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## ORIGINAL ARTICLE

# *In vitro* biological activities and preliminary phytochemical screening of different extracts from *Achillea sintenisii* Hub- Mor



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**Abstract** This study was designed to investigate *in vitro* biological activities and phytochemical composition of aqueous and ethanolic extracts from *Achillea sintenisii* Hub- Mor. (AS). To determine the chemical composition of AS extracts, phytochemical analyses were performed by using HPLC–ESI-Q-TOF-MS-MS. Afterwards, both extracts were investigated in terms of their effect on fibroblast proliferation, collagen synthesis, and hydrogen peroxide-induced damage. In addition to cell culture analysis, antibacterial, antioxidant, hyaluronidase inhibitory activities and total phenolic contents of the extracts were analyzed in cell-free systems. Our results demonstrated that the aqueous and ethanolic extracts did not show cytotoxic activity on fibroblasts, on the contrary, promoted fibroblast proliferation. Both AS extracts potently inhibited hyaluronidase activity and the inhibitory effect of ethanolic extract was comparable with the positive control, especially at high

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concentrations. The aqueous extract was the potent stimulator of collagen synthesis at 200 µg/mL concentration. Although the ethanolic extract showed antibacterial activity against all gram-positive bacteria, the aqueous extract was only effective against *K. pneumoniae* and *B. subtilis*. The ability of AS extracts, which have a rich phenolic compound content ( $\geq 50$  mg GAE/g), to scavenge free radicals and protect fibroblasts against hydrogen peroxide-induced damage can be considered as a result of their antioxidant potential. Our findings scientifically support the widespread use of this plant, by demonstrating the pharmacological properties of the extracts.

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## 1. Introduction

In parallel with the growth of the world population, the increase in diversity of diseases and the inadequacy of conventional therapies have accelerated researchers on discovering novel therapeutic agents in the field of medicine (Mena et al., 2013). In this scope, bioactive constituents isolated from plants have gained attention as one of the major sources to provide safe/ effective molecules to modern medicine and scientific research has focused more on natural remedies. On the other hand, people have turned to low-cost and easily accessible natural products especially in developing countries for the treatment of many ailments including cold, gastrointestinal disorders and skin diseases (Khan and Ahmad, 2019). Natural treatment options are used not only in developing countries as well as preferred in developed countries to avoid the undesirable effects associated with modern treatments (Bagde et al., 2010). In this context, plant-based extracts have been widely used for centuries and this tendency is continuously growing. The traditional use of herbal preparations is also encouraged by the World Health Organization (WHO) directives. According to WHO reports, approximately 80 % of the population uses herbal treatment options for primary health care in developing countries (WHO 2005). In addition, WHO reports point out that 25 % of modern medicines prescribed are of plant origin (WHO 2005; Khan and Ahmad 2019). Despite the widespread use of plants for medicinal purposes, only 15 % of the plant species in the world have been evaluated in terms of their pharmacological activity (Süntar 2020). Therefore, the lack of sufficient scientific evidence supporting the claimed efficacy is the crucial problem limiting their rational use in the health field.

The genus *Achillea* L. (Asteraceae), which is widespread in many parts of the world, mainly in Europe, Asia, North America and South Australia, has approximately more than 130 species (Paduch et al., 2008; Agar et al., 2015). Turkey is considered one of the most important diversity centers for the genus *Achillea* L. (Agar et al., 2015). According to the Turkish Plants Data Service, this genus is represented by 48 taxa in the Turkish flora and the rate of endemism is over 50 % (TUBİVES, 2018). *Achillea sintenisii* Hub- Mor. (AS), which is the subject of the present study, is one of these endemic species and is widely distributed in the Central Anatolia region of Turkey. The species of the genus *Achillea* are enormously rich in different types of secondary metabolites and accordingly have valuable biological properties, especially for dermatological applications (Strzpek-Gomólka et al., 2021). Scientific studies have shown that major constituents of *Achillea* species such as chlorogenic acid, apigenin-7-O-glucoside, and schaftoside stimulate collagen expression through different mechanisms (Dorjsembe et al., 2017). Chlorogenic acid has the ability to inhibit matrix metalloproteinase (MMP)- 9, which is one of the proteases associated with the inflammatory process and also plays an important role in the degradation of collagen (Jin et al., 2005; Benedek et al., 2007; Bor et al., 2022). Apigenin and luteolin isolated from *Achillea* species have been reported to suppress the expression of inflammatory mediators and contribute to the prevention of long-term inflammation, which is an important strategy for wound treatment (Dorjsembe et al., 2017). Also, *A. millefolium* extracts are known

as powerful antioxidants in relation to the phenolic components they contain including luteolin-7-O-glucoside, chlorogenic acid, and rutin (Vitalini et al., 2011). It has also been suggested that isovitexin flavonoid isolated from some *Achillea* species, such as *A. alpina* L., has the potential to promote skin whitening associated with down-regulation of intracellular tyrosinase activity (Lee et al., 2019). In this context, various extracts prepared from this genus have been used as active ingredients in skin healing and conditioning products in the pharmaceutical industry (Strzpek-Gomólka et al., 2021). Besides their industrial use, many *Achillea* species, including *Achillea sintenisii* Hub-Mor., have been used in traditional medicine as a natural remedy for pain, inflammation, wounds, and bleeding (Strzpek-Gomólka et al., 2021; Sezik et al., 2001). In addition to the traditional use of *Achillea* species, the wound healing (Küveli Akkol et al., 2011; Agar et al., 2015), antinociceptive (Küveli et al., 2007), antimicrobial (Candan et al., 2003; Karaalp et al., 2009), antispasmodic (Karamenderes and Apaydin, 2003), anti-inflammatory (Gómez et al., 1999) and antioxidant (Konyalioglu and Karamenderes, 2005; Anlas et al., 2017) activities of several *Achillea* species have been confirmed by scientific reports. Previous studies on AS have demonstrated that the methanolic extract and essential oil from this species possess strong antimicrobial activity against various bacteria and yeast (Sökmen et al., 2003). However, to the best of our knowledge, there is not any comprehensive study concerning the biological activities of AS extracts.

In the present study, it was aimed to provide scientific support to the traditional use of AS extracts with pharmacological activity studies. It is known that the type of extraction solvent and technique may have a significant impact on the quantity and variety of isolated bioactive compounds and simultaneously on the biological activity of extract (Rafińska et al., 2019). In this context, we used two extracts (ethanol and water) obtained from AS by different extraction techniques and primarily performed the phytochemical screening of the extracts by using high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS-MS). Afterwards, the antibacterial, antioxidant and hyaluronidase inhibitory activities of the extracts were assessed in cell-free systems; as well as the effects on fibroblast proliferation, collagen synthesis, and protection against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced damage were investigated in *in vitro* systems.

