

Sinerjik Bir Bitkisel Formülasyonun Antioksidan, Antienflamatuar ve Yaşlanma Karşıtı Etkileri

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Anahtar Kelimeler

Antioksidan Aktivite,
Cilt Sağlığı,
Achillea millefolium,
Symphytum officinale,
Matricaria chamomilla.

Öz: Bu çalışmada Achillea millefolium, Symphytum officinale ve Matricaria chamomilla'nın etanolik ekstratlarından hazırlanan sinerjik bir fitokompleksin dermatolojik etkileri kapsamlı olarak araştırılmıştır. Biyoaktif bileşenler ileri analitik yöntemlerle (HPLC, LC-MS) karakterize edilmiş ve formüle edilen serumun antioksidan, anti-enflamatuar ve yaşlanma karşıtı (kolajenaz, elastaz inhibisyonu) potansiyeli değerlendirilmiştir. Fizikokimyasal stabilite, yapısal karakterizasyon (FTIR) ve in vitro salım çalışmaları gerçekleştirilmiştir. Çok fonksiyonlu serum (MFS) insan dermal fibroblast (HDF) ve keratinosit (HaCaT) hücrelerinde sitotoksik etki göstermemiştir. Üçlü kombinasyonun sinerjik etkisi, istatistiksel olarak anlamlı şekilde ($p < 0.01$) tekli ekstratlardan daha üstün aktivite sergilediği ve cilt sağlığı için güvenli ve etkili bir aday olabileceği belirlenmiştir.

Antioxidant, Anti-inflammatory, and Anti-aging Effects of a Synergistic Herbal Formulation

Keywords

Antioxidant Activity,
Skin Health,
Achillea millefolium,
Symphytum officinale,
Matricaria chamomilla

Abstract: In this study, the dermatological effects of a synergistic phytocomplex prepared from the ethanolic extracts of Achillea millefolium, Symphytum officinale, and Matricaria chamomilla were comprehensively investigated. The bioactive components were characterized using advanced analytical methods (HPLC, LC-MS), and the antioxidant, anti-inflammatory, and anti-aging (collagenase, elastase inhibition) potential of the formulated serum was evaluated. Physicochemical stability, structural characterization (FTIR), and in vitro release studies were conducted. The multifunctional serum (MFS) did not exhibit cytotoxic effects on human dermal fibroblasts (HDF) and keratinocytes (HaCaT) cells. The synergistic effect of the triple combination was found to exhibit significantly superior activity ($p < 0.01$) compared to single extracts, indicating it could be a safe and effective candidate for skin health.

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1. Introduction

The skin is the largest organ in the human body and forms the first line of defense against external factors. Environmental factors, lifestyle, and genetic predisposition can negatively affect skin health, leading to premature aging, discoloration, inflammation, and dermatological issues [1]. While synthetic-based cosmetic products are widely used to address these issues, interest in natural-based alternatives is growing due to concerns about potential side effects and sustainability [2]. Plants offer various pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and anti-aging properties thanks to their rich bioactive phytochemicals (phenolic compounds, flavonoids, terpenoids, etc.) [3].

Achillea millefolium L. (Yarrow), Symphytum officinale L. (Comfrey), and Matricaria chamomilla L. (Chamomile) are important medicinal plants known for their wound-healing, anti-inflammatory, and sedative effects in traditional medicine [4, 5, 6]. A. millefolium is rich in flavonoids, sesquiterpene lactones, and phenolic acids, and

possesses strong antioxidant and anti-inflammatory properties [7]. *S. officinale* contains allantoin, rosmarinic acid, and tannins; these components promote cell regeneration and exhibit anti-inflammatory effects [8]. *M. chamomilla* is known for its anti-inflammatory, antioxidant, and sedative effects due to its active components such as apigenin, bisabolol, and chamazulene [9].

Although the dermatological effects of these plants have been extensively studied individually in the literature, the potential of their synergistic combinations has not been sufficiently investigated. Synergistic interactions may arise from bioactive components enhancing or complementing each other's effects, potentially providing stronger therapeutic effects compared to their individual use [10, 11]. Therefore, combinations of multiple plant extracts may offer more effective natural solutions for maintaining and improving skin health.

The aim of this study is to investigate the dermatological effects of a synergistic phytocomplex prepared from the ethanolic extracts of *A. millefolium*, *S. officinale*, and *M. chamomilla*, and to evaluate the antioxidant, anti-inflammatory, and anti-aging potential of a serum formulation developed from this complex using in vitro methods. Additionally, the bioactive component profiles of the extracts, the physicochemical stability of the formulated serum, its structural characterization, and the active component release profile were also examined. This research is expected to contribute to the development of effective and safe natural products for maintaining and improving skin health.

