



## *In vitro* assessment of dermatological activity potential of *Achillea clypeolata* Sm. in H<sub>2</sub>O<sub>2</sub>-treated human dermal fibroblasts

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### ABSTRACT

Members of *Achillea* L. genus are widely used against dermatological disorders in traditional medicine. An increasing number of experimental studies indicated that these species are an important source of ailments against such conditions. Besides, clinical studies yield supportive results about the dermatological effects of the genus. However, the number of studies stating the mechanisms of actions is quite scarce. *A. clypeolata* Sm. grows naturally in the Thrace region of Türkiye, and is used against various health problems in folk medicine. The methanolic extract prepared from the aerial parts of the plant (ACM) was subjected to a series of tests focusing on the mechanisms of dermatological activity. First, *in vitro* antioxidant screening tests, including DPPH, CUPRAC, FRAP and TOAC were applied. Then, the inhibitory potential of ACM against skin-related enzymes such as collagenase, elastase and hyaluronidase was measured. For a more detailed profiling of the activity, human dermal fibroblast cells (HDFs) treated with H<sub>2</sub>O<sub>2</sub> were given varying concentrations of ACM. WST-1 analysis was carried out for the assessment of cell viability, and DCFDA (2',7'-dichlorofluorescein diacetate) method was employed for the measurement of cellular antioxidant activity. In addition, the inhibitory potentials of ACM against matrix metalloproteinase -2 and -9 enzymes, which are responsible for the degradation of extracellular matrix components in the skin, were also tested. The total phenolic (26.02 ± 1.04 mg GAE/g dry extract) and flavonoid (12.85 ± 0.69 mg QE/g dry extract) contents of ACM were calculated with the activity tests. Also, for a comprehensive quantitative analysis, LC-MS/MS analysis was carried out. All of the data gained from the mentioned tests and analysis revealed the high capacity of ACM in dermatological conditions such as the inhibitory potential enzymes associated with skin aging. Phytochemical profiling studies showed that the extract is rich in phenolic compounds, specifically chlorogenic acid, hesperidin and hyperoside. Data obtained from this study suggest that *A. clypeolata* may act as a natural source of skin-active ingredients.

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### 1. Introduction

Genus *Achillea* is a member of the Asteraceae family and has been a very important source of various folk remedies throughout history due to its rich secondary metabolite profile (Cirak et al., 2022). There are more than 120 species included in the genus, and they are widely distributed across the globe, such as in America, Europa, Asia, and Australia. Türkiye is one of the most important *Achillea* regions in the world, with forty-eight recorded species in the Turkish flora, half endemic (Toplan et al., 2022). *Achillea* species are utilized in folk

medicine against various health disorders such as pneumonia, inflammations, gynaecologic problems, gastrointestinal disorders etc. Furthermore, one of the most prominent uses of *Achillea* species is against various skin problems. Ethnobotanical studies demonstrate that species from *Achillea* genus are used against wounds, rashes, psoriasis, sprains and as an astringent for skin conditions such as edema and erythema (Barda et al., 2021). Clinical studies based on traditional uses also showed promising results for *Achillea* utilization against skin problems (Salehi et al., 2020). In the light of the ethnobotanical and bioactivity studies, interest in *Achillea* species as wound healers and cosmetic ingredients is on the rise (Strzypek-Gomółka et al., 2021). They can be regarded as good candidates for antiaging formulations due to their phytochemical profile rich in phenolic compounds, which are known for their antioxidant and inhibitory potential for particular enzymes related to the skin aging process.

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Literature demonstrates various *Achillea* species are rich sources of numerous phenolic compound particularly phenolic acids (i.e. chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, etc.) and flavonoids (i.e. naringenin, luteolin, quercetin, kaempferol, apigenin and their glycosides, etc.) are prominent for their potent antioxidant properties (Salehi et al., 2020).

Skin is the outermost layer of defense for humans against foreign features. As people age, skin experiences some alterations caused by both internal and environmental impacts which may lead to skin aging. Some of the most obvious signs of skin aging are wrinkles, sagging skin, age spots, dryness, as well as fat loss that causes the skin to lose its natural smoothness (Melfa et al., 2020). The primary structural elements of the dermal extracellular matrix (ECM) are collagen, elastin, and hyaluronic acid. Collagen, constituting 80% of the dry weight of the skin, gives the skin its tensile strength. Elastin fibers are brittle when dry but flexible and elastic when moist and preserve skin elasticity. Hyaluronic acid, a mucopolysaccharide, maintains the viscoelasticity, smoothness, and hydration of the skin. Chronic UV radiation exposure is the primary cause of extrinsic skin aging, which increases the formation of reactive oxygen species (ROS) and causes structural alterations in the ECM. It is clearly demonstrated that with increased ROS, the expression of proteolytic enzymes such as matrix metalloproteinases (MMPs) as well as serine proteases like elastase and glycosidase enzyme hyaluronidase. These are important enzymes in the aging skin process breaking down collagen, elastin, and hyaluronic acid, causing the ECM to remodel and the skin has a lesser amount of elasticity (Senol Deniz et al., 2020; Ibrahim et al., 2022; Senol Deniz et al., 2021).

