

Evaluation of the Biological and Wound Healing Activities of *Centaurea virgata* Lam.

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ABSTRACT

Objective: Plants have significant potential effects in treating wounds. Some plants are also used in traditional medicine for their wound healing properties. The aim of this study was to investigate the antioxidant and *in vitro* wound healing activities of *Centaurea virgata*.

Methods: The antioxidant activities of extracts were examined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) methods. The total phenolic content was determined using the Folin-Ciocalteu reagent (FCR) method. In addition, the antiproliferative activity of different extracts on fibroblast cells was performed by MTT method and *in vitro* wound healing activity of ethanol extract of *C. virgata* was determined by scratch assay.

Results: Ethyl acetate fraction of ethanol extract of *C. virgata* (CVEA) had the highest DPPH radical scavenging activity with an IC_{50} value of 138.7 $\mu\text{g/mL}$. According to ABTS results, it was determined that the CVEA had a stronger radical scavenging activity than the other extracts. Hexane (CVH) and aqueous ethanol fractions (CVAE) revealed poor antioxidant activity with IC_{50} values of 824.8 $\mu\text{g/mL}$ and 610.3 $\mu\text{g/mL}$, respectively. On the other hand, among all of the extracts analysed, it was found that phenolic content of CVEA was higher than other extracts. Except for CVAE, other extracts showed antiproliferative activity depending on the dose.

Conclusion: According to the obtained results, our finding suggests that CVAE has a migratory effect on fibroblasts and that CVAE might be a potential therapeutic agent for wound healing.

Keywords: *Centaurea virgata*, antioxidant activity, wound healing

1. INTRODUCTION

Wound healing is a collection of highly coordinated biological events consisting of three intertwining phases such as inflammation, proliferation and remodeling (1). Arachidonic acid metabolites released from thrombocytes collected in the wound area, proteases, and various growth factors contribute to the formation of the inflammation process. At the end of this phase, the number of immunologic cells begins to decrease; endothelial cells and fibroblasts emerged in the wound area and they initiate the proliferative phase (2). Within 2-3 days of injury, angiogenesis is indicated as the beginning of the proliferative phase. In angiogenesis, new blood vessels are formed from the epithelial cells to provide nutrients and oxygen for new cells. Epithelial cells proliferate into the wound areas. On the other hand, fibroblasts proliferate and differentiate into myofibroblasts. At the same time, fibroblasts secrete collagen and fibronectin to form new connective tissue (3). In the course of wound healing, keratinocytes differentiate into dermis so that the remodeling phase begins. The most important feature of this phase is the increase of collagen accumulation in the wound area (4).

Despite the great success achieved through research on the mechanism of wound healing, the development of effective therapeutics for wound healing remains a complex issue for scientists.

Plants have significant potential effects wound treatment. Also, there are reports showing that some plant species have been traditionally used by the public to treat wounds (5-8). Secondary metabolites found in plants such as phenolic acids, tannins, flavonoids and triterpenes are known to have wound healing activities (5,9). These natural products or compounds accelerate the regeneration and healing of tissue loss through complex mechanisms. Today, many phyto-pharmacology laboratories are working extensively to isolate the active components of various medical plants and to determine their mechanism of action. The screening of herbal extracts is of great interest to scientists for the discovery of new effective drugs in the treatment of wounds. For this purpose, *in vitro* wound healing activity of *C. virgata*, which is used by the people for wound healing purposes, was investigated in our current study.

The genus *Centaurea*, the fourth largest genus of the Asteraceae family, has about 400-700 species on the earth. They usually spread around the Middle East and east of the Northern hemisphere (10). *Centaurea* genus is widely distributed in Turkey. It has many medicinal properties including anti-inflammatory, antidiabetic, digestive, antipyretic, cytotoxic, anti-dandruff and antibacterial (11-13). *Centaurea virgate* Lam. is a species used in the traditional medicine in Turkey for the treatment of stomach pain, jaundice, headache, kidney stone and also as a diuretic and wound healing. (14). As far as we know, there is no report on the wound healing activity of *C. virgate*. Therefore, we aimed to determine *in vitro* antioxidant and wound healing activities of *C. virgate* in this study.