## 2. Materials and methods

### 2.1. Plant material

AS was collected from Sivas, Turkey (Between Sivas- Hafik, Topçuyenköy- 1384 m) in June 2013 (geographic coordinates: 39°46'23.0"N 37°34'48.1"E). A voucher specimen is deposited in the herbarium of Cumhuriyet University, Faculty of Science, Department of Biology (CUFH-AA 5044). Before starting the extraction process, the collected plant samples were dried at room temperature (20–24°C).

## 2.2. Extraction process

In the extraction process, water and ethanol (96 %) were used to isolate different bioactive constituents from the aerial parts of AS. The aqueous extract (ASA) was prepared by boiling 25 g of the powdered plant material in deionized water (500 mL) during 5 min (Albayrak et al., 2012). Then the extract was allowed to stand for 10 min at room temperature. To obtain the ethanolic extract (ASE), the same amount of plant material was macerated with ethanol for 10 consecutive days, at room temperature (Ustuner et al., 2019). All these extracts were concentrated at 40 °C under reduced pressure and dried by freeze-dryer. The extracts were stored at -20 °C until analysis. The extraction yields of ASA and ASE were 7.9 % and 3.2 % respectively.

## 2.3. Phytochemical screening (HPLC ESI-Q-TOF MS-MS analysis)

To identification of bioactive compounds in AS extracts, HPLC ESI-Q-TOF MS-MS analyses were performed using Agilent 1260 series coupled with Agilent 6550 iFunnel high-resolution mass spectrometry (Agilent Technologies, Inc., CA, USA). Jet stream electrospray ionization technique was used in negative ionization mode. Agilent TC C-18 (4.6 mm × 150 mm × 5 µm) analytical column was used for chromatographic separation. Data interpretation was carried out by using Agilent MassHunter software B.06.01 and Metlin Metabolite database. HPLC ESI-Q-TOF MS-MS conditions were summarized in Table 1. Deionized water (1 mL) and methanol-water mixture (1 mL) were used to dissolve ASA

and ASE (10 mg), respectively. All the samples were filtered (0.45 µm) before the injection.

## 2.4. Total phenolic content (TPC) estimation

The content of phenolics in AS extracts were quantitatively determined using the Folin-Ciocalteu (FC) assay (Dominquez et al. 2005). Briefly, 10 µL of the methanolic solutions of the extracts (at a concentration of 2 mg/mL) were mixed with 150 µL of FC reagent diluted with water (1:4, v/v). After 3-min incubation, 50 µL of sodium carbonate solution was added and this mixture incubated for 2 h in the dark. Finally, the absorbance of each well was read at 725 nm. Gallic acid (0, 0.05, 0.1, 0.2 and 0.4 mg/mL) was used as the standard phenolic compound and the calibration curve of gallic acid was drawn to calculate the total phenolics. TPCs of AS extracts were expressed as milligram (mg) of gallic acid equivalent (GAE) per gram (g) of extract.

## 2.5. 2,2-diphenyl-1 picrylhydrazyl (DPPH) scavenging assay

DPPH free radical scavenging ability of AS extracts was determined following the method described by Yang et al. (2009). Serial dilutions of AS extracts (25 to 200 µg/mL, 200 µL) and ascorbic acid (positive control) were mixed with freshly prepared DPPH solution (0.15 mM, 50 µL). After 30-min incubation in the dark, the absorbance of each well was measured by a microplate reader (at 517 nm wavelength). The IC<sub>50</sub> values, defined as the extract concentration needed to decrease the initial free radical by 50 %, were calculated using a log-dose inhibition curve.

## 2.6. Antibacterial activity (Agar dilution assay)

The agar dilution assay was carried out to determine the antibacterial activities of the extracts following the procedure described by the Clinical and Laboratory Standards Institute (CLSI, 2006). In this context, minimum inhibitory concentrations (MICs) of the extracts against both gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Staphylococcus epidermidis*) and gram-negative bacteria (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) were detected. Gentamicin sulfate was used as the reference standard for antibacterial activity against both gram-positive and gram-negative bacteria, as it is a broad-spectrum antibiotic (Kimna et al., 2018). For antibacterial analysis, the extracts were dissolved at a concentration of 80 mg/mL and diluted with cation-adjusted Mueller Hinton broth to obtain final concentrations of 0.015625 to 8 mg/mL. Each inoculum (1 mL) was poured into petri dishes and mixed with 9 mL of Muller-Hinton agar (45–50 °C) by gentle circular rotation. Then, the mixture was kept cool at room temperature (25 ± 2 °C). A bacterial suspension (adjusted to about 10<sup>7</sup> CFU/mL) was prepared and added to each well of the microplate. The replicator was placed into the microplate to soak the pins and transfer them onto the agar plate. The MIC values were determined as the lowest extract concentrations that prevent bacterial growth after 24 h of incubation at 37 °C.

**Table 1** Working conditions of HPLC ESI-Q-TOF MS-MS.

HPLC conditions	
Column temperature	30 °C
Injection volume	5.0 µL
Analysis time	82 min
Mobile phase A- B	10.0 mM ammonium acetate in water-Acetonitrile
Flow rate	0.65 mL/min
Solvent composition timetable	5 % B (0 min) 5 % B (4 min) 10 % B (12 min) 10 % B (15 min) 20 % B (28 min) 40 % B (48 min)
Detection wavelength	260 nm
MS conditions	
Ionization mode	Negative
Drying gas flow, N <sub>2</sub>	14.0 L/min
Drying gas temperature	200 °C
Nebuliser	40 psi
Sheat gas flow, N <sub>2</sub>	11.0 L/min
Sheath gas temperature	375 °C
Capillary voltage	1100 V
Nozzle voltage	2000 V
Mass range	20–1500 amu
Reference ion	966.000725

### 2.7. Hyaluronidase inhibitory activity

The inhibitory effect of AS extracts on the hyaluronidase, an enzyme responsible for the degradation of hyaluronic acid, was measured by a turbidimetric method. The method is based on the remaining hyaluronic acid interacts with the albumin solution and creates turbidity (Kass and Seastone, 1944; Tolksdorf and McCready, 1949). In this experiment, tannic acid was used as a reference, and albumin was used as a blank. The samples were run in three parallel. First, 20  $\mu\text{L}$  of samples at different concentrations (50, 100, and 200  $\mu\text{g}/\text{mL}$ ) were added to the wells on the ELISA microplate. Hyaluronic acid (0.4  $\text{mg}/\text{mL}$ , 10  $\mu\text{L}$ ) was added to the samples and incubated at 37  $^{\circ}\text{C}$  for 4–5 min. Then, hyaluronidase enzyme (0.05  $\text{mg}/\text{mL}$ , 10  $\mu\text{L}$ ) was added to the mixture and stored for 10 min. Finally, 180  $\mu\text{L}$  of albumin solution was added to the microplate wells brought to room temperature and incubated for further 10 min. The absorbance was read in a microplate reader at 540 nm. The percentage inhibition was calculated according to this formula:

Percentage of hyaluronidase inhibition (%) = [(Sample absorbance - Control absorbance) / Control absorbance]  $\times$  100.