*Achillea clypeolata* Sm. grows naturally in the flora of Türkiye, particularly in the Thrace region. Previous studies underlined its antimicrobial properties and protective effects on H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in human erythrocytes and leucocytes (Karaalp et al., 2009; Konyalioglu and Karamenderes, 2005). Phytochemical studies were employed to evaluate terpenic compounds, essential oils and some flavonoids together with coumarins of *A. clypeolata* (Djurdjević et al., 2013; Simić et al., 2005; Werner et al., 2007). Anti-aging potential and detailed phytochemical investigation for the phenolic profile of *A. clypeolata* have not been reported previously, to our knowledge. In the light of these information, antioxidant and enzyme inhibitory bioactivities of *A. clypeolata* methanolic extract were evaluated, in addition with detailed phytochemical investigation with LC-MS for this study. DPPH, FRAP, CUPRAC and TOAC assays were conducted to evaluate *in vitro* antioxidant potential of the extract while DCFDA assay were carried out to reveal its cellular antioxidant properties. Inhibition potential of aging-related enzymes such as elastase, hyaluronidase and collagenase, were determined by *in vitro* assays. Also, the inhibitory potential of the methanolic extract prepared from the aerial parts of *A. clypeolata* (ACM) on matrix metalloproteinases of HDFs, and its influence on cell viability were tested.

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo Fischer (USA). Coomassie Brilliant blue R-250 was purchased from Bio-Rad (USA). All other chemicals, reagents, enzymes and references used in the experimental protocols were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The quality of all chemicals was of analytical grade or purer.

### 2.2. Plant material

The aerial parts of *A. clypeolata* were collected from Dereköy, Kırklareli in June 2019. Botanical identification of plant samples was carried out by Prof. Dr. Galip Akaydin. A voucher sample of the plant was

kept in the Herbarium of Faculty of Education, Hacettepe University, Ankara, Türkiye (Akaydin 16,312).

### 2.3. Preparation of the extract

70 g of dried and powdered aerial parts of *A. clypeolata* were macerated in 700 mL of methanol and were shaken with an orbital shaker device for 24 h. The maceration process was repeated on the remains twice by shaking for 24 h. Following the elimination of the marc, methanol was completely evaporated by a rotavapor and the residue was lyophilized. The yield of the extract (ACM) was calculated as 6.34%.

### 2.4. In vitro investigation of the phenolic profile

*In vitro* evaluation for phenolic profile of ACM was done with previously published colorimetric methods. Total phenol content study was done with Folin–Ciocalteu reagent and the blue molybdenum-tungsten complex which occurs in the presence of phenolics were measured at 765 nm and results were given as mg gallic acid equivalents per g dried extract. Total flavonoid content was determined by measuring the absorbance of acid stable complexes after the reaction between flavonoids and AlCl<sub>3</sub>. Absorbance was measured in 415 nm and results were expressed as quercetin equivalents. Both assays were done in triplicates (Barak et al., 2022a).

### 2.5. In vitro evaluation of antioxidant potential

*In vitro* antioxidant potential of ACM was determined via previously published methods based on metal reducing and free radical scavenging capacities. Metal reducing activity of the extract was measured with CUPRAC, FRAP and TOAC assays. FRAP assay is based on reduction of ferric ions with a reducing agent. Ferric tripyridyl triazine complex was reduced to ferrous form at low pH and absorbance was determined at 593 nm. Results were given as mM FeSO<sub>4</sub> per g dry extract. CUPRAC and TOAC assays were based on reduction potential of samples for cupric and Mo (VI) ions, respectively. Ascorbic acid was used as the standard compound for both assays and results were given as mg ascorbic acid equivalent per g dried extract. Absorbances were measured at 450 nm for CUPRAC and 695 nm for TOAC assay. All measurements were made in triplicates and standard deviation was stated in the results (Bardakci et al., 2020).

### 2.6. Determination of phenolic compounds by LC-MS/MS

Determination of phenolic profile of ACM, high-performance liquid chromatography-mass spectrometer - mass spectrometer (Agilent 1260 Triple Quadrupole MS/MS) was used. Each analysis was performed with three replications. HPLC column C18 ODS was used in the analyses (25 × 4.6 mm × 5 μm). Injection volume for analysis: 2 μL. Water/0.1% formic acid (A), methyl alcohol (99.9%) (B) was used as a carrier phase. The gradient method is as follows: 3 min 2% B, 6 min 25% B, 10 min 50% B, 14 min 95% B, 17.5 min 2% B. Flow rate: 0.4 mL/min. The identification of compounds was performed in positive and negative modes (Gören et al., 2009).

### 2.7. Inhibition of skin enzymes associated with ECM turnover

The time-dependent inhibition potential of ACM at 0.5 mg/mL on collagenase, elastase and hyaluronidase enzymes was tested by colorimetric methods. 0.25 mg/mL EGCG was used as positive control in anti-collagenase and anti-elastase tests, while tannic acid was used in anti-hyaluronidase test (Ersoy et al., 2019; Barak et al., 2022b). In this context, the effects of ACM on anti-collagenase, anti-elastase and anti-hyaluronidase enzymes were performed according to the

protocol described by Barak et al. (2022b), and inhibition levels of enzymes were determined at five different time points.

## 2.8. Cell culture and WST-1

Human dermal fibroblast cells, which we used in our previous publication, were purchased commercially from ATCC (Kurt-Celep et al., 2020). HDF cells were grown in a sterile cell incubator at 37 °C containing 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The cells were grown in complete high glucose-Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma-Aldrich. When the HDF cells covered approximately 80% of the growth plates, they were passaged with a detachment solution containing 0.25% Trypsin and 0.53 mM EDTA. They were stocked for the next experiment in liquid nitrogen. WST-1 reagent was used to determine the nontoxic concentrations of H<sub>2</sub>O<sub>2</sub> and ACM applied to HDF cells and their effect on cell viability. HDF cells were seeded at a density of  $1 \times 10^4$  in a 96-well plate. Then, the cells were treated with different concentrations of ACM prepared in serum-free medium for 24, 48 and 72 h, respectively. A similar procedure was applied to determine the nontoxic concentration of H<sub>2</sub>O<sub>2</sub> and HDF cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 2 h. At the end of the incubation processes, cells were incubated with 1:10 diluted WST-1 reagent for 45 min and then absorbance measurements were taken at 570 nm wavelength (Kurt-Celep et al., 2021, 2022a, 2023).

