

T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY

**PHARMACOLOGICAL AND ANALYTICAL
COMPARISON OF THE WOUND HEALING
EFFECT OF THE *HYPERICUM PERFORATUM* L.
OLEATES PREPARED BY SUN MACERATION
TECHNIQUE IN DIFFERENT FIXED OILS**

DOCTOR OF PHARMACOGNOSY THESIS

CİHAN ÇAPAR, Pharm.

İstanbul-2023

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APPROVAL

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material that has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

16.07.2023

Cihan ÇAPAR



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ABBREVIATIONS

AFLP: Amplified Fragment Length Polymorphism

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

BF₃: Boron Triflorür

CI: Collagenase Inhibition

COX: Cyclooxygenase

CRS: Cold Reduces Stress

ECM: Extracellular Matrix

EI: Elastase Inhibitory Values

EVOO: Extra Virgin Olive Oil

FALGPA: 2 mM N-[3-(2-Furyl) acryloyl]-Leu-Gly-Pro-Ala

FDA: American Food and Drug Administration

FGF: Fibroblast Growth Factors

GC-MS: Gas Chromatography-Mass Spectrometry Chromatography

HAO: Sunflower (*Helianthus annuus*) Fixed Oil

HI: Hyaluronidase Inhibitory Values

HIV: Human Immunodeficiency Virus

HNE: Human Neutrophil Elastase

HP+HAO: Sunflower (*Helianthus annuus*) Fixed Oil Macerate of *H. perforatum*

HP+NSO: Black seed (*Nigella sativa*) Fixed Oil Macerate of *H. perforatum*

HP+SO: Sesame (*Sesamum indicum*) Seed Fixed Oil Macerate of *H. perforatum*

HPLC: High-Performance Liquid Chromatography

HPO: Olive (*Olea europaea*) Fixed Oil Macerate of *H. perforatum*

ISSR: Inter-Simple Sequence Repeat

ITS: Internal Transcribed Spacer

LC-MS: Liquid Chromatography-Mass Spectrometry

LD: Left Down

LOX: Lipoxygenase

LU: Left Up

MAAPVN: N-Methoxysuccinyl-Ala-Ala-Pro-Val p-Nitroanilide

MALDI-IMS: Matrix-Assisted Laser Desorption/Ionization - Imaging Mass Spectrometry Analysis

MAO: Monoamine Oxidase

MMP: Matrix Metalloproteinase

MMPs: Matrix Metalloproteases

NIRS: Near-Infrared Reflectance Spectroscopy

NMDA: N-methyl-D-aspartic acid

NMR: Nuclear Magnetic Resonance

Nrf2: Nuclear Factor Erythroid 2

NSO: Black seed (*Nigella sativa*) Fixed Oil

OO: Olive (*Olea europaea*) Fixed Oil

O/W: Oil-in-Water

PASI: Psoriasis Area Severity Index

PGE2: Prostaglandin E2

(PPAR- α): Proliferator-Activated Receptor-Alpha

RAPD: Random Amplified Polymorphic DNA

RD: Right Down

ROS: Reactive Oxygen Species

RU: Right Up

SO: Sesame (*Sesamum indicum*) Seed Fixed Oil

Subsp: Subspecies

T.E.W.L: Transedipermal Water Loss

TGF- α : Transforming Growth Factor Alpha

TGF- β : Transforming Growth Factor Beta

TIC: Total Ion Chromatogram

TIME: Tissue management, Infection or Inflammation, Moisture Balance, and the Edge of the Wound

TIMP: Tissue Inhibitor of Metalloprotease

TXA2: Thromboxane A2

UV: Ultra-Violet Spectroscopy

VEGF: Vascular Endothelial Growth Factor

ABSTRACT

Çapar, C. (2023). Pharmacological and analytical comparison of the wound healing effect of the *Hypericum perforatum* L. oleates prepared by sun maceration technique in different fixed oils. Yeditepe University, Institute of Health Sciences, Department of Pharmacognosy, Ph.D. Thesis, İstanbul.

St. John's wort (*Hypericum perforatum* L.) has been recognized as one of the most popular medicinal plants worldwide. Among a wide range of healing benefits wound healing effect of its oleates in various edible fixed oils, particularly in raw olive oil, have been used traditionally for centuries externally in wound healing (wounds, scars, etc.) or orally for the treatment of peptic ulcer. Although the wound healing effect of various types of *H. perforatum* oleates prepared in edible fixed oils, *i.e.*, in olive (*Olea europaea*) oil, sesame (*Sesamum indicum*) seed oil, sunflower (*Helianthus annuus*) oil, and black seed (*Nigella sativa*) oil, have been proven both by experimental and clinical studies, efficiency of these oleates in wound healing has not been comparatively investigated so far. This experimental study aimed to reveal the wound healing potencies of *H. perforatum* oleates in various edible oils (olive oil, sesame seed oil, sunflower oil, and black seed oil) comparatively, both in analytical and pharmacological means. Firstly, quality specifications of the edible fixed oils used in maceration were analyzed by GC-MS chromatography, and the specific marker components (*i.e.*, oleuropein in olive oil and thymoquinone in black seed oil) were determined by HPLC or sesamin and sesamol were measured by UV spectroscopy in sesame seed oil. Then macerates were prepared with the traditional sun maceration technique. The amounts of the main active components, namely hypericin, hyperforin, and pseudohypericin, were determined by LC-MS and UV methods. Since fixed oils have also known to possess some wound healing activity *in vitro* and *in vivo* studies were conducted to determine the wound healing effects of both the fixed oils and the oleates comparatively. The results demonstrated that the wound-healing activity of all oleates were significantly higher comparing to that of the fixed oils used as vehicle. *In vivo* experiments showed that oleates in olive oil and sesame oil provided the best synergetic effects among the others.

Key words: *Hypericum perforatum*, Fixed oils, Histopathology, Wound Healing, Oleates

ÖZET

Çapar, C. (2023). Farklı sabit yağlarda güneşte maserasyon tekniğiyle hazırlanan *Hypericum perforatum* L. oleatlarının yara iyileştirici etkisinin farmakolojik ve analitik yöntemlerle karşılaştırması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi Anabilim Dalı, Doktora Tezi, İstanbul.

Sarı Kantaron (*Hypericum perforatum* L.), dünya çapında en popüler tıbbi bitkilerden biri olarak kabul edilmektedir. Çok sayıda iyileştirici faydasından biri olarak çeşitli yenilebilir sabit yağlarda, özellikle de ham zeytinyağında, oleatlarının yara iyileştirici etkisi geleneksel olarak yüzyıllardır harici olarak yara iyileştirmede (yara, yara izi vs.) veya oral olarak peptik ülserin tedavisinde kullanılmaktadır. Zeytinyağı (*Olea europaea*), susam (*Sesamum indicum*) yağı, ayçiçek (*Helianthus annuus*) yağı ve çörek otu (*Nigella sativa*) gibi yenilebilir sabit yağlarda hazırlanan çeşitli *H. perforatum* oleat türlerinin yara iyileştirici etkisinin deneysel ve klinik çalışmalarla kanıtlanmış olmasına karşın bu oleatların yara iyileştirmedeki etkililiği şimdiye dek karşılaştırmalı olarak araştırılmamıştır. Bu deneysel çalışma, çeşitli yenilebilir yağlardaki (zeytinyağı, susam yağı, ayçiçek yağı ve çörek otu yağı) *H. perforatum* oleatlarının yara iyileştirici etkilerini analitik ve farmakolojik açıdan karşılaştırmalı olarak ortaya çıkarmayı amaçlamaktadır. İlk olarak, maserasyonda kullanılan yenilebilir yağların kalite standartları, GC-MS kromatografisi ile analiz edilmiş olup belirli işaretlenmiş bileşenleri (zeytinyağında oleuropein ve çörek otu yağında timokinon) HPLC ile belirlenmiş ve susam yağında bulunan sesamin ve sesamol, UV spektroskopi ile ölçülmüştür. Daha sonra maseratlar, geleneksel güneş maserasyonu tekniğiyle hazırlanmıştır. Ana aktif bileşenlerin, yani hiperisin, hiperforin ve psödohiperisinin miktarları, LC-MS ve UV yöntemleriyle belirlenmiştir. Sabit yağların birtakım yara iyileştirici etkinliğine de sahip olduklarının bilinmesinden dolayı, karşılaştırmalı olarak hem sabit yağlar hem de oleatlardaki yara iyileştirici etkileri belirlemek adına *in vitro* ve *in vivo* çalışmalar yürütülmüştür. Sonuçlar tüm oleatların yara iyileştirici etkinliğinin taşıyıcı olarak kullanılan sabit yağlarla karşılaştırıldığında anlamlı ölçüde daha yüksek olduğunu göstermiştir. *In vivo* deneyler zeytinyağı ve susam yağındaki oleatların, diğerleri arasında en iyi sinerjetik etkileri sağladığını ortaya koymuştur.

Anahtar Kelimeler: *Hypericum perforatum*, Sabit yağlar, Histopatoloji, Yara iyileştirme, Oleatlar

1. INTRODUCTION AND AIM OF THE STUDY

Wound is a very common health problem in the daily life of a human. Therefore, many medicinal remedies have been developed and used for the treatment of such injuries since the prehistoric times. Among these, St.John's wort (*Hypericum perforatum* L.) oleate prepared in various edible fixed oils has gained much popularity in due time. People simply left fresh flowering aerial parts of the plant for maceration inside an edible fixed oil for an uncertain time and keep at home as a home remedy to use in case of wound, externally for skin injuries, burns or internally for oral and gastro-esophageal injuries. Although olive oil is the most frequently preferred vehicle in such formulations, other common fixed oils, *i.e.*, sesame seed oil, black seed oil, sunflower seed oil, have also been used. Preparation techniques and duration for maceration show variation, but Sun maceration technique and 15-45 days for duration period (1) is the most frequently referred technique.

Olive (*Olea europaea*) fixed oil macerate of *Hypericum perforatum* (HPO) contains phloroglucinol derivatives hyperforin, adhyperforin and furohyperforin. It is thought that protohyperisin and pseudoprotoghyperisin from naphthodiantrons are transformed into a stable form hypericin and pseudohypericin (2). Many pharmacological studies have proven anti-inflammatory and antimicrobial effects of these substances in HPO. Although pharmacological efficiency potentials of the oleates in other fixed oils, *i.e.*, sesame (*Sesamum indicum*) seed fixed oil (SO), black seed (*Nigella sativa*) fixed oil (NSO) and sunflower (*Helianthus annuus*) fixed oil (HAO) have also been experimentally explored any comparative study between these oleates both in analytical content and wound healing effects have not been reported.

The aim of this study is to comparatively investigate the analytical contents and wound healing effects, both in *in vitro* and *in vivo*, of the fixed oils used as vehicle and their oleates. The fresh flowering aerial parts of *H. perforatum* was macerated separately in four selected fixed oils, and left maceration under sunlight in glass jars for 40 days, according to the well-known traditional Sun-maceration technique. Then, the amounts of hypericin, hyperforin and pseudohypericin were quantitatively by Liquid Chromatography–Mass Spectrometry (LC-MS) and Ultraviolet Spectroscopy (UV) methods. The quality of the fixed oils used as vehicle were also analyzed by Gas Chromatography-Mass Spectrometry (GC-MS), and their specific marker components, *i.e.*, oleuropein for olive oil, thymoquinone for black seed oil were analyzed by High-

Performance Liquid Chromatography (HPLC), and sesamin and sesamolin contents in sesame seed oil were determined by UV. For comparative evaluation of the wound healing potentials of both vehicle oils and oleates, *in vivo* and *in vitro* studies were performed. Following the *in vivo* wound healing experiments histopathological evaluation of the tissue samples and effects of the fixed oils and oleates on the extracellular matrix (ECM) enzymes were studied.





