	Stem methanol extract
Control	100±2.4	100±4.2	100±3.7	100±3.9
0.6 mg/mL	117±3.5	118±5.1	114±4.6	117±5.0
1.2 mg/mL	125±2.8	128±3.6	118±4.3	123±4.7

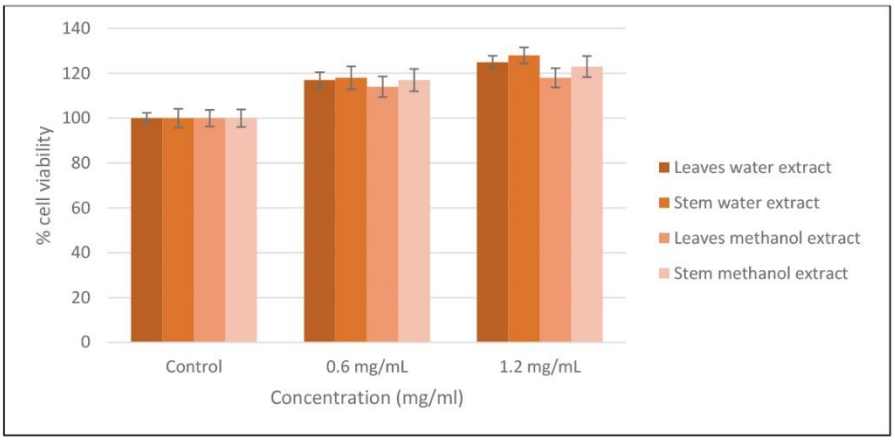

Figure 1. % Viability graph of L929 fibroblast cells.

In vitro Cell Proliferation Assay: The effect of *C. coggygria* (leaves and stem) aqueous extracts on cell proliferation was analyzed with the xCELLigence RTCA system. Proliferation graphs of L929 fibroblast cells to which water extracts were applied (*C. coggygria*-leaves) are shown in figure 2 and (*C. coggygria* stem) in figure 3. Water extracts were applied at 3 different concentrations (1.2 mg/ml- 0.6 mg/ml- 0.3 mg/ml). The first application was made at the 18th hour when the cells showed a logarithmic increase. After the first application of *C. coggygria* leaves-water extract, a rapid increase in proliferation was observed until the 25th hour, especially in cells where 1.2 mg/ml concentration was applied (Figure 2). It was observed that after the second application of the plant extracts at the 42nd hour, the concentration of 0.3 mg/ml increased the cell proliferation more rapidly compared to the control group. In the first application at the 18th hour, it was observed that *C. coggygria* stem-water extract increased the proliferation of L929 fibroblast cells at a concentration of 1.2 mg/ml. The increase in proliferation continued until the 25th hour (Figure 3). The increase in cell proliferation continued until the 45th hour with the second application of the plant extracts at the 42nd hour. It was seen that there was no previous study in the literature on the effect of *C. coggygria* plant extracts on L929 fibroblast cell proliferation. After applying *C. coggygria* leaves and stem extracts with the xCELLigence RTCA real-time cell analysis system, it was observed that it caused a faster increase in proliferation compared to the control group.