## 2.9. Detection of intracellular reactive oxygen species levels with DCFDA reagent

Following the determination of nontoxic ACM and H<sub>2</sub>O<sub>2</sub> concentrations in HDF cells with WST-1, 2',7'-dichlorofluorescein diacetate (DCFDA) assay was used to detect H<sub>2</sub>O<sub>2</sub>-driven ROS levels in cells and to elucidate the role of ACM samples in reducing the amount of cellular ROS. HDF cells were induced with 75 μmol/L H<sub>2</sub>O<sub>2</sub> for two hours then the cells were treated with ACM at concentrations of 75 μg/mL and 100 μg/mL for 24 and 48 h. EGCG was used as a positive control. After the incubations of ACM and EGCG were finished, complete-DMEM was removed from these cells, and the cells were incubated for 45 min with 10 μM DCFDA reagent in serum-free DMEM. At the end of the incubation, fluorescence measurement was taken with the help of Thermo Scientific™ Varioskan™ LUX instrument in the wavelength range of 485 nm between 535 nm (Kirindage et al., 2022; Lee et al., 2022).

## 2.10. Gelatin zymograph assay

The gelatinase zymography assay was used to test the ECM degradation and the change in MMP levels caused by the H<sub>2</sub>O<sub>2</sub>-driven increased amount of ROS (Kurt-Celep et al., 2022b). Different concentrations of ACM (75 and 100 μg/mL) were applied to HDF cells treated with H<sub>2</sub>O<sub>2</sub>. After 48 h of ACM incubation, the medium was collected for the detection of gelatinase MMPs released into the extracellular environment of the cells, and the levels of MMP-2 and MMP-9 were determined with the help of an electrical field in native-PAGE containing 1% gelatine.

## 2.11. Statistical analysis

The presented experiments within the scope of the article were performed at independent times and by repeating them at least three times. All experiments were performed with GraphPad Prism software version 8. While 2-way ANOVA multiple comparisons tab was used in WST-1 experiment for cell viability and nontoxic concentration determination, one-way ANOVA statistical analysis was performed in skin enzymes assay (anti-collagenase, anti-elastase, and anti-hyaluronidase) and gelatinase zymography (MMP-2 and MMP-

9) experiment. In addition, 2-way ANOVA multiple comparisons analysis was used for the DCFDA assay in which the cellular oxidative stress level was measured.

## 3. Results and discussion

### 3.1. Phytochemical evaluation

An extensive phytochemical evaluation was performed on ACM to reveal its phenolic profile with sufficient amount of detail. Initially, the total phenolic and total flavonoid *in vitro* assays were conducted for obtaining a general overview of phenolic profile of the extract. Results showed that ACM had a total phenolic content of  $26.02 \pm 1.04$  mg GAE/g dry extract total phenolic content and  $12.85 \pm 0.69$  mg QE/g dry extract total flavonoid content. In a previous study, Konyalioglu and Karamenderes (2005) investigated infusions of 15 different *Achillea* species including *A. clypeolata*, which were also collected from northwest Türkiye for their total phenol and flavonoid contents. Similarly, *A. clypeolata* showed significant amount of total phenol and flavonoid content in that study,  $109.09 \pm 1.82$  mg/L and  $0.131 \pm 0.0086\%$ , respectively. The total phenolic content assay was conducted on various *Achillea* species collected from different parts of Türkiye in previous studies and results revealed that *Achillea* species have a significant amount of phenolic content (Agar et al.; 2015, Eruygur et al.; 2019). The phenolic ingredients have been observed to be responsible for most of the physiological bioactivities of the *Achillea* species (Strzpek-Gomóika et al., 2021). Thus, such evaluation of phenolic ingredients is essential for the accurate conception of potential bioactivities of extracts. For this reason, extensive evaluation with LC/MS was conducted to determine a phytochemical profile of the extract and quantitative results were given in Table 1.

**Table 1**  
Content (ng/mL) of bioactive compounds in ACM.

Compound	Transition	RT	Content
Gallic acid	168.9 -> 125.0	8.891	256 ± 3
3-hydroxytyrosol	153.0 -> 123.0	10.368	22 ± 0
Protocatechuic acid	152.9 -> 108.9	10.699	1736 ± 14
3,4-Dihydroxyphenylacetic acid	167.0 -> 123.0	11.005	21.3 ± 0.6
Pyrocatechol	109.0 -> 52.9	11.033	5.6 ± 1.5
(+)-Catechin	289.0 -> 245.0	11.412	ND
Chlorogenic acid	355.0 -> 163.0	11.869	60,605 ± 2121
3-Hydroxybenzoic acid	137.0 -> 93.0	13.022	10.6 ± 0.5
4-Hydroxybenzoic acid	136.9 -> 93.1	12.249	532 ± 1.73
2,5-Dihydroxybenzoic acid	152.9 -> 109.0	12.173	287 ± 6
(-)-Epicatechin	291.0 -> 139.1	12.437	6 ± 0
Homovanillic acid	181.0 -> 137.1	12.742	164 ± 30
Caffeic acid	179.0 -> 135.0	12.760	1525 ± 23
Syringic acid	196.9 -> 181.9	12.866	765 ± 25
Vanillic acid	166.9 -> 122.6	12.668	460 ± 24
Vanillin	151.0 -> 136.0	13.191	531 ± 25
Verbascoside	623.0 -> 160.8	13.535	ND
Taxifolin	303.0 -> 285.1	13.814	ND
2-Hydroxycinnamic acid	162.9 -> 119.1	14.653	ND
p-Coumaric acid	162.9 -> 119.0	13.910	34 ± 1.5
Sinapic acid	222.9 -> 207.9	13.874	ND
Ferulic acid	193.0 -> 134.0	14.033	94 ± 5
Luteolin 7-glucoside	447.1 -> 285.0	14.332	1133 ± 6
Hesperidin	611.1 -> 303.0	14.521	45,515 ± 1206
Rosmarinic acid	359.0 -> 160.9	14.542	21.6 ± 1.5
Hyperoside	465.1 -> 303.1	14.564	13,321 ± 204
Apigenin 7-glucoside	433.1 -> 271.0	14.799	270 ± 12
Pinoresinol	357.0 -> 151.0	15.020	27.6 ± 4.7
Eriodictyol	287.0 -> 134.9	15.147	ND
Quercetin	301.0 -> 151.0	15.637	591 ± 3
Luteolin	287.0 -> 153.1	15.876	3811 ± 23
Kaempferol	285.0 -> 229.1	16.189	23.3 ± 2
Apigenin	271.0 -> 153.0	16.312	478 ± 9

