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**PHARMACEUTICAL TECHNOLOGY**

# CYTOTOXIC ACTIVITY AND EVALUATION OF *IN VITRO* RELEASE OF APIGENIN 7-O-GLUCOSIDE FROM THE TOPICAL PHARMACEUTICAL FORMULATION CONTAINING *MATRICARIA RECUTITA* L. FLOWER EXTRACT

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**Abstract:** The extracts from flower heads of *Matricaria recutita* L. have various biological effects, most notably anti-inflammatory. The most important flavonoid is apigenin, which is mostly found in the form of apigenin 7-O-glucoside. Apigenin 7-O-glucoside is assessed as a marker in *Matricaria recutita* L. extracts due to its anti-inflammatory effect. In this study, a new, cost-effective, and accurate HPLC-PDA method was developed and implemented for the quantification of apigenin 7-O-glucoside in liquid extracts of the flowers of *Matricaria recutita* L. grown in the Bandırma/Balıkesir and Arnavutköy/Istanbul, Türkiye regions. The extraction solvent was ethanol 95.4% v/v (containing 0.12% sodium hydroxide, 0.22% sodium acetate). The apigenin 7-O-glucoside content of the extracts was detected. The total phenolic and flavonoid content of the extracts was analyzed. Since the extract prepared from the herb grown in the Arnavutköy/Istanbul region has higher apigenin 7-O-glucoside and total phenolic and flavonoid content, a topical cream containing this extract was formulated. An in vitro release test was performed for apigenin 7-O-glucoside to examine the pharmaceutical quality and performance of the cream compared to the reference cream. The test cream showed a faster release than the reference product. In addition, the physicochemical stability of the test product was monitored for 90 days. It is expected to be more effective than the reference product in in vivo studies due to the faster release of the test product. The cell viability profile and apoptotic effect of the extracts and the test cream were evaluated on fibroblast (CCD-1079Sk) and melanoma (SK-MEL-28) cell lines.

**Keywords:** topical product; in vitro release; *Matricaria recutita* L. extract; apigenin 7-O-glucoside; activity.

*Matricaria recutita* L. *(M. recutita)* (Asteraceae) is among the most extensively employed therapeutic herbs, the effectiveness of which has been verified in numerous research studies [1, 2] but requires substantiation in clinical studies [3, 4]. *M. recutita* (chamomile), referred to locally as "Papatya", is extensively prevalent across all areas of Türkiye and grows naturally along roadsides and in unused fields [5]. With an annual global production of 4000 tons of chamomile, this herb has gained significance on

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a global scale, both in terms of its economic and medical importance [6]. *M. recutita* is a botanical species that has been frequently employed in contemporary and conventional medicine for its anti-inflammatory, antispasmodic, and antimicrobial properties [7, 3, 8]. It is not only utilized as a component in various traditional and medicinal formulations, but is also used in the cosmetic and pharmaceutical industries [9].

*M. recutita* has also been discovered to include a variety of biological phenolic compounds, such as phenylpropanoids (caffeic acid, chlorogenic acid), coumarins (umbelliferone, herniarin), flavones (apigenin (APG), apigenin 7-O-glucoside (AP7G), luteolin, luteolin 7-O-glucoside), flavonols (quercetin, rutin), and flavanones (naringenin). Numerous studies have indicated that these phenolic compounds are associated with the medicinal effects of this plant [10]. Within these compounds, both APG and AP7G have demonstrated anti-inflammatory effects [11, 12]. APG is found as the primary flavonoid compound present in *M. recutita*, with a significant amount of this compound existing in a glucoside form, while only a minor amount is present as a free compound [13, 14]. Thus, AP7G is regarded as a useful and suitable marker for the *M. recutita* flower extract [10]. The methanolic and aqueous extracts derived from chamomile demonstrated apoptotic and anti-proliferative effects on different human cancer cells while minimally affecting normal cells. It has also been shown that AP7G is the primary component in *M. recutita.* Nevertheless, additional research is required to explore its potential as an anticancer agent [13].

According to the European Medicines Agency (EMA) Monograph, medicinal products containing liquid extract are used for adjuvant therapy of irritations of mucosa and skin in the genital and anal region and the treatment of minor inflammation of superficial wounds, small boils (furuncles) and skin (sunburn) [15]. According to its bioactive components and traditional use, various extract types and formulation have been used to provide new therapeutic options for dermal disorders. It can be seen that several formulations were developed containing *Matricaria* species to be used in the treatment of skin diseases. It is known that some of the patented inventions have already been produced and made available by the pharmaceutical industries, and their products are accessible to the general population [16]. The results proved that a hydrogel containing APG prepared with gellan-chitosan gum seems to be highly suitable for wound healing due to its unique properties of biocompatibility, biodegradability, moist nature, and antioxidant effectiveness [17,18]. Although there is an increasing trend in the number of new bioproduct discoveries based on *Matricaria* species for the treatment of skin diseases, there are not enough studies in the literature to evaluate the in vitro release of topical chamomile products depending on the amount of APG and AP7G.

In the present study, *M. recutita* flower heads were grown and collected in the Arnavutköy/Istanbul and Bandırma/Balıkesir, Türkiye regions, and the extracts were prepared from these flower heads. The study aimed to prepare *M. recutita* extract within the EMA Monograph, to develop and validate a new HPLC-PDA method to quantify AP7G in extracts, and eventually to formulate a new topical product containing this extract that is non-cytotoxic and demonstrates appropriate in vitro release. The cream formulation can be reliably used as an alternative product for dermatological purposes. Additionally, the developed and validated HPLC-PDA method can be utilized for the quality control of *M. recutita* extracts.

## EXPERIMENTAL

### Materials

AP7G standard (≥97%) for HPLC was supplied from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol (96%), acetonitrile, orthophosphoric acid, citric acid monohydrate, folin-ciocalteu's phenol reagent, aluminum chloride, sodium acetate, sodium hydroxide, and ammonium acetate were supplied from Merck (Darmstadt, Germany). Sodium carbonate was supplied from Isolab Chemicals (Eschau, Germany). Kamillosan® Cream product was supplied from MEDA pharma (Hamburg, Germany).

The purity and sources of the standard compounds for LC-HRMS are presented as below. Ascorbic acid (≥ 99%), fumaric acid (≥ 99%), chlorogenic acid (≥ 95%), caffeic acid (≥ 98%), 2,5-dihydroxybenzoic acid (> 99%), naringin (≥ 95%), vanillic acid (≥ 97%), rutin (≥ 94%), hispidulin-7-glucoside (≥ 98%), quercetin (≥ 95%), apigenin 7-metilat (≥ 98%), naringenin (≥ 95%), salicylic acid (≥ 99%), luteolin (≥ 98%), APG (≥ 95%), pinocembrin (≥ 95%), and chrysin (≥ 97%) were supplied from Sigma-Aldrich (Stenheim, Germany). AP7G (≥ 97%) and caffeic acid phenethyl ester (≥ 97%) were supplied from EDQM CS (Strasbourg, France). (-)-Epicatechin gallate (> 97%), (+)-*trans*-taxifolin (> 97%), hyperoside (> 97%), quercitrin (> 97%), 3'-O-methyl quercetin (> 97%), hispidulin (> 97%), and acacetin (> 97%) were supplied

from TRC (Toronto, Canada). Penduletin (> 97%), cirsilineol (≥ 95%), isosakuranetin (> 97%), and dihydrokaempferol (> 97%) were supplied from Phytolab (Vestenbergsgreuth, Germany). Apigenin 7-O-acylglucoside (≥ 98%) was supplied from Solarbio (Beijing, China). Luteolin 7-glucoside (≥ 99%) was supplied from AppliChem (Darmstadt, Germany). Chrysoeriol (≥ 99%) was supplied from Extrasynthese (Genay, France). Herniarin (≥ 98%) was supplied from Carl Loth GmbH (Karlsruhe, Germany). Luteolin 7-rutinoside (> 97%) was supplied from Carbosynth (Berkshire, United Kingdom). 6-OH-luteolin 7-glucoside (≥ 98%) was supplied from Aobious (Gloucester, MA, USA).

**Plant Collection**

Flower heads of *M. recutita* grown in Türkiye (Arnavutköy/Istanbul) were collected. Flower heads of *M. recutita* grown in the Bandırma/Balıkesir, Türkiye region were purchased from Balat Gıda firm. *M. recutita* from Asteraceae family was detected by Dr. Ebru Özdemir Nath, Altınbaş University, Türkiye. Herbarium specimens of the plant were stored at the Altınbaş University Herbarium (Herbarium number: HERA 1236, HERA1237).