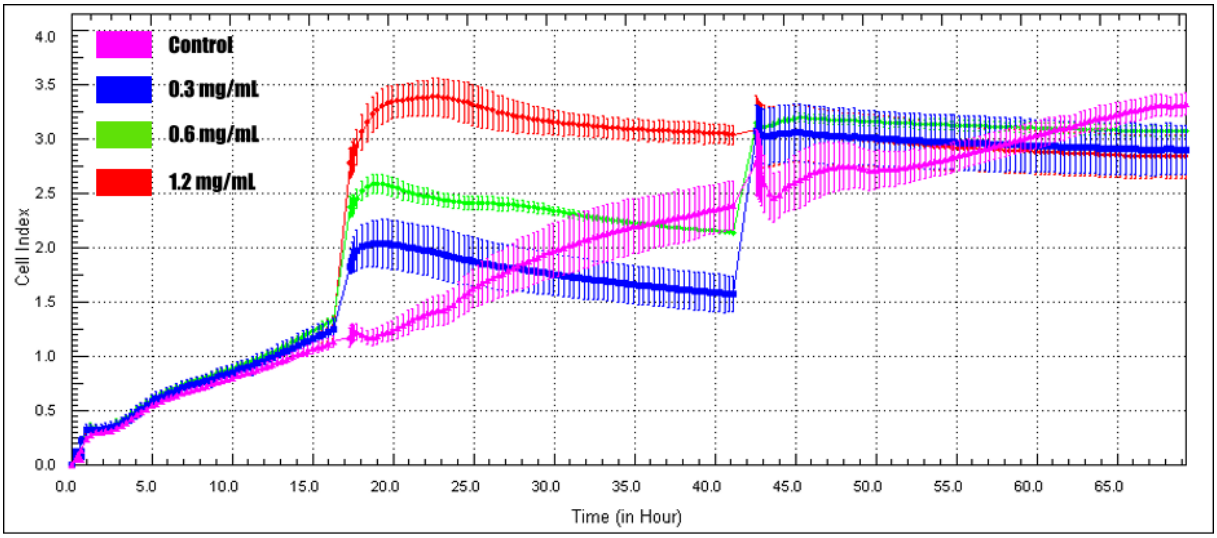

Figure 2. Proliferation graph of L929 fibroblast cells to which *C. coggygria* leaves water extracts were applied.

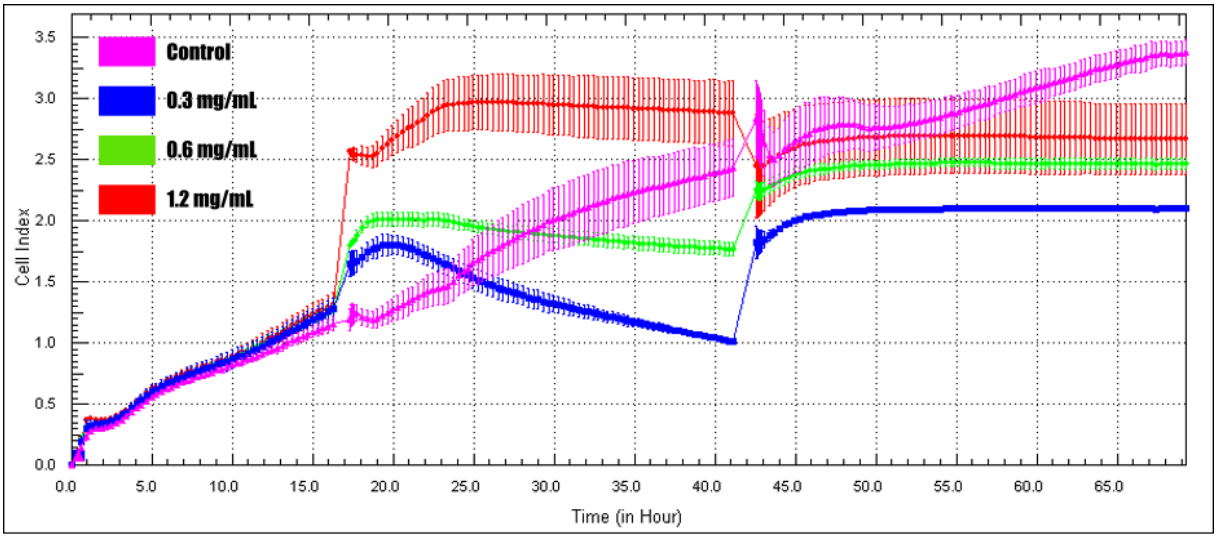

Figure 3. Proliferation graph of L929 fibroblast cells to which *C. coggygria* stem water extracts were applied.

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In vitro Genotoxicity Assay: The genotoxic effect of *C. coggygria* leaves - stem water extracts was determined by the OECD 487 micronucleus test. The genotoxicity rate was determined by calculating the number of binucleated cells, multinucleated cells, micronuclei in the binucleated cells and the total number of cells in the treated wells (Figure 4). The medium was used as a negative control. Mitomycin C was used as a positive control. When the negative control and water-extracted cells were evaluated microscopically, it was observed that there was no difference in terms of cells with micronuclei. When the positive control and water extract treated CHO cells were compared microscopically, there was a difference between the two groups in terms of cells with micronuclei. According to the micronucleus test results, it was determined that *C. coggygria* water extracts had no genotoxic effect on CHO cells.