2. THEORETICAL INFORMATION

2.1. Botanical Information

2.1.1. Botanical Properties

Division	Spermatophyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Subclass	Magnolipsida
Ordo	Theales
Family	Hypericaceae
Genus	<i>Hypericum</i> L.
Species	<i>Hypericum perforatum</i> L.

Hypericaceae family (formerly Clusiaceae) constitutes the largest genus of the family, with more than 400 species (3). There are 107 taxa (96 natural species) and 49 endemics under 20 sections in Turkey(3). All species and subspecies growing naturally in Turkey are perennial. The floristic characteristics of the *Hypericum* genus from The Flora of Turkey (Vol 2) are described as follows '*shrubs or herbs are usually with translucent glands containing essential oils and sometimes red or black glands containing hypericin. Leaves simple, opposite, or rarely whorled. In Turkey, all species and subspecies growing naturally are perennial*' (4–6).

This family consists of shrubs or mostly herbaceous plants that are usually evergreen or deciduous in winter. The flower is at the established end, sometimes in cymes, rarely in the leaf axils, or in one flower. Sepals (2-) 4-5 and petals (3-) 4-5 and free. Stamens are free, bundled, or sometimes compounded at the base. The ovary is superior and has 3-5 eyes. The fruit is berry- or capsule- shaped. The seeds have no endosperm (7).

The most commonly represented species of the genus *Hypericum* in Turkey are *H. perforatum*, *H. triquetrifolium* T., *H. calycinum* L., *H. empetrifolium* W., *H. scabrum* L., and *H. tedrapetum* F. (8), (9).

In Robson's "Monography of *Hypericum*" ((10), it is stated that 30 sections represent the genus *Hypericum*. These sections are Campylosporus, Psorophytum, Ascyreia, Takasagoya, Androsaemum, Inodora, Bupleuroides, Hypericum, Olympia, Campylopus, Origanifolia, Drosocarpium, Oligostema, Thasia, Crossophyllum, Hirtella, Taeniocarpium, Arteriocarpium, Arterophyllum, Humifusoideum, Adenosepalum, Elodes, Brathys, and Spachium (10).

The genus *Hypericum* contains more than 400 species, which can be divided into two subgenera; one is primarily from the Old World, where dark hypericin glands are present, and the other is primarily from the New World, where no such glands exist. Genus *Hypericum* is divided into 36 sections based on differences in morphological characteristics. *H. perforatum* belongs to the *Hypericum* series, along with ten other species closely related to *Hypericum*. Geographically and morphologically, it lies between *H. attenuatum* and *H. maculatum*. Crockett and Robson describe four related subgroups of *H. perforatum*: subspecies (subsp.) *drill*, subsp. *chinense*, subsp. *songaricum* and subsp. *veronense*, but are rarely mentioned in the literature or published databases (7). The endemic distribution of *H. perforatum* subsp. *chinense* is China; subsp. *songaricum* has two different populations, one in Ukraine and bordering southern regions of Russia, the other from Kyrgyzstan, Kazakhstan, and Xinjiang, China, and subsp. *veronense* draws from Turkey in southern Europe to Macaronesia in the west and Saudi Arabia and Sudan in the south. Therefore, *H. perforatum* subsp. *perforatum*, endemic to Central Siberia and northwest Turkey in Europe, its native range overlaps with subsp. *veronense*, in many places. *H. perforatum* is a round, stolon-like perennial herb with yellow flowers that usually grow from a woody branch base to 1-3 feet tall, with star-shaped yellow flowers (0.75 d.-1.5 in.). Flowers of *H. perforatum* have five yellow petals spotted with black dots (hypericin glands), pistils have three styles and a center boss of bushy has yellow stamens. The stem-clasping is elliptic to oblong leaves (1.25" long) with translucent dots and black marginal punctations. In addition, the foliage of *H. perforatum* has an unpleasant aroma when bruised or rubbed (3,10).

Differences in plant essential oil composition between species are important for studying chemotaxonomic relationships between genera and species. For example, researchers use black glands found in the vegetative organs of most species belonging to the genus *Hypericum* as a distinctive feature in species classification (11).

2.1.2. Identification and Distinction using Macro-anatomical Characteristics

The genus *Hypericum* is an herbaceous perennial and shrub. Leaves perfoliate or not, rarely auriculate, always translucent dotted, sepals and petals 5.5 free, petals usually yellow, stamens in 5 groups, and anterior to petals. The ovary is 3.5 or unilocular, with multiple ovules. Its fruit is a pesticide capsule or rarely groceries. There are longitudinal lines or transverse ridges on the capsule. The gland's configuration and distribution are vital in classification. According to the Flora of Turkey Volume 11 (4), 'glands are described as marginal, where they interrupt the contour of the organ; intramarginal, where they occur near the margin without interrupting the contour and superficial, where they occur away from the margin. Narrow glands in the ovary and capsule walls are termed vittae, and short swollen glands are described as vesicles.' The genus is generally distributed in Iran, Turan, and the Mediterranean region (4,6,8).

H. perforatum is a herbaceous perennial plant endemic to Europe and has been introduced to many other parts of the world. The plant can grow up to 1 meter tall and has small yellow flowers that bloom in the summer. *H. perforatum* can be identified using macro-anatomical characteristics such as the stem, leaves, and flowers. The specific characteristics of *H. perforatum* are simple and opposite leaves, rarely alternating, elliptic-oblong or linear, 5-35 mm, narrowly ovate or lanceolate, sessile, and, each time is, translucent. stipula is absent. The flowers are five-part golden yellow petals bordered by black-mottled glands, usually actinomorphic and hermaphrodite. Perianth, decussate consists of 2-6 sepals and 4-12 petals. It carries many stamens. The gynaecium is syncarpous and consists of a single pistil of 3-5 or more carpels and an ovary bearing 3-5 or more loci. The fruit is of the septicide/loculicide capsule or drupe type (3,6). The appearance and distribution of secretion pockets in the plant are essential in determining the systematic position of the species. Pockets are defined as marginal around the organ, intermarginal when near the periphery, and superficial when away from the periphery (12). Stems 10-110 cm, erect, ascending with branches, perennial, and herbaceous. It grows wild all over Turkey and Europe. It is on field roads, forest shores, hills, meadows, and blooms from July to September (13).

The plant begins to sprout on the soil with the weather's warming in March in Turkey. This plant continues to bloom from the second half of June to August. From the beginning of July, capsule-type fruits are formed. These capsules, green at first and brown

at maturity, mature from the beginning of August and crack and shed seeds (5,14). The plant tends to reproduce more vegetatively.

The distinction between different species of *Hypericum* can be made by comparing the macro-anatomical characteristics of the plants. For example, *H. perforatum* can be distinguished from *H. maculatum* by the shape of the leaves. *H. maculatum* has elliptical leaves with a pointed tip and no transparent dots, whereas *H. perforatum* has oblong leaves with rounded tips and transparent dots. Additionally, *H. maculatum* has reddish stems, while *H. perforatum* has greenish stems. In conclusion, the identification and distinction of *H. perforatum* and other species of *Hypericum* can be made using macro-anatomical characteristics such as stem, leaves, and flowers. The transparent dots on the leaves are a unique feature of *H. perforatum* and can be used to distinguish it from other species (3,5,6,15).

2.1.3. Identification and Distinction Using Micro-Anatomical Characteristics

Micro-anatomical characteristics of *H. perforatum* can be helpful for its identification and distinction from other plant species. Some of the micro-anatomical characteristics of *H. perforatum* include: (16)

1. Stomata: The leaves of *H. perforatum* have numerous small openings called stomata. These openings are essential for gas exchange and transpiration.
2. Glandular hairs: *H. perforatum* has numerous glandular hairs that produce and store the plant's active constituents. These hairs are often found on the surface of the leaves and stem.
3. Oil glands: The leaves and flowers of *H. perforatum* contain oil glands, which are tiny structures that produce and store essential oils. These oils are responsible for the plant's characteristic fragrance.
4. Vascular tissue: *H. perforatum* has a special arrangement of vascular tissue in its stems and leaves. This can be observed under a microscope and can be used to distinguish St. John's Wort from other plant species.
5. Epidermal cells: The epidermis of *H. perforatum* leaves comprises polygonal cells with irregularly thickened walls. These cells can be observed under a microscope to identify the plant.

This information is vital for correctly identifying the plant, especially in traditional medicine, where the correct species is crucial for its therapeutic efficacy (3).

2.1.4. Organoleptic Identification

Organoleptic identification is a sensory evaluation method used to identify and distinguish different substances based on their physical properties, such as color, odor, taste, and texture. In the context of *H. perforatum*, organoleptic identification involves using the senses to identify the plant and its characteristics. For example, to identify *H. perforatum* using organoleptic methods, one would look for the following characteristics(17):

- a) **Color:** *H. perforatum* has bright yellow flowers that bloom in summer. The leaves of the plant are green and slightly oval-shaped.
- b) **Odor:** The plant has a distinctive fragrance that is often described as sweet and slightly spicy. The odor is powerful when the plant is crushed or bruised.
- c) **Taste:** *H. perforatum* has a bitter taste that can be unpleasant for some people. It is not typically consumed in large quantities due to its bitter taste.
- d) **Texture:** The leaves of *H. perforatum* are slightly fuzzy, while the flowers are smooth and delicate.

Organoleptic identification, *H. perforatum* can also be identified through other methods, such as chemical analysis or microscopic examination.

2.1.5. Genetic Identification and Distinction

H. perforatum is known to have a broad genetic variability, which can affect its bioactive compound content, and, consequently, its therapeutic potential. Genetic identification and distinction of *H. perforatum* can be helpful for various purposes, such as plant breeding, conservation, and quality control of herbal products.

There are several molecular markers available for the genetic analysis of *H. perforatum*, such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP). One study used AFLP analysis to examine the genetic diversity and population structure of *H. perforatum* in Europe. The results showed high genetic diversity within populations and that distinct genetic groups can be identified based on geographic location. This suggests that regional

differences in environmental conditions may have influenced the genetic variation in *H. perforatum* (18).

Another study used ISSR markers to analyze the genetic diversity of *H. perforatum* populations in China. The study found high genetic diversity among the populations and observed genetic differentiation between populations from different geographic regions. The authors suggest that this genetic variation may be due to differences in climate, topography, and soil properties. In addition to genetic identification and distinction, DNA barcoding can also be used to authenticate *H. perforatum* and its products. DNA barcoding involves amplifying and sequencing a specific DNA region, such as the internal transcribed spacer (ITS) region, to identify a species. This method can be useful for the quality control of herbal products and for detecting adulteration or substitution. The genetic variability of *H. perforatum* may also have implications for its therapeutic potential and should be considered when using this plant for medicinal purposes (19).