The results were given as mean ± standard deviation ( $n = 3$ ). ACM: Methanolic extract of *A. clypeolata* aerial parts. ND: not detected.

The presence of 34 different phytochemical ingredients were investigated with LC/MS method, and 27 were found in the extract. Results showed that chlorogenic acid, a well-known quinic acid derivative, is the major metabolite of ACM ( $60.6 \pm 2.1 \mu\text{g/mL}$ ) in the extract. In addition, two flavonoid glycosides hesperidin and hyperoside were found in considerable amounts ( $45.5 \pm 0.1$  and  $13.3 \pm 0.2 \mu\text{g/mL}$ , respectively). Alongside these major metabolites, some other phenolic compounds were found at significant levels. Luteolin, a flavone aglycone was found to be  $3.811 \pm 0.02 \mu\text{g/mL}$  and two other phenolic acids, protocatechuic acid and caffeic acid were determined as  $1.73 \pm 0.01$  and  $1.53 \pm 0.02 \mu\text{g/mL}$ , respectively. In addition, flavonoid glycosides such as luteolin-7-glucoside and apigenin-7-glucoside were found in noteworthy amounts ( $1133 \pm 6$  and  $270 \pm 12 \text{ ng/mL}$ , respectively), a flavonoid aglycone quercetin was also quantified in the extract as  $591 \pm 3 \text{ ng/mL}$ .

In previous studies, essential oil, diterpenes and sesquiterpenes of *A. clypeolata* were investigated (Karaalp et al., 2009). Additionally, Werner et al. (2007) isolated centaureidin and scopoletin, a flavonoid and a coumarin, respectively. Nonetheless, this is the first study that focused on the assessment of the phytochemical profile of *A. clypeolata* with extensive LC-MS method, to our knowledge. However, there are several studies investigated other species from *Achillea* genus collected from Türkiye. Agar et al. (2015) conducted LC-MS analysis on three *Achillea* species growing in Türkiye: *A. coarctata*, *A. kotschyi* and *A. lycaonica*. Results revealed that chlorogenic acid is a major metabolite of methanolic extracts from aerial parts of the plants. In addition, hesperidin and hyperoside were detected in significant amounts in *A. kotschyi* and *A. lycaonica*, on the contrary, neither of the compounds was detected in *A. coarctata*. Results of the study showed a relatively inferior phenolic profile in *A. coarctata*, compared to other species. In addition, luteolin and caffeic acid were detected in three extracts, in significant amounts, while protocatechuic acid was not detected in *A. coarctata*. LC/MS results of the study conducted by Agar et al. (2015) showed considerable correspondence to our findings. In another study, different extracts of *A. monocephala* were investigated with a validated LC/MS method (Yilmaz et al., 2018). Results showed that chlorogenic acid is the most abundant ingredient of the ethanolic extract obtained from the aerial parts ( $12.7 \text{ mg/g}$ ), consistent with the results of the present study. Corresponding to our findings, luteolin, protocatechuic acid, hesperidin and caffeic acid were found in notable amounts (2343, 351, 314 and  $8.36 \mu\text{g/g}$ , respectively). In addition, various studies showed that chlorogenic acid was abundant for several species in *Achillea* genus, including *A. alpina*, *A. asiatica*, *A. biebersteinii*, *A. coarctata*, *A. distans*, *A. kotschyi*, *A. ligustica*, *A. lycaonica*, *A. millefolium*, *A. multifidi*, *A. schurii*, *A. setacea*, *A. vermicularis* and *A. wilhelmsii*. Caffeic acid and luteolin were also detected extensively in the genus, numerous studies showed their presence (Barda et al., 2021). Within this context, results of our study are in agreement with previously published studies and it can be seen that the methanol extracts of the *Achillea* species are fairly rich in terms of phenolic compounds.

### 3.2. In vitro antioxidant screening tests

Reactive oxygen species (ROS), either in radical or non-radical form, are inevitable end-products of oxidative stress, which is the result of a normal metabolic process. They impose critical negative effects on various bodily tissues including the skin. The aggregation of ROS together with insufficient antioxidant defense mechanisms of the body leads to a myriad of modifications in the skin including the oxidation of lipid molecules in cell membranes and binding with proteins or nucleic acids. This will result in impairment in the permeability of cell membranes and affect cellular transport. Therefore, keeping the ROS production and ROS-related damage at bay is a potent way of tackling such effects on the skin itself (Zi et al., 2018). Various frequently used *in vitro* antioxidant tests with different mechanisms

**Table 2**

*In vitro* preliminary tests for the antioxidant potential.

	DPPH <sup>1</sup>	TOAC <sup>2</sup>	FRAP <sup>3</sup>	CUPRAC <sup>2</sup>
ACM*	$290.8 \pm 11.27$	$154.57 \pm 5.99$	$0.99 \pm 0.02$	$138.01 \pm 6.40$

<sup>1</sup> Results were expressed as the mean of triplicates  $\pm$  standard deviation (S. D.) and as mg butylated hydroxyl toluene equivalents (BHTE) in 1 g sample.