### 2.8. Cell culture conditions

Cell culture analyses were carried out on fibroblast cell line (3 T3-Swiss albino mouse, ATCC-CCL-92). Briefly, the cells were nurtured in Dulbecco's Modified Eagle's Medium-F12 supplemented with penicillin/streptomycin solution (1 %) and fetal bovine serum (10 %). A humidified ambience (37  $^{\circ}\text{C}$ , 5 %  $\text{CO}_2$ ) was set up for the growth of the cells. The culture medium was changed every 48 h until the cultures reached confluence. For the cell culture analysis, fibroblasts were detached from the culture flask using trypsin-EDTA solution (0.25 %). The stock solutions of plant extracts were prepared by dissolving the extracts in dimethyl sulfoxide (DMSO) and diluting with cell culture medium. The final DMSO concentration did not exceed 0.2 % throughout the study and the control cells were treated with the corresponding amount of DMSO.

#### 2.8.1. Cell proliferation assay (MTT assay)

The effects of *Achillea sintenisii* Hub- Mor. extracts on fibroblast proliferation were determined by MTT (3- [4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. For this purpose, mouse fibroblasts were seeded to the wells of a 96-well plate at a density of  $1 \times 10^4$  cells per well and incubated for 24 h for cell attachment. Then, the medium was gently removed from the wells and the cells were further incubated with varying concentrations of (50, 100, and 200  $\mu\text{g}/\text{mL}$ ) AS extracts for 72 h. The MTT cell proliferation assay kit was used according to the manufacturer's instructions (Roche Applied Science). The absorbance of each well was read at 595 nm using the multimode microplate reader. The effects of AS extracts on fibroblast proliferation were assessed as the percent cell viability where untreated cells were considered as 100 % viable. The cell viability percentage was calculated as follows:

Percentage of cell viability (%) = (Absorbance of treated cells / Absorbance of control cells)  $\times$  100.

#### 2.8.2. Hydroxyproline assay

To quantify the effect of *Achillea sintenisii* Hub- Mor. extracts on collagen production, hydroxyproline assay was carried out following to the method published by Jorge et al. (2008). 3 T3 mouse fibroblasts were seeded at a density of  $1 \times 10^3$  cells per well, and the plates were incubated at 37  $^{\circ}\text{C}$  for 24 h. After incubation, the culture medium was discarded and cells were exposed to serial dilutions of extracts (50, 100, and 200  $\mu\text{g}/\text{mL}$ ) in fresh medium for 72 h. In the positive control group, fibroblasts were treated with ascorbic acid (25  $\mu\text{g}/\text{mL}$ ). The supernatant of each well was collected and hydrolyzed with 6 N hydrochloric acid (HCl) at 120  $^{\circ}\text{C}$  by using an autoclave. Chloramine-T solution was added to the hydrolyzed samples first, and after 20 min of the incubation period at room temperature, Ehrlich's reagent was added to the mixture. The absorbance of the samples was measured at 550 nm by a multimode microplate reader, following 15 min of incubation at 65  $^{\circ}\text{C}$ . To determine the hydroxyproline content in the samples, a standard curve was prepared by hydroxyproline standard in concentrations ranging from 0 to 10  $\mu\text{g}/\text{mL}$ . The results were expressed as  $\mu\text{g}/\text{mL}$  of hydroxyproline.

#### 2.8.3. Antioxidant activities of the extracts on mouse fibroblasts

The effect of AS extracts on the  $\text{H}_2\text{O}_2$ -induced oxidative damage was investigated by the method published by Annan and Dickson (2008). The cells were seeded in a 96-well plate ( $5 \times 10^3$  cells/well) and incubated at 37  $^{\circ}\text{C}$  overnight. Afterwards, the culture medium was discarded and two independent experiments were performed on the cells. In the first experiment, fibroblasts were pre-treated with the extracts at different concentrations (50, 100, and 200  $\mu\text{g}/\text{mL}$ ) for 24 h and subsequently incubated with  $\text{H}_2\text{O}_2$  ( $10^{-4}$  M) for 3 h. In the second protocol, fibroblasts were treated with the same concentrations of AS extracts and  $\text{H}_2\text{O}_2$  ( $10^{-4}$  M) simultaneously for 3 h. In both experiments, MTT assay was used to measure the protective effect of AS extracts against cellular damage induced by  $\text{H}_2\text{O}_2$  in fibroblasts. As the positive control group, fibroblasts were treated with Catalase (250 IU/mL) in both experiments.

### 2.9. Statistical analysis

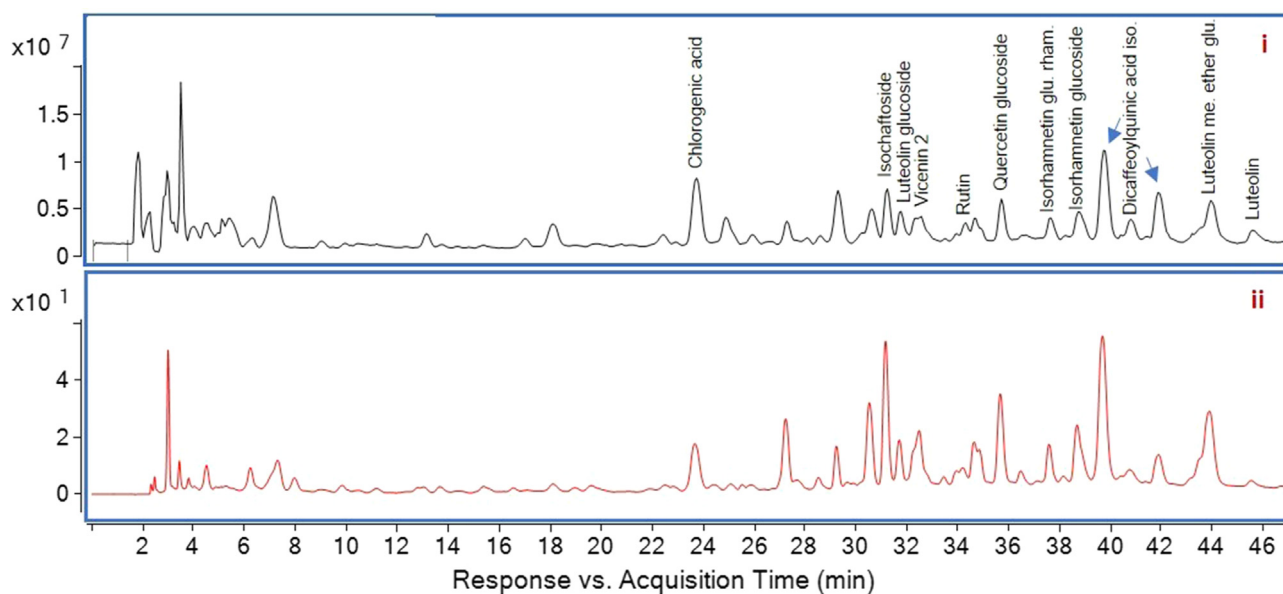
Results were presented as mean  $\pm$  standard error of the mean (SEM) from three independent experiments. Data were analyzed using one-way (ANOVA) followed by Student's *t*-test. (SPSS 15.0, SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at  $P < 0.05$ .