2. MATERIAL AND METHODS

2.1. Plant Material Collection and Extraction

Plant samples were collected in the flowering periods from the Çatalça district of Istanbul province of Turkey in 2010 and were identified by Dr. İsmail Şenkardeş, a botanist of the Faculty of Pharmacy, University of Marmara. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (Mare No: 22438)

Dried and ground aerial part of *C. virgate* (16.18 g) were extracted with (6 × 200 mL) EtOH, using an ultrasonic bath. After filtration and evaporation, the ethanol extract (CVE) was dissolved in 30 mL 60% aqueous ethanol, and subjected to solvent-solvent partition between n-hexane (5 × 50 mL), chloroform (3 × 50 mL), and ethyl acetate (2 × 50 mL). The n-hexane, chloroform, ethyl acetate and aqueous ethanol fractions of ethanol extracts of *C. virgate* obtained by this method were coded as CVH, CVC, CVEA and CVAE, respectively. All extracts were stored under refrigeration for further analysis.

2.2. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) Radical Scavenging Activity

Free radical scavenging capacity of *C. virgate* extracts were evaluated according to the previously reported procedure using the stable DPPH (15). Briefly, 10 µL of extracts in DMSO at different concentrations (250-0,048 µg/mL) were added to 190 µL methanol solution of DPPH (0,1 mM) in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 517 nm. The percent radical scavenging activity of extracts and standard against DPPH were calculated according to the following:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control (containing all reagents except the test compounds), and A_1 is the absorbance of the extracts/standard. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. Butylated hydroxyanisole (BHA), ascorbic acid and trolox were used as positive control.

2.3. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Radical Scavenging Activity

Free radical scavenging capacity of *C. virgate* extracts were evaluated according to the previously reported procedure (15). ABTS radical cations were prepared by mixing equal volume of ABTS (7 mM in H₂O) and potassium persulfate (4.9 mM in H₂O), allowing them to react for 12-16 h at room temperature in the dark. Then, ABTS radical solution was diluted with 96% ethanol to an absorbance of about 0.7 at 734 nm. 10 µL of extracts in DMSO at different concentrations (250-0.048 µg/mL) were added to 190 µL of ABTS radical solution in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 734 nm. The percent radical scavenging activity of extracts and standard against ABTS were calculated according to the following:

$$\text{ABTS radical-scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control (containing all reagents except the test compounds), and A_1 is the absorbance of the extracts/standard. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. BHA, ascorbic acid and trolox were used as positive control.

2.4. Determination of Total Phenolic Content (TPC)

Total phenolic content of *C. virgate* extracts was measured using Folin-Ciocalteu reagent (16). The assay was adapted to the 96 well microplate format. 10 µL of extracts in various concentrations (151.52-18.94 µg/mL) were mixed with 20 µL Folin-Ciocalteu reagent (Sigma), 200 µL of H₂O, and 100 µL of 15% Na₂CO₃, and the absorbance was measured at 765 nm after 2 h incubation at room temperature. Gallic acid was used as a standard and the total phenolic content was expressed as 'mg gallic acid equivalent (GAE)/g extract'.

2.5. In-vitro Fibroblast Cell Proliferation Assay

The effects of plant extracts on fibroblast cell proliferation were determined by MTT method. The test samples were prepared by dissolving extracts in DMSO and followed by dilution with supplemented DMEM medium to obtain the final concentration of the extracts of *C. virgate* (10, 25, 50, 100 and 200 µg/mL, respectively). Briefly, NIH3T3 fibroblasts were seeded at 1x10⁴ cells/well into 96-well plate in DMEM containing 10% FBS. The final DMSO concentration in the assay was 0.1%. After 48h, cells were exposed to five different concentrations (10-200 µg/mL) of plant extracts and were then incubated for 48h at 37°C in a humidified atmosphere containing 5% CO₂. The MTT solution (5 mg/mL) was added directly to the each well and the plate was then incubated at 37°C for 4h. At the end of the incubation, 100 µL SDS was added to each well and then incubated for another 12 h solubilize the formazan formed in the viable cells. The absorbance of the samples was measured at 570 nm using

a microplate reader. The percentage of cell proliferation was calculated and compared to a negative control.

2.6. Scratch Assay

Briefly, NIH3T3 fibroblasts were seeded at 5×10^4 cells/well in DMEM containing 10% FBS were seeded into each well of 24 well plate and incubated at 37°C with 5% CO_2 . After the confluent monolayer of NIH3T3 fibroblasts was formed, two horizontal scratches were generated using a sterile pipette tip of 100 μL in each well. Any cellular debris was removed by washing with PBS and replaced with 1 mL of fresh medium in the absence or presence of plant extracts. Photographs were taken as two views on the left and right of each well at 10x magnification using a microphotograph (Olympus CK2, Japan) on day 0; then, plates were incubated at 37°C with 5% CO_2 and photographs were taken at 24 h.