<sup>2</sup> Results were expressed as the mean of triplicates  $\pm$  standard deviation (S. D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.

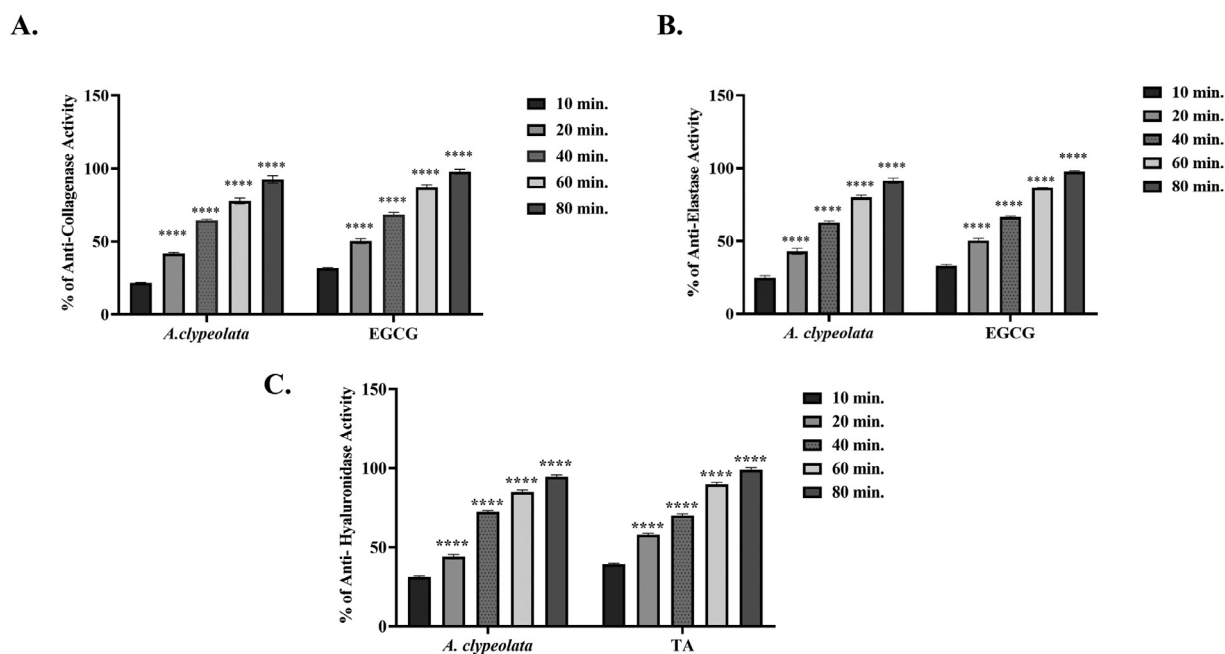
<sup>3</sup> Results were expressed as the mean of triplicates  $\pm$  standard deviation (S. D.) and as mM FeSO<sub>4</sub> equivalents in 1 g sample.

\* Methanolic extract prepared from the aerial parts of *A. clypeolata*.

such as 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) scavenging activity, ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC) and total antioxidant capacity (TOAC) were applied for preliminary screening of the plant extract. Results shared in Table 2. indicated that the mentioned extract possesses both radical scavenging activity and metal reducing capacity, which are major mechanisms of antioxidant activity. Recently, *in vitro* antioxidant potential of various extracts from different *Achillea* species growing naturally in Türkiye were studied (Toplan et al., 2022). The results of this comparative study similarly indicated the highest antioxidant potential was spotted in the methanolic extracts of *Achillea* spp. Another study similarly found methanolic extract of *A. millefolium* from Northern Anatolia also possessed strong free radical scavenging and metal reducing activities (Barut et al., 2017). These results cumulatively manifested the high antioxidant potential of alcoholic extracts prepared from *Achillea* plants. Such high activities after methanolic extraction are generally attributed to the presence of phenolic compounds in different structure.

### 3.3. Inhibitory activities on skin-related enzymes

Extracellular matrix (ECM) comprises the dominant part of dermal layer, responsible for skin elasticity, smoothness and development. Major components of ECM include matrix metalloproteins such as collagen and elastin and also proteoglycans such as hyaluronic acid. ECM is prone to degradation not only by chronological aging but also by extrinsic factors causing increased production of ROS, and eventually to oxidative stress. This process brought about a series of changes to the skin including loss of moisture, roughness and also wrinkles. Such physiological and structural changes are arisen due to the loss of ECM molecules. For a healthy skin, the amount of these molecules should remain at an optimum level. Inhibition of enzymes that are responsible for the breakage of these molecules is stated as a strategy to preserve the structural integrity of the skin (Ersoy et al., 2019). Fig. 1 shows the anti-collagenase, anti-elastase and anti-hyaluronidase activities of *A. clypeolata* extract at different time points. The results indicate that the statistically significant inhibitory activities ( $p < 0.01$ ) of the extract increase with time. Similarly, hyaluronic acid, an important ECM component with glycosaminoglycan structure, tends to be prone to degradation by the negative influence of ROS. Predictably, anti-hyaluronidase inhibition plays a considerable role in the treatment of many pathological conditions of the skin, and can be deemed an effective strategy in the mitigation of such problems. Therefore, the inhibitory activities of numerous plant extracts against these enzymes have been widely investigated (Barak et al., 2022b). A recent study on another member of *Achillea* genus (*A. sintensii*) showed that aqueous an ethanolic extracts of this plant concentration-dependently inhibited hyaluronidase enzyme. Similarly, the same study indicated such extracts significantly stimulate collagen synthesis in mouse fibroblasts (Anlas et al., 2023). Also, it should be noted that these activities are very close to EGCG, which was used as the reference compound. Several studies also focused on the inhibitory potentials of plants or plant-related products (Boran, 2018;



**Fig. 1.** Determination of inhibition potential of ACM on skin remodeling dependent enzymes at five different time points. The time-dependent inhibition effect of 0.5 mg/mL ACM extract on collagenase, elastase and hyaluronidase enzymes is presented in Figures A, B and C, respectively. While 250  $\mu\text{g/mL}$  EGCG concentration was used as positive control in collagenase and elastase inhibition experiments, 250  $\mu\text{g/mL}$  TA was used in hyaluronidase inhibition experiment. One-way ANOVA statistical analysis was used for anti-collagenase, elastase and hyaluronidase enzymes activity. The statistical p-value ( $\leq 0.0001$ ) was displayed by \*\*\*\*.

