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FELLOWSHIP FINAL REPORT

Chemical analysis of some plants used for cosmetic purposes in Turkish ethnobotanical studies and their in vitro physiological effects on human origin cell lines

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REPORT INFO

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ABSTRACT

Türkiye has long been known for its wealth of natural medicinal and cosmetic resources. In terms of plant diversity, Türkiye is one of the wealthiest countries on Earth. Thirty percent of the 10,500 plant species identified in Türkiye to date are endemic. This richness translates into a high use of plants for medicinal or cosmetic purposes. As a result of ethnobotanical research conducted in Türkiye, several plant genera widely used by local people for cosmetic purposes have been identified. This study aims to rationalize the traditional cosmetic uses of plants in Türkiye by conducting ethnobotanical studies across different provinces, collecting plants, screening for biological activities, assessing cytotoxic effects on skin cell lines, and conducting in-depth phytochemical analysis of the most promising plants. This is to develop new cosmetic formulations using local plants.

1- Introduction

Türkiye has long been known for its wealth of natural medicinal and cosmetic resources. In terms of plant diversity, Türkiye is one of the wealthiest countries on Earth. Thirty percent of the 10,500 plant species identified in Türkiye to date are endemic. This richness translates into a high use of plants for medicinal or cosmetic purposes [1]. The study of traditional plant knowledge as a part of human-plant relationships is known as ethnobotany. It includes the traditional understanding of how to classify, grow, and use plants for food, medicine, and cosmetics. Ethnobotany has grown in popularity worldwide as a result of the discovery and documentation of obscure traditional medicinal and cosmetic uses of wild flora, and nowadays, many medications and cosmetics contain plant-based ingredients derived from these studies. Moreover, these ethnobotanical studies on documenting indigenous knowledge are essential for the exploitation and conservation of biological resources, as well as for the tribes' economy [2].

Herbarium of Altınbaş University, Faculty of Pharmacy (HERA) is an international herbarium registered with the New York Botanical Garden. In light of information from local people, identifying the scientific names of plant species and preserving them in herbariums is a vital aim to ensure that the chemical or biological studies are carried out with the correct plant species. Natural resources are of great importance in the development of new cosmetic products. Today, major cosmetic companies are seeking to identify new plant species and the chemical compounds derived from them. Ethnobotanical studies revealed that *Thymus* L. and *Helichrysum* Mill. And *Sideritis* L. species are used for cosmetic purposes. *Thymus brachychilus* Jalas, *Thymus leucostomus* Hausskn. & Velen., *Thymus migricus* Klokov & Des.-Shost., *Thymus sipyleus* Boiss., *Sideritis congesta* P.H.Davis & Hub.-Mor. and *Sideritis montana* L., *Sideritis trojana* Bornm., *Sideritis germanicopolitana* subsp. *germanicopolitana* Bornm., *Helichrysum graveolens* (M.Bieb.) Sweet, *Helichrysum plicatum* subsp. *plicatum* DC., *Helichrysum arenarium subsp. aucheri (Boiss.), Helichrysum arenarium* subsp. *erzincanicum* P. H. Davis & Kupicha were selected for this study [3].

As the primary aim of the study is to develop cosmetic products, human dermal cells derived from L929 Mouse fibroblasts, HuVeC Human Endothelial cells, and HaCat Human Keratinocyte cells were used, given the potential for the substance to penetrate the dermis. With the cell culture method, the effect of 0-0.5 mg/ml doses of plant extracts on cell viability and the level of contribution to proliferation by inducing metabolic activity was investigated. The level of toxicity and the dose that does not cause toxicity but induces proliferation are determined.

For decades, bioguided fractionation and isolation of compounds have been the main workflows for identifying plant compounds and determining which are bioactive. It is still widely used. Although it allows for the discovery of new bioactive compounds, this workflow, which is very time-consuming, primarily when minor compounds drive the activity, often yields compounds already known for these activities, and it cannot be conclusively determined whether the activities result from synergistic effects. Nowadays, metabolomics-based approaches using UHPLC-HRMS are increasingly utilized to dereplicate the extract, i.e., identify the molecules already known in the extract. This is to account for the complex composition of crude natural extracts and to consider the possibility of synergistic effects. These approaches are still in development to integrate taxonomic and/or bioactivity data; indeed, these data are primordial for effective dereplication of the extract's compounds or for prioritizing the isolation of key compounds. Using this methodology, extracts from selected plant species were fully dereplicated. The collected data enabled an improved understanding of these species' chemistry.