3. RESULTS

The DPPH and ABTS radical scavenging activity results are shown in Table 1 as comparable with known antioxidants such as ascorbic acid, trolox and BHA. CVEA had the highest DPPH radical scavenging activity with an IC_{50} value of 138.7 $\mu\text{g}/\text{mL}$. On the other hand, CVH and CVAE revealed a poor antioxidant activity with IC_{50} values of 827.8 $\mu\text{g}/\text{mL}$ and 610.3 $\mu\text{g}/\text{mL}$, respectively. When the results obtained from ABTS radical scavenging activity were evaluated, it was determined that the CVEA had stronger radical scavenging activity than the other extracts. Similarly, CVH had the lowest radical scavenging activities.

Table 1. Antioxidant Activities of Various Extracts Obtained From Different Parts of *Centaurea virgate* ($n=3$).

Extracts*/Standards	DPPH radical scavenging activity	ABTS radical scavenging activity
	IC_{50} ($\mu\text{g} \cdot \text{mL}^{-1}$) Mean \pm SD	IC_{50} ($\mu\text{g} \cdot \text{mL}^{-1}$) Mean \pm SD
CVE	200.3 \pm 1.49	240.5 \pm 1.70
CVH	827.8 \pm 13.72	487.4 \pm 3.11
CVC	293.2 \pm 1.74	234.6 \pm 0.57
CVEA	138.7 \pm 2.48	72.89 \pm 0.44
CVAE	610.3 \pm 12.8	288.8 \pm 2.12
Ascorbic acid	17.6 \pm 0.37	14.5 \pm 0.32
Trolox	14.54 \pm 0.18	13.00 \pm 0.21
BHA	57.15 \pm 0.09	17.06 \pm 0.58

*SD: standard deviation; CVE, CVH, CHC, CVEA, CVAE show the ethanol extract and its n-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the leaves of *Centaurea virgate*, respectively. BHA: Butylated hydroxyanisole.

Table 2 presents total phenolic contents obtained for all the *C. virgate* extracts. Among all of the extracts analysed, it was found that phenolic content of CVEA was higher than that of the other extracts. While the content for CVH could not be determined, it was revealed that the CVAE extract had very low phenolic content.

Table 2. Total Phenolic Contents of Various Extracts Obtained From *C. virgate*.

Extracts*	TPC (mg GAE/g extract)**
CVE	23.83
CVH	-
CVC	24.05
CVEA	51.30
CVAE	2.88

*CVE, CVH, CHC, CVEA, CVAE show the ethanol extracts and its n-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the leaves of *Centaurea virgate*, respectively. ** Results were expressed as gallic acid equivalent (GAE). -: could not be determined.

MTT results revealed that CVC had the highest cytotoxic effect on NIH3T3 cells with an IC_{50} value of 23.07 $\mu\text{g}/\text{mL}$ (Table 3). Except for CVAE, other extracts also showed cytotoxic effects depending on the dose.

Table 3. The Proliferative Effect of Extracts on NIH3T3 Cells.

Extracts	200 μg	100 μg	50 μg	25 μg	10 μg	IC_{50} (μg)
	% Proliferation					
CVEA	33.41	43.9	61.04	80.91	110.91	96.8
CVE	20.67	31.4	49.57	73.05	114.94	65.31
CVAE	95.06	95.79	97.4	104.8	107.8	4517.25
CVC	18.96	19.15	37.93	53.29	72.68	23.07
CVH	53.9	66.1	77.68	88.7	89.09	393.8

*CVE, CVH, CHC, CVEA, CVAE show the ethanol extract and its n-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the leaves of *Centaurea virgate*, respectively.

As a result of MTT analysis, wound healing experiments were studied with this extract since it was determined that only CVAE had no cytotoxic effect on the NIH3T3 cells. Depending on the dose, it was determined that CVAE increased the migration of fibroblasts in the scratch assay (Figure 1).