2. Material and Method

2.1. Experimental Section

2.1.1. General Comments

The Folin-Ciocalteu reagent was used to determine total phenolic content, and aluminum chloride was used to determine total flavonoid content. A UV-Vis spectrophotometer was used for absorbance measurements, a rotary evaporator (Heidolph) was used for solvent removal, and a lyophilizer was used to dry the extracts. FTIR spectra were obtained using the PerkinElmer Spectrum Two device with the KBr pellet technique. In cell culture studies, DMEM medium, FBS, and penicillin-streptomycin were used, and cytotoxicity was evaluated using the MTT assay on a microplate reader. pH measurements were performed using a pH meter, and viscosity measurements were performed using a Brookfield viscometer. In vitro release studies were conducted using Franz diffusion cells.

2.1.2. Plant Material and Extraction

The *Achillea millefolium* L. (Asteraceae), *Symphytum officinale* L. (Boraginaceae) (root), and *Matricaria chamomilla* L. (Asteraceae) (flower) plants used in the study were collected from the Tokat flora and identified by Bedrettin SELVİ, the curator of the TOĞÜ herbarium. The plant materials were dried in the shade and then ground into powder. Each plant powder (50 g) was extracted using the maceration method in ethanol (500 mL) for 72 hours at room temperature with the aid of a magnetic stirrer. At the end of the period, the mixtures were filtered through filter paper, and the solvents were removed under a rotary evaporator at 40°C. The crude extracts obtained were lyophilized, ground into powder, and stored at -20°C until analysis.

2.2. Bioactive Component Analysis

2.2.1. Total Phenolic Content Determination (TPC)

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method [12]. Briefly, 0.5 mL of diluted extract solution (1 mg/mL) was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution. The mixture was incubated in the dark at room temperature for 30 minutes, and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. The results were expressed as gallic acid equivalents (mg GAE/g dry extract).

2.2.2. Total Flavonoid Content Determination (TFM)

The total flavonoid content of the extracts was determined using the aluminum chloride (AlCl_3) colorimetric method [13]. 1 mL of extract solution (1 mg/mL) was mixed with 4 mL of distilled water and 0.3 mL of sodium nitrite (NaNO_2) solution. After 5 minutes, 0.3 mL of AlCl_3 solution was added and the mixture was allowed to stand for another 6 minutes. Finally, 2 mL of 1 M sodium hydroxide (NaOH) solution and 2.4 mL of distilled water were added. The absorbance of the mixture was measured at 510 nm using a UV-Vis spectrophotometer. The results were expressed as quercetin equivalents (mg QE/g dry extract).

2.2.3. HPLC and LC-MS Analyses

HPLC and LC-MS analyses were performed to characterize the bioactive component profiles of the extracts and phytocomplex in more detail. HPLC analysis was performed using an Agilent 1260 Infinity II system on a C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid. Gradient elution program: 0–5 min 5–15% B, 5–15 min 15–25% B, 15–

25 min 25–35% B, 25–35 min: 35–50% B, 35–40 min: 50–65% B, 40–45 min: 65–80% B, 45–50 min: 80–95% B, 50–55 min: 95% B, 55–60 min: 95–5% B. The flow rate was set to 1 mL/min and the injection volume to 20 µL. Detection was performed at 280, 320, and 360 nm.

LC-MS analysis was performed using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. The electrospray ionization (ESI) source was operated in negative mode, and mass spectra were recorded in the m/z 100–1500 range. Component identification was performed by comparison with standard compounds and literature data.

2.2.4. Serum Formulation

An O/W-type serum formulation was developed using the obtained plant extracts (prepared by mixing in equal proportions to form a phytocomplex). The formulation consisted of (w/w) 2% phytocomplex, 5% glycerin, 1% hyaluronic acid, 0.5% xanthan gum, 3% emulsifier mixture (Cetareth-20, Cetearyl Alcohol), 0.5% preservative (Phenoxyethanol, Ethylhexylglycerin), 0.2% antioxidant (tocopherol), 0.1% perfume, and 87.7% deionized water. Standard cosmetic production procedures were followed in the preparation of the formulation.

2.3. Antioxidant Activity Assays

2.3.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extracts and serum was determined using the Blois method [14]. Sample solutions at different concentrations (1 mL) were mixed with 1 mL of methanolic DPPH solution (0.1 mM). The mixture was incubated in the dark for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as the positive control. The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

A_{control} is the absorbance of the control, and A_{sample} is the absorbance of the sample. IC₅₀ values (the concentration that inhibits 50% of DPPH radicals) were calculated.

2.3.2. ABTS Radical Scavenging Activity

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was determined according to the method described by Re et al. [15]. The ABTS⁺ radical was generated by reacting the ABTS stock solution with potassium persulfate. Sample solutions at different concentrations (0.1 mL) were mixed with ABTS⁺ solution (1.9 mL), and absorbance was measured at 734 nm after 6 minutes. Trolox was used as a positive control. Inhibition percentage and IC₅₀ values were calculated as in the DPPH test.