2.2. Utilisation in Medical Practice

Hypericum species have been used in treating many diseases since the time of the ancient Greeks. Among the crucial physicians in history, Galen, Dioscorides, and this herb was frequently preferred by Hippocrates as diuretics, wound healing, menstrual disorders, and intestinal disorders. Throughout history, people have used *H. perforatum* to treat mental distress, mild depression, fatigue, and exhaustion. Extracts of the plant stand out among natural therapeutic agents in calming depression (20).

In the London Pharmacopoeia, written in 1618, the oil obtained from the flowers of *H. perforatum* and its benefits were mentioned (21). People who immigrated to America contributed to the recognition of the plant.

In Turkey, the oil obtained by putting the plant's flowers in olive (*Olea europaea*) fixed oil (OO) and keeping them in the sun is applied externally as a wound healer (8,22). Furthermore, one teaspoon of the dried flowers is added to 200 cc of boiled water. After brewing for 3-4 minutes, it can be filtered, and a maximum of 2-3 glasses can be drunk daily. *H. perforatum* tea is used for stomachache and intestinal disorders or respiratory ailments such as bronchitis in traditional medicine (23). Several utilization records are found in a reference survey for the macerates are as follows;

For preparing the tincture, add two handfuls of sun-dried and finely chopped herbs (stem, leaves, and flowers) into 1L of cognac. The bottle is left under the sunlight for 14 days and shaken occasionally. At the end of the period, it is filtered, and the resulting tincture is transferred to dark-colored bottles and stored in cool environments (24).

According to Swiss Pharmacopoeia, the crumbled fresh flowering tops of *H. perforatum* are macerated with 40.0 g refined HAO. The mixture is reshuffled regularly; then fermentation and extraction appear at 15-30 °C. The herbal substance is extracted 50-80 days later, removing the water layer. It is allowed to dilute the oil to a maximum of 80 g of HAO; the expected hypericin value is at least 0.001% (spectrophotometric determination) (25).

According to the folk-medicine technique used in Albania, the preparation of ethanolic extract is fulfilled by maceration. Dried stems and flowers, reduced to a crude powder, were left in a shady place for maceration with ethanol 80, 70 and 50%. Six weeks later, the mixtures were filtered into a funnel and provided with a piece of cotton, and

extracts were reserved in dark glass bottles. The ratio between the plant material and the solvent was 1:9 (26).

HPO; To prepare it, traditionally, approximately 100 g of *H. perforatum* branch tips are placed in a glass jar with 250 g of OO. The prepared mixture is shaken every few days and kept under sunlight for 15-45 days. After the time is up, it is filtered through cotton (8). In another, less preferred method, a water bath is used. Approximately 100 g of *H. perforatum* flower and 1000 g of OO are placed in a bowl. The mixture is kept in a water bath for two hours with occasional agitation and filtered through cotton. Although this method yields faster HPO, it is not common in Turkey (8). Apart from these two methods, oil is also prepared from seeds. The seeds are kept in 1/10 OO for a week and filtered. This oil obtained from the seed is also used to treat sunburns (8,27).

2.3. Chemical Composition and Bioactivities

Hypericum species contain many secondary metabolites, such as organic acids, essential oils, tannins, amino acids, naphrodiantrons, phloroglucinols, and flavonoids, responsible from the medicinal effect (23). The rate of presence of these bioactive agents in *H. perforatum*, which is used as a drug, depends on the amount and quality of sunlight in the region where the plant grows, the area where it was collected, the gathering season, and the date it was collected.

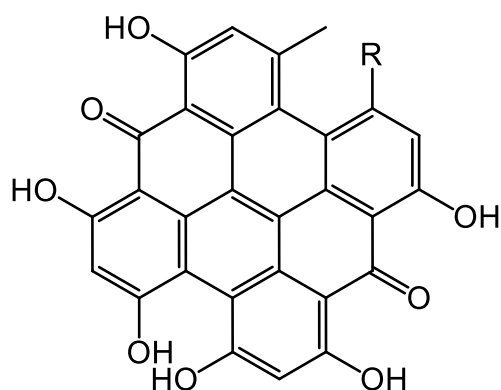
2.3.1. Naphthodiantrons

Naphrodiantrons are causing the red color and phototoxic properties of *H. perforatum*. The most important component of this group is hypericin. Other naphrodiantron derivatives are hypericin-like pseudohypericin, protohypericin, pseudoprotiohypericin, isohypericin, and cyclopseudohypericins (28) (Figure 1).

Total hypericin, together with pseudohypericin and isohypericin in the naphthodiantron structure, has a ratio of 0.06–0.4% in the plant (29). It dissolves in pyridine, and other organic bases to give a red fluorescent solution. It is generally insoluble in many organic solvents. Hypericin dissolves in alkaline aqueous solvents; below pH 11.5, its solution is red; above pH 11.5, it is turned to green (red fluorescent) (30).

Although hypericin was first discovered by Buchner in 1830, this compound was named by Cerny in 1911. Cerny presented the formula of the molecule as $C_{16}H_{10}O_5$ (31).

The compound's chemical structure was elucidated as "10,11-dimethyl-1,3,4,6,8,12-hexahydroxynaphthodiantron" by Brockmann and Sanne in 1953.

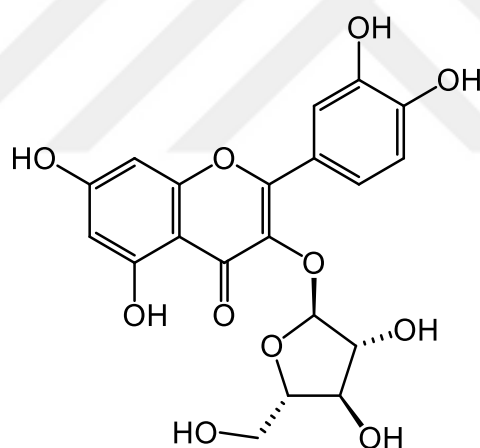


Hypericin

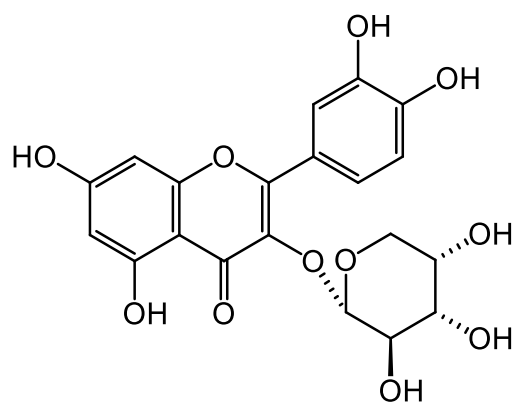
R= CH₃

Pseudohypericin

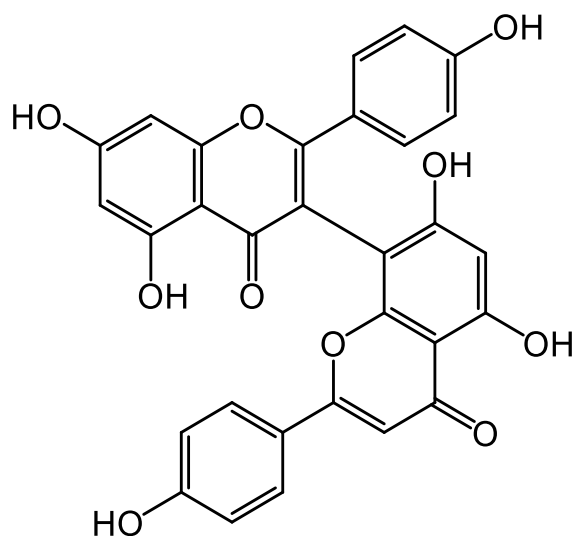
R= CH₂OH



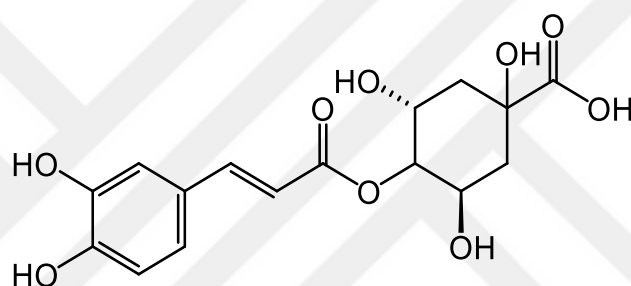
Avicularin



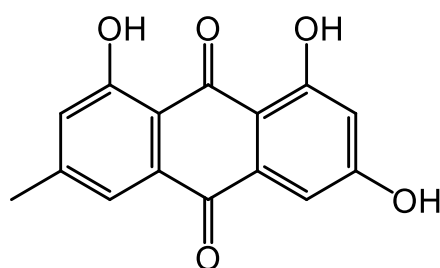
Guaiaverin



13, 118-Biapigenin



4-O-Caffeoylquinic acid



Emodin

Figure 1. Representatives of the main classes of secondary metabolites in *H. perforatum* and other *Hypericum* species

The rate of hypericin in the drug for *H. perforatum* may vary depending on harvest time, cutting height, and especially genotype (32). The red color is revealed when the dark glands, especially on the edges of the plant and flower leaves, are rubbed a mildly, because of this feature it has got its name "Bloodgrass". This red color is due to hypericin.

When the plant is consumed excessively, hypericin-induced sensitivity to light occurs. For this reason, the plant is also called "kuzukiran" among the people because it poisons to small cattle. The red color in macerate is generally thought to be due to hypericin and its derivatives. However, recently, it has been thought that compounds called I3, II8-biapigenin and 1,3,6,7 tetrahydroxyxanthone may also be responsible for the red color and fluorescence of the oil (27,31). The MALDI (Matrix-Assisted Laser Desorption/Ionization) -IMS (Imaging Mass Spectrometry analysis) results of a study by Kusari et al. on the secretion pockets in leaves, hypericin and its derivatives, pseudohypericin and protohypericin, were detected in the black-red colored glands in the leaf. In addition, hyperforin was detected in the lighter-colored transparent glands (33).

Hypericin is thought to have antidepressant and anxiolytic effects. In several studies on its mechanism of action, it has been reported that it acts similar to synthetic antidepressants (SSRI and MAOI) and has an affinity for sigma receptors.(20,34). In Europe and America, drugs containing hypericin are frequently prescribed by physicians in treating mild to moderate depressions (35).

Apart from the antimicrobial effects of hypericin, various pharmacological effects are reported. A study determined that hypericin has a strong photodynamic effect on tumor cells and viruses. This photodynamic effect increases with light (36). Hypericin and pseudohypericin acts by inhibition of protein kinase C and prevent the overgrowth of mammal cells. This may gain an antiretroviral property to molecules (37). The effect of hypericin on the apoptosis of tumor cells has not yet been fully clarified, and its photosensitizer characteristic might play a contributory role. It is thought that hypericin has an antiproliferative effect at low doses, while at high doses, it destroys tumor cells through apoptosis and necrosis (38).

Hypericin and pseudohypericin are highly effective in their antiviral effects against DNA and RNA viruses. It has been reported that these substances are very effective in preventing the semination of the Human Immunodeficiency Virus (HIV) virus (39,40).

Hostettmann et al. (31) claimed that *H. perforatum* might cause side effects, such as weakness, confusion, gastrointestinal complications, dizziness, dry mouth, loss of hair, manic disorder, hyperactivity, irritation, allergy, and photosensitivity. In addition, photosensitivity is a well-known side effect of hypericin (31).