Ersoy et al., 2019). Those rich in polyphenolic compounds seem to manifest high inhibition potential due to the specific conformations of these molecules. Chlorogenic acid, which was found to predominate in the phenolic profile of the extract, was previously shown to be responsible for high anti-collagenase (Boran, 2018; Mandrone et al., 2015), and anti-hyaluronidase activities (Lee and Kim, 2010). Anlas et al. (2023) also pinpointed chlorogenic acid among the major metabolites of *A. sintensis*. Besides, EGCG, which is frequently used as a reference compound for such activity studies, including the current one, also has a polyphenolic structure.

### 3.4. WST-1

ECM ingredients are predominantly synthesized by fibroblasts in the dermis. Therefore, HDF cells are frequently used as a cellular model to appraise the effects of ROS-related cellular damage. Moreover,  $\text{H}_2\text{O}_2$  is a reactive oxygen species (ROS), which is highly produced by-product of electron chain reaction taking place in mitochondria. Since it does not have an ionic charge, it is readily able to diffuse across the cellular membrane resulting in damage by reacting with crucial macromolecules.  $\text{H}_2\text{O}_2$  is directly linked with skin damage and associated skin problems caused by both intrinsic and extrinsic factors. Concerning all of these data,  $\text{H}_2\text{O}_2$ -induced cellular damage in HDF cells is commonly used as a cellular model for studying potential activity against skin aging (Hao et al., 2022; Wen et al., 2017). In the current study, WST-1 assay, which depends on the reduction of tetrazolium salts through the catalyses by enzymes located in the mitochondria, was first employed to pinpoint the non-toxic concentration for use in further tests. The extract was applied to HDF cells at six different concentrations for 24, 48 and 72 h, and cell viability was calculated as 100% at each time point (Fig. 2).

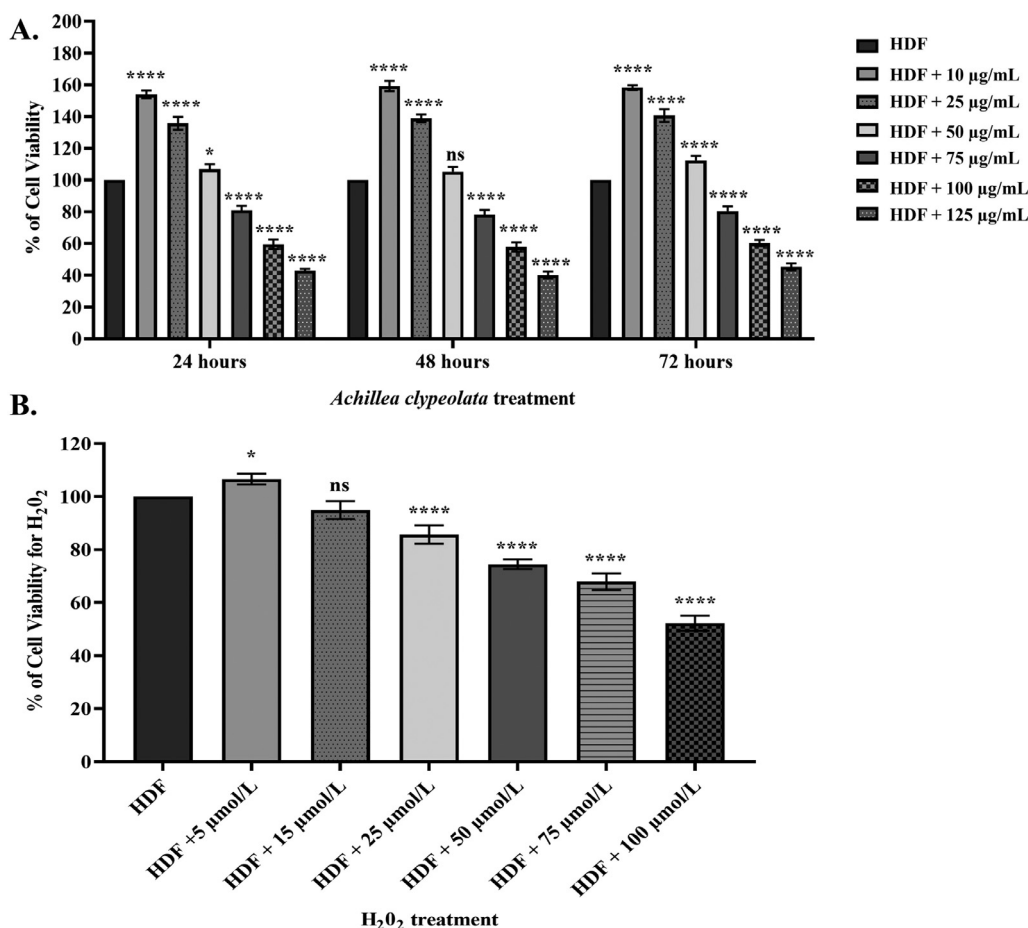
### 3.5. Determination of intracellular ros level