## 3. Results and discussion

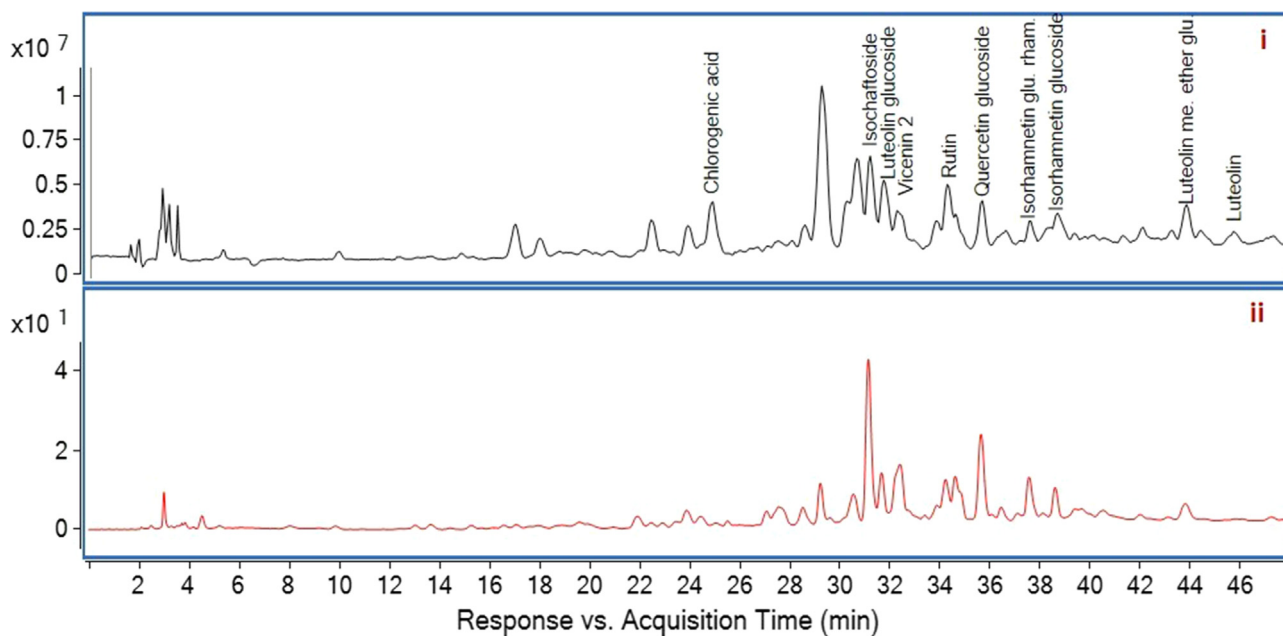
### 3.1. HPLC ESI-Q-TOF MS-MS analysis

In this study, the compounds were putatively identified by their mass spectrometric ions through comparison with the literature and library database search from the instrument software. NIST library matching percentage was higher than 99.99 % for identification of all compounds. The MS spectrum and HPLC chromatogram of ASA and ASE were shown in Fig. 1 and Fig. 2, respectively. Chlorogenic acid, isoschaftoside isomers, luteolin derivatives (glucoside and methyl ether glucuronide), vicenin 2, rutin, quercetin glucoside, isorhamnetin





**Fig. 1** MS spectrum (i) and HPLC chromatogram (ii) of ASA. ASA, Aqueous extract; HPLC, high-performance liquid chromatography; MS, mass spectrometry.



**Fig. 2** MS spectrum (i) and HPLC chromatogram (ii) of ASE. ASE, Ethanolic extract; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

derivatives (glucoside and glucoside rhamnoside) and dicafeoylquinic acid isomers were identified in ASA. Almost all the compounds found in ASA were also identified for ASE, except dicafeoylquinic acid and its isomers. The difference between AS extracts can be clearly seen in Fig. 1 at mainly 39.65 min and minorly 40.79; 41.86 mins.

Separated compounds were identified by HPLC ESI-Q-TOF MS-MS in negative ionization mode. All identified compounds including retention time, molecular weight, relative abundances (%) and fragments were summarized in Table 2. Chlorogenic acid showed  $[MH]^-$  value at  $m/z$  353.0881 with

the fragment ions at  $m/z$  191 and 179 due to the loss of a cafeoyl group and a quinic group, respectively (Chen et al., 2012). Isochaftoside showed  $[MH]^-$  value at  $m/z$  563.1407 with the fragment ion at  $m/z$  311 and luteolin glucoside showed  $[MH]^-$  value at  $m/z$  447.0863 with the fragment ion at  $m/z$  285. Both product ions prove the lack of a glucoside group (Plazonic et al., 2009). Vicenin 2 showed  $[MH]^-$  value at  $m/z$  593.1504. Rutin showed  $[MH]^-$  value at  $m/z$  609.1409 with the fragment ion at  $m/z$  301 by losses of rutinose group (Tohma et al., 2016). Quercetin glucoside showed  $[MH]^-$  value at  $m/z$  463.0814 and gave fragment ion at  $m/z$  301. Isorham-

**Table 2** Identified compounds in aqueous extract of AS by HPLC ESI-Q-TOF MS-MS.

Compounds	tR(min)	[M–H] <sup>−</sup>	Fragment ions	Relative abundance for ASA (%)	Relative abundance for ASE (%)
Chlorogenic acid	23.67	353.0881	191, 179	6.53	2.19
Isochaftoside	31.16	563.1407	311	3.04	6.94
Luteolin glucoside	31.76	447.0863	285	1.58	5.31
Vicenin 2	32.57	593.1504	456, 474, 576	2.32	3.02
Rutin	34.64	609.1409	301	1.47	6.20
Quercetin glucoside	35.71	463.0814	301	2.50	2.59
Isorhamnetin glucoside rhamnoside	37.58	623.1641	477, 316	1.58	1.17
Isorhamnetin glucoside	38.78	477.0990	316	2.83	3.72
Dicaffeoylquinic acid isomers	39.65	515.1310	353, 191, 179	15.59	0.89
Luteolin methyl ether glucuronide	43.86	477.0966	299, 285, 199, 175	5.73	3.04
Luteolin	45.53	285.0405	199, 151, 133	1.19	1.27

ASA: *A. sintenisii* Hub- Mor. aqueous extract, ASE: *A. sintenisii* Hub- Mor. ethanolic extract.

netin glucoside rhamnoside and isorhamnetin glucoside showed [MH]<sup>−</sup> value at *m/z* 623.1641 and 477.0990, respectively, which are interpreted from the instrument database. Dicaffeoylquinic acid showed [MH]<sup>−</sup> value at *m/z* 515.1310 with fragment ions 353 (chlorogenic acid), 191, 179 (Bejaoui et al., 2003). The obtained spectra showed the [MH]<sup>−</sup> molecular ion at *m/z* 477.0966 gave fragment ions 299, 285, 199, 175 which was attributed to the luteolin methyl ether glucuronide by interpretation from the instrument database. Luteolin gave [MH]<sup>−</sup> value at *m/z* 285.0405 with fragment ions at *m/z* 199, 151 and 133 (Tohma et al., 2016).