**Preparation of Extracts**

The flower parts were dried in the dark, at 30°C ± 5°C and 65% ± 5% RH. Dried flower heads of chamomile were powdered between approximately 20 mm to 40 mm in size by a grinding machine (G1, Yazıcılar, Türkiye). The 40 g of powdered flower parts was taken and extracted with a mechanical stirrer at 1000 rpm (W3000-D, Wiggens, China) for two hours in 500 mL of the extraction solvent at 25°C ± 2°C (Dried Herb/Extraction Solvent : 1 : 12.5). The extraction solvent used was ethanol 95.4% v/v (containing 0.22% sodium acetate, 0.12% sodium hydroxide) with a drug extract ratio (DER) 2.7-5.5:1. The extract was filtered through filter paper (20 µm, 40 cm × 40 cm, Isolab, Germany), and evaporated using a rotary evaporator (G3, Heidolph, Germany) at 45°C until 10 g remained. The evaporation process took four hours. The extract obtained at the targeted rate was stored in amber bottles at 4°C.

The extracts were prepared, and are presented in Table 1. The drug extract ratio (DER) indicates the ratio of the amount of dried herb material to the amount of the obtained extract. All extracts were prepared with a drug extract ratio (DER) of 4 : 1 (yield 25%), and which is within the target range of 2.7-5.5 : 1.

**Quantitative Analysis and Validation of AP7G in Extracts by HPLC Method**

According to the International Committee on Harmonization (ICH) guidelines, the HPLC-PDA method for AP7G in extracts was developed and validated by precision, recovery, limit of detection and quantification, specificity, robustness, and linearity [19]. Determination of only Apigenin glucosides as quantitative is difficult, as many reference compounds are not available commercially. It is difficult to separate each one and analyze. In this process, many acetylated derivatives of AP7G were converted to AP7G by alkaline hydrolysis, and it was quantified by the HPLC-PDA method [20].

In a sample preparation, 1 mL of liquid extract was put into a 50 mL Erlenmeyer flask. Then, 10 mL of methanol and 10 mL of sodium hydroxide solution (8.5% g/mL) were added to it. The mixture was heated under a reflux condenser on a magnetic mixer for 1 hour. It was cooled and diluted to 50.0 mL with of 0.5% orthophosphoric acid in water and acetonitrile 3 : 1 (*v/v*). Next, 1.5 g of citric acid monohydrate was added to 50.0 mL of the solution. It was shaken for 5 min and then filtered with 0.45 µm RC before analysis.

**Chromatographic Conditions**

HPLC equipment (Shimadzu LC-2030C HPLC, Japan) and a degasser, a pump (Shimadzu Quaternary LC Pump, Japan), and a controller coupled to a Shimadzu PDA Plus photodiode-array detector equipped with an automatic injector interfaced were used. C18 column (4.6 × 250 mm, 5 µm, Perkin Elmer, USA) was used. The mobile phase contained 0.5% orthophosphoric acid in water (*v/v)* (A) and acetonitrile (B). Separation was carried out using a gradient system—0-9 min: 25% B; 9-19 min: 25% B-75% B; 19-24 min: 75% B; 24-25 min: 75%

Table 1. Drug extract ratio (DER) of the prepared extracts.

| **Extract** | **Drug extract ratio (DER)** (Bandırma/Balıkesir) | **Drug extract ratio (DER)** (Arnavutköy/Istanbul) |
|---|---|---|
| Liquid extract (DER 2.7-5.5 : 1), extraction solvent: ethanol 95.4% V/V (containing 0.12% sodium hydroxide, 0.22% sodium acetate) | 4 : 1 | 4 : 1 |

B-25% B; 25-50 min: 25% B—and the injection volume was 20 µL. The column temperature was 40°C, and the flow rate was 1 mL/min. The wavelength was 340 nm.

**Phytochemical Analysis of Extracts by LC-HRMS**

Phytochemical analysis of extracts was done by LC-HRMS method. Measurements of LC-HRMS were conducted on a Thermo ORBITRAP Q-EXACTIVE (Bremen, Germany) mass spectrometry-equipped ESI ion source and with Dionex LC system. Environmental conditions were set as: temperature 22.0 ± 5.0°C and relative humidity (50 ± 15)% rh. A Troyasil C18 column (150 x 3 mm i.d., 5 µm particle size, Istanbul, Türkiye) was used for the separation of compounds. The scan range was fixed to m/z 100-900 amu and the other mass parameters were used as follows: aux flow rate of gas: 10; flow rate of gas: 45; temperature of capillary: 320°C; heater of aux gas temperature: 320°C; sprey voltage: 3.80 kV; and Slens RF was 50. Mobile phases A and B were mixed with 1% water-formic acid and 1% methanol-formic acid, respectively. The gradient program was used and it was 0-1.00 min 50% A and 50% B, 1.01-6.00 min 100% B, and 6.01-15 finally min 50% A and 50% B. The flow rate of the mobile phase was 0.35 mL/min, and the column temperature was set to 22°C. A previously established and validated LC-MS/MS method was applied to detect the contents in the *M. recutita* extracts, and the compounds were identified by comparing the retention times and target ions of the compounds in Bezmialem Vakif University's Drug Application and Research Center (ILMER) Library [21, 22].

**Total Phenolic and Total Flavonoid Compounds of Extracts**

The total phenolic compounds in the extracts were detected spectrophotometrically using a modified Folin Ciocalteu method. Measurements were made on the plate using UV-VIS. A total of 104 µL of water and 8 µL of the sample were added to the microplate. Then, 8 µL of Folin Ciocalteu Reagent (FCR) was added. After waiting for 5 minutes, 80 µL of 10% sodium carbonate ($Na_2CO_3$) was added. It was kept in the dark for 90 min. Measurements were taken at 725 nm in the UV-VIS spectrophotometer. The amount of gallic acid corresponding to the absorbance values of the samples was detected using the standard graphic equation. The determination of the total amount of phenolic compounds was made with the gallic acid calibration equation. Results were calculated in percent of gallic acid equivalent [23].

The total flavonoid compounds in the extracts were detected as spectrophotometric using the aluminium chloride method. Measurements were made on the plate using UV-VIS. A total of 134 µL of water and 20 µL of the sample were added to the microplate. Then, 6 µL of 10% aluminum chloride ($AlCl_3$) and 40 µL of 1M ammonium acetate ($CH_3COONH_4$) were added. After 10 minutes, absorbance was measured at 425 nm in a UV-VIS spectrophotometer. The amount of quercetin corresponding to the absorbance values of the samples was detected using the standard graphic equation. The determination of the total amount of flavonoid compounds was made with the quercetin calibration equation. Results were calculated in percent of quercetin equivalent [23].

**Preparation of Topical Cream Formulation**

A cream (Lot no:M1) containing an extract prepared from the Arnavutköy/Istanbul region equivalent to the AP7G content of Kamillosan® Cream (Lot no:326670) was formulated. In the review published by Srivastava and Siddiqui, it was stated that factors such as the addition of emulsifiers, antimicrobial preservatives, and pH agents ensure product stability for a herbal cream formulation [24]. Considering these factors, emulsifiers (glyceryl monostearate, cetyl alcohol, stearic acid), an antimicrobial preservative (methyl 4-hydroxybenzoate), and a pH agent (sodium hydroxide) were used in the formulation of the test cream. Uddin et al. developed a cream formulation containing a polyherbal extract using a manufacturing method that involved preparing the oil phase, preparing the water phase, and subsequently adding the water phase to the oil phase. They reported that the cream exhibited physicochemical stability for one month at 40°C [25]. This manufacturing method was similarly employed in the present formulation development studies. The topical cream produced contained liquid extract, stearic acid, glyceryl monostearate, cetyl alcohol, glycerin, sodium hydroxide, methyl 4-hydroxybenzoate, and distilled water, and the unit formula is presented in Table 2.