2- Experimental details

Herbarium studies, plant material extraction, and activity studies were conducted at the Altınbaş University Natural Products R&D Center. Chemical analysis of the plants was conducted at ICOA laboratories.

2.1. Ethnobotanical studies and obtaining the plants

2.1.1. Plant Material

Plant material was obtained by visiting different settlements in the Marmara region of Türkiye and by using information provided by local people. During ethnobotanical studies, a survey form specific to cosmetic uses was used to get information about cosmetic plants.

2.1.2. Extraction of Plant Materials

The aerial parts of selected taxa commonly used in cosmetics were air-dried at room temperature in the shade, then ground into powder. The plant material powders were macerated with a ratio of 1 part (g) of plant to 20 parts (ml) of 96% ethanol, in a tightly closed container for 3 days, protected from light, and stirred frequently. The

solvent was evaporated to dryness under a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator) at 40 ºC and 120 rpm.

2.2. In-depth phytochemical study

A first characterization by HPTLC (high-performance thin-layer chromatography) was performed to establish a raw metabolic profile and thus determine the molecular families present in these extracts. Subsequently, UHPLC (ultra-high-performance liquid chromatography) hyphenated to high-resolution mass spectrometry (HRMS) was used to perform a more detailed analysis of their molecular composition. These HRMS/MS profiles of extracts were reprocessed using a non-targeted workflow in MZmine3 before constructing a Feature-Based Molecular Network (FBMN) in the web-based mass spectrometry ecosystem GNPS (Global Natural Product Social Molecular Networking). Dereplication of the chemical composition of the extracts was performed using different computational annotation tools, such as SIRIUS, which converts spectra into metabolite structures, and Taxonomically Informed Metabolite Annotation (TIMA), which considers taxonomic information of the samples to process annotation. This workflow is particularly suitable for the phytochemical characterization of plants for which other species of the same genus have already been described.

2.3. Biological activities screening and cytotoxic effect on skin cell lines

2.3.1. Cell Culture

L929 Mouse fibroblasts, HuVeC Human Endothelial cells, and HaCat Human Keratinocyte cells stored in the nitrogen tank will be cultured in DMEM medium within an incubator containing 5% CO_2 at 37°C with humidified air. A total of 500 mL of medium was prepared by adding 1% Penicillin/Streptomycin, 50 µg/mL Gentamicin, 1% Glutamine, and 10% Fetal Bovine Serum. Each cell type that reached a sufficient number for conducting experiments with added active substances was seeded into 96-well plates at 0.01 × 10^6 cells per well. When cells reached at least 95% confluence, active substances were added to form the experimental group [4].

2.3.2. Determination of Viability Rate with 2,5-diphenyl-2H-tetrazolium bromide (MTT) Method

A MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] 12 mM stock solution was prepared as described by Mosmann (1983). Cells were seeded onto 96-well plates in a volume of 100 µL (approximately 10^4 cells per well). This method determined how much the applied substances affect the viability rate of cells.

2.3.3. Determination of IC_{50} Value for Plant Species

The determination of the effective concentration that causes a 50% reduction in cell proliferation (IC_{50}) is essential for understanding the biological and pharmacological properties of the substance. A concentration, expected to increase cell proliferation at least as much as the control group within the range of 0 µg/ml to the determined IC_{50} value, was selected (Prolifmax). When this selected concentration was applied, an increase in cell proliferation was anticipated. The identified concentration was used in the Scratch assay (wound-healing experiment).

2.3.4. Determination of Oxidant and Antioxidant Levels

Upon application of the IC_{50} and Prolifmax concentrations identified through MTT, the difference in oxidant levels between cell lines will be assessed by examining malondialdehyde (MDA), nitric oxide (NO), and Total Oxidant Status (TOS). To determine the effect of the Prolifmax concentration on antioxidant levels in cells, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Glutathione (GSH), and Total Antioxidant Status (TAS) were examined. ELISA was

performed to determine all protein levels according to the procedures provided with the commercially available ELISA kits.

2.3.5. Detection of DNA Damage Level

An increase in reactive oxygen species can damage DNA, leading to the release of 8-hydroxy-2'-deoxyguanosine (8-OHdG). 8-OHdG was used as a biomarker to determine DNA damage levels [5]. In this study, DNA damage in cells treated with the IC_{50} concentration was measured by ELISA using 8-OHdG levels. The method will be applied in accordance with procedures specified by commercially available ELISA kits.