The phenolic contents of water and water–ethanol extracts obtained from AS have been previously investigated and similar to our results, the presence of quercetin glucoside and luteolin glucoside in both extracts has been reported (Şabanoglu et al., 2017). However, the presence of different phytochemicals including chlorogenic acid or dicaffeoylquinic acid, which are the main bioactive constituents of many *Achillea* species, in AS extracts were demonstrated for the first time in this study.

### 3.2. DPPH scavenging capacities and total phenolic content of *A. sintenisii* Hub- Mor. extracts

Free radicals are intermediates formed during cell metabolism and essential for critical cellular functions (such as leukocyte adhesion, signal transduction, platelet aggregation, etc.) under normal conditions (Bruce and Buehler, 2012). However, their excessive production can cause oxidative stress, which can damage to cellular reactions and lead to various chronic diseases (Zhang et al., 2020). For example, excessive amounts of free radicals generated on the wound surface in response to skin injury may also delay the healing process by destroying lipids, proteins, and essential components of extracellular matrix (Ustuner et al., 2019). It was suggested that treatment options which act to modulate free radical formation can be a beneficial approach in the management of degenerative disorders (Henriques et al., 2006). In this context, subsequent scientific studies have reported that plants with high antioxidant capacity may down-regulate degenerative processes and effectively reduce the incidence of many disorders related with oxidative stress (Sultana et al., 2009). The oxidation reducing properties of plant-derived antioxidants may occur by inter-

rupting the free radical chain reaction or by directly scavenging reactive oxygen species. In this direction, we investigated the antioxidant activities of AS extracts by determining their DPPH free radical scavenging abilities.

The percentage of DPPH radical scavenging activity ranged from 19.19 % to 50.67 % for ASE and from 16.9 % to 53.56 % for ASA. On the other hand, the standard compound, ascorbic acid, inhibited the DPPH radical more strongly up to 95.79 %. ASA and ASE demonstrated moderate and very similar activity against DPPH free radical with IC<sub>50</sub> values of 183.13 ± 1.10 µg/mL and 184.31 ± 0.80 µg/mL, respectively (Table 3), however, this activity was not as strong as ascorbic acid (27.63 ± 1.12 µg/mL). In previous studies conducted on other *Achillea* species, high variation has been reported among DPPH radical scavenging capacities of the extracts due to various factors such as temperature or altitude difference in the region where the plants were collected and genetic differences (Gharibi et al., 2013). Our results demonstrated that the aqueous and ethanolic extract of AS has similar radical scavenging potential to the ethanolic extracts of *A. wilhelmsii* and *A. cucullata* with close IC<sub>50</sub> values (118.90 µg/mL and 132.55 µg/mL, respectively) (Nickavar et al., 2006; Eruygur et al., 2019). Similar to the results of our study, it is noteworthy that the DPPH scavenging activities of *A. wilhelmsii* and *A. cucullata* extracts were not as pronounced as rutin and gallic acid, which are used as reference compounds for radical scavenging activity. These results can be interpreted as the fact that although the extracts belonging to *Achillea* species have DPPH free radical scavenging activity, these effects are not as strong as standard antioxidants. On the other hand, the reduction of DPPH radical is

**Table 3** Antioxidant activities of *A. sintenisii* Hub- Mor. extracts expressed by DPPH scavenging activity and total phenolic content.

Extracts	DPPH scavenging activity [IC <sub>50</sub> (µg/mL)]	TPCs [mg GAE/g Extract]
ASA	183.13 ± 1.10	58.62 ± 0.95
ASE	184.31 ± 0.80	110.0 ± 0.84

ASA: *A. sintenisii* Hub- Mor. aqueous extract, ASE: *A. sintenisii* Hub- Mor. ethanolic extract, GAE: Gallic acid equivalent, TPCs: Total phenolic contents.

highly correlated with the presence of hydroxyl groups (phenolic or non-phenolic), which act as hydrogen donors to stabilize the free radicals (Mensor et al., 2001). The results of the phytochemical analysis in this study evidenced the presence of phenolic compounds (rutin, luteolin, quercetin, etc.) containing hydroxyl groups in AS extracts. In this context, the free radical scavenging capacities of AS extracts can be attributed to the presence of these compounds, which have the ability to stabilize free radicals.

In the present study, we also determined the amount of TPC. The TPCs of ASA and ASE were determined as  $58.62 \pm 0.95$  mg GAE/g and  $110 \pm 0.84$  mg GAE/g, respectively. According to Agourram et al. (2013), the TPC of plant extracts could be subdivided as follows: high content ( $\geq 50$  mg GAE/g), medium content (50–20 mg GAE/g), and low content ( $< 20$  mg GAE/g). Considering this classification, it can be concluded that both AS extracts have a rich content of phenolic compounds that act as highly effective scavengers of various oxidizing molecules and free radicals (Montoro et al., 2005).

### 3.3. Antibacterial activities of *A. Sintenisii* Hub- Mor. Extracts

Antibacterial activities of AS extracts were investigated against four strains of gram-positive and three strains of gram-negative bacteria. The antibacterial activities of AS extracts expressed with MIC values are summarized in Table 4. ASE exhibited antibacterial activity against all of the tested gram-positive bacteria (*B.subtilis*, *S.aureus*, *S.epidermidis*, *B.cereus*) with MIC values ranging from 0.5 to 8 mg/mL, while ASA was only effective against *K. pneumonia* and *B. subtilis* with a MIC value of 4 mg/mL. *B.subtilis* was sensitive to both ASE and ASA, while *P.aeruginosa* and *P.mirabilis* were resistant to both AS extracts. Our findings are consistent with the results of the study conducted by Sökmen et al. (2003) who reported that the essential oil of this plant did not show any antibacterial activity against *P. mirabilis* and *P. aeruginosa*. On the other hand, our findings reveal that ASE has a better antibacterial profile against *B.cereus* and *S.aureus* with lower MIC values than those reported for the essential oil of the same plant (Sökmen et al., 2003).

According to the classification reported by Fabry et al. (1998), crude plant extracts should have MIC values  $< 8$  mg/mL, to be considered as useful therapeutically. In this direction, we can conclude that especially ASE has sufficient antibacterial activity against many gram-positive bacteria. This activity can be attributed to the presence of bioactive molecules with previously demonstrated antibacterial potential, such as quercetin or chlorogenic acid (Yang et al., 2020; Lou et al., 2011). It is noteworthy that, although ASA contains these molecules, it showed no antibacterial activity against

most gram-negative and gram-positive bacteria tested. This situation may be a result of the inefficient diffusability of aqueous extracts in the agar medium and the low ability of aqueous extracts to damage bacterial cell walls, as previously reported by Kaczorova et al. (2021).