The manufacturing process included the steps as follows. The oil phase (stearic acid, glyceryl monostearate, cetyl alcohol) weighed in the main tank was melted by heating to 70°C. Sodium hydroxide was dissolved in some water in the auxiliary tank. Then, glycerin was added and mixed for 5-10 minutes. This phase was heated until it reached the same temperature as the oil phase. The mixture in the auxiliary tank was transfered to the oil phase, and the mixing was continued on a heater. In

another auxiliary tank, methyl 4-hydroxybenzoate at 95°C, and liquid extract at 70°C were added to the remaining part of the water and mixed. When it was at 70°C, it was added to the main tank. When the addition process was completed, it was mixed until it cooled down.

The reference Kamillosan® Cream is used in follow-up treatment following local corticosteroid therapy for inflammatory skin diseases such as various forms of eczema including contact dermatitis, industrial eczema, eczema in children, and neurodermatitis (atopic eczema). Kamillosan® Cream contains liquid *M. recutita* extract (ethanol 95.4% v/v (containing 0.22% sodium acetate trihydrate, 0.12% sodium hydroxide) with a drug extract ratio (DER) of 2.7-5.5:1, methyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, cetyl stearyl alcohol, hydrogenated peanut oil, wool wax alcohols, glycerol monostearate, sodium cetyl stearyl sulfate, isopropyl myristate, glycerin, and purified water.

**Quantitative Analysis of AP7G of Topical Product by HPLC Method and Monitoring Stability**

Stability was evaluated for the test cream containing liquid extract stored in aluminum tubes at 25°C/60% RH and 40°C/75% RH temperature for 90 days. The stability assessment of the test product included an examination of the visual appearance, pH, rheological behavior, and quantification assay of AP7G.

For the product analysis, the HPLC method was used as the analytical method. For the quantitative analysis of AP7G in the topical product, approximately 500 mg of the topical product was placed in a 100 mL Erlenmeyer flask. Then, 20 mL of methanol and 2 mL of sodium hydroxide solution (8.5% g/mL) were added. The mixture was heated under a reflux condenser on a magnetic mixer for one hour. It was cooled and diluted to 100.0 mL with 0.5% orthophosphoric acid in water and acetonitrile 3 : 1 (*v/v*). Next, 1.0 g of citric acid monohydrate was added to the 100.0 mL of the solution. The mixture was shaken for 5 min and then filtered with a 0.45 µm RC before HPLC analysis.

The product appearance (i.e., odor, color, consistency) was evaluated visually. The product pH was measured by a calibrated pH meter at 25 ± 2°C (Ohaus ST300, USA).

**Rheological Measurements**

To examine the flow properties of the test and reference products, methods in the literature were modified and studied. The rheological properties of the test and reference products were comparatively analyzed using the "shear rate ramp" test with a Thermo Scientific HAAKE RS1 rheometer and cone plate sensor (C35 TiL, 1° cone angle, 53 µm gap width). In this test method, approximately 1 gram samples were placed on the plate to measure the flow properties of the samples. The temperature was set at 25°C ± 2°C. The temperature of the samples was allowed to stabilize for 60 seconds. The tests were carried out so that 120 data points were taken in the sliding speed range of 0-1000 1/s rpm for 120 seconds. N = 3 were studied for each sample.

**In Vitro Release Test**

In vitro drug release studies were carried out via a modified Franz type diffusion cell with an effective diffusion area of 1.77 $cm^2$ and a rotation speed of 500 rpm (PermeGear, V6A Stirrer, USA), which are commonly used for in vitro performance studies of semi-solid dosage forms. The receptor chamber was filled with 12 mL of PBS pH 7.4 and ethanol (50:50) maintaining sink condition. The dialysis membrane (Sigma Aldrich, Dialysis tubing cellulose membrane Lot 3110; USA) was hydrated

Table 2. Unit formula of test product.

| Raw materials | Unit formula (% w/w) |
|---|---|
| Liquid Extract | 3.14 |
| Stearic acid | 17.0 |
| Glyceryl monostearate | 1.0 |
| Cetyl alcohol | 1.0 |
| Glycerin | 6.0 |
| Methyl 4-hydroxybenzoate | 0.15 |
| Sodium hydroxide | 0.25 |
| Distilled water | 71.46 |
| **Total** | **100.0** |

in a receptor medium for at least 18 hours. A sample size of 400 mg was placed on donor cells to completely cover the effective surface area [26]. The receptor phase was maintained at 32°C under constant stirring with a magnetic bar. The test cream containing liquid extract (Lot no:M1) and reference product Kamillosan® Cream (Lot no:326670) were placed upwards into the donor compartment of the diffusion cells, completely covering the effective surface area (n = 6). The donors were capped by parafilm immediately after the addition of the formulation to prevent evaporation.

The drug release of the formulations was followed for six hours. Samples of 1 mL were removed at appropriate time intervals (1, 2, 3, 4, 5, and 6 h) for analysis and replaced immediately by a fresh receptor medium [27]. The samples were filtered through 0.45 µm HPTFE filters (CHROMAFIL®XTRA H-PTFE-45/13). The samples were analyzed for their AP7G content by a validated HPLC method.

**Cell Culture and Treatment**

SK-MEL-28 (human melanoma cells, ATCC, HTB-72) and CCD-1079Sk (human normal skin fibroblast cells, ATCC, CRL-2097) cell lines were initially seeded into culture flasks and grown in DMEM supplemented with 10% FBS (Sigma-Aldrich, St Louis, MO), 1% penicillin/streptomycin (Thermo Fisher Scientific, Carlsbad, CA). The cell lines were maintained in a humidified atmosphere of 5% $CO_2$ at a temperature of 37°C. The culture medium was changed every two days until the cells reached 80% confluence.

**Cell Viability Assay**

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used to assess the cytotoxicity of the extracts, AP7G, and APG. The cells were grown in 96-well plates at a density of $5\times10^3$ cells per well. In total, 70% to 80% of confluent cells (after 24 hours) were treated with different concentrations of extracts, AP7G, and APG (prepared in a medium containing 1% DMSO) in the growth medium for 24 and 48 hours. At the end of the incubation periods, MTT solution was added to the wells to give a final concentration of 0.5 mg/mL. The cells were then incubated at 37°C for a further 3.5 hours. To solubilize the formazan crystals, 100 µL of DMSO was added to each well. The optical density was read at 540 nm using an ELISA microplate reader and directly correlated with the number of viable cells. For each concentration, the data for which the measurement was repeated at least three times were compared. The relative% cell viability and the $IC_{50}$ value were determined by plotting the graph as a function of concentration. Untreated cells were considered the control and medium without cells as the background control. To evaluate the cytotoxic effect of the compounds on cell viability, untreated cells with compounds were regarded 100% viable and cell viability was calculated according to the following formula:% Cell Viability= Sample/Control×100.

For the determination of the cytotoxicity of the test cream and Kamillosan® Cream, SK-MEL-28 and CCD-1079Sk cells were seeded in a 24-well plate and incubated until they were semi-confluent (24 h). The samples were then applied to the surface of a wax paper for direct contact and placed in the wells with the test substance on the cell surface. The wax papers were prepared to match the 1:10 surface area of the wells and sterilized. After 24 and 48 hours of incubation, the formation of formazan was determined for each sample, and the values were compared with those obtained from the control cultures. The viability of the cells was directly related to the amount of blue-violet formazan, which was read as the optical density at 540 nm [28].

**Apoptosis Assay**

The apoptotic effects of samples were analyzed using a flow cytometer (BD Accuri C6) with a FITC Annexin V Apoptosis Detection Kit with PI. For apoptosis assay, CCD1079-Sk and SK-MEL-28 cells were separately seeded in 24-well plates ($2\times10^5$ cells/well) and incubated for 24 h. Then, the cells were treated with samples. After the 24 h and 48 h incubation period, the growth medium containing the samples was removed and rinsed with PBS twice then once with binding buffer. The cells were harvested with trypsinization and resuspended in Annexin V binding buffer at $1x10^6$ cells/mL. Next, 2.5 µL of FITC Annexin V was added to 100 µL of cell suspension. Cells were incubated for 15 minutes in the dark at room temperature. The cells were centrifuged for 5 minutes and the supernatant was discarded. Then, 2.5 µL PI was transfered to 100 µL cell suspension. After an incubation period of 15 min, the cells were analyzed by a flow cytometer [29].

