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Chlorogenic Acid: HPLC Quantification and In Vitro Assessment of Proliferative and Migration Effects on Human Dermal Fibroblast Cells

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ABSTRACT

Chlorogenic acid (CA) exhibits diverse biological activities, including antioxidant and antiinflammatory effects. This research aims to develop, optimize, and validate an HPLC method to quantify CA in methanol and investigate its *in vitro* proliferative and cell migration effects on human-dermal-fibroblast (HDF) cell lines in a dose-dependent manner. The HPLC experimental conditions were optimized using the central composite design (CCD) method for determining CA. Chromatographic separation occurred at a wavelength of 330 nm. Under the optimized conditions, the method exhibited linearity across a concentration range of 0.1-100 µg/mL, demonstrating sensitivity (LOQ:0.1µg/mL), precision (RSD%≤3.32), and accuracy (RE%≤4.05). To evaluate the in vitro proliferative and cell migration effects on HDFs, we employed the XTT cell proliferation assay and TAS-TOS commercial kits. The XTT assay revealed that CA displayed a proliferative effect within the concentration range of 75-250 µM (P <0.01), and at a concentration of 125 µM, TAS levels increased significantly (P<0.05). The scratch assay demonstrated that HDF cell migration increased at 12 h, with substantial closure of the wound area at 24 h when treated with CA concentrations between 75-125 µM. The results demonstrate that pure chlorogenic acid extracted from plants exhibits dose-dependent effects on cell proliferation, antioxidant, and cell migration.

Keywords: Chlorogenic acid, HPLC, Experimental design, Central composite design, Proliferative and migration effects.

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Introduction

Phenolic acids have recently played an important role in human health due to their various pharmacological and biological effects. Additionally, chlorogenic acid (CA), one of the polyphenols found in large amounts in the human diet, is found in large amounts in foods, certain plant species, fruits, and coffee types, especially in green coffee [1]. CA [1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid 3-(3,4-Dihydroxycinnamate], named 5-O-caffeoylquinic acid (5-CQA) by the International Union of Pure and Applied Chemistry (IUPAC), is an ester from the hydroxycinnamic acid family (Figure 1).

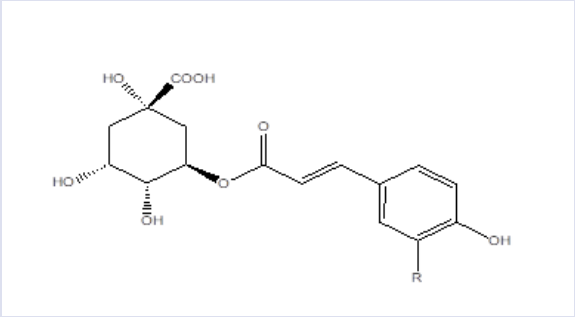

Figure 1. Structure of Chlorogenic Acid

It has been reported that CA has an antimicrobial effect against various bacteria and antifungal effect against *Candida albicans*, and it has been found that it is suitable as an ideal preservative and food additive with these properties. It has been reported that, similar to other phenolic acids, OH groups and numbers on CA positively affect antioxidant activity. In addition, it has been reported that CA, interleukin-8 (IL-8), and mRNA expression significantly inhibit their secretion due to oxidative stress. Thus, it is a compound with antioxidant and antiinflammatory potential [2]. In addition, it has antibacterial, antiviral, antifungal, antidiabetic, and anticarcinogenic properties [3-5]. Furthermore, it was observed that chronic use of CA in high doses for a long time increased the lipid peroxidation level of liver and kidney samples without affecting the heart and muscle tissue [6,7]. Therefore, it is crucial to determine the amount of CA in plants and foods containing CA. Obviously, a simple and rapid analytical method is needed for the routine measurement of this substance. A review of the literature reveals numerous analytical methods, such as high-performance liquid chromatography (HPLC) with UV detection, employed to determine chlorogenic acid (CA), the primary phenolic acid, in different *Helichrysum* species [8-10], other plants [11], and various foods, particularly coffee [12, 13], following diverse

extraction processes [14-17]. In many HPLC methods for CA determination, separation is achieved by adding acid to the mobile phase [8-13]. Researchers commonly reported that the peak area, retention times, and peak resolution of CA are significantly influenced by the mobile phase solvent ratio and acid percentage in HPLC analysis. Typically, single-factor optimization, lacking interactions between factors, was employed, which was time-consuming and necessitated numerous experiments to determine the optimal level for each factor. Currently, green analytical chemistry is a prominent topic in analytical chemistry, focusing on environmentally friendly approaches to enhance the quality of analytical results [18]. Chemometric techniques, incorporating experimental design and principles of green analytical chemistry (such as reducing interferences of the analyzed substance and using fewer chemicals and time while maintaining acceptable accuracy and precision), have been recently applied to determine experimental conditions in chemistry. The application of the CCD method allowed for the simultaneous determination of various parameters with their interrelation [19].