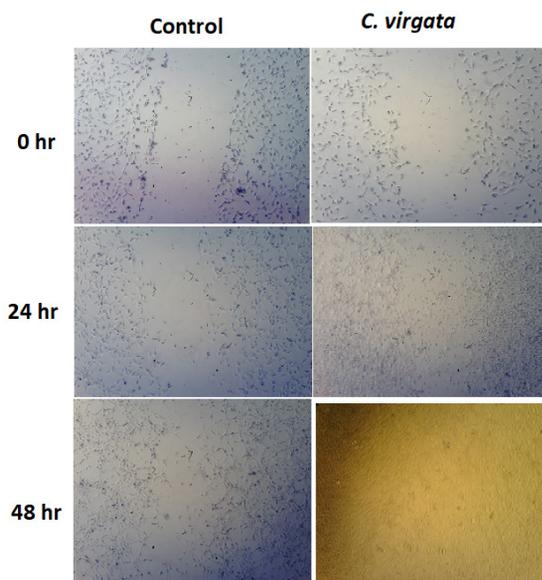


Figure 1. CVAE promoted the migration and wound closure of NIH3T3 cells in a wound scratch assay.

4. DISCUSSION

Plants have rich medical value and sources of molecules with potential wound-healing activity due to the phytochemical compounds they contain. Today, plant extracts are seen as an effective alternative for wound healing due to their widespread availability (8,17,18). In the current study, *in vitro* wound healing activity of *C. virgata* was revealed for the first time.

Wound healing is a rather complicated repair mechanism that begins after injury. Inflammation during the healing process leads to reactive oxygen species (ROS) formation, which is known for its harmful effects. The wound healing improving effects of various plant extracts can be attributed to the antioxidant and antimicrobial properties of the phytocomponents (17). Studies have shown a positive role between the free radical scavenging effect and the wound healing process.

Dermal fibroblasts are defense cells that respond to injury. Proliferation and migration of fibroblasts to the wound site are important events in the wound healing process. It is a useful model for stimulating fibroblast cell growth and studying wound healing activity *in vitro*. Therefore, isolation of active compounds found in natural products that affect migration of fibroblasts can help improve cutaneous wound healing. It has been reported that flavonoids, tannins, saponins and terpenoids in plants show wound healing activity by stimulating the growth of fibroblasts (19,20).

Interestingly, CVC exhibited the highest antiproliferative effect on fibroblast cells depending on the dose. Studies have shown that *C. virgata* predominantly contained flavonoids such as apigenin and eupatorin (21). In activity studies with apigenin, it has been found that it inhibited osteoblastogenesis and osteoclastogenesis (22). In addition, apigenin and its derivatives are known to have antiproliferative activity (23). Similarly, various studies have shown that eupatorin has an antiproliferative effect (24). Tüzün et al (2017) reported that the chloroform extract of *C. virgata* predominantly contained apigenin and eupatorin flavonoids. In line with this study, in our study CVC showed the highest antiproliferative effect (25).

CVAE, in particular, promoted the growth of fibroblasts at concentrations of 10 and 25 µg/mL. This mitogenic effect is a positive event for the wound healing process because fibroblasts are important cells involved in wound contraction and ECM production.

In our study, we determined that none of the extracts had significant antioxidant activity. Moreover, the obtained results for DPPH are in agreement with the phenol contents determined for each sample. The low antioxidant activities of these extracts might be attributable to their low phenolic contents.

In a study performed by Keser et al. (2020), it was demonstrated that *C. virgata* ethanol extract at concentration of 500 µg/mL had antioxidant activity with percent inhibition values

of 70.15% and 57.24% against ABTS and DPPH radicals, respectively. (21). When we compared these results with our current study, CVC with IC₅₀ values of 200.3 µg/mL and 240.5 µg/mL showed a better antioxidant activity. These differences in the antioxidant activity of *C. virgata* may be due to the fact that different parts of the plant have been studied and/or different extraction methods have been used.

5. CONCLUSION

According to the obtained results, our finding suggests that CVAE has a migratory effect on fibroblasts and that CVAE might be a potential therapeutic agent for wound healing. It is also reported in the literature that a *Centaurea* species, *C. lycopifolia*, is used by public for wound healing purposes (26). This information supports the wound healing activity of the aqueous ethanol extract of *C. virgata* which have found in our current study. However, it is necessary to isolate the active compounds responsible for the activity in order to understand the mechanism of action. Also, our results corroborate the use of *C. virgata* in traditional medicine as an wound healing.

Conflict of Interest

The author declares no conflict of interest.

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