In vitro Antimicrobial Activity: *C. coggygria* (leaves and stem parts) water and methanol extracts were impregnated on empty anti discs to be placed in petri dishes. Extract-impregnated discs and antibiotic discs were placed in petri dishes in which *S. aureus*, *E. coli* and *C. albicans* were cultivated, and the zone diameters of the formed zone were observed after 24 hours of incubation. Table 3. and the measured zone diameters and images are given in figure 5. Gentamicin was used for *E. coli* and *S. aureus*, ketoconazole was used for *C. albicans* as positive control. No inhibition zone was observed in microorganisms (*S. aureus* and *C. albicans*) to which water and methanol extracts were applied. The zone diameters formed in *E. coli* were calculated as 9 mm in 1.2 mg/ml methanol (leaves and stem) extract and 8 mm in 0.6 mg/ml water extract (leaves and stem).

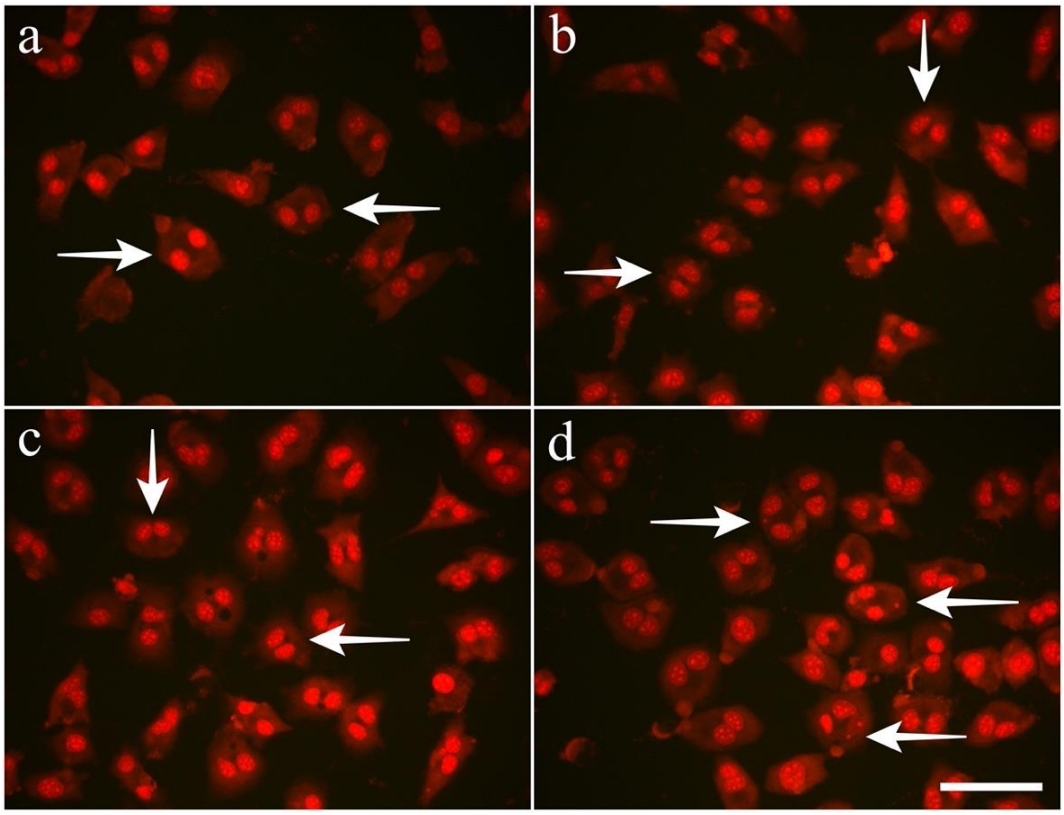

Figure 4. Binucleated and micronucleated CHO cells. a) Binucleated CHO cells treated with 1.2 mg/mL *C. coggygria* (leaves) water extract. b) Binucleated CHO cells of 1.2 mg/mL *C. coggygria* (stem) water extract applied. c) CHO cells treated with medium alone as negative control. d) CHO cells treated with mitomycin C as positive control. Cells with micronuclei are indicated by the arrow. Scale bar = 100 µm.