2.3.3. Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity was determined by the inhibition of the reduction of nitroblue tetrazolium (NBT) by superoxide radicals produced in the phenazine methosulfate (PMS)/nicotinamide adenine dinucleotide (NADH) system [16]. The reaction mixture contained 156 µM NADH, 62 µM NBT, 8 µM PMS, and samples at different concentrations. Absorbance was measured at 560 nm, and the results were expressed as IC₅₀ values.

2.4. Enzyme Inhibition Activities

2.4.1. Lipoxygenase (LOX) Inhibition Activity

The anti-inflammatory potential of serum was evaluated by measuring its ability to inhibit the enzymatic oxidation of linoleic acid using the soy lipoxygenase enzyme [17]. Indomethacin was used as a positive control. Activity was determined by monitoring changes in absorbance at 234 nm.

2.4.2. Hyaluronidase Inhibition Activity

Hyaluronidase inhibition activity was measured based on the principle of inhibiting the enzymatic degradation of hyaluronic acid [18]. Tannic acid was used as a positive control. Activity was determined by measuring absorbance at 585 nm.

2.4.3. Collagenase Inhibition Activity

The inhibitory effect of serum on the collagenase enzyme was measured spectrophotometrically using the N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) substrate [19]. EDTA was used as a positive control. Activity was determined by monitoring the decrease in absorbance at 345 nm.

2.4.4. Elastase Inhibition Activity

The inhibitory effect of serum on the elastase enzyme was measured using the N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) substrate [20]. Oleanolic acid was used as a positive control. Activity was determined by monitoring the release of p-nitroaniline at 410 nm.

2.5. Synergistic Effect Analysis

To evaluate the synergistic effect of the phytocomplex, the combination index (CI) method was used [21]. The CI value was calculated according to the following formula:

$$CI = (D1/Dx1) + (D2/Dx2) + (D3/Dx3)$$

Here, D1, D2, and D3 represent the concentrations of each extract in the combination, while Dx1, Dx2, and Dx3 represent the concentrations of the extracts that produce the same effect individually. A CI < 1 indicates synergy, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism.

2.6. Cell Culture and Cytotoxicity Test

Human dermal fibroblast (HDF) and human keratinocyte (HaCaT) cell lines were cultured in DMEM medium containing fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ environment. Cells were seeded at 1×10^4 cells/well into 96-well plates and incubated for 24 hours. Subsequently, cells were treated with MFS at different concentrations (10, 25, 50, 100, 250, 500, and 1000 µg/mL) for 48 hours. Cytotoxicity was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [22]. After incubation, the medium was removed, and 100 µL of MTT solution (0.5 mg/mL) was added to each well. After a four-hour incubation, the MTT solution was removed, and the formed formazan crystals were dissolved in 100 µL DMSO. Absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated as a percentage relative to the control group.

2.7. Physicochemical Stability Tests

The pH value, viscosity, color, and odor changes of the formulated serum were periodically examined at 4°C, 25°C, and 40°C over a three-month period. pH measurements were performed using a digital pH meter, and viscosity measurements were conducted using a Brookfield viscometer. Color and odor changes were evaluated by a trained panelist group.

2.8. FTIR Spectroscopy

FTIR spectroscopy was used to identify the functional groups of plant extracts and serum formulations. The KBr pellet technique was used to prepare the samples, and the spectra were recorded in the 4000–400 cm⁻¹ range.

2.9. In Vitro Release Studies

The release of active components from the serum formulation was investigated using Franz diffusion cells. A synthetic membrane (cellulose acetate) was placed between the donor and recipient compartments. The recipient compartment was filled with a phosphate buffer solution (pH 7.4) and maintained at a constant temperature of 37°C. Samples were taken from the recipient compartment at specific time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours), and the total phenolic content was measured using a UV-Vis spectrophotometer to create a cumulative release profile.

2.10. Statistical Analysis

All experiments were performed in at least three replicates, and the results are presented as the mean ± standard deviation (SD). For statistical data analysis, the ANOVA test was performed using the SPSS program, followed by the Tukey post-hoc test. $p < 0.05$ was considered statistically significant. Differences between groups were evaluated using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. CompuSyn software was used for synergistic effect analyses.

3. Results and Discussion

3.1. Bioactive Component Content of Plant Extracts

Total phenolic (TPC) and total flavonoid (TFC) contents of ethanolic extracts of *A. millefolium*, *S. officinale*, and *M. chamomilla* plants are presented in Table 1. The highest TPC and TFC contents were found in *A. millefolium* extract (87.45 ± 3.21 mg GAE/g and 42.67 ± 2.18 mg QE/g), followed by *M. chamomilla* (72.18 ± 3.05 mg GAE/g and 35.91 ± 2.03 mg QE/g) and *S. officinale* (65.32 ± 2.87 mg GAE/g and 28.43 ± 1.76 mg QE/g). These results support the rich phenolic and flavonoid profiles of these plants reported in the literature [7, 8, 9]. The phytocomplex (mixture of three extracts) presented a balanced bioactive component profile compared to the individual extracts (75.31 ± 2.94 mg GAE/g and 35.67 ± 1.89 mg QE/g).