2.3.6. Effect on Intercellular Tight Junction Proteins

The levels of tight junction complex proteins, Occludin, Zonula Occludens 1 (ZO-1), Claudin-1, and Claudin-4, in keratinocyte cells treated with each plant extract at 0 µg/ml and at Prolifmax concentrations were demonstrated using immunocytochemistry [6].

2.3.7. Measurement of Cell Migration Rates via In Vitro Scratch Assay Method

Media prepared containing 2% FBS for the purpose of measuring migration rates. 1 ml of cell suspension at 100,000 cells/ml was added to 12-well plates and incubated. Once the cells had adhered to the plate surface as a single layer, straight horizontal and vertical lines were drawn in each well with a long pipette tip, forming a "plus" sign. To remove the medium and debris, the wells were washed with 1 ml of fresh medium. After the concentrations of interest (Prolifmax) were added to the cells, 1 ml of medium containing 2% FBS was added. Measurements were taken from the upper region of the intersection point of the drawn lines, ensuring accurate measurements at each time point. Images were captured under a microscope at 0 hours and then returned to the incubator. Images were taken at 0-, 24-, 48-, and 72-hours using phase-contrast microscopy. The closure rate of the scratch was quantitatively compared.

2.3.8. Statistical Analysis

GraphPad (Prism 8) software was used for statistical analysis. Multiple comparisons were conducted using the Tukey procedure, and a p-value <0.05 will be considered statistically significant. Additionally, variance analysis was used to assess the apoptotic index within each group.

3- Results and discussion

As a result of ethnobotanical research conducted in Türkiye, several plant genera widely used by local people for cosmetic purposes have been identified. *Thymus* L., *Helichrysum* Mill. and *Sideritis* L. species were commonly used for cosmetic purposes. The species from these genera collected during field studies were pressed, dried, and prepared as herbarium specimens. A HERA code number is given in the Herbarium of Altınbaş University, Faculty of Pharmacy. Plant species were determined using the Flora of Türkiye.

A phytochemical study of four *Thymus* extracts was conducted using UHPLC-HRMS/MS. Data were represented using molecular networking and annotation tools, including SIRIUS4 [7] and Tima-R [8], to increase annotation confidence and refine compound identification. This methodology allowed us to identify the main compounds in these extracts as triterpenoids, such as Uvaol, known for improving endothelial cell function [9], and flavonoids, including polymethoxylated flavonoids, known for modulating endothelial cell function [10]. To better understand their uses, the metabolic activities of ethanolic extracts from these 4 species have been evaluated in Keratinocyte (HaCat) and Endothelial (HuVeC) cells. In contrast, cytotoxicity has been evaluated in L929 fibroblast cells. The extracts lacked cytotoxicity and were ineffective against HaCat cells but did activate HuVeC, which could be promising for wound-healing treatments.

Helichrysum species contain a diverse group of phenolic and terpenic compounds. The four *Helichrysum* species collected were extracted

with ethanol and then analyzed by UHPLC-DAD-ELSD. These profiles show very different chemical compositions among the four species, with *Helichrysum graveolens* having higher phenolic content and *Helichrysum plicatum* subsp. *plicatum* having higher terpenic content. This study also evaluates the cytotoxic effects of *Helichrysum* species extracts on HaCat (human keratinocyte) cells and assesses their potential as alternative cosmetic bioactive ingredients to *H. italicum* (Roth) G.Don. While *H. italicum* demonstrated the highest cytotoxic activity, *H. graveolens* exhibited notable proliferative effects at specific concentrations, suggesting their suitability for applications promoting skin health. Overall, these findings indicate that *H. graveolens* and *H. plicatum* may serve as promising, less cytotoxic alternatives to *H. italicum*, with *H. graveolens* offering additional proliferative benefits for cosmetic applications aimed at enhancing skin regeneration.

UHPLC-DAD-ELSD has analysed four *Sideritis* taxa's extracts, which are mainly composed of flavonoids and terpenoids. Analysis has shown a significant difference in terpenoid composition. UHPLC-HRMS/MS analysis is in progress to refine the compound identification and better understand the differences in the physiological effects of these extracts. The ethanolic extracts of these *Sideritis* species were tested for cytotoxicity against L929 fibroblast cells and for metabolic activity against keratinocyte (HaCat) and endothelial (HuVeC) cells to better understand their uses. *Sideritis congesta* P.H.Davis & Hub.-Mor. *Sideritis montana* L. has proven ineffective on keratinocytes but demonstrated activation of dermal cells. Intriguingly, *Sideritis trojana* Bornm. selectively promote the division of HuVeC cells, while *Sideritis germanicopolitana* subsp. *germanicopolitana* Bornm. stimulate greater proliferation of fibroblast cells than the negative control group.