Table 3. Inhibition zones of bacteria and fungi treated with *C. coggygria* extracts.

C.coggyria	***E. coli*** **(mm)**	**Ant(*E. coli*) Gentamicin (mm)**	***S. aerus*** **(mm)**	**Ant(*S. aerus*) Gentamicin (mm)**	***C.Albicans*** **(mm)**	**Ant(*C.Albicans*) Ketokonazol (mm)**
Leaves methanol extract	9	18	-	12	-	18
Leaves water extract	8	18	-	12	-	18
Stem methanol extract	9	18	-	12	-	18
Stem water extract	8	18	-	12	-	18

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In vitro Hemolysis Assay: Hemolysis is the release of hemoglobin from the cell by damaged erythrocyte membranes, showing a deterioration in RBC membrane integrity. Hemolysis test is used in blood compatibility evaluation according to TS EN ISO 10993-4 standard. The method we apply is the hemolysis test performed according to the ASTM F756, to which the TS EN ISO 10993-4 standard refers. According to ASTM F756-0 standard evaluation criteria, if the hemolytic index is <2%, is not hemolytic. The hemolytic index for the *C. coggygria* leaves water extract was 1.25%, and the hemolytic index for the *C. coggygria* stem water extract was calculated as 1.56%. In addition, the hemolytic effect of C. coggyria was investigated for the first time according to the literature search and it was determined that have an anti-hemolytic effect.

In vivo Burn Wound Healing: Wound healing activity of *C. coggygria* (leaves-stem) water extracts was determined by measuring burn wound areas in rats on certain days. Wound sites were followed for 21 days. Wound contraction percentage of plant extracts and control group is shown in Figure 6. Photographs of wounds during the treatment period are shown in Figure 7. At the end of 21 days, it was observed that the wounds healed faster in the *C. coggygria* (leaves-stem) applied group compared to the control group. When the burn wound was created, the wound diameter was measured as 1.8 cm. The recovery rate was 11.1±0.3% in animals treated with leaf extract on the 7th day, 38.9±0.2% in animals treated with stem extract, and 11.1±0.4% in the control group. On the 7th day, there was a statistically significant difference between the stem and control groups. At the end of the 21st day, the recovery rate was 55.6±0.1% in the leaf extract applied group, 50.0±0.1% in the stem extract applied group, and 27.8±0.5% in the control group. *C. coggygria* (leaves-stem) extracts are thought to accelerate wound healing.

Table 4. *In vivo* wound healing statistical analysis results of plant extracts applied at 0.6 mg/ml concentration on days 1, 3, 7, 14 and 21 (n=3).

	Mean±SE	Median	IQR	Mean Rank	P value
Group					0.55
Leaves	1.14±0.15	1.5	0.8	22.43	
Stem	1.08±0.15	1.1	0.8	20.73	
Control	1.32±0.11	1.5	0.7	25.83	
Day 1					0.96
Leaves	1.67±0.33	1.7		4.83	
Stem	1.67±0.33	1.7		4.83	
Control	1.67±0.08	1.7		5.33	
Day3					0.78
Leaves	1.60±0.06	1.6		5.67	
Stem	1.57±0.09	1.6		5.17	
Control	1.43±0.18	1.5		4.17	
Day7					0.04
Leaves	1.47±0.33	1.5		5.67	
Stem	1.10±0.06	1.1		2.00	
Control	1.57±0.07	1.5		7.33	
Day14					0.12
Leaves	0.77±0.14	0.8		3.00	
Stem	0.93±0.09	0.9		4.50	
Control	1.27±0.17	1.2		7.50	
Day21					0.37
Leaves	0.20±0.06	0.2		5.00	
Stem	0.13±0.03	0.1		3.50	
Control	0.67±0.28	0.9		6.50	