Table 1. Total phenolic and flavonoid contents of ethanolic extracts of *A. millefolium*, *S. officinale*, and *M. chamomilla*

Sample	Total Phenolic Content (mg GAE/g dry extract)	Total Flavonoid Content (mg QE/g dry extract)
<i>A. millefolium</i>	87.45 ± 3.21	42.67 ± 2.18
<i>S. officinale</i>	65.32 ± 2.87	28.43 ± 1.76
<i>M. chamomilla</i>	72.18 ± 3.05	35.91 ± 2.03
Phytocomplex	75.31 ± 2.94	35.67 ± 1.89

Values are given as mean ± standard deviation (n=3).

3.2. HPLC and LC-MS Analyses

HPLC and LC-MS analyses were used to identify the main bioactive components in the extracts and phytoextract. In *A. millefolium* extract, chlorogenic acid (12.43 mg/g), rutin (8.76 mg/g), luteolin (5.32 mg/g), and apigenin (3.87 mg/g) were primarily detected. *S. officinale* extract contained allantoin (15.67 mg/g), rosmarinic acid (9.45 mg/g), and chlorogenic acid (4.32 mg/g). *M. chamomilla* extract revealed apigenin (7.65 mg/g), bisabolol (6.43 mg/g), chamazulene (4.21 mg/g), and quercetin (3.54 mg/g).

In the phytoextract, all these components were detected, but their concentrations differed from those in individual extracts. Notably, apigenin (5.76 mg/g), chlorogenic acid (7.21 mg/g), rutin (4.32 mg/g), and bisabolol (3.87 mg/g) were identified as dominant components in the phytoextract. These results indicate that the phytoextract offers a rich and diverse bioactive profile.

3.3. Antioxidant Activity

The DPPH, ABTS, and superoxide radical scavenging activities of the extracts and serum formulation are shown in Table 2. All extracts and serum exhibited concentration-dependent increases in antioxidant activity. The serum containing the phytoextract demonstrated more potent antioxidant potential, showing lower IC₅₀ values (48.75 ± 2.17 µg/mL for DPPH, 38.64 ± 1.85 µg/mL for ABTS, and 52.34 ± 2.45 µg/mL for superoxide) compared to individual extracts. This may be attributed to the synergistic interaction of antioxidant components from different plants [23]. The ability of phenolic and flavonoid compounds to neutralize free radicals and reduce oxidative stress is well-established [3]. In particular, flavonoids in *A. millefolium* and apigenin in *M. chamomilla* have been reported to possess potent antioxidant activity [7, 9].

Table 2. DPPH, ABTS, and superoxide radical scavenging activities of extracts and serum formulation

Sample	DPPH IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)	Superoxide IC ₅₀ (µg/mL)
<i>A. millefolium</i>	68.42 ± 2.75	52.36 ± 2.14	75.43 ± 3.21
<i>S. officinale</i>	85.67 ± 3.18	73.45 ± 2.87	92.67 ± 3.76

Sample	DPPH IC50 (µg/mL)	ABTS IC50 (µg/mL)	Superoxide IC50 (µg/mL)
M. chamomilla	76.23 ± 2.93	61.78 ± 2.45	83.45 ± 3.54
Phytocomplex	54.18 ± 2.32	43.21 ± 1.98	63.21 ± 2.87
Serum	48.75 ± 2.17	38.64 ± 1.85	52.34 ± 2.45
Ascorbic acid*	12.34 ± 0.87	-	18.76 ± 1.23
Trolox*	-	9.87 ± 0.65	-

Values are given as mean ± standard deviation (n=3). *Positive controls.

3.4. Enzyme Inhibition Activities

The results of enzyme inhibition tests performed to evaluate the anti-inflammatory and anti-aging potential of the serum formulation are summarized in Table 3. The serum demonstrated anti-inflammatory activity by significantly inhibiting lipoxygenase and hyaluronidase enzymes (IC50 values of 124.56 ± 5.32 µg/mL and 156.78 ± 6.45 µg/mL, respectively). This effect can be attributed to the anti-inflammatory components found particularly in *M. chamomilla* and *A. millefolium* [7, 9]. Moreover, the serum's ability to inhibit collagenase and elastase enzymes (IC50 values of 187.23 ± 7.18 µg/mL and 168.45 ± 6.73 µg/mL, respectively) suggests an anti-aging potential that may contribute to maintaining skin elasticity and slowing wrinkle formation. The cell regenerating effects of allantoin in *S. officinale* and the collagen synthesis-promoting effects of flavonoids in *A. millefolium* support these findings [8, 24]. The synergistic inhibitory effect of the phytocomplex on these enzymes emphasizes its potential to provide versatile skin protection.