As mentioned above in detail, ROS act directly on the aging process due to both environmental factors and natural means. They are known to trigger a cascade of pathways leading to structural

degradation in ECM of dermal layer. Therefore, HDF cells were chosen for the appraisal of the influence of the extract on intra cellular  $\text{H}_2\text{O}_2$ -induced ROS production and subsequent formation of oxidative stress. Accordingly, ROS-specific fluorescence staining DCFDA was used to detect the intracellular ROS levels in HDF cells treated with  $\text{H}_2\text{O}_2$  and subsequently with the extract. The fluorescence intensity measured at 24 and 48 h was given in Fig. 3. The results showed that  $\text{H}_2\text{O}_2$  treatment tremendously increased ROS levels in the cell lines as expected. Dose-dependent application of the extracts to these cells appears to diminish this level. Especially at 100  $\mu\text{g/mL}$ , the extract induced a nearly total scavenging of intracellular ROS (nonsignificant difference compared to the control group,  $p > 0.05$ ), similar to the reference compound, EGCG. The same method was reported to be used to determine the activity of a flavone molecule, isolated from *A. fragrantissima* on the ROS production of amyloid beta-induced neuroblastoma cell line (Elmann et al., 2011) and likewise, significant decrease in the accumulation of ROS levels was reported. In another analogous study, ROS levels in  $\text{H}_2\text{O}_2$ -treated PC12 cell line were found to decrease following the application of an aqueous extract of *Glechoma hederaceae* L. plant. The extract was also reported to be a major source of chlorogenic acid, akin to ACM (Chao et al., 2022). In a comprehensive study focusing on the effect of infusions prepared from various *Achillea* species on the levels of antioxidant enzymes on human erythrocytes and leukocytes, *A. clypeolata* infusion was found to be protective against  $\text{H}_2\text{O}_2$ -induced oxidative damage (Konyalioglu and Karamenderes, 2005).

### 3.6. Inhibitory activities on MMP-2 & -9

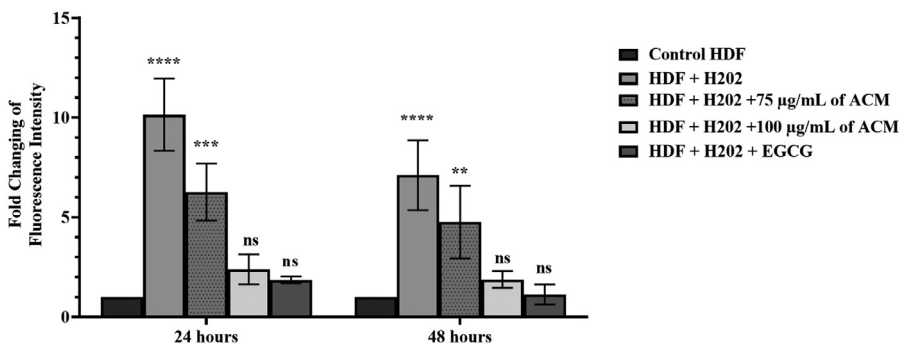
Matrix metalloproteinases (MMPs) constitute a family of structurally analogous enzymes. A myriad of pathophysiological conditions such as inflammation, wound healing, photoaging, arthritis, etc. include MMP activation. They are responsible for the degradation of ECM components and their remodeling in the dermis. MMP secretion is mainly conducted by fibroblasts. In the case of elevated oxidative stress, the expression and activation of MMPs scale up and the breakage of collagen, elastin, gelatine, etc. occurs. Therefore, the inhibition



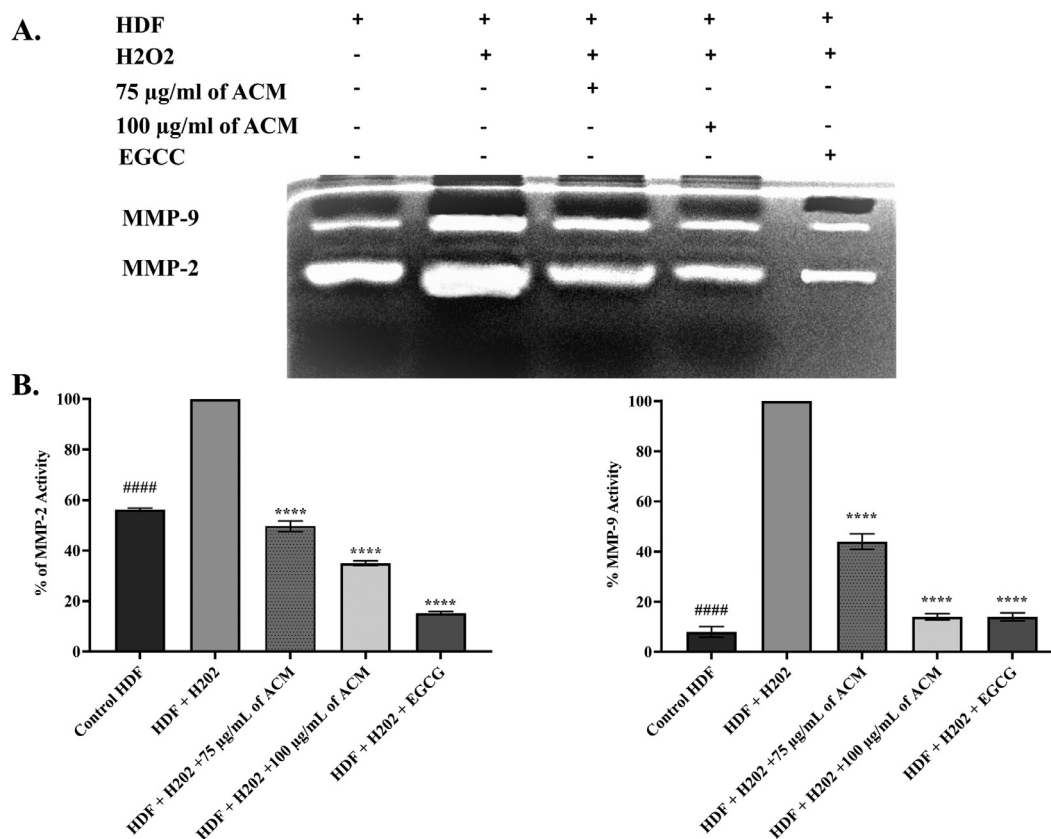
**Fig. 2.** Detection of time-dependent nontoxic concentrations of ACM and H<sub>2</sub>O<sub>2</sub> at increasing concentrations applied to HDF cells by WST-1. The nontoxic concentration of increasing concentration of ACM extract applied to HDF cells at three different time points was determined in Fig. 2A. The nontoxic concentration of increasing concentration of H<sub>2</sub>O<sub>2</sub> applied to HDF cells for two hours was determined in Fig. 2B. In the WST-1 experiment, which was repeated three times, the 2way ANOVA multiple comparisons tab of the GraphPad Prism 8 software program was used for statistical evaluation. The smallest significant p value ( $\leq 0.01$ ) is denoted by \*, while  $p \leq 0.0001$  value is denoted by \*\*\*\*, and ns indicates nonsignificant.