4- Conclusion

The results of the studies, including detailed phytochemical and activity studies conducted on *Thymus*, *Sideritis*, and *Helichrysum* species, will be published in articles. *Helichrysum* species, in particular, have been found to have potential for the cosmetics industry. However, more detailed phytochemical studies are needed to elucidate the mechanism of action and isolate the active substances.

5- Perspectives of future collaborations with the host laboratory

In the future, more detailed studies are planned to isolate active substances from these species and elucidate the mechanisms of their biological activities. Furthermore, it is planned to prepare anti-aging product formulations by obtaining more effective extracts through different extraction methods that increase the yield of the active substance group.

6- Articles published in the framework of the fellowship

The article titled "Phytochemical screening and investigation of the physiological effects of four *Thymus* L. species from Türkiye" has been prepared but is still in the publication process.

The conference presentations presenting the results of the project team's collaborative work during and before this fellowship are shared below.

1. Özdemir Nath, E., Campos, P. E., Gündoğan, G. I. (July, 2024). In vitro physiological effects and phytochemical exploration of four *Sideritis* L. species from Türkiye. International Congress on Natural Products Research, Krakow, Poland (Poster presentation).

2. Campos, P.E., Le Cabec, A., Özdemir Nath, E., Sönmez, S., Gündoğan, G. İ., Destandau, E. (July, 2024). Phytochemical exploration and biological evaluation of four *Thymus* L. species from Türkiye. International Congress on Natural Products Research, Krakow, Poland (Poster presentation).

3. Özdemir Nath, E., Campos, P.E., Gündoğan, G. İ., Destandau, E. (February, 2025). Phytochemical exploration and biological evaluation of three *Helichrysum* species from

Türkiye. 32. Young Research Fellows Meeting, Paris, France (Poster presentation).

4. Özdemir Nath, E., Reset, L., Gündogan, G.İ., Da Silva, D., Colas, C., Lesellier, E., Destandau, E., Campos, P.E. (July, 2025). Phytochemical Investigation of *Helichrysum arenarium* subsp. *aucheri* (Boiss.) with Promising Wound Healing Potential and Targeted Extraction of Bioactive Compounds by Sequential Selective Supercritical Fluid Extraction (S3FE). 4th International Symposium AFERP-STOLON, Bordeaux, France (Poster presentation).

5. Özdemir Nath, E., Campos, P.E., Da-Silva, D., Gündoğan, G.İ., Çetin, A., Hano, C., Destandau, E.. (October, 2025). Phytochemical screening and investigation of physiological effects induced by *Helichrysum arenarium* subsp. *erzincanicum* in endothelial, keratinocyte and fibroblast cell lines. PSE meeting, Madeira Island, Portugal (Poster presentation).

6. Campos, P.E., Özdemir Nath, E., Da Silvaa, D., Sönmez, S., Gündoğan, G.İ., Destandaua, E. (October, 2025). Exploring Turkish Medicinal Plants: From Traditional Uses to Phytochemical and Dermocosmetic Investigations. PSE meeting, Madeira Island, Portugal (Oral presentation).

The seminars and workshops held at Le Studium, the University of Orleans, ICOA, and Muséum d'Orléans pour la Biodiversité et l'Environnement during and after the Le Studium fellowship are listed below.

1. Özdemir Nath, E. The Importance of Ethnobotany and Herbarium Studies as the First Step in Phytochemistry and Biological Activity Research, ICOA, Université d'Orléans, France (6.3.2025).

2. Özdemir Nath, E. Herbarium workshop, ICOA, Université d'Orléans, France (13.03.2025).

3. Özdemir Nath, E. Integration of ethnobotany, phytochemistry, and biological activity studies in the design of phytocosmetic formulations. Le Studium Advance Research Institute, Orleans, France (03.04.2025)

4. Özdemir Nath, E. The importance of Ethnobotany and Herbarium studies as the first step in Phytochemistry and Biological activity research,12. Rencontres botaniques du Centre-Val de Loire, MOBE, Muséum d'Orléans pour la Biodiversité et l'Environnement, Orléans, France (15.11.2025).

5. Özdemir Nath, E. An ethnobotanical approach to phytocosmetics, Master's program in Chemistry, specializing in Bioactive and Cosmetic Products, ICOA, Université d'Orléans, France (17.11.2025).

7- Acknowledgements

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