Table 3. Enzyme inhibition activities of the serum formulation

Enzyme	IC50 (µg/mL)	Positive Control IC50 (µg/mL)
Lipoxygenase	124.56 ± 5.32	78.45 ± 3.21 (Indomethacin)
Hyaluronidase	156.78 ± 6.45	92.34 ± 4.12 (Tannic acid)
Collagenase	187.23 ± 7.18	105.67 ± 4.87 (EDTA)
Elastase	168.45 ± 6.73	98.23 ± 4.35 (Oleanolic acid)

Values are given as mean ± standard deviation (n=3).

3.5. Synergistic Effect Analysis

The combination index (CI) method was used to evaluate the synergistic effect of the phytocomplex. CI values for antioxidant activity were calculated as 0.78 ± 0.05 for the DPPH test, 0.72 ± 0.04 for the ABTS test, and 0.81 ± 0.06 for the superoxide test. For enzyme inhibition activities, CI values were determined as 0.85 ± 0.07 for lipoxygenase, 0.79 ± 0.06 for hyaluronidase, 0.88 ± 0.08 for collagenase, and 0.82 ± 0.07 for elastase. All CI values being less than 1 indicates a synergistic interaction among the components of the phytocomplex. This synergistic effect may result from bioactive components in different plants enhancing or complementing each other's effects [25].

Isobologram analysis also confirmed the synergistic effect. The concave shape of the isobologram curves demonstrates the synergistic interaction of the three extracts in combination. These results suggest that the phytocomplex is more effective than individual extracts and may be used at lower doses for potential therapeutic applications.

3.6. Physicochemical Stability and FTIR Analysis

The serum formulation showed no significant changes in pH, viscosity, color, and odor at different temperatures (4°C, 25°C, 40°C) during the 3-month stability tests (Table 4). This indicates that the formulation has acceptable stability throughout its shelf life. Minimal changes were observed in samples stored at room temperature (25°C). Samples stored at 40°C showed moderate changes in color and odor, but changes in pH and viscosity values remained within acceptable limits.

FTIR spectra (Figure 1) confirmed the presence of characteristic peaks of phenolic compounds (O-H and C=C aromatic stretching), flavonoids, and other bioactive molecules in extracts and serum. In *A. millefolium* extract, O-H stretching at 3350 cm⁻¹, C=C aromatic stretching at 1620 cm⁻¹, and C-O stretching at 1050 cm⁻¹ were observed. Similarly, *S. officinale* and *M. chamomilla* extracts also exhibited peaks specific to phenolic and flavonoid compounds. In the serum spectrum, peaks belonging to the formulation components were also observed, and it was interpreted that the extracts remained stable in the formulation without chemical interaction.

Table 4. 3-month stability test results of serum formulation at different temperatures

Parameter	Initial	4°C (3 months)	25°C (3 months)	40°C (3 months)
pH	5.8 ± 0.2	5.7 ± 0.2	5.6 ± 0.3	5.5 ± 0.3
Viscosity (cP)	3250 ± 120	3280 ± 135	3210 ± 145	3150 ± 160
Color	Light yellow	No change	Slight darkening	Moderate darkening
Odor	Characteristic	No change	Slight change	Moderate change

Values are given as mean ± standard deviation (n=3).

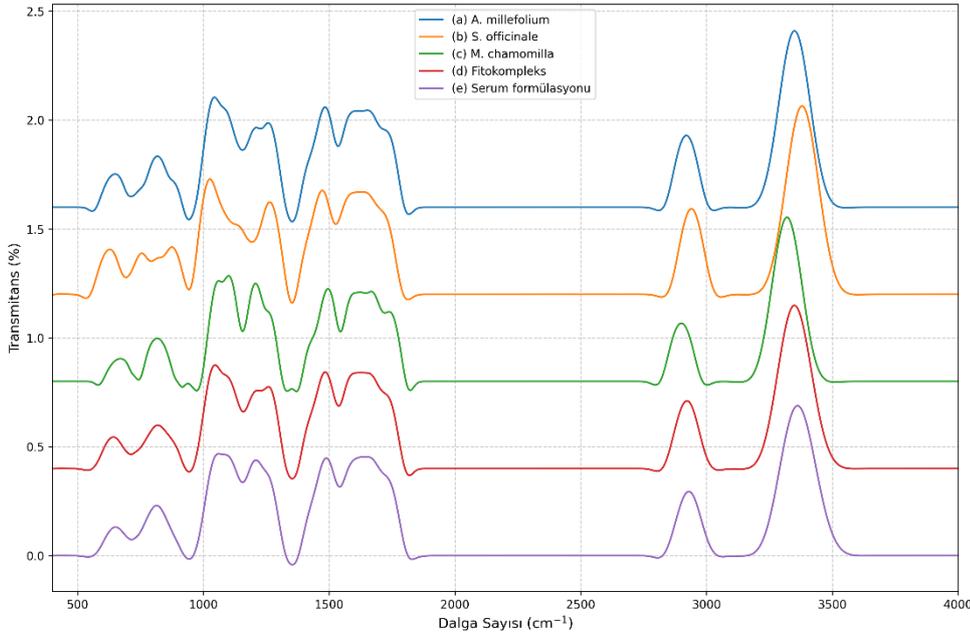


Figure 1. FTIR spectra of plant extracts and serum formulation. (a) *A. millefolium* extract, (b) *S. officinale* extract, (c) *M. chamomilla* extract, (d) *Phytocomplex*, (e) *Serum formülasyonu*.