Wound healing is a complex process that includes hemostasis, inflammation, cell proliferation, extracellular matrix synthesis, and remodeling. Different factors are involved in regulating and controlling these phases [20]. Therefore, when investigating the sufficient attributes for wound healing, it is evident that cell proliferation and cell migration are particularly effective on the wound. This is because wounds cannot be successfully repaired without an adequate level of cell division [21]. Therefore, the substance used as a wound healer should affect the factors that play an essential role in wound healing (inflammatory cells, platelets, cytokines, extracellular matrix, etc.). In addition, it should shorten the duration of these periods and provide ideal neovascularization and scar tissue formation [22]. Antioxidants and antiseptics are used to increase the number of fibroblast cells in wound healing. The wound is cleansed in the fibrin matrix by the release of oxygen radicals, which kill the invading foreign organisms. This accelerates the healing process. Fibroblasts play an essential role in wound healing. They initiate the proliferative repair phase. Today, to accelerate and regulate wound healing, studies on the use of many medical and traditional drugs are still being carried out, and new products are being researched. In this context, the studies conducted on CA showed that it has different pharmacological effects containing antimicrobial, anti-inflammatory, and antioxidant properties ([11], [23-25])

In an *in vivo* study evaluating the effectiveness of CA on wound healing, it was reported that CA has a high wound healing capacity due to its potent antioxidant capacity and accelerates the wound healing process by increasing collagen synthesis in different phases of wound healing [6]. Another study stated that CA increased glutathione, catalase, and superoxide dismutase and decreased lipid peroxidation, thus having strong antioxidant activity. It has been shown that CA increases the wound healing process by increasing collagen synthesis and antioxidant activity through TNF-α and TGF-β upregulation during wound healing [7]. However, there is no study of an *in vitro* dose-dependent proliferative and cell migration effects on the HDF cell line of CA.

Therefore, this study aims to develop an optimized HPLC method using CCD, an experimental design method, and investigate the effects of CA on proliferation, oxidative stress, and *in vitro* cell migration using HDF cell line.

Materials and Methods

Reagents

CA was procured from Sigma (USA). Methanol (HPLC grade) and acetonitrile (HPLC grade) were supplied by Merck (Germany), while analytical grade orthophosphoric acid (OPA) was acquired from Sigma–Aldrich (USA). A Milli-Q system (Thermo Scientific, USA) was used to obtain ultrapure water. ATCC® PCS-201-012™ was used as Adult HDFa. The commercial TAS-TOS kits were obtained from REELASSAY (Turkey). XTT kits, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), phosphate buffer (PBS), L-glutamine, Pen-strep solution, and trypsin-EDTA were provided by Sigma (USA).

HPLC Method

Preparation of Stock, Working, and Quality Control Solutions of Chlorogenic Acid

Stock solutions of CA were prepared with methanol at a 10 mg/mL concentration. Working solutions (WC; 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, 60, 80, and 100 µg/mL) and quality control (QC; 0.5, 10, and 80 µg/mL) solutions for CA were prepared in acetonitrile. All solutions were stored at +4°C pending analysis.

Optimization of Mobile Phases in HPLC Analysis by Central Composite Design (CCD)

In the central composite design (chemometric experimental design method, CCD), the percentage concentration of acetonitrile (A), which is effective in obtaining the highest peak area and the most regular peak shape in HPLC analysis, and the percentage concentration of OPA in water (B) were selected as significant factors of CCD in the mobile phase for the determination of CA. The A and B factor ranges were 30%-95% and 0.01%-0.5%, respectively. CA solutions (10 µg/mL) were prepared to monitor the response variables.

HPLC Apparatus and Analytical Conditions

An Agilent 1200 Series HPLC instrument was equipped with a G1311A/Quat Pump, and a G1314B/VWD detector was used. Chromatographic separation was achieved on a BRISA LC2 C18 column (250 × 4.6 mm, 5-µm particle size). Isocratic elution with a mobile phase consisting of acetonitrile and water containing 0.2% OPA (95:5 v/v) was used for HPLC analysis. The detection wavelength was set at 330 nm. Chromatographic separation was performed at

a 7 min run time using a 1 mL/min flow rate, 10 µL injection volume and ambient column temperature.

Cell Culture Experiments

The medium used in the cell culture study to be carried out in *in vitro* environments should contain the necessary substances suitable for cell growth, survival, and proliferation. Therefore, this study used DMEM containing 10% FBS, 2 mM L-glutamine, 20 IU/mL penicillin, and 20 mg/mL streptomycin to grow HDFs. Routine cell culture steps such as thawing, propagation, passaging, and freezing of the cells were performed on HDFs.