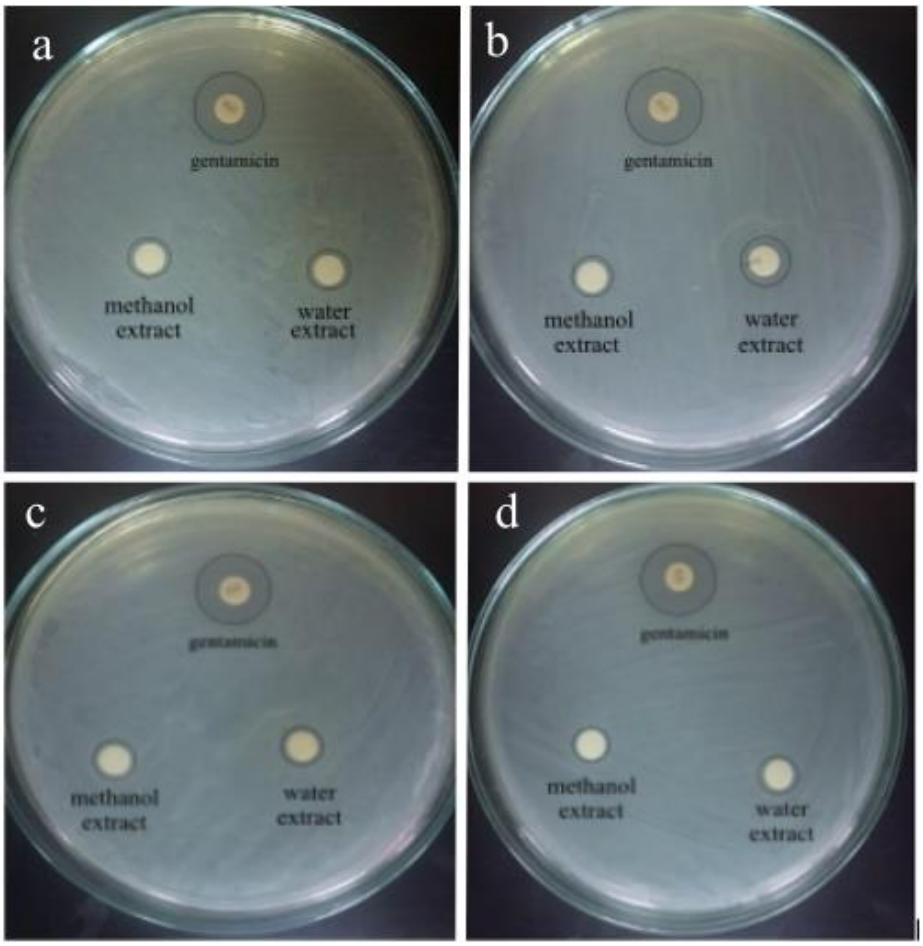

Figure 5. Inhibition zones formed by *C. coggygria* extracts in *E. coli*.
a) Inhibition zones formed by 1.2 mg/ml stem extract.
b) Inhibition zones formed by 0.6 mg/ml stem extract.
c) Inhibition zones formed by 1.2 mg/ml leaves extract.
d) Inhibition zones formed by 0.6 mg/ml leaves extract.