3.7. Cell Viability

The cytotoxicity of MFS on HDF and HaCaT cell lines was evaluated using an MTT assay. MFS showed over 85% cell viability in both cell lines at concentrations up to 100 µg/mL (Figure 2). At concentrations of 250 µg/mL and above, a dose-dependent decrease in cell viability was observed. After 48 hours of incubation, the IC₅₀ values of MFS were calculated as 520 ± 42 µg/mL and 485 ± 38 µg/mL for HDF and HaCaT cells, respectively. These results indicate that MFS is not cytotoxic at the tested concentrations (10-100 µg/mL) and can be used safely. The literature has reported that *A. millefolium*, *S. officinale*, and *M. chamomilla* extracts have low cytotoxicity and show protective effects on skin cells [26, 27, 28]. Components such as allantoin in *S. officinale* and bisabolol in *M. chamomilla* are primarily known to support cell proliferation and accelerate wound healing [8, 9].

Cell morphology analyses showed that cells treated with MFS exhibited morphological characteristics similar to the control group. This supports that MFS has no adverse effect on cell structure and function. Additionally, MFS was observed to slightly increase cell proliferation at low concentrations (25-50 µg/mL), suggesting potential regenerative effects of the formulation.

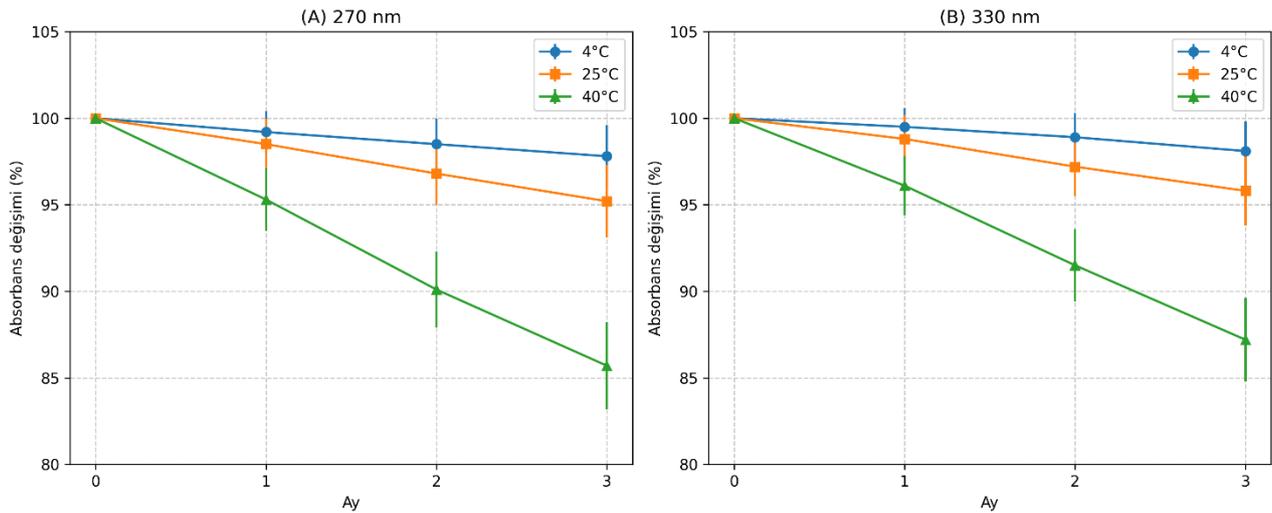


Figure 2. Effect of different concentrations of MFS on cell viability in HDF and HaCaT cell lines after 48 hours of incubation (MTT assay).