XTT Assay for Chlorogenic Acid

Cell viability was determined by inducing the effect of CA on cell proliferation using the XTT kit. HDF cells (10000/well) were seeded into 96-well plates, and then these cells were incubated under 5% CO_2 for 24 h at 37°C and grown to 80% confluence. Cell counts were made in all experimental steps to ensure experimental standardization. The cells were treated with control (C; untreated cells), 0.5% DMSO, and CA (final concentration 10-1000 µM in each well) dissolved in 0.5% DMSO culture media and incubated under 5% CO_2 for 24 h and 48 h at 37°C to determine a dose-dependent manner. After cultivation for 24 h and 48 h, XTT reagent solution was added to the medium as recommended by the manufacturer, followed by the measurement of absorbance at 450 nm with a correction wavelength of 630 nm in a microplate reader.

Determination of Total Oxidant Status (TOS) and Total Antioxidant Status (TAS)

TOS and TAS of CA were determined with commercial kits according to methods developed by Erel [26]. First, HDFs (10000/well) were seeded in 96-well plates and incubated under 5% CO2 for 24 h at 37°C. After that, 10, 25, 50, 75, 100, 125, 250, 500, and 1000 µM concentrations of CA in HDF cells were added and incubated for 24 and 48 h. After incubation, supernatants of HDF cells were separated (number of repetitions: 3) and stored at -20°C until analysis.

TOS analysis: The method measures the color intensity in the presence of oxidants in samples with spectrophotometry. For this purpose, supernatants removed at 24 h and 48 h were analyzed as recommended by the manufacturer using the TOS commercial kit to determine the TOS levels of CA. The method was calibrated with H_2O. The method's precision was expressed as RSD% and found to be ≤ 2%. The obtained results are given in µmol H_2O_2 Equiv./L sample.

TAS analysis: The method is based on the OH* radicals produced by the Fenton reaction and its reaction with the colorless substrate O-dianisidine to produce dianisyl radicals by antioxidants in samples against potent free radical reactions. For this purpose, supernatants removed at 24 h and 48 h were analyzed as recommended by the manufacturer using a TAS commercial kit to determine the TAS of CA. The obtained results were given as µmol Trolox Equiv./L sample.

Scratch Assay

HDF cells were used for the *in vitro* wound healing model for cell migration by performing a scratch assay. HDF cells were seeded in 6-well plates (3×10^5 cells/well) and incubated until 100% confluence was achieved in DMEM solution (containing 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine) under 5% CO_2 at 37°C. Later, the scratch was formed in one direction of approximately the same size using a micropipette tip (0.1-10 µL). Then, the floating cells and the medium were exchanged with fresh medium. Different concentrations of CA (containing 10, 25, 50, 75, 100, 125, 250, and 500 µM concentrations) were added to each well, and the microscopic image was monitored with an inverted fluorescence microscope (Leica, ebq 100-04, Germany) at 0, 12, 18, and 24 h. The experiment was terminated when cell migration was completed entirely in one group [26].

Statistical Analyses

Experimental design and statistical analyses (one-way ANOVA with Tukey's post hoc test) were performed using Design-Expert8.0 (Stat-Ease Inc., Minneapolis, MN, USA) and SPSS 20 (SPSS, Chicago, IL, USA) software, respectively. *P* values<0.05 were taken into consideration to indicate statistical significance.

Results

Chlorogenic Acid Analysis with HPLC

Optimization of HPLC Analysis with the CCD Method:

CA was prepared at a 10 µg/mL concentration by dilution with acetonitrile. Chromatographic separation was achieved on a C18 (250x4.6 mm, 5 µm) column at 330 nm. To determine the mobile phase composition consisting of acetonitrile, water, and OPA%, the CCD method was applied to determine the percentages of acetonitrile (A %) and OPA in water (OPA %), which were selected as mobile phase components in the HPLC method. The ranges of the A and B factors are specified in Section 2.3. The peak area of CA (10 µg/mL) was monitored as a response variable, and 13 different experiments were carried out. The peak area of CA experimentally determined from CCD is shown by the response surface curve (three-dimensional graph) (Figure 2A). Experimental data were placed on the response surface to obtain optimum conditions. The resulting quadratic regression model is shown below with coded terms:

$$RF = 301.96 – 22.80A -43.34B + 8.58 AB-11.81 B -31.81B$$

where RF is the response factor corresponding to the peak area of CA. A and B factors. According to the parameters obtained from the ANOVA statistical method

for the model, the *P* value was 0.0057. Since this *P* value was significant at < 0.05, the A and B factors significantly affected the peak area of CA. The coordinates producing the maximum desirability were A: 95% and B: 0.2%. The predicted response peak areas from the numerical optimization are 319.52. Experiments (n=10) were performed to compare the point predicted values with the experimental results, and the response peak area values were recorded. Based on the predicted outcome, the mean recovery of the obtained results was calculated as 100.26 ± 0.8023%. In all HPLC conditions mentioned above, CA showed a retention time of 2.7 min with a maximum peak at 330 nm (Figure 2B).