### 3.4. Hyaluronidase inhibitory effects of *A. sintenisii* Hub- Mor. extracts

Hyaluronic acid, one of the main glycosaminoglycans found in the extracellular matrix of connective tissues, plays an essential role in many pathological and physiological processes including embryogenesis, angiogenesis, wound healing, cell differentiation/proliferation, and inflammation (Girish et al., 2009; Gonzalez- Rico et al., 2019). As natural component of extracellular matrix, hyaluronic acid is the focus of scientific research especially related with the skin-health (Li et al., 2020). Hyaluronidase-mediated degradation of hyaluronic acid can increase the permeability of connective tissue, impair the structural integrity of the extracellular matrix, as well as affect progression of cancer, spread of toxins or microbial pathogenesis (McCook et al., 2015; Girish and Kemparaju 2007; Li et al., 2020). Therefore, inhibition of hyaluronidase activity is critical in many pathological conditions including skin wounds, allergy or inflammation, and is considered as a promising strategy in reducing the progression of diseases which are associated with up-regulation of this enzyme (Li et al., 2020; Arunkumar et al., 2021). Hyaluronidase inhibitory activities of many plant species have been demonstrated by scientific reports (Kim et al., 1995; Liyanaarachchi et al., 2019). However, no study has been found in the literature on the hyaluronidase inhibitory activities of *Achillea* species, which are especially used in traditional medicine to treat skin inflammation and wounds. In the present study, it was thought that the investigation of hyaluronidase inhibitory activity could contribute to the elucidation of the mechanism of action of *Achillea* species on skin disorders and AS extracts were evaluated for their *in vitro* hyaluronidase inhibitory activities.

The inhibitory effects of AS extracts on hyaluronidase enzyme were presented in Table 5. Both of the extracts exhibited concentration-dependent and significant hyaluronidase inhibitory activity at all concentrations, compared with the control. The inhibitory activity of ASE was very close to tannic acid, especially at high concentrations. Previous studies have reported that identified flavonoid glycosides in AS extracts such as quercetin and luteolin glycosides can able to inhibit the hyaluronidase activity and lead to a more stable extracellular matrix (Kim et al., 2005; Gendrisch et al., 2021). Hence, it is thought that these phytochemicals contained in the extracts may be responsible for the hyaluronidase inhibitory activity. In

**Table 4** Antibacterial activities of *A. sintenisii* Hub-Mor. extracts.

Plant material	Minimum Inhibitory Concentrations (mg/mL)						
	<i>B.subtilis</i>	<i>B.cereus</i>	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>P.mirabilis</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
ASA	4	NI	NI	NI	NI	NI	4
ASE	2	4	8	0.5	NI	NI	NI
Gentamicin <sup>a</sup>	0.0005	0.0008	0.0005	0.0005	0.00025	0.00025	0.00025

ASA: *A. sintenisii* Hub- Mor. aqueous extract, ASE: *A. sintenisii* Hub- Mor. ethanolic extract, NI: not inhibition, <sup>a</sup>: positive control.

**Table 5** Percentage of hyaluronidase inhibitory activities (%) of *A. sintenisii* Hub-Mor. extracts.

Plant material	Concentrations		
	50 µg/mL	100 µg/mL	200 µg/mL
ASA	64.5 ± 2.73 <sup>***</sup>	71.77 ± 1.71 <sup>***</sup>	72.8 ± 1.08 <sup>***</sup>
ASE	69.45 ± 1.83 <sup>***</sup>	83.69 ± 2.06 <sup>***</sup>	84.16 ± 2.25 <sup>***</sup>
Tannic acid	79.94 ± 1.05 <sup>***</sup>	82.18 ± 2.45 <sup>***</sup>	85.95 ± 1.49 <sup>***</sup>

ASA: *A. sintenisii* Hub- Mor. aqueous extract, ASE: *A. sintenisii* Hub- Mor. ethanolic extract.

<sup>\*\*\*</sup> P < 0.001, versus control.

particular, the inhibitory activity of ASE is very close to tannic acid, indicating that this extract may serve as a potent natural hyaluronidase inhibitor and play an important role in hyaluronidase-mediated pathological processes.

### 3.5. Effect of *A. sintenisii* Hub- Mor. extracts on fibroblast proliferation

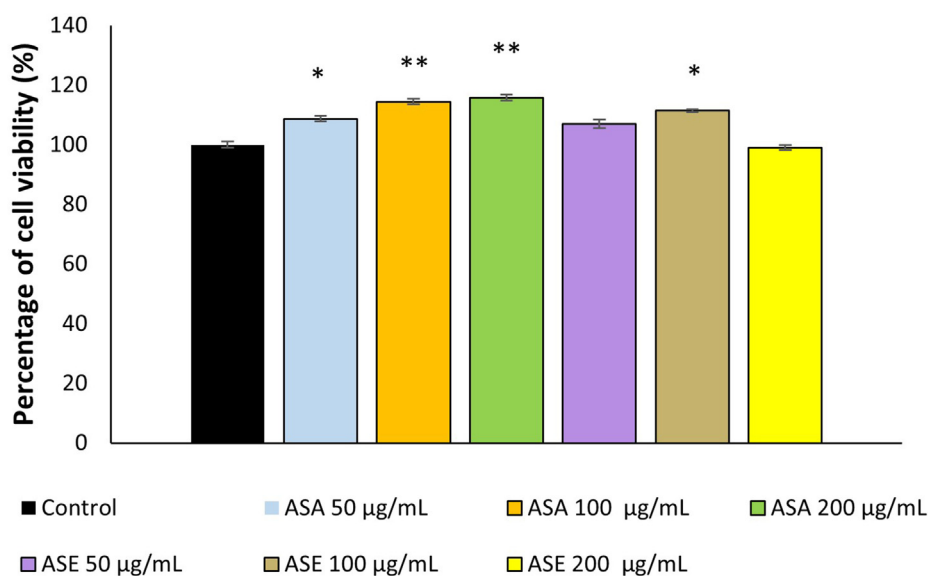
In the present study, the effects of AS extracts on the proliferation of fibroblasts were investigated by MTT assay, which is a useful method used for screening the cytotoxic or proliferative effects of plant extracts (Mektrirat et al., 2020). The viability of fibroblasts treated with various concentrations of AS extracts was presented in Fig. 3. As shown in the figure, the percentage of cell viability treated with ASA and ASE varied between 108.70 % and 115.73 % and 99.09 %- 111.4 %, respectively. ASA (at all concentrations) and ASE (at 100 µg/mL concentration) significantly stimulated fibroblast proliferation compared with the control group. The results of the MTT assay also contributed interpretation of the cytotoxic activity of AS extracts on fibroblasts. None of the extracts significantly reduced fibroblast viability compared with non-treated cells at the applied concentrations. Considering these results it can be concluded that AS extracts have a good safety profile

on healthy cells by not adversely interfering with cell viability, as well as promote the healing process of skin by stimulating fibroblast proliferation.