2.3.2 Phloroglucinols

Phloroglucinol has a skeleton and a lipophilic isoprene chain. The most known phloroglucinol derivatives are hyperforin, furohyperforin, and adhyperforin. Depending on the age of the drug, phloroglucinol derivatives are between 0.2 and 4%. Hyperforin content is reported between 1.5% and 2.4%. The suggested daily hyperforin dose for oral administration should not exceed 1 mg (25).

The substance formed by adding a methyl group in the lateral position of hyperforin is adhyperforin. Other fluoroglycinol derivatives are furanohyperforin, hyperoside, and leukocyanin (41). Phloroglucinols are found in the flowers, fresh buds, and carpels of the plant (42).

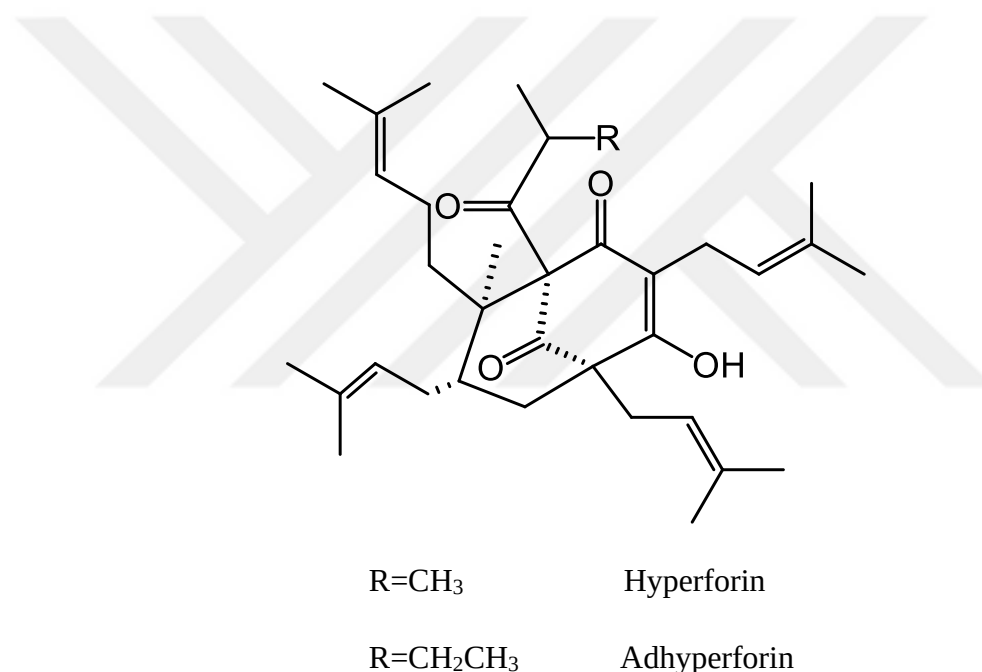


Figure 2. Chemical structures of hyperforin and adhyperforin

Hyperforin is one of the major bioactive compounds found in the leaves and flowers of *H. perforatum*. The sources say that about 4% of the dry weight of leaves and flowers consists of hyperforin and its derivatives. However, hyperforin is challenging to work with, as it is a compound that is easily degraded by light. In addition, hyperforin is prone to air oxidation and is unstable in most solvents (43).

Hyperforin is highly lipophilic, temperature-sensitive, and sensitive to photodegradation, and it has a structure that decomposes rapidly in apolar solvents such as *n*-hexane (30). According to the studies conducted for this, following carbon dioxide

extraction, it should be isolated by High Speed-Counter Current Chromatography technique after dissolving in methanol and stored at 20°C. The reason why the compound is unstable is the presence of the enol-shaped β -dicarbonyl group in its structure (44).

The antibacterial effect of *H. perforatum* in its traditional use against infections has long been known (28). Russian researchers found Hyperforin between 1971 and 1975, and its antibacterial properties were studied. The same group obtained the crystal structure of the compound, and its configuration was confirmed by X-ray analysis. Although hyperforin is chemically similar to humulon and lupulon, it is a compound found only in *H. perforatum*. Its chemical instability prolongs the duration of many studies on hyperforin (15).

According to the experiment of Schempp et al., *H. perforatum* showed an antibacterial effect against *Staphylococcus aureus* and several other gram-positive bacteria due to its hyperforin content, while it was ineffective against Gram-negative bacteria or *Candida albicans* (45). Later studies found that alcoholic extracts of the plant were more effective than aqueous extracts. It has been reported that the antibacterial effect of *Hypericum* extracts is mainly due to hyperforin and hypericin, which are more potent in opposition to Gram-positive bacteria than Gram-negative ones (3).

Furthermore, hyperforin is one of the rare compounds that naturally contain cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LOX) inhibitors noted to date (46). The dual inhibitory effect may clarify the therapeutic findings of *H. perforatum* and the use of hyperforin for topical applications in inflammatory skin diseases. Recently, hyperforin has been delineated as an anti-inflammatory effect on leukocytes (47).

It has been reported that it also helps inhibition of reactive oxygen species and release of the elastase enzyme (48). It is also known that hyperforin has an effect on the increase of T lymphocytes by showing lymphocyte effects on various epidermal cells (49).

Since hyperforin is fat-soluble, it is expected to penetrate the central nervous system. Many antidepressant drugs act as selective inhibitors to prevent over- or under-uptake of one or more neurotransmitters (mainly serotonin). In this inhibition, hyperforin acts as a competitive inhibitor at the binding sites of carrier proteins. It acts as a suitable inhibitor in inhibiting the uptake of serotonin, dopamine, noradrenaline, gamma-aminobutyric acid (GABA), and glutamate (50,51).

Different carrier proteins have a common point in inhibiting neurotransmitter uptake; they all use the sodium gradient as a driving force, while hyperforin inhibits inhibitory proteins by reducing the sodium gradient (52).

In addition, hyperforin indirectly inhibits the response via GABA, N-methyl-D-aspartic acid (NMDA), and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, regulates the opening of presynaptic calcium channels, and leads to the release of neurotransmitters into the inter-synaptic area (51).

Hyperforin is thought to have a remarkable effect on β -amyloid precursor proteins (53). It is well-known that there is large-scale accretion and aggregation of extracellular β -amyloids within the amyloid slabs of Alzheimer's patients (50). Hyperforin rapidly affects amyloid precursor proteins, reducing intracellular precursor concentration and increasing secretion rate. Current information shows that hyperforin affects β -secretases, which should not be ignored (53). It is being investigated whether hyperforin has a potential inhibitory role in increasing α -secretase/ β -secretase ratios in Alzheimer's patients.

A crystal form of hyperforin was reported to successfully prevent the spread and metastasis of cancerous cells (43,50). The antineoplastic effect of hyperforin, that is, cell-wide divisions of cancer cells, is based on numerous anticarcinogenic, anti-proliferate, proapoptotic, anti-invasive, and antimetastatic effects (49) of hyperforin; it also had an antiproliferant effect on mammalian cancer cell caps, including epithelial cells, harmful cell tumors, bad cell brain tumors, fibroblast-derived tumors, and bone marrow cancer. At the same time, hyperforin also begins to increase apoptosis in tumor cells (48,49).

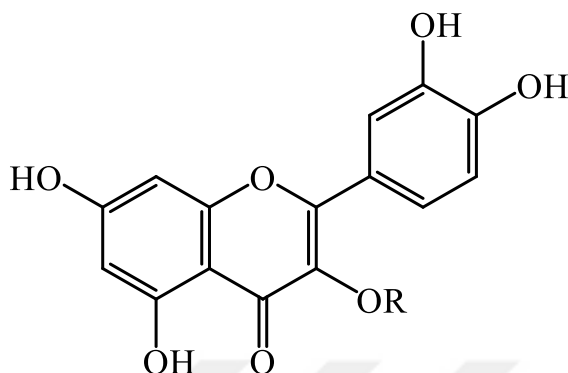
In the first cells, hyperforin was rapidly reduced, and the amplitude of mitochondrial membrane capture during apoptosis in tumor cells was also rapidly reduced. It affects permeability by causing morphological changes and ensures cytochrome C release from mitochondria (49). An animal study done in 2005 proved that hyperforin does not inhibit or combat angiogenesis *in vitro* or *in vivo* (50).

The inhibitory effects of hyperforin in infectious and inflammatory diseases are also targeted (54). In addition to these effects, hyperforin also has an antimalarial effect (55).

Hyperforin can be protected from oxidation when it is stored under a nitrogen atmosphere, while it is not oxidized within the plant matrix (56).

2.3.3. Flavonoids

Flavonols in this group in *H. perforatum* are: kaempferol, luteolin, myricetin, and quercetin. Those included in the group of flavonol glycosides are hyperoside, quercitrin, isoquercitrin, amentoflavone (I3, II8-biapigenin), luteolin, and rutin (31) (Figure 3).



R=H	Quercetin
R=Rhamnose	Quercitrin
R=Glucose	Isoquercitrin
R=Rhamnose-Glucose	Rutin
R=Galactose	Hyperoside

Figure 3. Quercetin and its glycosides

Analytical studies have been accomplished that testing reverse phase-high pressure liquid chromatography (RP-HPLC) equipped with various detectors such as diode array detector (DAD), fluorescence, electrochemical, and UV for the quantification of hypericin, hyperforin, and flavonoids in *H. perforatum* samples with electron-spray and nuclear. LC-MS methods carrying various types of detectors, such as nuclear magnetic resonance (NMR), are preferred (57).

It has been reported that quercitrin has an antidepressant effect by inhibiting MAO-A *in vitro*. Rutin is also an antidepressant (58). Quercitrin is also crucial for both the control of the phototoxic and anti-inflammatory activity of the plant (59). It shows anti-inflammatory activity by inhibiting protein kinase C and reducing lipopolysaccharide

and cytokines (60). In addition, it was shown that quercetin and apigenin suppress the growth of some tumor cells and induce apoptosis (61).

Since amentoflavone is a serotonin reuptake absorption inhibitor, it is thought that the 3', 8''-biapigenin and hyperoside, which are in the amentoflavone structure, are responsible for the sedative effect of the plant. In addition, 3', 8''-bi-apigenin shows binding activity to benzodiazepine receptors *in vitro*. It has also been shown to have sedative, anti-inflammatory, and antioxidant effects, inhibits ulcer development, and be effective in wound healing (62,63).

Proanthocyanidins and procyanidins are found in *H. perforatum* in the above-ground parts; dimers, trimers, tetramers, and catechin and epicatechin polymers are found in the plant's stem, fruits, and sprouts (64).

2.3.4 Essential Oils

Among the essential oils found in *H. perforatum*, the main components mainly vary according to the part used, harvesting season, and locality (65,66) α and β -pinene, β -myrcene, limonene, α and β -selinene, α -verbenol, β -caryophyllene, 2-methyl octane, n-decane, n-tetradecanol, n-undecane, n-nonane, 2-methyl terpenes such as -decane and 2-methyl-dodecane, geraniol, β -farnesene, humulene, and germacrene D have been detected (64,66,67).

2.4. Analytical Studies

Many studies (68) have been aimed at the analytical methods to identify *H. perforatum*, and determine its quality and evidence of adulteration. Not all the described methods are applicable for all these targets or all forms of *H. perforatum* in the marketplace. All reports discussed the *H. perforatum* above-ground components primarily unless otherwise stated.