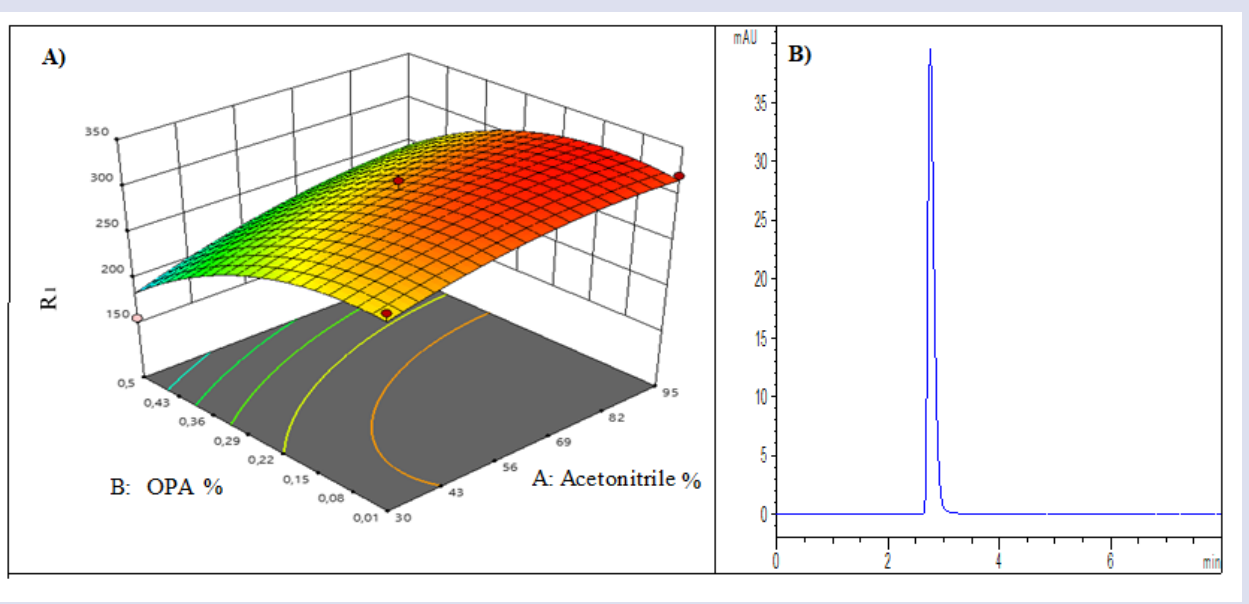

Figure 2. A) Three-dimensional plot of the response surface for peak area (R1) shown as a function of A and B. B) HPLC chromatogram of CA (10 µg/mL)

HPLC Method Validation: The calibration curve was obtained by plotting the peak areas and CA concentrations. The equations for calibration curves in the 0.1-100 µg/mL concentration range for determination of CA were obtained by:

PA = 32.421x – 8.530 (Sa: 0.325, Sb: 0.068 and R^2 = 0.9999; n: 6)

where PA is the peak area of CA, Sa is the standard deviation of the shift in the calibration curve, Sb is the standard deviation of the slope in the calibration curve, and R is the correlation coefficient.

The method precision and accuracy were determined by analyzing the QC samples (0.5, 10, and 80 ng/mL) with intraday (6 times per day) and interday (6 times once daily for 6 days) measurements. The method's precision was given as the relative standard deviation (RSD %: (standard deviation/mean) × 100), and it was < 3.320%. The accuracy of the method was given as relative error (RE %: [(found concentration-known concentration)/known concentration × 100] and found to be less than ± 4.052 (Table 1). With acceptable values, the method is accurate and precise for the determination of CA.

Table 1. Precision and accuracy of CA using the proposed method.

Added (µg/mL)	Intraday			Interday		
	Found ± SD (µg/mL)	Accuracy (RE)	Precision (RSD %)	Found±SD (µg/mL)	Accuracy (RE)	Precision (RSD %)
0.5	0.479± 0.016	-4.052	3.320	0.501± 0.009	0.191	1.806
10	9.970± 0.283	1.129	2.835	10.018±0.023	1.605	0.232
80	79.887± 1.191	-0.165	1.491	80.047±0.741	0.068	0.925

SD: standard deviation (n=6). RE: relative error, RSD: relative standard deviation.

The limits of detection (LOD) and the limit of quantification (LOQ) were calculated as 3xσ (standard deviation of y-intercepts)/S (slope of the calibration curve) and 10xσ/S and found to be 0.031 µg/mL and 0.1 µg/mL, respectively.