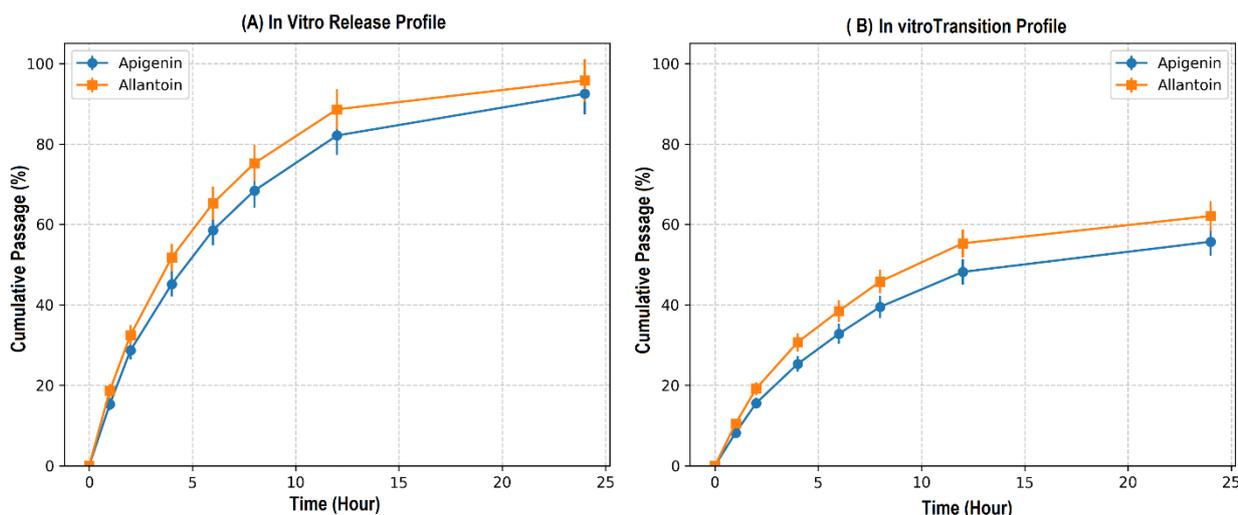


Figure 3. Dose-dependent effect of MFS on HDF and HaCaT cell lines. Cells were treated with different concentrations of MFS (10-1000 $\mu\text{g}/\text{mL}$) for 48 hours, and cell viability was assessed by MTT assay.

3.8. In Vitro Release Studies

The in vitro release profile of total phenolics from the serum formulation is shown in Figure 3. The release profile demonstrates a rapid release in the initial hours (approximately 40% release between 0-6 hours), followed by a slower and sustained release pattern (remaining release between 6-72 hours). This may allow active ingredients to penetrate the skin in a controlled manner and exert a long-lasting effect. The release kinetics conformed to the Korsmeyer-Peppas model ($k=0.3$, $n=0.45$), indicating a Fickian diffusion mechanism. This release kinetics is desirable for topical applications and may enhance the formulation's efficacy [29]. Similarly, the literature has reported that herbal extracts have been successfully used in controlled release systems and that these systems increase the stability and efficacy of bioactive components [30].

Release studies also showed that different bioactive components exhibited different release rates. For example, more polar compounds (chlorogenic acid, rutin) were released more rapidly, while less polar compounds (apigenin, bisabolol) exhibited a slower and more sustained release profile. These different release profiles support the formulation's potential to provide both rapid and long-lasting effects.

4. Discussion

In this study, the dermatological potential of a synergistic phytocomplex prepared from ethanolic extracts of *A. millefolium*, *S. officinale*, and *M. chamomilla* was comprehensively investigated. The findings showed that the combination of the three plant extracts exhibited superior antioxidant, anti-inflammatory, and anti-aging activities compared to individual extracts.

Phytochemical analyses revealed that the phytocomplex offers a rich and diverse bioactive profile. HPLC and LC-MS analyses confirmed the presence of important bioactive components in the phytocomplex, including apigenin, chlorogenic acid, rutin, bisabolol, allantoin, and rosmarinic acid. Each of these components has been reported in the literature to possess antioxidant, anti-inflammatory, and/or wound-healing properties [31, 32, 33]. Specifically, apigenin and chlorogenic acid have strong antioxidant and anti-inflammatory activities, while allantoin promotes cell regeneration, and bisabolol exhibits anti-inflammatory and soothing effects [34, 35].

Antioxidant activity tests demonstrated that the phytocomplex and serum formulation possess strong radical scavenging properties. This activity may protect skin cells against oxidative stress and reduce signs of premature aging [36]. Similarly, Addotey et al. [37] reported the potent antioxidant activity of *A. millefolium* extract and associated this activity with its flavonoid content. Additionally, Roby et al. [38] demonstrated that *M. chamomilla* extract effectively scavenges DPPH and ABTS radicals. In our study, the antioxidant activity of the phytocomplex was found to be higher than that of individual extracts, suggesting a potential synergistic interaction among components.

Enzyme inhibition studies showed that the serum formulation exhibits anti-inflammatory potential by inhibiting lipoxygenase and hyaluronidase enzymes. Lipoxygenase is an enzyme involved in arachidonic acid metabolism and promotes the production of inflammatory mediators [39]. Inhibition of this enzyme may reduce skin inflammation and aid in the treatment of various skin disorders. Similarly, hyaluronidase inhibition may preserve skin moisture and elasticity by preventing the degradation of hyaluronic acid [40]. Chauhan et al. [41] reported the lipoxygenase inhibitory activity of *M. chamomilla* extract and attributed this activity to components such as apigenin and bisabolol. In our study, the lipoxygenase and hyaluronidase inhibitory activity of the phytocomplex was found to be higher than that of individual extracts, supporting a synergistic interaction among components.