2.4.1. UV Spectrometry

This technique is often used to analyze natural products, including *H. perforatum*. *H. perforatum* contains several active compounds, including hypericin and hyperforin, which are believed to be responsible for their therapeutic effects. Hypericin and hyperforin both absorb UV light at specific wavelengths, and the concentration of these compounds can be determined by measuring the volume of light absorbed by a sample at these wavelengths. UV spectrometry can also be used to measure the purity of an *H.*

perforatum extract. Impurities in the extract may absorb UV light at different wavelengths than the active compounds, allowing them to be detected and quantified. UV spectrometry is a proper analytical technique in the context of *H. perforatum* analysis, providing information on both the concentration of active compounds and the purity of the extract (69).

2.4.2. Thin-layer Chromatography (TLC) / High-Performance Thin-layer Chromatography (HPTLC)

Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are analytical techniques used to separate and identify compounds in a mixture. These techniques are commonly used, particularly in pharmaceutical analysis, to identify the active components in herbal medicines, including *H. perforatum*. The active components of *H. perforatum* are believed to be hypericin and hyperforin, although other compounds in the plant may contribute to its therapeutic effects (70).

TLC is an easy and inexpensive method that involves spotting a few samples onto a thin layer of an adsorbent material (such as silica gel or alumina) that has been coated onto a glass plate. HPTLC is a more advanced version of TLC that uses a thinner layer of adsorbent material and a higher-quality stationary phase, which allows for better resolution and sensitivity. HPTLC is also automated and can be used to analyze multiple samples simultaneously, making it a more efficient and reliable technique for pharmaceutical analysis. In the context of *H. perforatum*, TLC and HPTLC can be used to identify the plant extract's active components and ensure that the extract is of high quality and purity. By comparing the chromatographic profile of a *H. perforatum* extract to that of a standard reference, it is possible to determine the concentration and purity of hypericin and hyperforin in the extract. This information is essential for ensuring that the extract is safe and effective for use in medicinal products (71).

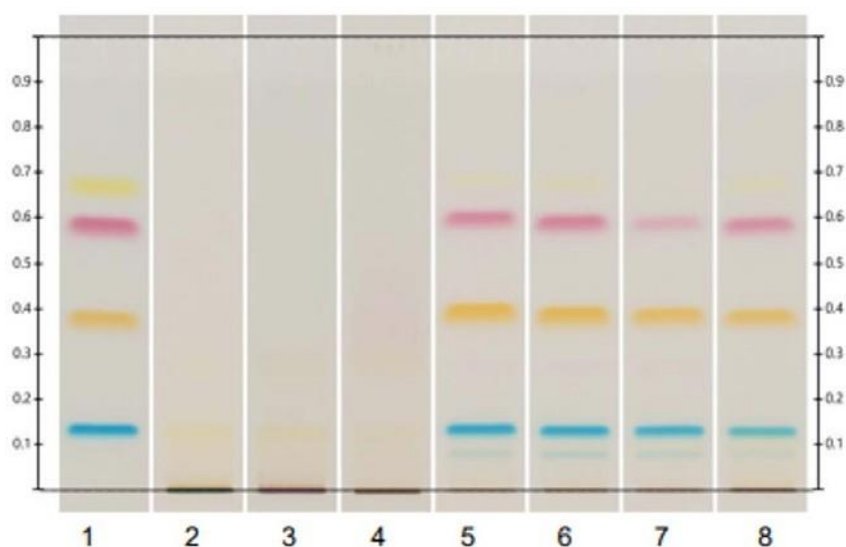


Figure 4. HPTLC system for the detection of food dyes. Lane 1: Brilliant Blue FCF, Sunset Yellow FCF, Amaranth, and Tartrazine (with increasing R_f); lane 2: *H. perforatum* herb; lanes 3-4: *H. perforatum* herb extract; lanes 5-7: *H. perforatum* herb bulk extract containing food dyes; lane 8: Commercial *H. perforatum* dietary supplement containing food dyes—conditions as described by the HPTLC Association (3)

2.4.3. LC-MS (Liquid Chromatography-Mass Spectrometry)

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) is a chromatography type that combines the mass analysis capabilities of LC and the physical separation capabilities of MS. HPLC can be used to detect phloroglucinols and naphthodianthrone, which are pharmacologically active groups of the *H. perforatum* to identify a broader range of constituents belonging to various classes of compounds in the extracts. HPLC analysis may necessitate a longer gradient elution, which can take up to 60 minutes. However, if the analysis targets explicitly a particular group of metabolites, it is possible to achieve a shorter analysis time. Flavonoids, chlorogenic acid derivatives, hypericin, pseudohypericin, and hyperforin were identified by their distinctive m/z values, UV spectra, and retention times (68).

2.4.4. GC (Gas Chromatography) and GC-MS (Gas Chromatography-Mass Spectrometry)

Gas chromatography (GC) is a widely used technique for separating and analyzing gaseous or easily evaporable fractions in a mixture. In general chromatography, there is a stationary phase (capillary column) and a mobile phase (gas). The sample is taken into suitable solvents, and the materials free of fibers from the column are separated. The

flame ionization detector (FID) is one of the most used detectors in GC. FID uses a flame to ionize organic gas containing carbon (68).

GC-MS is an analytical method that combines the features of gas chromatography and mass spectrometry for those containing different substances in a test sample. GC analysis is primarily preferred for separating volatile components, and MS provides quantitative information after chromatographic separation with GC.

The utilization of GC-FID and GC-MS for *H. perforatum* is generally for the characterization of essential oils (68).

2.5. Fixed Oils Used as Vehicle in Oleate Formulations

2.5.1. Olive (*Olea europaea*) Fixed Oil

OO is a type of oil extracted from the fruit of *Olea europaea* L. (Olive). It is commonly used in cooking and as a dressing for salads and other dishes. OO is a critical component of the Mediterranean diet, which has been linked to various health benefits. OO is a high source of monounsaturated fatty acids, considered healthy type of oil. It also contains antioxidants, such as vitamin E, which could help to protect the body against impairment from free radicals. Some studies have suggested that consuming OO can also help lower the risk of heart disease, stroke, and certain types of cancer. When using OO in cooking, choosing the right type of oil for the dish is essential. Extra virgin olive oil (EVOO) is best for cold dishes or drizzling overcooked food, as it has a strong, overpowering flavor when heated. Virgin or refined OO is better suited for high-heat cooking, such as frying or sauteing (72).

Globally OO production has averaged 2.91 million tons over the past five seasons. Countries where individual OO is produced the most include Spain, Italy, Greece, Turkey, Tunisia, and Morocco (73). There are 182076 olive trees in our country. Of these, 154037 are fruit-bearing trees, and 28039 are non-fruiting trees (74). Of the total olive production of 1525000 tons, 415000 tons are used as table olives, and 1110000 tons are used for oil production (74). It has also become widespread in countries such as Australia and Japan. OO has antioxidant, anti-inflammatory, anti-atherogenic, anti-ulcer, anticarcinogenic, antimicrobial, diabetic, nerve system protective, skin protective, and anti-aging effects. OO, which contains an essential monounsaturated fatty acid (oleic acid), is known for its effects on heart health, and it has a high content of single-bound unsaturated fatty acids. Consuming ten grams of EVOO daily rather than margarine,

butter, mayonnaise, and dairy fat was associated with 8%-34% reduced risk of total and cause-related mortality (75,76).

According to the International olive oil council, OO is classified as;

a) Extra Virgin Olive Oil

EVOO is indeed the most qualified form of all olive oils, which is obtained by cold-pressing technique to avoid the essential ingredients composed by exposure to high temperature. EVOO has a reduced acidity level compared to its standard virgin form. EVOO is considered the best form because it absorbs most of the natural flavor and aroma. This form is also the most frequently used type of OO for medical purposes, such as preventing colon cancer. Since it can be used for medical purposes, it is recommended to be consumed directly without mixing with any ingredient (77).

b) Virgin-Olive Oil

This virgin form of OO is unrefined. The cold-pressing method used to extract Virgin olive oil provides a more natural aroma and flavors than other methods. In addition, it contains a moderately increased acidity level of 1 to 4 percent. This unique form of OO is also resistant to the elevated temperatures. Compared to the EVOO, this type of oil has a light taste and higher acidity. Therefore, this form of OO is proper for low-heat cooking purposes (77).

c) Refined Olive Oil

This form of OO is extracted from black and ripe *Olea europea* fruits. Among other forms, it has the least amount of virgin olive oil. OO is produced from virgin olive oils by refining methods that do not cause changes in the basic glyceridic structure. As a result, it has a free acidity, indicated as oleic acid, of less than 0.3 grams per 100 g. However, it is still considered low-quality OO and only suitable for cooking, especially at high temperatures. In addition, it still includes the same amount of fat and calories as other forms (77).

d) Pure Olive Oil

Unlike its name, this form of OO is completely purified. It is a mixture of EVOO or virgin-type olive oil with refined olive oil. The blending technique is frequently used when the result of extraction is beneath the specified quality standards. With the aim of enhancing the quality, the refined oil has to be blended with an improved quality form,

whether EVOO or virgin olive oil. The oil which is mixed will increase the vitamin E level and improve the flavor (77).

e) Olive Pomace Oil

Olive oil pomace is considered the worst quality among OO types. It is obtained from the residue that remains after the olive fruit is compressed. Subsequently, the olive fruits are compressed, and fruits leave oil and water residue behind. Once the water and oil are taken, the residue still includes some oil and could be extracted. Hence, it is derived only from the fruit residuals, and virgin oil is added to enhance the quality. As a result of these quality problems, the pomace type is the cheapest OO in the market and proper only for high-heat cooking (77).

2.5.1.1. Some Minor Compounds in Olive (*Olea europaea*) Fixed Oil and their Physiological Effects

OO is one of the critical components of the Mediterranean diet and contains many beneficial compounds that positively affect human health. These compounds include polyphenols, tocopherols, phospholipids, carotenoids, chlorophylls, sterols, and squalene. Most of these compounds found naturally in olives are transferred to OO as they are obtained purely physically.

2.5.1.2. Phenolic Components in Olive Oil

Most of the phenolic compounds found in the pulp of the olive are transferred to the OO. The most abundant phenolic compound in olive fruit is oleuropein. It constitutes 14% of the dry weight. During the ripening stage, oleuropein undergoes enzymatic and non-enzymatic hydrolysis to form simple phenolic compounds such as hydroxytyrosol in EVOO. This compound is an anti-inflammatory effect against intestinal and respiratory tract infections (75).

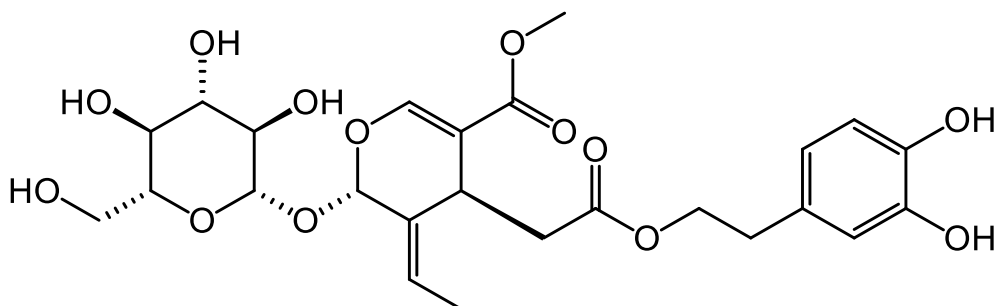


Figure 5. Chemical structure of Oleuropein

Oleocanthal is another organic compound isolated from EVOO and associated with oleuropein. Interestingly, it has been reported that oleocanthal, which has antioxidant properties, and also an anti-inflammatory effect similar to ibuprofen (a non-steroidal pain reliever). Long-term consumption of small amounts of oleocanthal in OO may reduce the risk of heart disease, which is partly associated with the Mediterranean diet. An average of 50 g OO per day is suggested to have a similar effect to 1/10 of an adult ibuprofen dose (78,79).