## RESULTS AND DISCUSSION

**Quantitative Analysis and Validation of AP7G in Extracts by HPLC-PDA Method**

In the present study, a new, cost-effective, accurate, and simple HPLC-PDA method was developed and validated. The linearity of the method was detected by evaluating analytical curves

Table 3. Validation parameters and results.

| Validation parameters | | Results |
|---|---|---|
| Linearity (µg/mL) | | 3.0-300.0 |
| Regression equation | | y = 60216288.48748x-130203.80970 |
| Regression coefficient ($r^2$) | | 0.9994 |
| Accuracy% | 80% | 99.61 |
| | 100% | 99.99 |
| | 120% | 98.24 |
| Precision | Repeatability (%RSD) | < 2.0 |
| | Intermediate precision (%RSD) | < 2.0 |
| Robustness (%RSD) | | < 2.0 |
| Limit of detection (µg/mL) | | 0.033 |
| Limit of quantification (µg/mL) | | 0.1 |

at six AP7G concentration levels (3.0, 1.0, 10.0, 50.0, 100.0, 300.0 µg/mL). The AP7G calibration curve was linear above the established range with $r$ of 0.9994 for the regression equation (y=602162 88.48748x-130203.80970). Accuracy, expressed as a percentage of mean recovery, was 98%-102%; precision was below 2% relative standard deviation (RSD). Validation parameters and results are given in Table 3. The validation studies were carried out according to the International Committee on Harmonization (ICH) guidelines. The results indicated that the HPLC-PDA method was more specific and sensitive for quantifying AP7G in this type of extract.

Haghi et al. studied the AP7G contents of *M. recutita* flower extracts using water, methanol, and 70% ethanol solvents. The AP7G contents of the extract were found to be 1.66, 1.69, and 2.67 mg/g of dried herb, respectively [20]. In another study, Baretta et al. examined the AP7G content of *M. recutita* flower glycolic extract, which was found to be 7.01 mg/g of dried herb [9]. Guzelmeric et al. evaluated the AP7G content of *M. recutita* flower aqueous extract, finding an AP7G content of 0.62 mg/g of dried herb [30].

In the present study, 40 g of dried flower parts was extracted in 500 mL of the extraction solvent at 25°C ± 2°C for two hours. The obtained extract was evaporated using a rotary evaporator for four hours at 45°C until 10 g remained. The AP7G content of the extracts was analyzed to be 1.61 mg/g of dried herb from the Arnavutköy/Istanbul region, and 1.24 mg/g of dried herb from the Bandırma/Balıkesir region. These results are presented in Table 4.

Although the same extraction solvent and method were used for both herbs in the preparation of extracts, it was revealed that the AP7G content of the extract prepared from herb grown in the Istanbul/Arnavutköy region was higher than that from the Bandırma/Balıkesir region. No study in the literature has been conducted with the extraction solvent used in the present study, and the AP7G contents of extracts prepared with different solvents exhibit varying results, including both higher and lower values.

Plant growth can be affected by environmental, climatic, and other factors. Therefore, the content of the herb may vary both qualitatively and quantitatively [31-36]. In the present study, many factors such as temperature, UV light, soil content, drought, and rainfall are thought to be potential factors contributing to the differences in AP7G content observed in extracts prepared from herbs grown in two different regions.

Table 4. AP7G content of extracts.

| Region | AP7G content (mg/g of dried herb) |
|---|---|
| Bandırma/Balıkesir | 1.24 ± 0.01 |
| Arnavutköy/Istanbul | 1.61 ± 0.01 |

*Values are mean ± SD (n = 3)

**Phytochemical Analysis of Extracts by LC-HRMS**

LC-HRMS analysis results of both extracts are presented in Table 5 along with their retention time. A previously developed and validated LC-HRMS method was implemented to detect the extract content, and the method parameters and results are presented in detail in Table 5. The validation parameters of this study were recovery, linearity, limits of quantification (LOQ), and limits of detection (LOD) [21].

Ascorbic acid, chlorogenic acid, caffeic acid, 2,5-dihydroxybenzoic acid, 6-OH-luteolin 7-glucoside, (+)-*trans*-taxifolin, luteolin 7-rutinoside, luteolin 7-glucoside, rutin, hyperoside, dihydrokaempferol, AP7G, hispidulin-7-glucoside, herniarin, quercetin, salicylic acid, luteolin, apigenin 7-O-acylglucoside, APG, hispidulin, chrysoeriol, caffeic acid phenethyl ester, and chrysin were found in both extracts. 3'-O-Methyl quercetin, cirsilineol, penduletin, pinocembrin, ısosakuranetin, acacetin, and apigenin 7-metilat were found only in the extract from Balıkesir. Fumaric acid, (-)-epicatechin gallate**,** naringin, vanilic acid, quercitrin, and naringenin were found only in the extract from Istanbul. Among all the detected compounds, AP7G, hispidulin-7-glucoside, herniarin, apigenin 7-O-acylglucoside, APG, and ascorbic acid were found to be in high amounts in the extracts.

Dihydrokaempferol compound detected in both extracts, and isosakuranetin and apigenin 7-metilat compounds detected in the extract from Balıkesir have not been reported previously in *M. recutita* flower extracts in the literature (Table 5).

**Total Phenolic and Total Flavonoid Compounds of Extracts**

Phenolic compounds constitute the primary group of phytochemicals, serving as the main source of antioxidant activity in plants and plant-derived products. Flavonoids comprise the most extensive group of existing phenolic compounds naturally, occurring in various plant parts both in their free state and as glycosides [54]. Many studies indicated that there is a linear positive correlation between the antioxidant activity of *M. recutita* extracts and their total phenolic, total flavonoid compounds.

Tarantilis et al. found that the total phenolic content of *M. recutita* flower aqueous extracts prepared at different temperatures ranged from 0.06% to 0.02% w/w [55]. The total

Table 5. Analysis results of LC-HRMS of extracts and analytical parameters.

| Identified compound | Balıkesir (mg/mL) | Istanbul (mg/mL) | Molecular formula | m/z | Polarity | Linear range | Equation | LOD/ LOQ | $r^2$ | Recovery | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ascorbic acid | 0.3805 | 0.4786 | $C_6H_8O_6$ | 175.02 | - | 0.5–10 | y = 0.00347x – 0.00137 | 0.39/1.29 | 0.9988 | 96.20 | [37] |
| Fumaric acid | - | 0.2638 | $C_4H_4O_4$ | 115.00 | - | 0.1-10 | y=0.00061x-0.000033 | 0.05/0.17 | 0.9991 | 97.13 | [37] |
| Chlorogenic acid | 0.2816 | 0.4841 | $C_{16}H_{18}O_9$ | 353.09 | - | 0.05-10 | y = 0.00817x + 0.000163 | 0.02/0.06 | 0.9994 | 96.68 | [7, 10] |
| (-)-Epicatechin gallate | - | 0.0013 | $C_{22}H_{18}O_{10}$ | 441.09 | - | 0.05-10 | y=0.00788x-0.0001875 | 0.01/0.03 | 0.9995 | 96.54 | [38] |
| Caffeic acid | 0.0908 | 0.0171 | $C_9H_8O_4$ | 179.03 | - | 0.3–10 | y = 0.0304x + 0.00366 | 0.08/0.27 | 0.9993 | 94.51 | [7, 20] |
| 2,5-Dihydroxybenzoic acid | 0.0462 | 0.0128 | $C_7H_6O_4$ | 153.02 | - | 0.5–10 | y = 0.06114x – 0.0102 | 0.17/0.57 | 0.9985 | 100.23 | [39] |
| 6-OH-luteolin 7-glucoside | 0.008 | 0.0223 | $C_{21}H_{20}O_{12}$ | 463.09 | - | 0.5–10 | y = 0.01482x – 0.001803 | 0.23/0.77 | 0.997 | 102.44 | [40] |
| (+)-*trans* taxifolin | 0.0004 | 0.0010 | $C_{15}H_{12}O_7$ | 303.05 | - | 0.3–10 | y = 0.0289x + 0.00537 | 0.01/0.03 | 0.9978 | 91.66 | [41] |
| Luteolin 7-rutinoside | 0.0011 | 0.0063 | $C_{27}H_{30}O_{15}$ | 593.15 | - | 0.1–10 | y = 0.00879x + 0.000739 | 0.01/0.03 | 0.9988 | 93.05 | [7] |
| Naringin | - | 0.0029 | $C_{27}H_{32}O_{14}$ | 579.18 | - | 0.05-10 | y=0.00576x-0.000284 | 0.01/0.03 | 0.9991 | 101.91 | [42] |
| Vanilic acid | - | 0.0256 | $C_8H_8O_4$ | 167.03 | - | 0.3–10 | y = 0.00133x + 0.0003456 | 0.1/0.33 | 0.9997 | 98.66 | [43] |
| Luteolin 7-glucoside | 0.0786 | 0.0497 | $C_{21}H_{20}O_{11}$ | 447.09 | - | 0.1–7 | y = 0.0162x + 0.00226 | 0.01/0.03 | 0.9961 | 96.31 | [10, 44] |