Regarding anti-aging potential, the serum formulation effectively inhibited collagenase and elastase enzymes. Collagenase and elastase are enzymes that degrade structural proteins such as collagen and elastin, respectively, and play important roles in skin aging [42]. Inhibition of these enzymes may contribute to preserving skin elasticity and slowing wrinkle formation. Thring et al. [43] investigated the collagenase and elastase inhibitory activities of various plant extracts and suggested that phenolic compounds may be responsible for these activities. In our study, the collagenase and elastase inhibitory activity of the phytocomplex was found to be higher than that of individual extracts, suggesting it as a potential synergistic formulation for anti-aging applications.

Synergistic effect analysis confirmed a synergistic interaction among the components of the phytocomplex. Combination index (CI) values being less than 1 in all tests indicates the presence of synergism [21]. This synergistic effect may result from bioactive components in different plants acting through different mechanisms and enhancing each other's effects [44]. For example, flavonoids in *A. millefolium* may exhibit strong antioxidant activity, while allantoin in *S. officinale* may promote cell regeneration, and bisabolol in *M. chamomilla* may exert anti-inflammatory effects. The combination of these components may provide a more comprehensive skin protection and improvement effect compared to their individual use.

Cytotoxicity studies revealed that the serum formulation does not exhibit toxic effects on human dermal fibroblast and keratinocyte cells at the tested concentrations (10-100 µg/mL). This suggests that the formulation may be safe for skin applications. Additionally, the slight proliferative effect observed at low concentrations (25-50 µg/mL) supports the formulation's potential regenerative properties. Similarly, Sharifi-Rad et al. [45] reported that *A. millefolium* extract has low cytotoxicity and may promote cell proliferation.

Physicochemical stability tests showed that the serum formulation exhibits acceptable stability throughout the 3-month test period. This suggests that the formulation is suitable for practical applications. FTIR analysis confirmed that the extracts remain stable in the formulation and do not interact chemically. This indicates that bioactive components are preserved in the formulation and may exert their potential effects.

In vitro release studies demonstrated that active components are released from the serum formulation in a controlled manner. The rapid release observed in the initial hours suggests that the formulation may exhibit a quick effect, while the slow and sustained release observed in subsequent hours supports a long-lasting effect potential. This release profile is desirable for topical applications and may enhance the formulation's efficacy [29].

Compared to the literature, our study is one of the first comprehensive investigations examining the synergistic combination of *A. millefolium*, *S. officinale*, and *M. chamomilla*. Previous studies have generally investigated the individual effects of these plants [7, 8, 9] or examined their binary combinations [46, 47]. Our study makes a significant contribution to the literature by revealing the synergistic effects of the triple combination. Additionally, our study provides a scientific basis for potential dermatological applications by comprehensively evaluating the antioxidant, anti-inflammatory, and anti-aging effects of the phytocomplex.

The strengths of our study include comprehensive phytochemical characterization of the phytocomplex, various biological activity tests, synergistic effect analysis, and detailed examination of the formulation's physicochemical properties. However, our study also has some limitations. Primarily, all tests were conducted under in vitro conditions, and animal and clinical studies are needed to confirm in vivo efficacy. Additionally, the skin penetration and bioavailability of the formulation should be examined in more detail. In future studies, the in vivo efficacy, long-term safety, and effects of the formulation on different skin types should be investigated.

5. Conclusion

This study comprehensively evaluated the dermatological potential of a synergistic phytocomplex prepared from *Achillea millefolium*, *Symphytum officinale*, and *Matricaria chamomilla* plant extracts and the efficacy of a serum formulation developed from this complex. The findings showed that the combination of the three plant extracts

exhibited superior antioxidant, anti-inflammatory, and anti-aging (collagenase and elastase inhibition) activities compared to individual extracts. The serum formulation demonstrated acceptable physicochemical stability and a controlled release profile. Furthermore, the formulation exhibited low cytotoxicity in human dermal fibroblast and keratinocyte cell lines, supporting its potential for safe use.

The synergistic effect of the phytocomplex was confirmed by combination index and isobologram analyses, demonstrating a significant synergistic interaction among components. This synergistic effect may allow the formulation to be more effective at lower doses and reduce potential side effects. HPLC and LC-MS analyses confirmed the presence of important bioactive components in the phytocomplex, including apigenin, chlorogenic acid, rutin, bisabolol, allantoin, and rosmarinic acid, suggesting that these components may contribute to the biological activities of the formulation.

These results suggest that the developed herbal serum may be a safe and effective natural product candidate with the potential to maintain skin health, delay signs of aging, and alleviate inflammatory skin conditions. Future research should focus on further elucidating the molecular mechanisms underlying the synergistic effects of this phytocomplex and confirming its efficacy in clinical studies. Additionally, the effects of the formulation on different skin types and conditions, its long-term safety, and stability should also be investigated.

This study provides a scientific basis for evaluating plants used in traditional medicine in modern dermatological applications and contributes to the development of natural-content cosmetic products. Better understanding of the synergistic effects of the phytocomplex may lead to the development of more effective and safe natural products and offer new therapeutic approaches in the field of skin health.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to the publication of this study.

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