Owen et al. reported that OO has a higher antioxidant capacity than other vegetable oils, and the antioxidant compound ratio is higher in EVOO than in refined virgin olive oil. Polyphenols in OO, such as oleuropein, hydroxytyrosol, tyrosol, and caffeic acid, have significant antioxidant and anti-inflammatory effects (80).

It is stated that these compounds have protective effects against the oxidation of LDL, which can cause cardiovascular occlusion. Studies have shown that people who regularly consume OO in their daily diet have a lower risk of developing cardiovascular and cancer diseases due to the antioxidant effect of phenolic compounds (81).

2.5.1.3. Tocopherols in Olive oil

The most important tocopherol in OO, also known as the vitamin E equivalent, is α -tocopherol. Tocopherols are also seen as protective in some chronic diseases, such as cardiovascular and cancer diseases, with their antioxidant effects. In a clinical study (82,83), they found that high doses of vitamin E (<67 mg α -tocopherol) used continuously for at least two years reduced coronary heart disease by 31-65%. Heinoen et al. reported that long-term (5-8 years) use of 50 mg of α -tocopherol per day in smoking men reduced prostate cancer by 32% and the death rate from prostate cancer by 42% (84). At the same time, vitamin E plays a role in metabolic events and in preventing neurodegenerative diseases such as Alzheimer's and Parkinson's. Vitamin E is used in cosmetics and skin-care products. Experimental studies show that using vitamin E externally or orally makes the skin layer barrier durable, prevents tumor formation, and protects against UV light (85).

2.5.1.4. Squalene content in Olive Oil

It is a metabolic precursor of the cholesterol pathway and, with other sterols, plays a role in synthesizing steroid hormones. When compared with other kind of vegetable oils, squalene content is higher in OO. OO contains 0.7% squalene, while other foods and

oils contain 0.002-0.030%. Although it is distributed throughout the body, the majority of squalene is carried to the skin and is used as a moisturizer and emollient in cosmetics and pharmaceuticals due to this feature. Squalene protects against UV-induced damage and prevents lipid peroxidation caused by UV-induced antioxidant activity in the skin. Similarly, squalene protects against radioactivity (80,86).

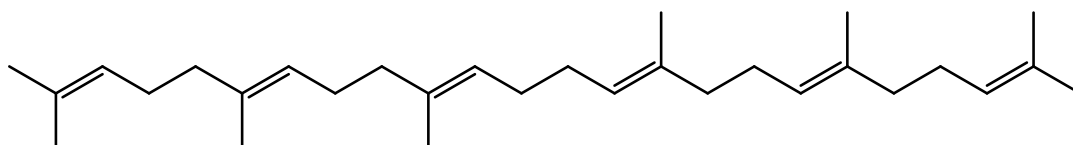


Figure 6. Chemical Structure of Squalene

The low incidence of skin cancer in countries that consume OO is partially attributed to the protective effect of squalene. In animal studies, squalene inhibits breast, pancreatic, and colon cancer cell proliferation and tumor growth by reducing the activity of the Ras oncogene, which causes uncontrolled excessive proliferation of cells. Squalene acts as a protective agent and reduces the side effects of chemotherapy. In addition, its use in drug delivery applications is being investigated, as it increases the immune response against various antigens that damage or weaken the body's defence system. Squalene, which also has the effect of oxygenating the cells, increases energy metabolism by providing more oxygen to the cells. Squalene also affects cardiovascular health and aging (87).

2.5.1.5. Sterol Components in Olive Oil

Sterols are tasteless and odorless and are generally temperature-sensitive. Phytosterols in the composition of vegetable oils are also used in identifying and detecting adulteration of oils since they are a characteristic feature. Studies show that many plant sterols prevent oxidative deterioration in frying oils. In particular, sterols, which seem inactive at low temperatures, are more active at high temperatures (80).

Some of the phytosterols and their esters are recommended in the diet due to their cholesterol-lowering effect on heart health. Phytosterols impact glucose and lipid metabolism, resembling cholesterol. Their role in modifying insulin resistance and daily glucose absorption lacks sufficient research. Phytosterols in OO may regulate insulin, preventing diabetes and related complications. In the USA, healthcare costs for diabetic foot reach 7,000–10,000 USD, increasing to 65,000 USD in complex amputation cases, highlighting the significant expenses involved (88).

2.5.1.6. Quality Control Parameters of Olive (*Olea europaea*) Fixed Oil

When obtained by cold-pressing at room temperature, OO is a shiny liquid. The color can range from amber to yellowish-green, and the intensity ranges from 0.913 to 0.920 at 20°C. The chemical composition of OO may differ according to the kind of olive, the width of the plant, and the harvest time. According to the international OO standard, the chemical components of EVOO can be divided into two types: the saponifier part (triglycerides, phospholipids, etc.). Therefore, the glyceride content of extra virgin OO is about 98%. It also contains polyunsaturated oleic acid, a minor amount of saturated fatty acids, and a minor amount of polyunsaturated fatty acids (80). The primary fatty acids found in OO are palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid.

Some standards set for OO are listed below;

National Standards,

TS 341 Edible OO Standard,

Turkish Food Codex Communiqué on Olive Oil and Olive Pomace Oil 2010/35,

International Standards (ISO, AOCS),

International Olive Oil Council Commercial Standard (IOCC),

European Union Standard (EEC),

European Pharmacopoeia

Codex Alimentarius

2.5.2. Black seed (*Nigella sativa*) Fixed Oil

Nigella sativa L. (Black seed) is a family member of the Ranunculaceae. Turkey is rich in diversity, as black seed, black cumin, or a grain of cornucopia is known. *N. sativa* is cultivated in North Africa, Central Asia, Southern Europe (89), and in Turkey, especially in Isparta, Konya, Afyon, Kütahya, Burdur, and Çukurova.

N. sativa has a very remarkable historical background. This plant has an Anatolian origin, and also native to Egypt, Syria, India, and some African countries (89). In the excavation studies, the 18th Pharaoh, in the tomb of Tutankhamen (1325 BC), in the valley of the ancient kings, *N. sativa* seeds were found possibly to wish for a good and

healthy after life. In addition, Cleopatra used NSO to look healthy and beautiful; it was used as a spice by the Romans and was also used in Ancient Mesopotamia (90).

N. sativa seeds were used by ancient Egyptian and Greek physicians for toothache, migraine, nasal congestion, and intestinal worms. In the works of Hippocrates and Dioscorides, it goes under the name "Melanthion." Hippocrates (460-370 BC) used NSO to reduce digestive system complaints and strengthen the liver. It has been used for therapeutic purposes and as a menstruation and lactation enhancer. In addition, In the European countries at the beginning of the Middle Ages, *N. sativa* gained importance from German kings Ludwig der Fromme and Karl the Great. *N. sativa* seed cultivation was started in their countries in 1031; Ibn Sina has an essential role in explaining the therapeutic benefits of *N. sativa* in his works. Having become a medicinal plant, *N. sativa* was popular among the people until the 1750s. Despite this glorious past, it has been forgotten for about 200 years in western societies. After being forgotten for so long, it was accidentally rediscovered in the early 21st century (91).

Studies conducted on *N. sativa* seeds and their components are provided to exert a extensive range of pharmacological activities, which include anticarcinogenic, antitumoral, antiulcerogenic, antibacterial, anti-inflammatory, analgesic (92), antioxidant (93), wound healing (94), hypoglycemic (95), and immune system booster activities (96).

NSO has been shown to have significant wound-healing properties. While NSO is generally considered safe for use on the skin, it is important to test a small area of skin first to reduce the hypersensitivity risk.

2.5.2.1. Chemical Properties of *Nigella sativa*

Based on the chemical composition of *N. sativa*, as in other aromatic plants, the place where the plant is grown, the harvest time, the geographical differences of the region, and the climate are said to be effective. Depending on the climate of the region, the composition and ratios of seeds may be subject to variations; essential oils (0.4-0.45%), fixed oils (32-40%), proteins (16-19.9%), amino acids, alkaloids, tannins, saponins, fibers (5.5%), carbohydrates (33.9%), minerals (1.79-3.44%), and vitamins (ascorbic acid, thiamine, niacin, pyridoxine, and folic acid). Still, oleic acid, one of the unsaturated fatty acids in the structure of the oil, linoleic acid, eicosadienoic, arachidonic acid, and linolenic acid, while saturated fatty acids, myristic acid, palmitic acid, and stearic acid exists. The structure of the essential oil consists of nigellone, besides

carvacrol, p-cymene, d-limonene, α , and β -pinene. The main pharmacologically active ingredients in the seeds, thymoquinone, dithymoquinone, thymohydroquinone, and thymol, are detected. Among the other compounds are nigellimine, nigellimine N-oxide, and nigellidine under indazole alkaloids (89,97).

The fatty acids in NSO can be found in saturated or unsaturated forms. These parts are in terms of fat oxidative stability and nutritional properties. Extraction methods determine the fatty acid composition, and the origin of the seeds may be affected. Total saturated and unsaturated fatty acids of cold-pressed oil 29.2% and 69.7%, were determined (98). Most abundant in *N. sativa* seeds, saturated fatty acids are palmitic acid, stearic and myristic acids, while primary unsaturated fatty acids are linoleic, oleic, and alpha-linolenic acids (99).

Thirty-two active compounds were determined in NSO. Approximately 46.7% of its composition is phenyl propanoids. Additionally, 26.7% monoterpenoid hydrocarbons, 26.9% monoterpenoid ketones, 4.0% monoterpenoid hydrocarbons, 4.0% monoterpenoid ketones, and 26.7% monoterpenoid hydrocarbons (100).

There is a carbonyl polymer of thymoquinone (C₁₀H₁₂O₂, 2-isopropyl-5-methyl-1, 4-benzoquinone), and it is isolated from *N. sativa* seeds. This polymer is much less toxic, but its active ingredient, thymoquinone, has potent pharmacological properties that retain most of its characteristics. Other ingredients isolated from the NSO; *p*-cymene (7.1-15.5%), carvacrol (5.8-11.6%), trans-anethole (0.25-2.3% or 38.3%), carvone (4.0%), longifolin (1.0-8.0%), limonene (4.3%), alpha-pinene (13.75%) and 4-terpineol (2.0-6.6%) (90,101)

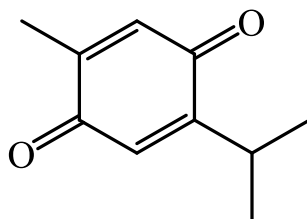


Figure 7. Chemical Structure of Thymoquinone

2.5.3. Sunflower (*Helianthus annuus*) Fixed Oil

Helianthus annuus (*H. annus*), whose homeland is Central America and which is claimed to have been domesticated further to the north, probably around 2000-2500 BC, is an industrial plant that has spread applications almost all over the world today and is cultivated because of its rich oil content in seeds.