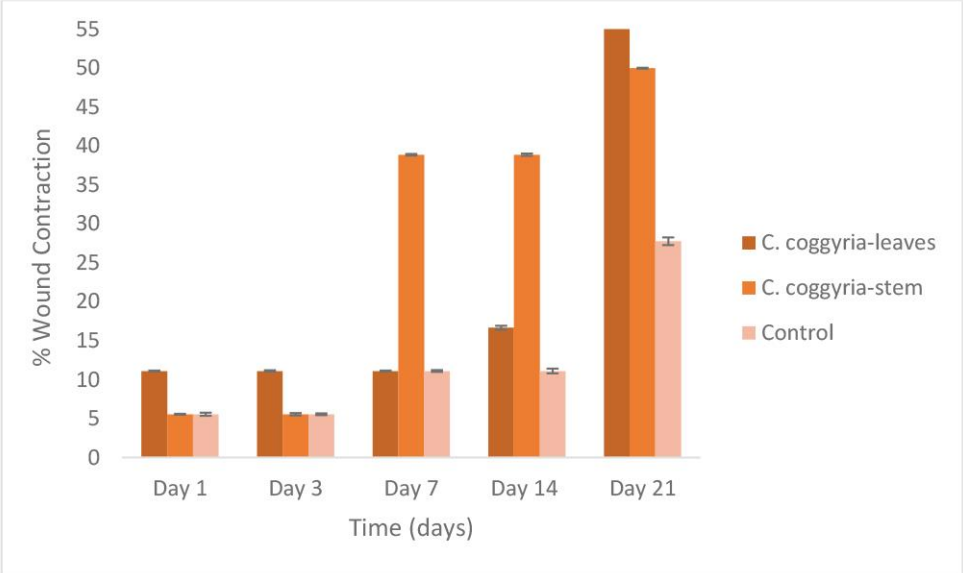

Figure 6. % Wound contraction of test, standard, and control groups at the 1st, 3rd, 7th, 14th and 21st days.

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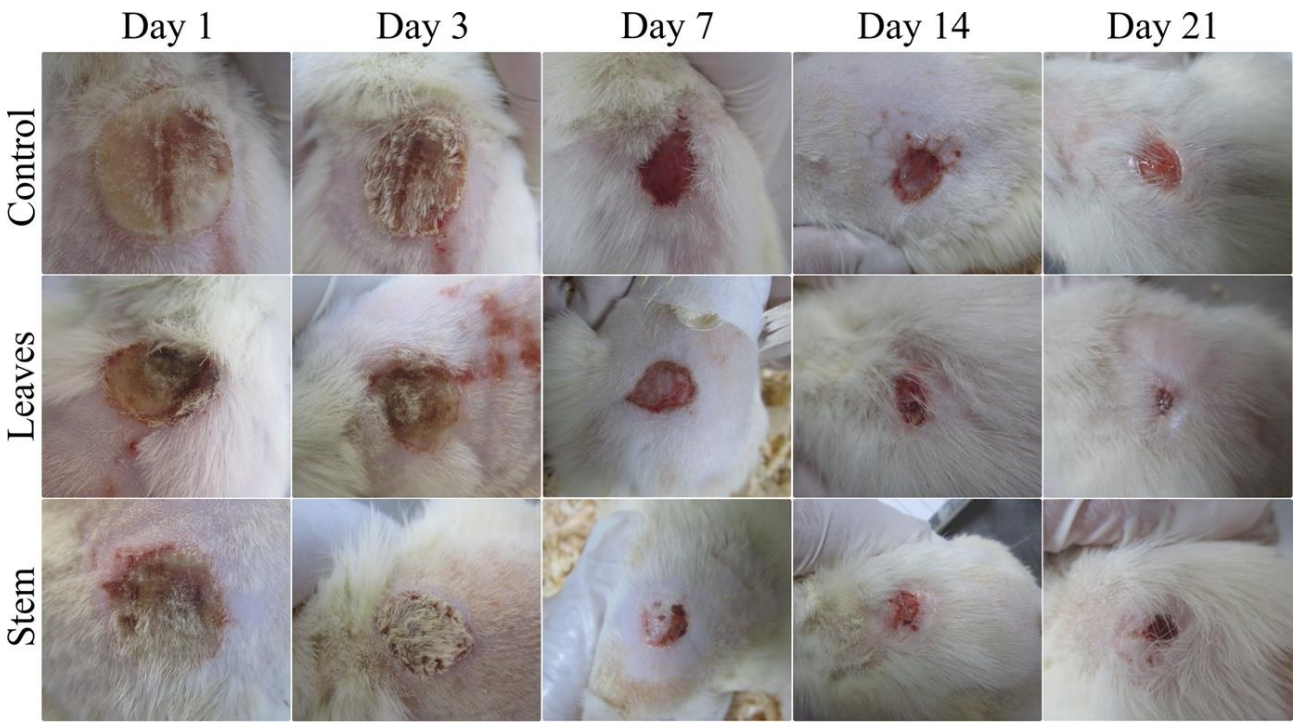

Figure 7. Photographs of 0.6 mg/ml *C. coggygria* extract applied to burn wounds on days 1, 3, 7, 14 and 21.