The extraction recoveries of CA in the extracts of plants were determined using the standard addition method of QC samples (0.5, 10, and 80 µg/mL) to plant solution. All of the experiments were repeated six times. The quantification of CA was calculated by comparing the found and added concentrations in each case. The

obtained recovery results for 0.5, 10, and 80 µg/mL CA were 99.85%, 101.04%, and 100.56%, respectively, and showed the good accuracy of the HPLC method.

To determine the stability of the stock and calibration solutions of CA during the study, the QC solutions of CA were kept at +24°C, at 6, 24, and 48 h at +4°C, analyzed at the end of these periods, and the recovery (%) was obtained by comparison with the immediately measured values. ±10 values were determined as the acceptance criteria. According to these criteria, CA was stable for at least 6 h at +24°C and at least 48 h at +4°C.

To showcase the suitability of the established method for plant samples, the developed and validated HPLC method was utilized to quantify chlorogenic acid (CA) isolated from the capitulum of *H. plicatum subsp. pseudoplicatum* plant using methanol. For this investigation, the extracted sample weighing 1 mg was diluted to 500 µg extract/mL (n:10) and subsequently analyzed with the developed and validated method. The relative quantity of CA was determined to be 9.770 ± 1.818 µg/mg extract.

Cell Culture Experiments for for Chlorogenic Acid

XTT Analysis Results for Proliferative Effect: The effects of CA on HDF cell proliferation were studied using the XTT assay. Figure 3 shows the XTT results obtained at between 10-1000 µM concentrations of CA in HDF cells at 24 and 48 h. We found that CA concentrations in a dose range of 75-250 µM have a significantly positive proliferative effect on HDF cells compared to control (untreated cells) at 24 and 48 h (P<0.01).

TOS-TAS Level Results for Oxidative Stress Activities: The effects of CA on oxidative stress were determined in HDFs using the TAS-TOS commercial assay. Figures 4 and 5 show the effects of CA at concentrations between 10-1000 µM on oxidative stress in HDF cells obtained after 24 and 48 h of incubation. It was determined that the TOS of CA decreased in HDF cells treated with 75-125 µM concentrations compared to the C group (untreated cells) at both 24 and 48 h (Figure 4). However, this decrease was not statistically significant (P > 0.05). The obtained TOS results were in line with our XTT results. It was determined that the TOS of CA at high concentrations increased while causing the highest increase in cell viability.

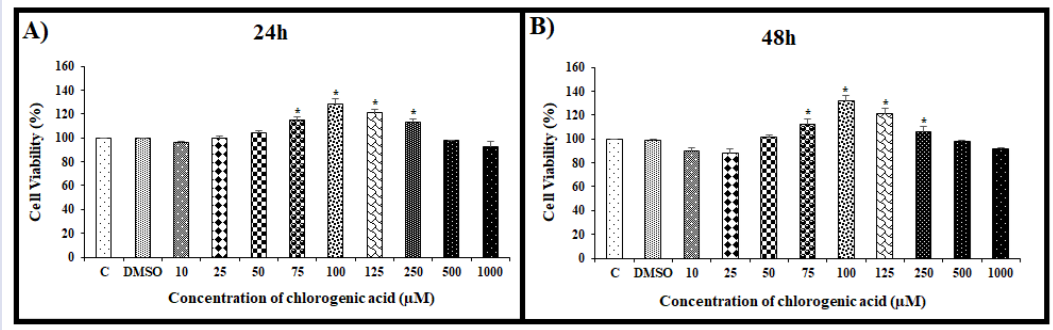

Figure 3: Proliferation effect obtained at different CA concentrations treated with HDF cells using XTT assay A) 24 h and B) 48 h (Results are given as the mean ± SD, n=8,*statistically significant compared with the control (untreated cells); C: control, DC: 0.5% DMSO (DMSO control)).

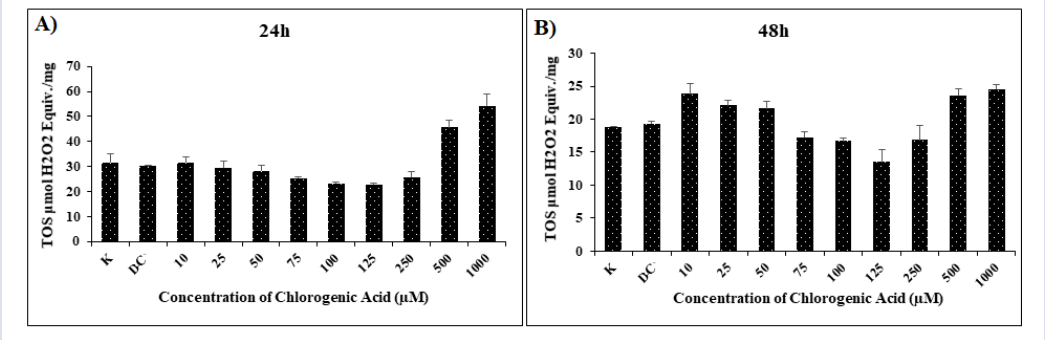

Figure 4: TOS levels of CA on HDF cells. A) 24 h and B) 48 h ((Results are given as the mean ± SD, n=8; C: control, DC: 0.5% DMSO))

The TAS of CA increased in HDF cells treated with 100-250 µM CA compared to the C group (untreated cells) at both 24 and 48 h (Figure 5). Only at 125 µM was the increase in CA statistically significant (P <0.05). Similar to the TOS results, the TAS results of CA were consistent with the XTT results. It was determined that 100-250 µM CA had the highest effect on cell viability and antioxidant capacity.