This plant, which has more than a thousand species in the world, was brought to the Old World by the Spanish after the 16th century. *H. annuus*, grown only as an ornamental plant until the 18th century, gained importance as a plant from which oil is obtained from its seeds, with the studies carried out in the same period. Therefore, it can be deduced that *H. annuus* was not used as an oil plant or had limited use when it was still in the Americas. Studies on Mesoamerican cultures can also confirm this inference. Indeed, when the Aztec and Maya cultures' eating and drinking habits and culinary cultures are examined from an archaeological point of view, there is no evidence of large-scale vegetable oil production. In archaeological excavations, no pottery was found suitable for cooking with oil. In addition, Spanish chronicles express the same issue about Mesoamerican peoples' eating and drinking cultures. In this case, it is possible to state that the *H. annuus* was bred to be suitable for use as an oil plant time after it reached the Old World. Before moving on to HAO, it would be helpful to briefly look at the historical and cultural development of the plant in the Old World (102).

2.5.3.1. Chemical Composition of Sunflower (*Helianthus annuus*) Fixed Oil

HAO is obtained from the seeds of *H. annuus*, the oil content of which varies between 40 and 52%. HAO contains 15% saturated, 85% unsaturated fatty acids, 14-43% of unsaturated fatty acids composed of oleic acid, and 44-75% of linoleic acid. Linoleic acid reduces the fat's saturation, increases digestion, and passes into the blood, making it more manageable. In addition, linoleic acid participates in the cell membrane's structure and lowers cholesterol levels. The high amount of linoleic acid (over 0.7%) in oils is considered qualified (103).

Table 1. Chemical composition of sunflower (*Helianthus annuus*) fixed oil

Fatty Acids	Chain length
Lauric	(12:0)
Myristic	(14:0)
Palmitic	(16:0)
Palmitoleic	(16:1)
Stearic	(18:0)
Oleic	(18:1)
Linoleic	(18:2)
Linolenic	(18:3)
Arachidic	(20:0)
Behenic	(22:0)

2.5.4. Sesame (*Sesamum indicum*) Seed Fixed Oil

Sesame, a hot climate plant, is suitable for temperature and precipitation. In Turkey, in May-July and October-November is harvested (104). The plant seeds, formed in the fruits, are peeled, and vegetable oil is collected by cold pressing the seeds with their extracted grains. Sesame (*Sesamum indicum*) seed oil (SO) produced with the cold press technique are exposed to high temperatures during the process. Trans fatty acids cannot be formed because they do not remain, and their bioactive compounds are also damaged. This situation is critical in terms of both health and nutritional value in foods. The cold press technique used in producing the highest quality vegetable oils comes first. The oils obtained by the cold press method are more expensive than those produced by other methods. Some substances, such as antioxidants, phenolic compounds, phosphatides, carotenoids, and phytosterols, have health benefits that come to the fore due to their content.

SO has been used for centuries as a traditional remedy for burns, and recent scientific research suggests that it may benefit burn injuries. SO contains anti-inflammatory compounds, which may help decrease swelling and inflammation in the affected area. Also, SO may also have analgesic or pain-relieving effects. When applied to a burn, it can help reduce the sensation of pain (105).

SO has moisturizing properties, which can help keep the skin hydrated and prevent it from becoming further damaged. In addition, SO consist of antioxidants that could protect the skin from further damage come from free radicals. This can help promote healing and reduce the risk of scarring. SO also has antibacterial properties, which can help prevent infection in the affected area (106).

2.5.4.1. Composition of Sesame (*Sesamum indicum*) seed Fixed Oil

A large percentage of SO (55%) includes protein, a coenzyme in the composition of sesame seeds Q10, carbohydrates, vitamins A, B₁ (thiamine), and E, calcium, magnesium, phosphorus, copper, zinc minerals, and sesamin, sesaminol, and sesamolin. At smaller concentrations, sterols include campesterol (18-19%), stigmasterol (6-7%), β -sitosterol (59-62%), Δ^5 -avenasterol (10-11%), and tocopherols: γ -tocopherol and δ -tocopherol (83% and 11% of total tocopherols, respectively). The fatty acid group of SO is as follows: C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid, C18:3 linolenic acid, C20:4 arachidonic acid (104).

2.6. Skin Anatomy and Physiology

Skin is the biggest organ in the body. It covers the entire outer surface of the body and acts as a vital barrier against pathogens, ultraviolet light, and chemicals. In addition, it forms a mechanical barrier against injury. It also regulates the temperature level and volume of water released into the environment (107).

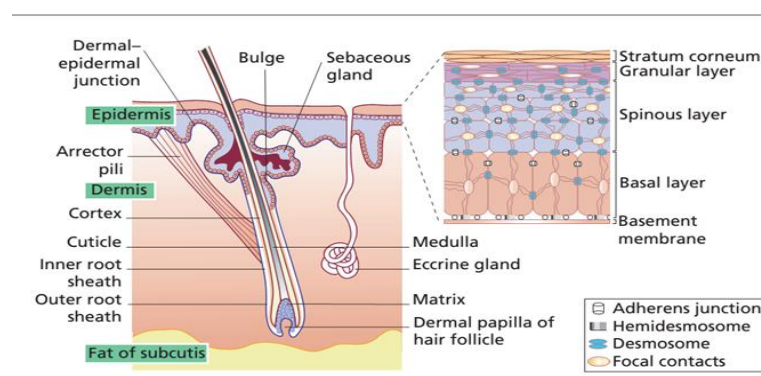


Figure 8. Structure of skin (108).

2.6.1. Epidermis

The epidermis is the skin's outermost layer, consisting of several layers. The number of layers varies in different body regions, with the thinnest skin having only three layers, while the thickest skin has up to five layers. Here are the five layers of the epidermis, listed from the outermost to the innermost:(109)

- a) ***Stratum corneum***: This layer is located at the outermost layer of the epidermis and is composed of dead skin cells called corneocytes. These cells are flat, compact, and filled with a protein called keratin, which provides strength and protection to the skin.
- b) ***Stratum lucidum***: This layer is merely presented in thick skin, such as the palms of the hands and soles of the feet. It is a thin, transparent layer of dead skin cells that supports the protection of the skin from friction and pressure.
- c) ***Stratum granulosum***: This layer contains granules that help produce keratin and other proteins that contribute to the strength and barrier function of the skin.
- d) ***Stratum spinosum***: This layer contains living skin cells that are actively producing proteins and lipids that contribute to the skin's barrier function.
- e) ***Stratum basale***: This layer is located at the innermost layer of the epidermis and comprises basal cells, actively dividing and producing new skin cells. In addition, this layer contains melanocytes, which produce melanin, the pigment that gives skin its color.

The epidermis constantly renews itself, with old skin cells shedding and new skin cells moving up from the inner layers to replace them. This process helps maintain the skin's strength and barrier function and protects the body from environmental factors such as UV radiation and pathogens (110).

2.6.2. Dermis

The dermis is the middle layer of the skin and is placed between the epidermis (the outermost layer) and the subcutaneous tissue (the deepest layer). It mainly comprises connective tissue and contains nerves, blood vessels, skin appendages, and other structures. In addition, the dermis is liable for providing strength and elasticity to the skin. The dermis is separated into two layers: the papillary dermis and the reticular dermis. The papillary dermis is the upper thinnest layer of the dermis, which is direct linked with the

epidermis. It contains loose connective tissue, tiny blood vessels, lymphatic vessels, and nerve endings. In addition, the papillary dermis is responsible for supplying nutrients to the epidermis and providing a foundation for the epidermis to rest on (111).

The reticular dermis is the thicker, lower layer of the dermis that lies below the papillary dermis. It consists of dense, irregular connective tissue and contains larger blood vessels, nerve fibers, and lymphatic vessels. The reticular dermis is liable for providing strength and elasticity to the skin. The dermis also contains different types of cells, including fibroblasts, which produce collagen and elastin fibers, and immune cells, such as macrophages and lymphocytes, which help protect the skin from infections. The hair follicles, sebaceous glands, and sweat glands are also located within the dermis, regulating body temperature and moisturizing the skin (112).

2.6.3. Hypodermis

The hypodermis, also named the subcutaneous layer and superfascia, is the layer directly beneath the dermis and connects the skin to the underlying fascia (fibrous tissue) of the bones and muscles. The thickness of the Hypodermis depends on the anatomical site, race, age, gender, endocrinal conditions, and nutritional habits of the individual. Subcutaneous adipose tissue is functionally and structurally firmly combined with the dermis through vascular and nerve networks and the continuity of epidermal extensions, such as hairs and nerve endings. The majority of subcutaneous adipose tissue is a lax composition of lipid-filled cells, the white adipocytes, held in a collagen fiber framework. However, merely one-third of adipose tissue consists of mature adipocytes, with the rest being stromal-vascular cells, consisting of fibroblasts, leukocytes, macrophages, and pre-adipocytes. Adipose tissue has limited ECM (Extra Cellular Matrix) when compared to other connective tissues (113).

2.7. Skin Injury and Wound Healing Process

A wound is a circumscribed trauma that is caused by external factors and can affect any tissue or organ (surgical or traumatic/accidental). The temporary or complete disappearance of cleaning, raising, and operating the working organs is called trauma. Trauma includes impact, injury, bruising, and local effects caused by impact. As the protection property of the skin is impaired by trauma, the risk of protection also increases. Therefore, finding the wound type is crucial for successful wound care (114).

2.7.1 Hemostasis

In the hemostasis process, the body ceases bleeding after a trauma or damage to a blood vessel. The process includes a series of phases that act together to form a clot and prevent further blood loss. The three main steps of hemostasis are;

- a) **Vascular constriction:** The smooth muscle in the vessel wall contracts to lower blood flow through the vessel; when a blood vessel is damaged. This action helps to limit blood loss from the damaged area.
- b) **Platelet plug formation:** Platelets, which are minor cell particles in the blood, form a plug at the site of trauma to facilitate stopping bleeding. They adhere to the exposed collagen fibers in the damaged vessel wall and each other, forming a temporary plug that seals the injured area.
- c) **Coagulation:** Coagulation is the process of forming a blood clot. It includes a complicated series of reactions in which clotting factors, a group of proteins in the blood, interact to form fibrin. This mesh-like substance reinforces the platelet plug and stabilizes the clot.

Hemostasis is a tightly regulated process, and any disruption in one or more of these steps can result in bleeding disorders or excessive clotting. In addition, several factors can affect hemostasis, including medications, genetic disorders, and underlying medical conditions such as liver disease or cancer (115).

2.7.2 Inflammation

Inflammation, which is the second phase of wound healing, is clinically seen as redness, sore, and heat, the classic “*rubor et tumor cum caloric et dolor*” often accompanied by pain. This phase usually lasts for four days after the injury. When using the metaphor of a broken-down house, the second assignment is to cleanse debris after the appliances are turned off, which is the work of unskilled workers. In wounds, these unskilled workers are neutrophils (116).