Table 5. Analysis results of LC-HRMS of extracts and analytical parameters (cont.).

| Identified compound | Balıkesir (mg/mL) | Istanbul (mg/mL) | Molecular formula | m/z | Polarity | Linear range | Equation | LOD/ LOQ | r² | Recovery | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rutin | 0.0032 | 0.0025 | $C_{27}H_{30}O_{16}$ | 609.15 | - | 0.05-10 | y = 0.00329x – 0.00005576 | 0.01/0.03 | 0.999 | 96.97 | [20] |
| Hyperoside | 0.0221 | 0.0273 | $C_{21}H_{20}O_{12}$ | 463.09 | - | 0.05-10 | y = 0.0072x – 0.00003096 | 0.01/0.03 | 0.9995 | 96.62 | [45, 39] |
| Dihydrokaempferol | 0.00060 | 0.0021 | $C_{15}H_{12}O_6$ | 287.06 | - | 0.3-7 | y=0.0756x+0.0118 | 0.01/0.03 | 0.9995 | 95.37 | N.A. |
| AP7G | 2.096 | 1.1615 | $C_{21}H_{20}O_{10}$ | 431.10 | - | 0.3–7 | y = 0.0246x + 0.00306 | 0.01/0.03 | 0.9962 | 96.07 | [7, 10, 20] |
| Hispidulin-7-glucoside | 1.453 | 0.4171 | $C_{22}H_{22}O_{11}$ | 461.11 | - | 0.5–10 | y = 0.0003758x – 0.00006441 | 0.57/1.91 | 0.9988 | 101.76 | [46] |
| Quercitrin | - | 0.0164 | $C_{21}H_{20}O_{11}$ | 447.09 | - | 0.05-10 | y=0.0179+0.0003331 | 0.01/0.03 | 0.9990 | 97.00 | [47, 39] |
| Herniarin | 2.348 | 0.8847 | $C_{10}H_8O_3$ | 177.05 | + | - | y = 5.23600 x + 0.72060 | 0.14/0.46 | 0.9980 | 106.8 | [10] |
| Quercetin | 0.0966 | 0.0061 | $C_{15}H_{10}O_7$ | 301.04 | - | 0.1–10 | y = 0.0509x + 0.00467 | 0.01/0.03 | 0.9978 | 96.41 | [7, 20] |
| Naringenin | - | 0.0113 | $C_{15}H_{12}O_5$ | 271.06 | - | 0.1–10 | y = 0.0281x + 0.00182 | 0.01/0.03 | 0.9995 | 86.65 | [7, 39] |
| Salicylic acid | 0.0695 | 0.0270 | $C_7H_6O_3$ | 137.02 | - | 0.3–10 | y = 0.0361x + 0.00245 | 0.01/0.03 | 0.9982 | 92.88 | [20] |
| Luteolin | 0.0306 | 0.0147 | $C_{15}H_{10}O_6$ | 285.04 | - | 0.1–10 | y = 0.117x + 0.00848 | 0.01/0.03 | 0.9981 | 96.98 | [20] |
| Apigenin 7-O-acylglucoside | 0.6940 | 0.2395 | $C_{22}H_{30}O_{11}$ | 473.11 | - | 0.5–10 | y = 0.01496x – 0.0006413 | 0.13/0.42 | 0.9986 | 99.12 | [48] |
| 3’-O-Methyl quercetin | 0.0191 | - | $C_{16}H_{12}O_7$ | 317.07 | - | - | y=0.01129X + 0.001507 | 0.22/0.75 | 0.9930 | 101.7 | [49] |
| APG | 0.3414 | 0.4786 | $C_{15}H_{10}O_5$ | 269.05 | - | 0.3–10 | y = 0.104x + 0.0199 | 0.01/0.03 | 0.9998 | 81.55 | [7, 10, 20] |
| Hispidulin | 0.0545 | 0.0193 | $C_{16}H_{12}O_6$ | 301.07 | + | 0.05-10 | y = 0.02614x + 0.0003114 | 0.01/0.03 | 0.9993 | 98.36 | [49, 50] |
| Chrysoeriol | 0.0241 | 0.0150 | $C_{16}H_{12}O_6$ | 299.06 | - | 0.5–10 | y = 0.1023x – 0.002224 | 0.15/0.5 | 0.9974 | 96.42 | [39, 51] |
| Cirsilineol | 0.0716 | - | $C_{18}H_{16}O_7$ | 313.07 | - | 0.5–10 | y = 0.1389x + 0.02817 | 0.22/0.72 | 0.9955 | 96.17 | [50] |
| Penduletin | 0.0966 | - | $C_{18}H_{16}O_7$ | 343.08 | - | 0.3-10 | y=0.0258x+0.00253 | 0.01/0.03 | 0.9991 | 83.43 | [52] |
| Pinocembrin | 0.0171 | - | $C_{15}H_{12}O_4$ | 255.07 | - | 0.5–10 | y = 0.5224x | 0.17/0.57 | 0.9971 | 101.89 | [51] |
| Isosakuranetin | 0.0411 | - | $C_{16}H_{14}O_5$ | 285.08 | - | 0.05-10 | y = 0.0026x + 0.0004973 | 0.23/0.77 | 0.995 | 106.37 | N.A |
| Caffeic acid phenethyl ester | 0.0031 | 0.00005 | $C_{17}H_{16}O_4$ | 283.10 | - | 0.3–7 | y = 0.255x + 0.0477 | 0.01/0.03 | 0.9964 | 94.42 | [44] |
| Chrysin | 0.050 | 0.00035 | $C_{15}H_{10}O_4$ | 253.05 | - | 0.05–7 | y = 0.0964x – 0.0002622 | 0.01/0.03 | 0.999 | 87.92 | [51] |
| Acacetin | 0.0166 | - | $C_{16}H_{12}O_5$ | 283.06 | - | 0.05–7 | y = 0.046x + 0.0001875 | 0.01/0.03 | 0.9995 | 87.52 | [53] |
| Apigenin 7-metilat | 0.0020 | - | $C_{15}H_{12}O_5$ | 283.06 | - | 0.5–10 | y = 0.3563x | 0.16/0.54 | 0.9949 | 97.10 | N.A. |

N.A.: Not available

phenolic content of the *M. recutita* flower methanolic extract was detected as 0.12% w/w, and the total flavonoid content was detected as 23.10% w/w by Selvam et al. [56]. In another study, the total phenolic content of the *M. recutita* flower methanolic extract was found to be 0.03% w/w by Elmastaş et al. [57]. Haghi et al. found that the total phenolic content of the *M. recutita* flower ethanolic extract was 2.43% w/w, and the total flavonoid content was 4.95% w/w [20]. Freitas et al. found that the total flavonoid content of the *M. recutita* flower hydroalcoholic extract was 4.11% w/w [58].

The analysis clarified that the total phenolic compounds in the extract prepared from the herb grown in the Bandırma/Balıkesir region was 0.14% w/w liquid extract, while that from the Arnavutköy/Istanbul region was 0.25% w/w liquid extract. In addition, the analysis clarified that the total flavonoid compounds in the extract prepared from the herb grown in the Bandırma/Balıkesir region was 0.06% w/w liquid extract, while that from the Arnavutköy/Istanbul region was 0.10% w/w liquid extract. No study in the literature has been conducted with the extraction solvent used in the present study, and the total phenolic and flavonoid contents of extracts prepared with different solvents exhibited varying results, including both higher and lower values.

The total phenolic compounds and total flavonoid compounds were detected as equivalents of gram gallic acid and quercetin per 100 g of extracts, respectively. The amounts of total phenolic compounds and total flavonoid compounds are shown in Table 6.

In the present study, the total phenolic and flavonoid compounds in the extract prepared from the herb grown in the Arnavutköy/Istanbul region was found to be higher than that from the Bandırma/Balıkesir region. As mentioned above, many factors such as environmental and climatic conditions are thought to be potential factors contributing to the differences in herb content [31-36]. As a result, the extract prepared from the plant grown in Arnavutköy/Istanbul is expected to show higher antioxidant activity.