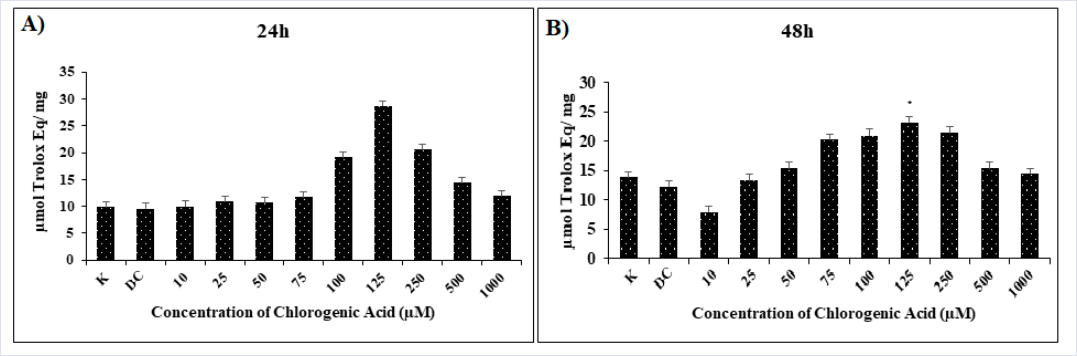

Figure 5: TAS levels of CA in HDF cells. A) 24 h and B) 48 h ((Results are given as the mean ± SD; n=8; *statistically significant compared with the control (untreated cells); C: control, DC: 0.5% DMSO).

Scratch Assay Results for Cell Migration Effects

In the current study, the effect of CA on cell migration was investigated in the range of 10-500 µM by using a starch assay test because it is toxic at concentrations above 500 µM according to XTT analysis. We found that CA had a dose-dependent effect on the HDF cell line.

We have demonstrated that CA at 50-250 µM concentrations had a stimulatory effect on cell migration, especially at 75-125 µM concentrations. CA increased cell migration at 12 h and significantly closed the wound area at 24 h (Figure 6). At the same time, it was determined that the cell migration results were parallel to the XTT results, and 75-125 µM CA had the strongest effect on cell viability and cell migration .