Discussion and Conclusion

The use of *C. coggygria* in medicine dates back to many years, especially among the public. It possesses antimicrobial and anti-inflammatory properties, as well as anti-hemorrhagic and wound healing properties. *C. coggygria* shows these therapeutic properties thanks to the phenolic compounds and flavonoids it contains (40). Matić et al.(26) reported that methanol extract of stem of *C. coggygria* contained 3.78 mg gallic acid in total phenolics, 8.29 mg rutin in total flavonoids. HPLC analysis also revealed that myricetin is the main component of *C. coggygria* extract along with varying amounts of hydroxyl derivatives of cinnamic acids (chlorogenic, caffeic, coumaric, ferulic and rosmaric acid) were identified in the extract. Previous research has shown that gallic acid, catechin and chlorogenic acid regulate wound healing. Gallic acid, which is found in almost all plants, is included in the literature with its anti-inflammatory, analgesic, anticancer and antidiabetic properties, as well as its powerful antioxidant effect. Yang et al. (43) reported that gallic acid reduces oxidative stress in human keratinocyte cells and speeds up wound healing. Reports have shown that chlorogenic acid is effective as an antidiabetic, antihypertensive, antitumor, and anti-inflammatory agent in the prevention of gastric lesions and liver injuries. In addition, chlorogenic acid regulates the secretion of collagens and matrix metalloproteinases, which are effective in scar tissue formation in the final stage of wound healing (29).

Flavonoids, which have antioxidant and antibacterial properties, also provide blood circulation in the body and prevent vascular occlusion. The antibacterial effect of catechins can suppress the proliferation of pathogenic bacteria in the wound area and prevents the possibility of infection in case of injury. In our study, flavonoids and phenolic compounds contained in *C. coggygria* were determined by TOF/LC-MS as gallic acid, catechin, protocatechin acid, vanillic acid, ellagic acid, rosmarinic acid, quercetin, 4-hydroxybenzoic acid, chlorogenic acid, rutin and apigenin 7-glycoside. The antioxidant, antibacterial, antidiabetic and anti-inflammatory properties of the flavonoid and phenolic compounds contained in *C. coggygria* show that they can be used in the healing of acute and chronic wounds. Ferrazzano et al. (16), the cytotoxicity of *C. coggygria* methanol extract in HGF-1 and Hacat cells was evaluated by XTT test. The study revealed that the water extract was less toxic than the ethanol extract. Iliev et al.(20) evaluated the antiproliferative effect of *C. coggygria* ethyl acetate extract in MCF-10A cells. They have reported an increase in cell proliferation at a concentration of 3 µg/ml. Artun et al.(4) the toxicity of *C. coggygria* methanol extract in Hela and vero cells was evaluated. While the IC50 value was calculated as 293 µg/ml in Hela cells, it was calculated as > 1000 µg/ml in vero cells. İn another study (43) it was reported that gallic acid, one of the phenolic compounds of *C.coggygria*, increased fibroblast proliferation. According to previous studies in the literature, *Cotinus* plant has different cytotoxic effects in different cell lines,