**Quantitative Analysis of AP7G of Topical Product by HPLC Method and Monitoring Stability**

The test cream (Lot no: M1) equivalent to the AP7G content of Kamillosan® cream (Lot no: 326670) was formulated, and its stability monitoring was carried out for 90 days. Initial AP7G content for the formulated cream and Kamillosan cream® was 0.63 mg/g.

An organoleptic test (ie, odor, color, appearance, etc.) is useful for quickly comparing the topical product and ensuring that the phases do not separate and there are no color/odor changes during storage. The test cream containing liquid extract had a characteristic chamomile extract odor and a brown-greenish cream color. No phase separation was observed after manufacturing. The pH can affect the stability of drug molecules, the rheological behavior of semi-solid products, and the effectiveness of preservatives in the products [59]. The pH of the test cream containing liquid extract was 6.31.

Stability assessment of the test product included the examination of the visual appearance, pH, rheological behavior, and quantification assay of AP7G. There were no changes during the 90 days in the chemical and physical characteristics at 25°C/60% RH and 40°C/75% RH. Initial and stability results are presented in Table 7.

Table 6. Total phenolic and total flavonoid compounds of extracts.

| Region | Total phenolic compounds % (w/w liquid extract) | Total flavonoid compounds % (w/w liquid extract) |
|---|---|---|
| Bandırma/Balıkesir | 0.14 ± 0.02 | 0.06 ± 0.00 |
| Arnavutköy/Istanbul | 0.25 ± 0.09 | 0.10 ± 0.02 |

*Values are mean ± SD (n = 3).

Table 7. Stability data of test cream (Lot no:M1) at 25°C/60% RH and 40°C/75% RH.

| Period | Appearance | pH | AP7G content (mg/g) |
|---|---|---|---|
| Initial | Brown-greenish cream | 6.31 | 0.63 ± 1.32 |
| 90th day (25°C/60% RH) | Brown-greenish cream | 6.35 | 0.65 ± 0.09 |
| 90th day (40°C/75% RH) | Brown-greenish cream | 6.40 | 0.64 ± 0.09 |

*Values are mean ± RSD (n = 3).

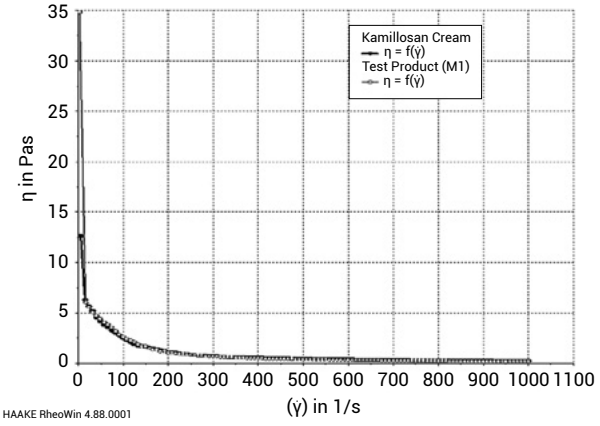



Figure 1.
Comparison of viscosity of Kamillosan® cream (Lot no: 326670) and test cream (Lot no:M1).

**Rheological Measurements**

The viscosity of Kamillosan® cream and the formulated cream was measured as described above. Viscosities were evaluated at shear rates (low, medium, and high) where the results plateaued and remained constant as seen in Figure 1.

As shown in Table 8 and Figure 1, the initial viscosity of the test product was higher at low shear stress and both of them show a plateau. At medium and high shear rates, viscosity values approached each other and overlapped in the graph. Both the test product and the reference product show decreasing viscosity and shear thinning behavior with increasing shear rate [60, 61]. The 90-day results at 40°C/75% RH (Figure 2) show that the viscosity of the test product decreased compared to the beginning. There was no significant difference between the initial viscosity values of the test product at 25°C/60% RH for 90 days (Unpaired t test ($P > 0.05$), GraphPad Prism 8.0.1).

**In Vitro Release Test**

There are studies in the literature evaluating the in vitro release of products containing mostly APG-loaded compounds [26, 62]. To our knowledge, no study has been conducted previously to evaluate

Table 8. Viscosity of Kamillosan cream (Lot no: 326670) and test cream (Lot no: M1) at three different shear rates.

| Shear Rate (1/sn) | Kamillosan® Cream (Lot no: 326670) | Test Cream (Lot no: M1) | Test Cream 25°C/60% RH-90th (Lot no: M1) | Test Cream 40°C/75% RH-90th (Lot no: M1) |
|---|---|---|---|---|
| | Viscosity (η; Pas) | Viscosity (η; Pas) | Viscosity (η; Pas) | Viscosity (η; Pas) |
| 15 | 6.18 ± 0.09 | 13.12 ± 5.38 | 13.54 ± 9.20 | 3.59 ± 1.50 |
| 500 | 0.40 ± 0.02 | 0.29 ± 0.07 | 0.48 ± 0.04 | 0.18 ± 0.02 |
| 1000 | 0.21 ± 0.01 | 0.13 ± 0.04 | 0.25 ± 0.01 | 0.09 ± 0.01 |

*Values are mean ± SD (n = 3).

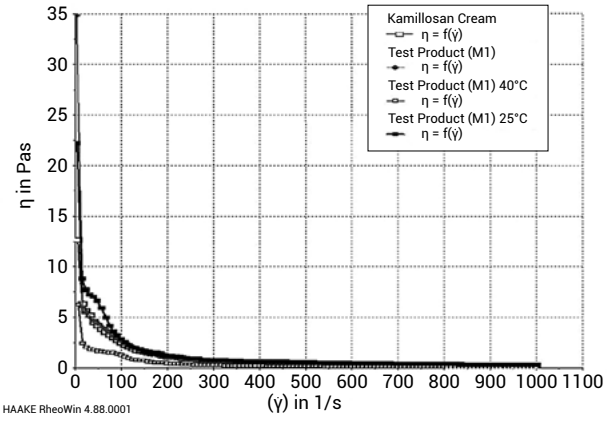



Figure 2.
Comparison of viscosity of test cream of 90-day stability (Lot no: M1).

Table 9. Cumulative amounts of AP7G released from the reference and test product through dialysis membrane.

| Time (h) | Kamillosan® cream (Lot no: 326670) | Test cream (Lot no: M1) |
|---|---|---|
| | µg/cm2 | µg/cm2 |
| **1** | 18.11 ± 0.44 | 22.41 ± 1.67 |
| **2** | 21.01 ± 0.75 | 26.26 ± 2.43 |
| **3** | 23.82 ± 0.75 | 29.56 ± 2.82 |
| **4** | 26.42 ± 1.10 | 32.36 ± 3.08 |
| **5** | 28.99 ± 1.23 | 35.09 ± 3.31 |
| **6** | 29.87 ± 1.27 | 35.76 ± 3.41 |

*Values are mean ± SD (n = 6)

in vitro AP7G release of a product containing the *M. recutita* extract used in this study, and the reference Kamillosan® Cream. Within the scope of in vitro release studies for the analysis of the reference product Kamillosan® cream and test product, the findings including cumulative AP7G amounts (µg) obtained against the square root of time (Higuchi kinetics) after HPLC analysis are given in Table 9.

From the profiles obtained as a result of in vitro release studies of the Kamillosan® cream and test cream using Franz diffusion cells and dialysis membrane, while formulated test cream release was analyzed as 35.76 ± 3.41 µg/cm$^2$, Kamillosan® cream release was analyzed as 29.87 ± 1.27 µg/cm$^2$ at the end of the sixth hour (Unpaired t test ($P > 0.05$), GraphPad Prism 8.0.1). The results were expressed as means ± standard deviations (n = 6). It revealed that the for AP7G cumulative release amount of the test cream was faster than Kamillosan® cream. The in vitro release profiles of the test and reference creams according to the amount of AP7G passing through the unit area are presented in Figure 3.