The results of our study were consistent with previous reports on the toxicity potential of different *Achillea* species on healthy cells. In the study conducted by Bali et al. (2015), the cytotoxicity potential of *Achillea teretifolia* Willd was investigated on human gingival fibroblasts and was reported that the methanolic and aqueous extracts did not induce cytotoxicity up to concentrations of 0.45 mg/mL and 1.4 mg/mL, respectively. In another study, evaluating the proliferative effect of hydroalcoholic extract from *Achillea millefolium* leaves on skin fibroblasts, Ghobadian et al. (2015) reported that the extract can stimulate fibroblast proliferation at < 20.0 mg/mL concentrations. Similarly, Agar et al. (2015) reported that *A. kotschy* Boiss. subsp. *kotschy* exhibited potent proliferative activity on mouse fibroblasts at 2.5–20 µg/mL concentration. It has been previously proven that many phytochemicals of AS, such as Vicenin-2, quercetin glucoside, and rutin, that we have identified both in ASA and ASE can promote fibroblast proliferation (Dinda et al., 2016; Tan et al., 2020; Muhammad et al., 2013). It is thought that individual or synergistic effects of these phytoconstituents can be responsible for the proliferative activities of AS extracts. The only notable difference between the phytochemical contents of the extracts was the presence of dicaffeoylquinic acid isomers in ASA. The significant and pronounced proliferative activity of ASA at all tested concentrations is likely to be related to dicaffeoylquinic acid isomers, which have been reported to act on fibroblasts in the skin healing process (Chiangnoon et al., 2022).

### 3.6. Effect of *A. sintenisii* Hub- Mor. extracts on collagen synthesis

Collagens are the principal structural protein of all connective tissues and are also present in the interstitial tissue of virtually



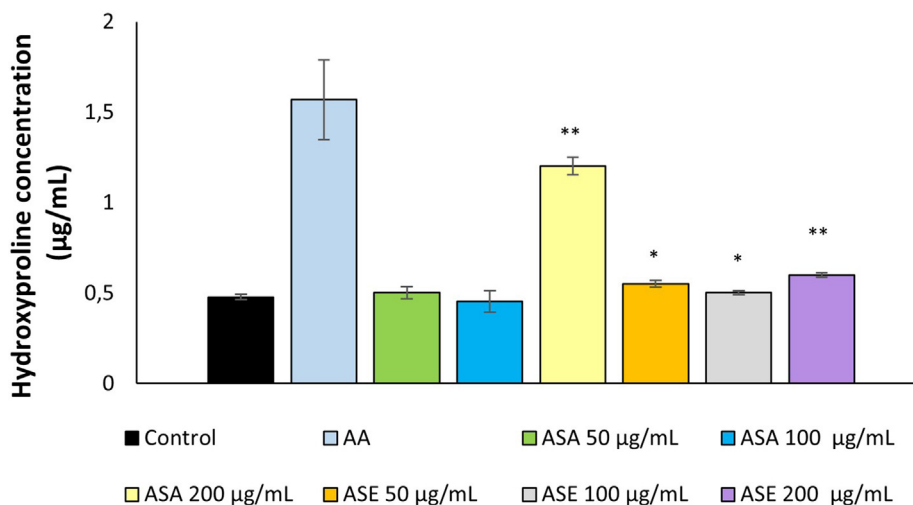
**Fig. 3** Percentage of fibroblast viability (%) following treatment with different extracts of *A. sintenisii* Hub-Mor., determined by MTT assay. ASA, Aqueous extract; ASE: Ethanolic extract. \* P < 0.05, \*\*P < 0.01 versus control cells.



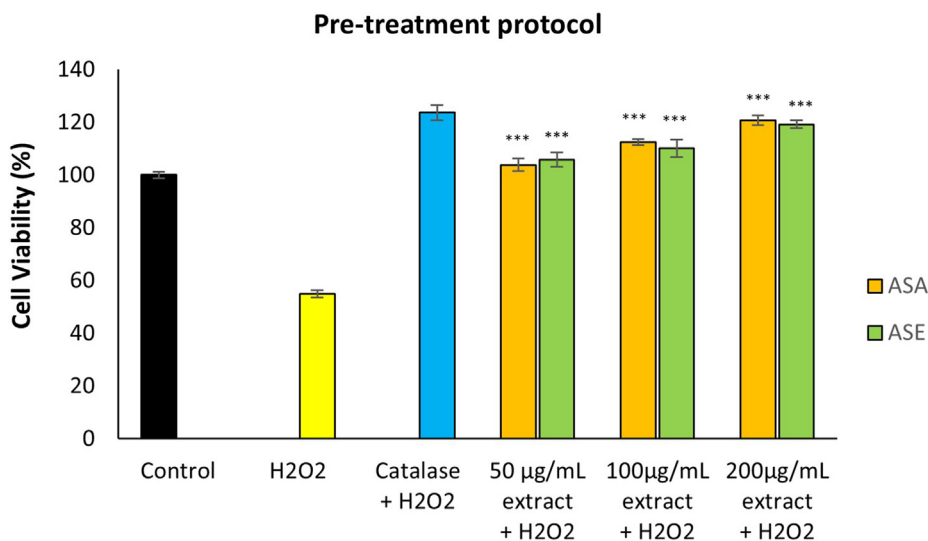
all parenchymal organs (Sandhu et al., 2012). Collagens provide support to many tissues including cartilage, tendon, bone, and ligaments and play a vital role in maintaining the structural integrity of these tissues (Rodriguez et al. 2018). Previous studies have demonstrated that extracts obtained from various *Achillea* species have the ability to stimulate collagen production in dermal fibroblasts (Agar et al., 2015). From this point of view, in our study, the effects of AS extracts on collagen production were evaluated by measuring hydroxyproline level, which is considered a marker for collagen biosynthesis (Anlas et al., 2019). The changes in hydroxyproline levels following treatment with ASA and ASE were demonstrated in Fig. 4. Treatment of cells with ASE resulted in an approximately 1.05 to 1.25-fold increase in hydroxyproline synthesis compared with untreated cells ( $P < 0.05$ ). On the other hand, ASA significantly stimulated hydroxyproline synthesis (2.51-

fold) compared with control only at the highest concentration ( $P < 0.01$ ).