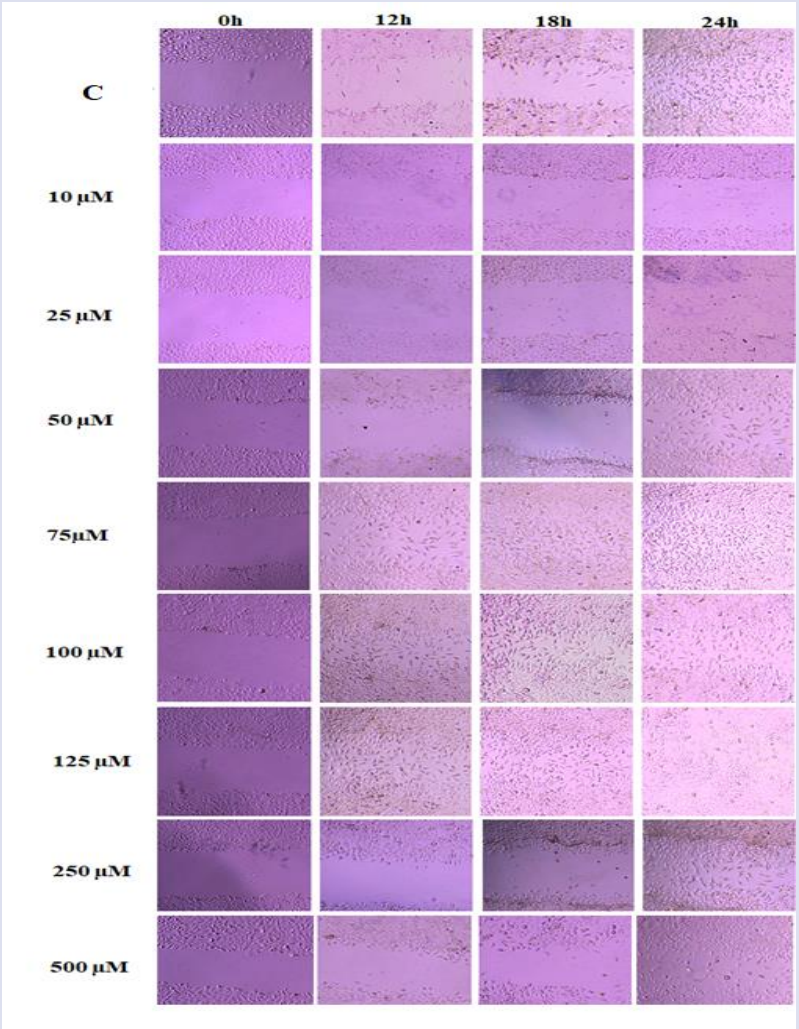

Figure 6. Photographs were taken at 0, 12, 18, and 24 hours by microscopy at 4× magnification to determine the effect of CA on HDF cell migration.

Discussion

In recent years, there has been an increasing focus on the study of phenolic compounds, which are vital bioactive compounds possessing various beneficial properties. Among these compounds, CA has garnered significant attention because it serves as the primary phenolic compound/metabolite present in plants and foods [27]. However, before recommending the traditional use of these bioactive compounds for wound healing, it is crucial to quantitatively determine their levels. While bioactive compounds undergo rigorous quality control, improper dosages may result in insufficient effects or even harmful consequences. Growing concerns about the potential carcinogenic effects of chemical preservatives in the food industry have spurred interest in natural and herbal alternatives. Natural antioxidants derived from various plants, which are well received by consumers, are preferred over synthetic chemical preservatives for food products. CA, in particular, stands out as an ideal natural and safe food additive due to its antioxidant properties [28, 29]. To harness the potential of plants as a source of CA, it is essential to optimize the isolation of CA from plant extracts, a task made possible through the selection of an appropriate analytical method. The HPLC method is widely used for the analysis of drug compounds, active components in plants, and residues in food [30]. Its broad applicability and effectiveness make it a valuable tool for these types of analyses. For quantitative determination of CA with the HPLC method, stock and dilution solutions of CA were prepared in water, acetonitrile, methanol or their different mixtures used in previous studies [8-13, 27, 31, 32]. To determine the solvent medium in which the CA chromatogram has the most regular and the highest peak in the HPLC. CA was prepared by dilution with the solvents mentioned above, and HPLC chromatograms were taken. Acetonitrile was selected as a solvent because CA was dissolved and achieved the highest peak. A C18 reversed-phase column was used for chromatographic separation of CA in HPLC studies in the literature for the determination of CA in food and plants and for good separation, 0.1% formic acid [13, 27, 31], 0.2% formic acid [8], 0.5% acetic acid [9], 0.1% acetic acid [10, 11], acetic acid [12], 0.05% trifluoroacetic acid [32], and 0.1% formic acid were added to the mobile phase, which usually contains acetonitrile or methanol and water. Experimental design methods determine the optimum experimental conditions by mathematical modeling. The CCD method is one of the experimental design methods. This method can determine the factors affecting the experiment and the interaction between experimental errors and optimum experimental conditions [33].

In our study, chromatographic separation was achieved on a C18 (250×4.6 mm, 5 µm) column at 330 nm, and different solvent mixtures of methanol-water and acetonitrile solvents as mobile phases were tried. The mobile phase mixture was selected as acetonitrile-water. It was stated that the mobile phase composition should be acidic to determine CA by the HPLC method. Preliminary experiments and prior knowledge of the literature were decisive for selecting these two parameters as significant. Therefore, OPA was added to water, one of the mobile phase components, to make the mobile phase acidic. At the end of 13 experiments with the CCD method, the selected parameters A% and OPA% were determined to be 9% and 0.2%, respectively, with the CCD method. Then, the optimization of the HPLC method was validated with different validation parameters following ICH Q2B guidance [33]. The validation study supported the HPLC method conditions by confirming that the assay was linear, specific, sensitive, accurate, and precise. The developed, optimized and validated HPLC method was demonstrated to be applicable by determining the amount of CA in the selected *H. plicatum subsp. pseudoplicatum* plant with capitulums. It can be easily applied to both plant and food samples if a suitable extraction process is found depending on the matrix.