As a result of the calculated release rate constant (k) values, the test product was 9.67 ± 1.22, Kamillosan cream® was 8.53±0.58 (Unpaired t test ($P > 0.05$), GraphPad Prism 8.0.1). The results were expressed as means ± standard deviations (n = 6). It was determined that the release rates of AP7G of the test cream was faster than Kamillosan cream®. The release rate constant (k) calculated as a result of

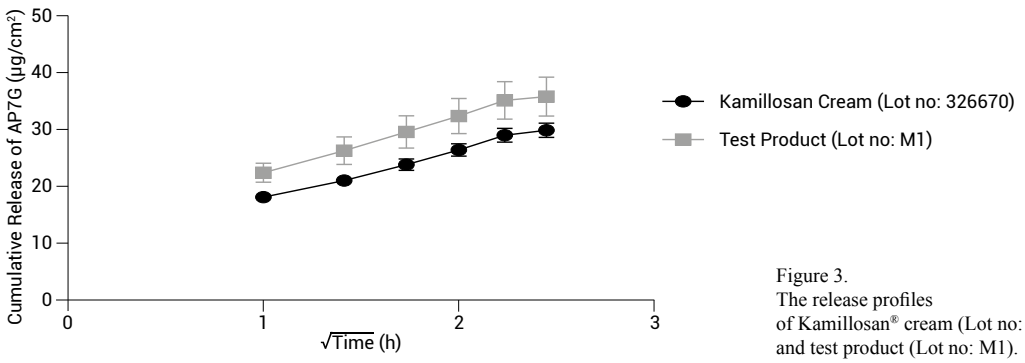



Figure 3.
The release profiles of Kamillosan® cream (Lot no: 326670) and test product (Lot no: M1).

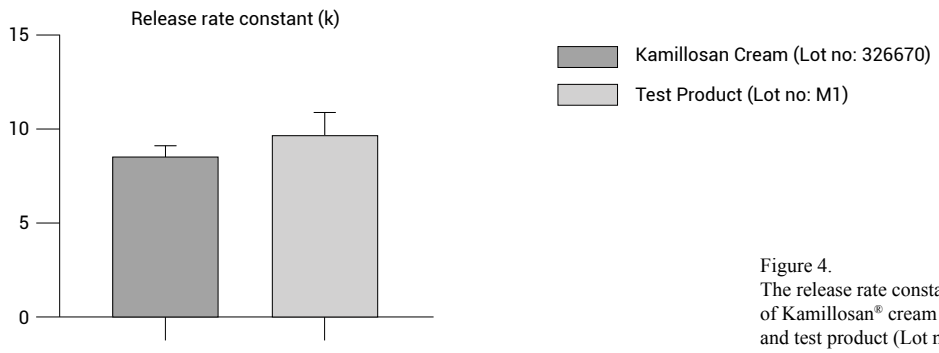



Figure 4.
The release rate constant of Kamillosan® cream (Lot no:326670) and test product (Lot no:M1).

the in vitro release test of Kamillosan® cream (Lot no: 326670) and the formulated test cream (Lot no: M1) is presented in Figure 4.

**Cytotoxicity Study**

The cytotoxic activity of the extracts, AP7G, APG, the test cream, and the reference cream against SK-MEL-28 tumor cells and CCD-1079Sk non-tumor cells was evaluated using MTT assay for 24 and 48 hours. When evaluating the cell viability plots (Figure 5 A, B), it can be seen that the extracts had no toxic effect at a concentration of 0.5 mg/mL. At a concentration of 0.031 mg/mL, cell viability decreased below 50% in both cell lines and periods. The concentrations of the extract and cream formulations were balanced according to the AP7G they contain. Surprisingly, there was no effect on cell viability at the higher concentrations. This could be due to the different compounds that are contained in the extract. Furthermore, no major differences were observed between the extract samples. The test cream sample also had a greater effect on viability against the cancer cell line at 48 hours. A positive effect on viability was observed in the cream samples at high concentrations, as in the extract samples. Figure 5 C shows the toxicity of AP7G against healthy and cancer cell lines at different concentrations. The highest cell death was observed at a concentration of 5 µg/mL. As the concentration decreased, cell viability increased. In cream formulations, 0.01 mg/g concentration decreased cancer cell viability more than CCD-1079Sk in 48 hours (Figure 5 D, E). Solutions of AP7G and APG (Figure 5 F) in DMSO were applied to the cells while cream and extract samples were applied with wax paper. The results were expressed as means ± standard deviations (n = 3). It is possible to observe differences due to the treatment method.

The cytotoxicity of extracts of this plant on SK-MEL cells was investigated in one publication. In this study, no cytotoxic effect was observed at a concentration of 0.8 mg/mL [63]. At a concentration of 0.5 mg/mL, which was the highest dose in the present study, no cytotoxic effect was observed. The results were consistent with a previous study. There are studies investigating the cytotoxic effects of *M. recutita* flower extracts on melanoma cell lines. In particular, research carried out on B16F10 melanoma cell lines showed that extracts of *M. recutita* have significant cytotoxic effects that lead to cell death in a dose-dependent manner [64]. *M. recutita* was also studied for its effects on other types of cancer. For example, a study on hepatocellular carcinoma (HepG2) cell lines found that chamomile extracts had anti-proliferative effects, significantly reducing cell viability [65]. In addition, the combination of *M. recutita* with 5-fluorouracil (5-FU) was shown to enhance antitumour activity and reduce the side effects of chemotherapy [66].

These findings highlight the potential therapeutic effects of *M. recutita* extracts on melanoma and other cancers. They also indicate the need for further research to elucidate the mechanisms and clinical applications of these effects.

**Apoptosis Assay**

The results of the flow cytometry analysis using AnnexinV-FITC and 7-aminoactino-mycinD (7-AAD) double staining against CCD-1079Sk and SK-MEL-28 cells are shown in Figure 6. The results indicate that the Istanbul extract had slightly lower toxicity than the Balıkesir extract, approximately 1.9% and 2.6% for CCD-1079Sk cells and 21.4% and 30.1% for SK-MEL-28 cells, which induced a total apoptotic/necrotic cell population after incubation for 24 h. Accordingly, Figure 6 shows that Kamillosan Cream and the test cream had no toxic effect on CCD-1079Sk cells after 24-h and 48-h incubation at a fixed concentration for AP7G (0.63 mg/g). However, we found that the treatment of cancer cells with Kamillosan® Cream and the test cream increased the number of apoptotic/necrotic cells. The test cream group induced a total apoptotic/necrotic cell population of 11.7% and 12.4% on SK-MEL-28 cells for 24 and 48 hours, respectively.

## CONCLUSIONS

In the present study, liquid extracts were prepared from the flower heads of the *M. recutita* grown and collected in the Arnavutköy/Istanbul and Bandırma/Balıkesir, Türkiye regions. A new HPLC-PDA method was developed and implemented for the quantification of AP7G in these liquid extracts. The total phenolic and flavonoid contents of the extracts were determined. Additionally, the cytotoxic activity of the extracts was examined. The extract prepared from the Arnavutköy/Istanbul region was chosen due to its higher AP7G, phenolic and flavonoid contents, and a topical cream containing this extract was formulated and an in vitro release test was performed for AP7G to examine the pharmaceutical quality and performance of the cream compared to the reference topical cream. In addition, the stability of the test product was monitored in terms of pH, visual appearance, AP7G assay, and rheological behavior, following storage