depending on the extract (such as water, ethanol, methanol) and concentrations. In present study, the cytotoxic effects of water and methanol extracts of leaves and stem parts of *C. coggygria* at two different concentrations (1.2 mg/ml - 0.6 mg/ml) were evaluated. It was determined that water and methanol extracts did not cause any toxicity (Table 2). Since the % viability was higher in L929 fibroblast cells treated with water extracts, only water extracts were applied *in vitro* cell proliferation, genotoxicity, hemolysis and *in vivo* wound healing assays. Varanka et al. (12) reported that *C. coggygria* methanol extract at 5% concentration was genotoxic and induced sex-linked recessive lethal mutations in the X chromosome of *Drosophila melanogaster* males. On the other hand, it has been reported that 2% methanol extract reduces genotoxicity. The mutagenicity of *C. coggygria* methanol extract was determined by in vivo SLRL and alkaline comet test. The SLRL test, 5% methanol extract has been reported to be genotoxic in spermatozoid. The comet test, the extracts were applied as 500, 1000, 2000 mg/kg (extract/body weight) and followed for 24 and 72 hours. It has been reported that 500 mg/kg extract was not significantly different from the control group in the group administered. Genotoxic damage was reported in the groups in which 1000 and 2000 mg/kg extract were applied (27). In our study, it was determined that water extracts of *C. coggygria* leaves and stem parts (1.2 mg/ml - 0.6 mg/ml) did not cause genotoxicity on CHO cells. When our results are compared with previous studies, the genotoxic and mutagenic effects of the extracts may vary depending on the applied method and concentration. Matic et al. (28) reported the antimicrobial activity of *C. coggygria* methanol extract was determined by macro broth dilution technique. Methanol extract was applied to different bacterial species and *C. albicans*. Inhibition zones of *C. coggygria* methanol extract in 150 ug and 300 ug concentrations in *E. coli* were determined as 29 mm and 17 mm, respectively. *C. coggygria* methanol extract has been reported to have the highest antimicrobial effect on *E. coli*. Dulger et al. (11), ethanol extract of *C. coggygria* was applied to some bacterial and yeast cultures. It has been reported that 10.4 to 22.8 mm inhibition zones are formed for bacteria, while inhibition zones ranging from 11.8 to 16.9 mm are formed for yeasts. In the study, it was shown that *S. aureus* is more sensitive to *C. coggygria* extract. In our study, water and methanol extracts (1.2 mg/ml and 0.6 mg/ml) of *C. coggygria* were prepared by impregnating 20 µl empty antibiotic discs and tested for antimicrobial activity and the extracts showed antibacterial activity against *E. coli* (Table 3). *Cotinus* shows anticancer, anticoagulant, hemolytic and antioxidant properties because of the phenolic compounds and flavonoids it contains (40). Some diseases and disorders, including cancers, are the result of excessive production of ROS and can aggravate others, such as hemolytic anemia. Antioxidant molecules derived from plants are effective in neutralizing ROS, thereby compromising their harmful effects on cells. Such studies in the literature provide evidence that plant extracts and derivatives have protective effects against ROS-mediated damage to red blood cells (6). *C. coggygria*'s lack of hemolytic effect shows it can be used in open wounds with bleeding. It was determined that the water extracts of *C. coggygria* plant (leaves and stem parts), whose anti-hemolytic effect was investigated for the first time in the literature, had no hemolytic effect. Wound healing is a complex process that includes hemostasis, inflammatory phase, proliferation phase, formation of granulation tissue, and epithelialization and remodeling phases. Ertaş et al. (14) *C. coggygria* has studied the effect of ethanol extract on burn wound healing. PGE2 levels, which play a role at the beginning of the inflammation phase and hydroxyproline levels, which extracellular protein in the granulation tissue, are measured by biochemical analyzes and speed up the wound healing of the ethanol extract. In this study, the effect of *C. coggygria* (leaves and stem) water extracts on wound healing was evaluated by measuring the burn site. At the end of the 21st day, it was determined that leaves and stem extracts accelerated wound healing compared to the control group.

In conclusion; the present study the phenolic compounds of the *C. coggygria*, were determined by TOF/LC-MS. It was determined by MTT test that *C. coggygria* had no cytotoxic effect on L929 fibroblast cells, and it increased L929 fibroblast cell proliferation with xCELLigence (Real Time Cell Analysis System). In addition, the hemolytic effect of *C. coggygria* was investigated for the first time in the literature and it was determined that it was anti-hemolytic. It was observed that there was no genotoxic effect by micronucleus test. It has been determined that it has an antimicrobial effect on *E. coli*. Finally, in the in vivo burn model, the rats were followed for 21 days, and it was observed that the extracts were effective in wound healing, and the wound area closed faster than the control group. As a result, it is thought that *C. coggygria* can lead to *in vivo* studies aimed at the use of various skin defects in the treatment.

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Ethical Statement

The study was approved by Kırıkkale University Animal Experiments Local Ethics Committee with the decision dated 16.12.2013, numbered 2013/15, meeting numbered 13/02.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

EB, MT, HE and SK conceived and planned the experiments. EB and HE carried out the experiments. EB and HE planned and carried out the simulations. EB and HE contributed to sample preparation. EB, MT, HE and SK contributed to the interpretation of the results. EB. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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