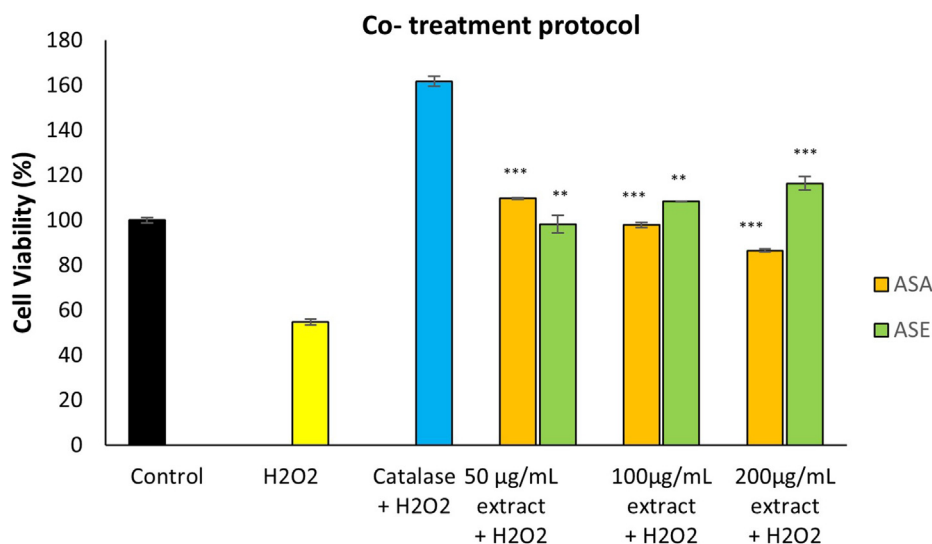
To the best of our knowledge, there is no study revealing the effect of *Achillea* species on collagen production, by directly measuring the hydroxyproline level in fibroblasts. On the other hand, our findings are consistent with the results of studies demonstrating the effects of various *Achillea* species on collagen synthesis by different *in vivo* and *in vitro* methods. In the study conducted by Agar et al. (2015), the morphometric analysis revealed that methanolic extracts of *Achillea kotschyi* Boiss. subsp. *kotschyi* and *Achillea coarctata* Poir. significantly increase the number of collagen granules in mouse fibroblasts. Similarly, histological observations on wound healing properties of *Achillea biebersteinii* Afan. indicated that especially the *n*-hexane extract has stimulated the formation of collagen fibers (Küpelı Akkol et al., 2011). Considering our



**Fig. 4** Hydroxyproline content in the groups treated with different extracts from *A. Sintensisii* Hub-Mor. AA, Ascorbic acid; ASA, Aqueous extract; ASE, Ethanolic extract. \*  $P < 0.05$ , \*\* $P < 0.01$  versus control cells.



**Fig. 5** Effect of *A. sintensisii* Hub-Mor. extracts on hydrogen peroxide-induced damage in fibroblasts following pre-treatment protocol-H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide; ASA, Aqueous extract; ASE, Ethanolic extract. \*\*\* $P < 0.001$  versus cells exposed to only H<sub>2</sub>O<sub>2</sub> without extract.



**Fig. 6** Effect of *A. sintenisii* Hub-Mor. extracts on hydrogen peroxide-induced damage in fibroblasts following co-treatment protocol-H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide; ASA, Aqueous extract; ASE, Ethanolic extract. \*\*P < 0.01, \*\*\*P < 0.001 versus cells exposed to only H<sub>2</sub>O<sub>2</sub> without extract.

results, it can be thought that AS extracts may contribute to the treatment of pathologies characterized by collagen disorders by promoting collagen synthesis, especially at high concentrations. The effect of AS extracts on collagen synthesis can be attributed to the presence of phenolics present in the extracts (such as rutin), which have previously proven to be effective on collagen biosynthesis in dermal fibroblasts (Jampa et al., 2022; Stipcevic et al., 2006).

### 3.7. Protective effect of *A. sintenisii* Hub-Mor. extracts on H<sub>2</sub>O<sub>2</sub>-induced damage

Oxidative stress has a crucial role in the development and pathogenesis of many diseases, by causing profound changes in biological structures, such as cellular membranes, proteins, lipids, and nucleic acids (Luo et al., 2018). In recent years, interest in plant-derived antioxidants which have protective properties against oxidative stress-induced damage caused by free radicals has increased (Sharathchandra and Rajashekhar, 2013). In this context, it has been revealed that many plant species have high antioxidant capacity, especially in relation to their polyphenol and flavonoid contents (Konyalioglu and Karamenderes, 2005; Ustuner et al., 2019). Previous reports on different *Achillea* species have demonstrated that this plant displayed pronounced antioxidant activity by protecting the cells against oxidative damage induced by H<sub>2</sub>O<sub>2</sub> (Varasteh-kojournian et al. 2016). Accordingly, in the present study, AS extracts were assessed for their ability to protect fibroblasts against H<sub>2</sub>O<sub>2</sub>-induced damage using two different treatment protocols.

As shown in Fig. 5 and Fig. 6, treatment with H<sub>2</sub>O<sub>2</sub> alone, reduced the viability of fibroblasts to approximately 54 %. The viability of cells pre-treated and co-treated with AS extracts was 1.89 to 2.20-fold and 1.58 to 2.12-fold higher respectively, compared with the cells treated with H<sub>2</sub>O<sub>2</sub> alone. In the pre-treatment protocol, ASA (at 200 µg/mL concentration) offered the highest protection against oxidative damage almost com-

parable with that of catalase (positive control). Both extracts exhibited significant protective activity against H<sub>2</sub>O<sub>2</sub>-induced damage in both co-treatment and pre-treatment protocols.

Our findings are similar to the results of the study conducted by Varasteh-kojournian et al. (2016), who reported that methanolic extract of *Achillea biebersteinii* can inhibit H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in fibroblasts. Additionally, Konyalioglu and Karamenderes (2005) have reported that different *Achillea* species protected the antioxidant enzyme levels of erythrocytes and leukocytes against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage, and this effect might be attributed to the rich phenolic contents of the extracts. Since AS extracts contain a wide variety of phytoconstituents with antioxidant properties (luteolin, quercetin, rutin, isorhamnetin, etc.), as demonstrated by our phytochemical analyses, it is thought that cytoprotective effects of the extracts may be a result of interactions of different antioxidants in the extracts (Gong et al. 2020; Boots et al., 2008; Ozgen et al., 2016).

## 4. Conclusion

Our findings scientifically support the traditional use of this plant by demonstrating its ability to proliferate fibroblasts, promote collagen synthesis, protect cells against oxidative damage, as well as exhibit antibacterial, antioxidant, and hyaluronidase inhibitory activities. In addition to the therapeutic potential of AS extracts, they did not exhibit cytotoxic activity on fibroblasts, suggesting their safety profile. The present study also provides important data regarding the phytochemical composition of two different AS extracts. Summarizing the results of this study, it can be concluded that AS extracts may have therapeutic potential in many pathological conditions associated with oxidative stress, hyaluronidase inhibition, collagen synthesis, or inflammation, as a result of their valuable pharmacological activities. Also presented results suggest that AS extracts could be used in the development of new cosmetics as a result of their effects, especially on collagen synthesis, cell proliferation, and hyaluronidase inhibition. This is the first report in the literature investigating various biological activities of AS extracts. However, further detailed *in vivo* studies are needed to reveal their effects on the biological system.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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