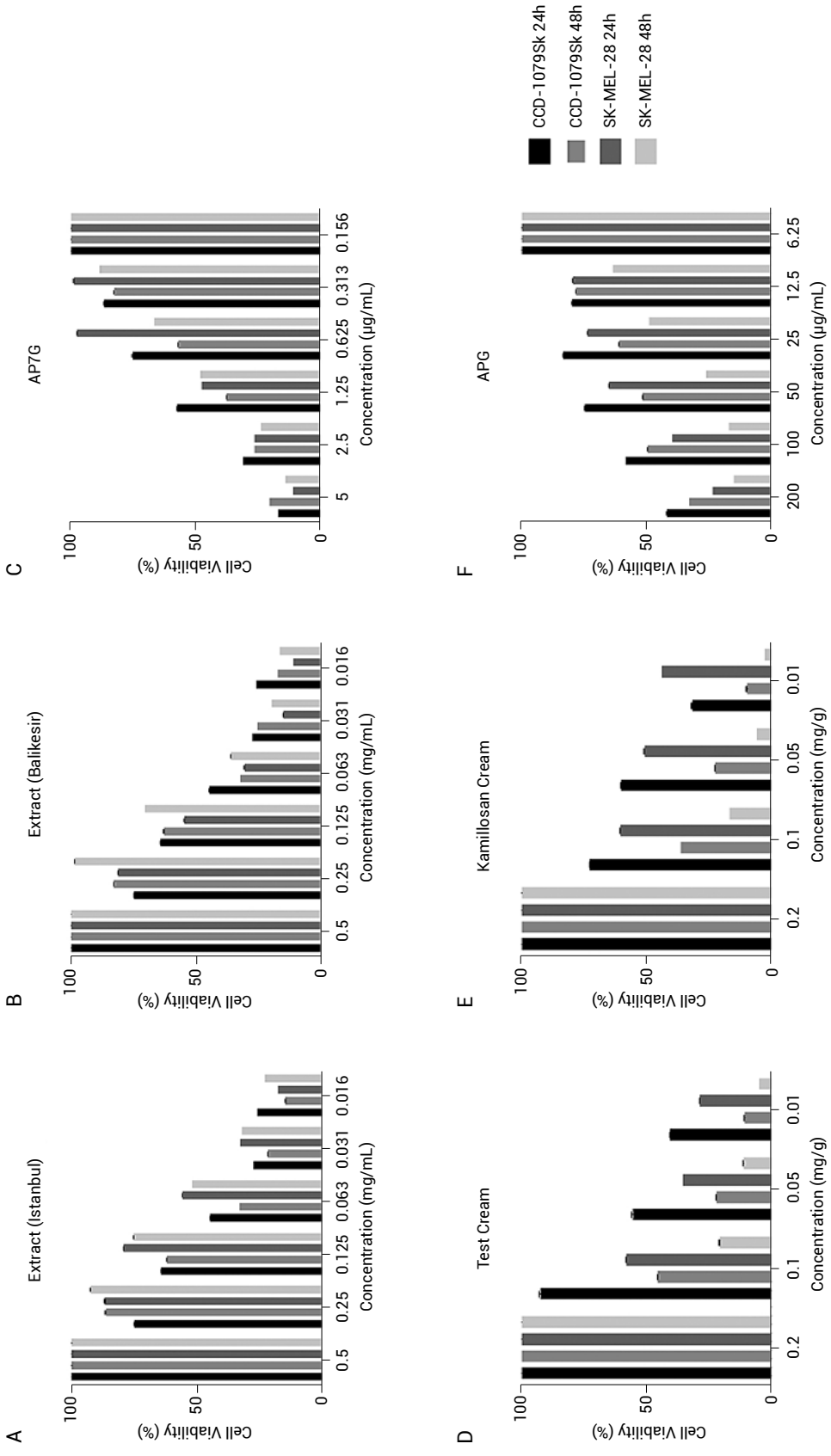



Figure 5. Viability of the samples on CCD-1079Sk and SK-MEL-28 cells after 24 h and 48 h of treatment.

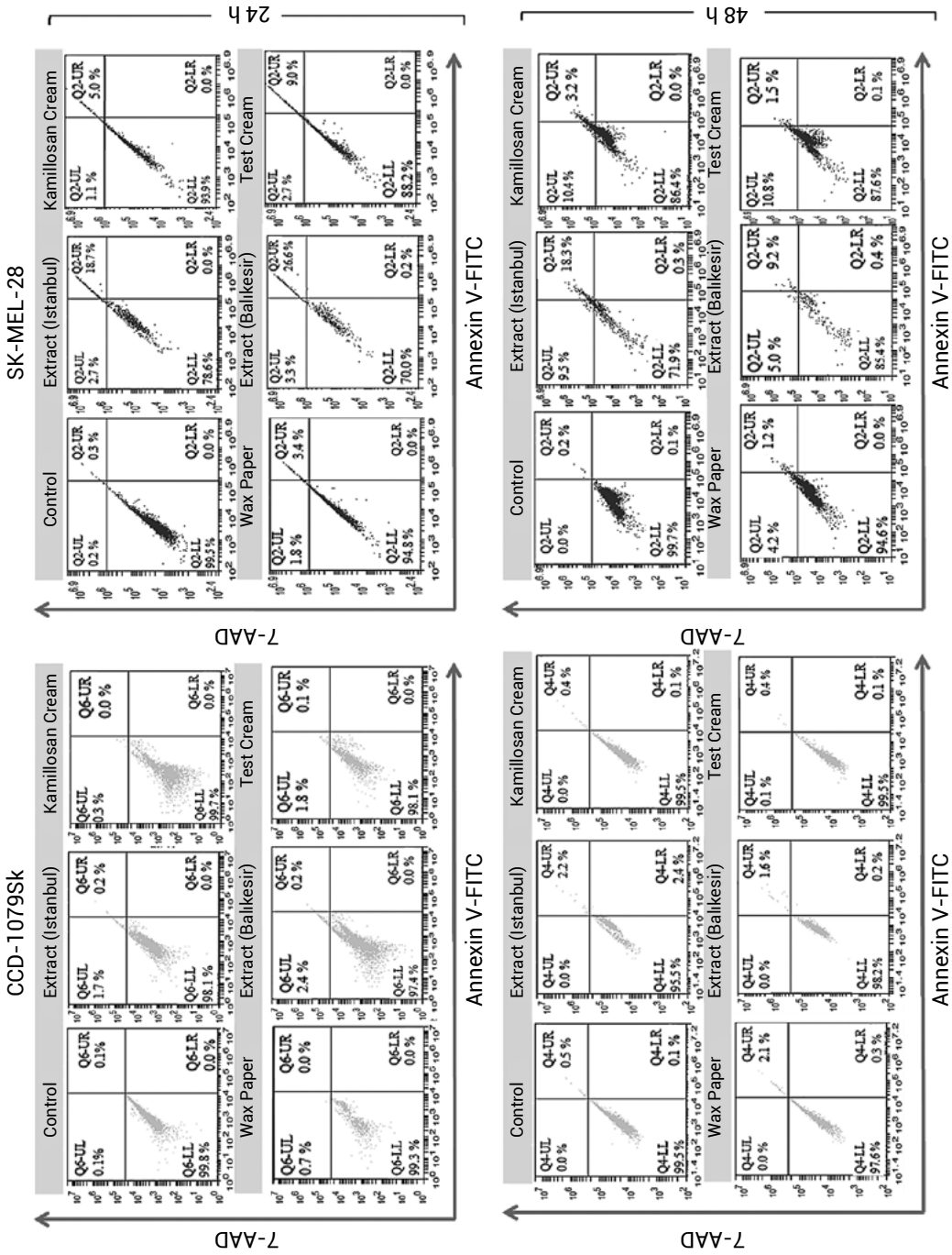



Figure 6. Apoptosis assay of the samples on CCD-1079Sk and SK-MEL-28 cells after 24 h and 48 h of treatment determined by flow cytometry.

at 25°C/60% RH and 40°C/75% RH for 90 days. A comparison of the in vitro release of AP7G was carried out between the test product and reference product. The test cream showed faster release than the reference product. As a result, in the in vitro release test, according to Higuchi kinetics, the maximum release of AP7G of the test product was better than that of the reference Kamillosan® cream. However, ex vivo and in vivo studies are required to obtain information on the permeation of formulations through the stratum corneum when applied to the skin. The in vitro cytotoxicity and apoptosis results showed that the Balıkesir extract had higher toxicity and apoptotic rate in SK-MEL-28 than the Istanbul extract. Similarly, compared to Kamillosan® cream, the formulated test cream was found to be slightly more toxic to melanoma cell lines. Considering the in vitro release,

stability, and cytotoxic activity results, the cream formulation is recommended as an alternative product for dermatological use.

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**Author's Contribution**

Conceptualization, M.C., Y.O., M.K., A.D., Z.C.; Methodology, M.C., Y.O. and M.K.; Software, M.C., H.A.I. and N.A.; Validation, M.K., Y.O. and M.C.; Formal Analysis, M.C., H.A.I., E.I., S.U., N.A., Z.C. ve A.D.; Investigation, M.C., H.A.I., E.I., S.U., Z.C. and A.D.; Resources, M.K., Y.O. and A.D.; Data Curation, M.K.; Writing – Original Draft Preparation, M.C.; Writing – Review & Editing, M.C., M.K., Y.O., A.D. and Z.C.; Visualization, M.C. M.K. and N.A.; Supervision, M.C.; Project Administration M.K.

**Conflict of Interest**

The authors disclosed no conflicts of interest related to this article.

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