Plants and food have had an important place in human life in terms of improving health since ancient times. Wound healing is characterized by epithelial, endothelial, inflammatory cells, platelets, and fibroblasts coming together and performing their normal functions in a particular order [35]. Plants and food contain antifungal, antibacterial, and bioactive antioxidant compounds, which provide coagulation, inflammation, collagen production, epithelial formation, and wound healing properties [34]. Bağdaş et al. [6] investigated the potential role of CA treatment on wound healing and oxidative stress markers and whether the treatment has side effects on vital organs and bone marrow using an experimental wound model *in vivo*. CA as IP at various doses was applied and showed that CA treatment accelerated wound healing. CA decreased malondialdehyde (MDA) and nitric oxide (NO) levels in the wound area, increased glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels, and increased hydroxyproline content in the scar tissue. It was concluded that CA treatment accelerated wound healing by increasing antioxidant defense. In addition, it has been shown that long-term high-dose chronic use of CA may cause side effects due to its pro-oxidant effect on the liver, kidney, and bone marrow. In another study, the effects of topical CA treatment on wound healing were investigated by Chen et al. [7], and it was found that CA caused a significant increase in wound contraction. The study showed that CA has potent antioxidant activity by increasing superoxide dismutase, catalase, and glutathione and decreasing lipid peroxidation. It causes upregulation of TNF-α and TGF-β levels in the inflammatory phase of wound healing (12-72 hours). It was concluded that it increases the wound healing process by increasing collagen synthesis and antioxidant activity through TNF-α and TGF-β upregulation. Antioxidant and antiseptic agents used in wound healing treatment cause an increase in the number of fibroblast cells. Because the release of oxygen radicals kills invading foreign organisms and the wound in the fibrin matrix is cleaned. In other words, it accelerates the

healing process. In this context, fibroblasts, which initiate the proliferative repair phase, have an important role in wound healing [11,25].

Various *in vitro* wound healing models utilizing cell lines and methods derived from human or murine (rodent) keratinocyte and fibroblast cells have been employed to explore potential therapeutic agents [36]. For instance, Moghadam et al. [37] demonstrated the wound-healing effect of *Parrotia persica* extract, which contains chlorogenic acid and myricetin-3-O-β-rhamnoside, using normal human keratinocyte and HDF cell lines *in vitro*. Similarly, Alexandru et al. [38] investigated the wound-healing activity of plant extracts containing polyphenols, such as chlorogenic acid, caffeic acid, luteolin, and apigenin, on L929 fibroblast cells. They observed an increase in collagen synthesis and antioxidant activity. Several studies have shown the *in vitro* wound healing and antioxidant activity of various plant extracts containing chlorogenic acid. However, the efficacy of CA isolated directly from the plant or pure CA remains unexplored. Additionally, a few studies have demonstrated the antioxidant and wound healing efficacy of CA *in vivo*. The scratch assay using an HDF cell line is one of the most commonly used wound models for cell migration in cell culture studies [39,40].

Nevertheless, the specific proliferative and migration properties of CA using human dermal fibroblast cells and its antioxidant activity using TAS-TOS have not been adequately explored in the literature.

In this line, our study evaluated the proliferative effect on cell viability with the XTT test after 24 and 48 incubations using various concentrations of CA (final concentration of 10-1000 µM in each well). The XTT results showed that CA had a significant dose-dependent proliferative effect in the dose range of 75-250 µM (Figure 3).

Since measuring oxidant species with different properties is expensive, time-consuming, and requires intensive labor, it is preferred to measure TAS-TOS, a reliable and sensitive method that researchers can easily apply in recent years with a short duration and high linearity. Therefore, in our study, the efficacy of CA on oxidative stress was evaluated using the TAS-TOS kit. It was observed that they have a dose-dependent antioxidant effect, similar to the literature [6, 7]. Additionally, CA significantly increased TAS compared with the control group, especially at 100-250 µM (Figure 5). These results were found to be compatible with the XTT results.

The effects of CA on *in vitro* cell migration were evaluated using the *scratch* assay. Our study found that CA increased cell migration at 50-250 µM, especially at 75-125 µM. CA increases cell migration at 12 h and significantly closes the wound area at 24 h. CA has the most substantial effect on cell viability, antioxidant effect, and *in vitro* cell migration effects, especially at concentrations of 75-125 µM. It has been shown that the cell migration effect of CA may depend on its antioxidant effect in a dose-dependent manner. In this study, we observed that the proliferative, antioxidant, and cell migration activities of CA exhibit dose dependency, and all these activities are evident within the same dose range. Consequently, it was hypothesized that the cell migration effect of CA is directly influenced by its antioxidant properties, as the observed effects seem to correlate with the varying doses applied.

Conclusion

This study aimed to develop and optimise an HPLC method using a central composite design (CCD) approach for the quantitative analysis of chlorogenic acid (CA). The method was carefully validated for accuracy, precision and sensitivity, establishing it as a robust tool for the determination of pure CA concentrations in isolated from *H. plicatum subsp. pseudoplicatum*. In addition, we investigated the dose-dependent effects of CA on cell proliferation and migration in HDF cell lines, highlighting its potential therapeutic applications in wound healing and tissue regeneration. The results of this research span analytical chemistry and pharmacology, providing a reliable means of quantifying CA and uncovering significant biological activities, laying the groundwork for future studies to explore the full therapeutic potential of CA.

Moving forward, it is recommended that future research focus on elucidating the specific mechanisms of action of CA, conducting clinical trials to validate its efficacy, and optimising its application in clinical settings.

Conflict of Interest

There is no conflict of interest among the authors.

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