of those enzymes is estimated to have a considerable effect on skin aging. MMP – 2 and –9 act as gelatinases responsible for degrading gelatine and denatured collagen. Interestingly, they have the exceptional ability to degrade all constituents of ECM (Baghel et al., 2022). Given their mentioned importance, the activities of MMP–2 and –9 were analysed in the current study. Gelatine zymography assay was performed for the assessment of H<sub>2</sub>O<sub>2</sub> – induced enhancement of MMP–2 and – 9 in HDFs, and the capacity of ACM to inhibit these

enzymes. The results were illustrated in Fig. 4. The graph demonstrates a nearly two – fold increase in MMP – 2, while there is a ten–fold increase in MMP–9 activities, following H<sub>2</sub>O<sub>2</sub> application. ACM was found to decrease the activities of both enzymes significantly. In particular, the inhibition capacity at 100 µg/mL application of ACM was observed to be very close to that of the reference compound, EGCG. Previously, *A. millefolium* and *A. filipendula* growing in Turkiye were reported to strongly inhibit different MMP members at



**Fig. 3.** Measurement of time-dependent variation of cellular oxidative stress levels with the DCFDA reagent. The oxidative stress created by H<sub>2</sub>O<sub>2</sub> at 75 µmol/L applied to control HDFs for 2 h, and then the oxidation scavenging potentials of ACM in increasing concentration at different time points applied to these cells were determined. The 2-way ANOVA multiple comparisons analysis of the GraphPad Prism 8 program was used for the statistical evaluation of the experiment, which was repeated three times independently. Accordingly, \*\* is used for  $p \leq 0.01$ , \*\*\* is used for  $p \leq 0.001$ , and \*\*\*\* is used for  $p \leq 0.0001$ . The value with no statistical difference is symbolized with ns.



**Fig. 4.** Observation of the effect of ACM applied in increasing concentration to HDF cells on gelatinase inhibition by zymography. The inhibition potential of ACM (at 75 and 100 µg/ml), and EGCC as positive control (250 µg/ml) applied to HDF on MMP-2 and MMP-9 synthesis was determined by gelatin zymography assay. The MMP-2 and MMP-9 enzyme's active forms were observed as white lytic bands on the black background gel (Fig. 4A). The band intensity of all treated and control HDF cells used in the zymography experimental setup was measured by Image J. One-way ANOVA statistical analysis was performed by comparison with samples with negative control H<sub>2</sub>O<sub>2</sub> (Fig. 4B). When H<sub>2</sub>O<sub>2</sub>-treated HDF was compared with control HDF, #### indicated  $p \leq 0.0001$  value. When the similar comparisons were performed for ACM (at 75 and 100 µg/ml) and EGCC (250 µg/ml) respectively, the  $p \leq 0.0001$  value was presented with \*\*\*\* (Fig. 4B).

100 µg/ml (Gonulalan et al., 2019). Also, both aqueous and alcoholic extracts of *Paeonia mascula* were reported to inhibit both MMP-2 and -9 at 50 µg/ml (Kurt-Celep et al., 2023). Similarly, a polar extract prepared from both rhizomes and aerial parts of *Primula auriculata* in HT – 29 colorectal cancer cell lines were found to inhibit MMP-2 and -9 at especially 100 µg/ml (Kurt-Celep et al., 2022a). All of these results indicate that natural products have the capacity to inhibit different members of MMP enzyme family at relatively low doses. Given that inhibition of MMPs have a substantial place in the treatment of various skin disorders, ACM appears to be a candidate for further studies on skin health products.

#### 4. Conclusion

The demand for natural remedies without side effects on dermatological conditions is on the rise, and likely to increase more in the near future. The current study yields data about the dermatological properties of *A. clypeolata*, which has been previously reported for such potential in folk medicine, together with many other *Achillea* species. The tested extract was found to exert cellular antioxidant activity on HDF cells with no negative effects on cell viability. Additionally, the results indicated that the extract inhibited the skin related enzymes and also the matrix metalloprotein enzymes in HDFs, which are responsible for the degradation of the extracellular matrix. Besides, both qualitative and quantitative LC-MS/MS analysis revealed that the extract is rich in terms of phenolic molecules. All of these data indicated that the plant might be considered as a potential

source of dermatological activity. Further studies on *Achillea* spp. in terms natural sources of skin effecting ingredients are required.

#### 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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