The inflammatory effect leads to leaking blood vessels and the deliverance of plasma and neutrophils into the enclosing tissue. Neutrophils phagocytose remains and pathogens and create the first line of defense against infection. When the pathogens and remains are digested, the neutrophils deplete and deliver intracellular enzymes into the enclosing matrix, which significantly digest the component. As fibrin is broken down as

part of a cleaning process, the breakdown products draw other affected cells, such as fibroblasts and epithelial cells. They are maintained by a local stem cell bank (116).

Wound healing demands correlated cellular activity and efficient cell-to-cell contact. Cells contact through cytokines and soluble proteins, which are called growth factors. The cell delivers these cytokines and growth factors and attaches to receptors on the target cell. When a cytokine attaches to a target cell, it triggers the cell to migrate.

In addition, growth factors induced the target cell to divide into parts and generate more cells or synthesize and deliver substances like collagen, which is essential to form the ECM. ECM also has a direct responsibility in wound healing by cooperating with the cells by receptors which are called integrins, bringing to platelet activation, epithelial migration, and fibroblast movement. Platelets are the first cells to be stimulated when a wound appears (117).

As a result of platelets' contact with collagen, proline, and hydroxyproline amino acids activate platelets. Activated platelets, platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), macrophages, and fibroblasts, platelet factor IV, and fibrinogen secrete many mediators that enable and initiate healing (118). These mediators and thrombin are secreted locally at the wound site, stimulating platelets to aggregate and form clots. Thrombin bleeding is stopped with the clot formed due to fibrinogen conversion to fibrin. Clotting creates a temporary matrix skeleton for the migration of cells (119). Then, fibronectin, monocytes, fibroblasts, and endothelium form together fibrin and this temporary matrix which functions to drain the cells. These migrating cells are bound together with the clot matrix. Twelve integrin receptors that recognize fibrin, fibronectin, and vitronectin to interact are used (118).

Prostaglandins (PGE and PGE₂) are inflammation mediators released in the area of inflammation and play a homeostatic role for leukocytes and fibroblasts (54).

In the wound healing process, the cells which are known as macrophages, function as “contractors.” Rotating monocytes transform into macrophages after they quit the blood vessels and get into connection with the ECM. Macrophages be capable of phagocytosing pathogens and contribute a second line of defense. Macrophages also produce extracellular enzymes to deteriorate necrotic tissue at the wound site. These enzymes comprise a family of substances called matrix metalloproteases (MMPs). MMPs need calcium to create a functional shape and zinc for the active site (120).

Around 20 diverse types of MMPs are produced by several particular cells, covering neutrophils, macrophages, epithelial cells, and fibroblasts, resulting in the impact of inflammatory cytokines like tumor necrosis factor-alpha and interleukin-1 and-6. MMPs carry out on all units of the ECM and are liable for eliminating weakened tissue, healing evaded or impaired tissue, and remodelling. MMPs are stabilized by tissue inhibitors of metalloproteases (TIMPs), which are secreted nearby by cells and inactivate MMPs by reversibly attaching to them. Dysregulated MMPs can harm newly composed tissue or damage growth factors (121).

Macrophages produce diverse cytokines and growth factors, such as fibroblast growth factor, epidermal growth factor, transforming growth factor beta, and interleukin 1, that occur to conduct the next phase of wound healing.

2.7.3 Proliferation

The proliferation phase begins about four days after the damage and generally lasts until the 21st day in acute wounds, depending on the wound size and the patient's health condition. It is defined by angiogenesis, collagen deposition, granulation tissue formation, wound contraction, and epithelialization. Clinically, proliferation is seen with red granulation tissue or collagen in the wound area and comprises dermal and sometimes subcutaneous tissue replacement in deep lesions and lesion contraction.

Within a few days, the necrotic tissue, blood clot, and infection in the wound begin to clear away due to the apoptosis process. Once at the injury site, monocytes are converted into macrophages, and macrophages then phagocytose the cell remnants and apoptotic bodies. Then, macrophages continue to take an active role in the proliferation process. Moreover, macrophages proliferating fibroblasts are essential in forming acute granulation tissue (122).

In the house analogy, after the rubble is removed under the direction of the contractor, the frame builders move to build the frame for the new house. Subcontractors could now set new plumbing and framing wires and build the roof and tile outside the house. These framework cells are fibroblasts. During this period, in most fibroblasts, granulation tissue formation in the wound, re-epithelialization, and wound contraction occur in the routine process of wound healing. Granulation tissue, especially in open wounds, is a barrier to infection. In addition, fibronectin and hyaluronic acid take advantage of their structure; the migration of fibroblasts and macrophage cells is like

scaffolding. The transitional matrix, cytokines, and growth factors are created throughout the inflammatory stage to assist in the granulation of tissue (123).

Activation of fibroblasts (fibroplasia) and angiogenesis, the neovascularization process is identified by the synthesis first of fibronectin, then type I and III collagen during granulation, with the migration of fibroblasts that produce collagen from edge to edge of the wound. As a result, collagen and fibroblast accumulation occurs (117,123–125). Most fibroblasts in granulation tissue are myofibroblasts. It both creates and guarantees the contraction of the wound due to the fibers that cover it (126). In addition, hydroxyproline in the collagen structure synthesized by the fibroblast restores its elasticity and strength (127).

Fibroplasia describes a process in which fibroblasts proliferate along the fibers in the fibrin clot, and new granulation tissue migrates into the wound along the developing capillaries, producing collagen and matrix proteins that contribute to the formation of the fibroblast 3-4 days after injury (119). As they migrate and multiply, they become contractile and become myofibroblasts. Myofibroblasts are liable for reorganizing the collagen into straight bundles to raise tensile strength, along with proteoglycan deposition to develop wound resilience against deformation. In addition, due to the actin microfilaments in their structure, they provide wound shrinkage (128).

Fibroblasts stimulated by various growth factors, such as TGF, IGF-1 (insulin-like growth factor-1), and PDGF, move into the temporary matrix and multiply. Fibroblasts use the fibrin clot as a temporary matrix, forming fibronectin, with the ECM containing structures such as laminin, hyaluronic acid, and collagen. Early during this period, while fibronectin increases around normal skin, collagen synthesis is reduced. As a result, fibronectin increases fibroblast migration, adhering to the matrix and leading to wound shrinkage (117,119,122). In addition, Fibroblasts are polarized along the fibronectin fibrils parallel to the epidermis. The forming of a firm type I and III fibrillar collagen structure is thought to depend on a pre-existing fibronectin network through a mechanism comprising integrins (128).

Angiogenesis is the process by which new blood vessels grow into tissue and form capillaries formation of veins. In new formations during angiogenesis, fibroblast proliferation decreases with vessels and increases oxygen-carrying capacity (119,129,130). Angiogenesis accompanies the proliferation of fibroblasts in wound

repair, microvascular endothelial response to injury cells, local activation of endothelial cells, and basement membrane degradation. Moreover, angiogenesis supports the sprouting of blood vessels into clots, cell proliferation, basal membrane remodelling, and tissue repair as newly formed blood vessels initiate an angiogenic process involving shrinkage (119,131). Wound cytoplasmic pseudopods extend from endothelial cells on the second day of formation, MMP is secreted, and migration of endothelial cells into the perivascular space occurs (119). Newly formed veins, providing nutrients, energy, and oxygen, contribute to forming granulation tissue. In addition, inflammatory cells must interact with blood vessels to enter the region.

Endothelial cells; activates collagenase during angiogenesis and plasminogen activator; they also promote the production of many mediators, such as cytokines and MMP. Angiogenesis is stimulated during wound healing; by the secretion of macrophages releasing cytokines; low oxygen tension; lactic acid; and biogenic wound production like amines. Vascular endothelial growth factor (VEGF), angiopoietin, Fibroblast growth factors (FGF), and Transforming growth factor beta (TGF- β) have a critical effect on angiogenesis. VEGF plays a vital role in angiogenesis as a vascular permeability factor known and secreted by several cell types (keratinocytes, fibroblasts, and endothelial cells). In addition, VEGF is a potent mitogen that stimulates the integrin receptor, allowing endothelial cell migration and vessel sprouting. During tissue injury, the low oxygen tension in hypoxic tissue is the most important stimulus of VEGF stimulation (132).

In the wound healing process, cells are divided under the control of growth factors and assemble new cells, which are directed by the cytokines and migrate to where they are needed. There is a harmony between MMPs and the Tissue Inhibitor of Metalloprotease (TIMP), resulting in the final production of new tissue. In chronic wounds, healing is interrupted, cell division is suppressed, and migration of the new cells is reduced. Therefore, there are high levels of inflammatory cytokines and MMPs and lowered levels of TIMPs in chronic wounds. Chronic inflammation: cells are generally older and unresponsive to growth factors, and this shortage of response has a significant indicator of the chronic inflammation state. Elevated bacterial contamination might lead to a chronic inflammatory state in the existence of devitalized tissue, chronic ischemia, or repetitive damage (114,133).

Re-epithelialization, or the excellent repair of the epidermis layer after a cutaneous injury, is the process of becoming. This process is the migration of interconnected keratinocytes to the wound site, and the proliferation of keratinocytes also involves repairing the membrane area. Keratinocytes are the first to form wounds migrating from the free edges of the wound to the wound area within the next 24 hours. Stable hair follicles in the wound area store the keratinocytes they serve. Approximately 12 hours after wound healing, keratinocytes undergo flattening and elongation, develop pseudopod-like protrusions, lose cell-cell and cell-matrix communication, and retract intracellular tonofilaments and keratinocytes. Then, a series of events occur, such as forming actin filaments at the margins of the cytoplasm. Keratinocyte migration process; ECM; integrin receptors; many matrix elements, such as metalloproteinases and growth factors, are involved. Fibrin, fibronectin, and the temporary matrix formed by type V collagen, the migration of keratinocytes, and the crust it forms allow the debris covering the wound underneath to be broken up. A wet or moist environment in wounds has been shown to promote reepithelization and reduce scar formation more than a dry environment. Due to integrin receptors on the keratinocyte's surface, fibronectin-rich transients are possible (121,134).

The direction of keratinocyte migration is determined by the new collagen found in the wound bed, and the binding of keratinocytes to integrin receptors on newly formed collagen determines the molecules. Dissolution of these bonds allows further migration of keratinocytes. MMPs play an essential role in this dissolution in keratinocyte migration. For example, MMP-1 is expressed by keratinocytes in the wound area. It facilitates the continuity of migration by preventing its attachment to collagen fibrils. As a result, keratinocytes proliferate enough cells to close the wound site required for migration. As a result of suppression of interaction, when it stops, the keratinocytes reattach to the substrate, the basal layer is restructured, and then the terminal differentiation process is restored as a result of the formation of the sequential epidermis and presence of single-layered keratinocytes toward the wound center and near the wound edges (122).

The presence of multi-layered and ordered keratinocytes is an indicator of this situation. Growth factor, Keratinocyte growth factor, and Transforming growth factor alpha (TGF- α); keratinocyte migration, proliferation, and differentiation are the most important factors stimulating the reepithelialisation process.

Wound contraction is the movement of the wound lips surrounding the wound towards the wound center. It is accomplished by diversified fibroblasts or myofibroblasts, which, in response to TGF- β , tissue tension and the existence of specific matrix proteins (113). Specialized fibroblasts have an important role for wound contraction. It is the process of facilitating wound closure by the skin of granulation tissue. As a result of pulling the edges inward, the area to be closed with reepithelialisation becomes smaller.

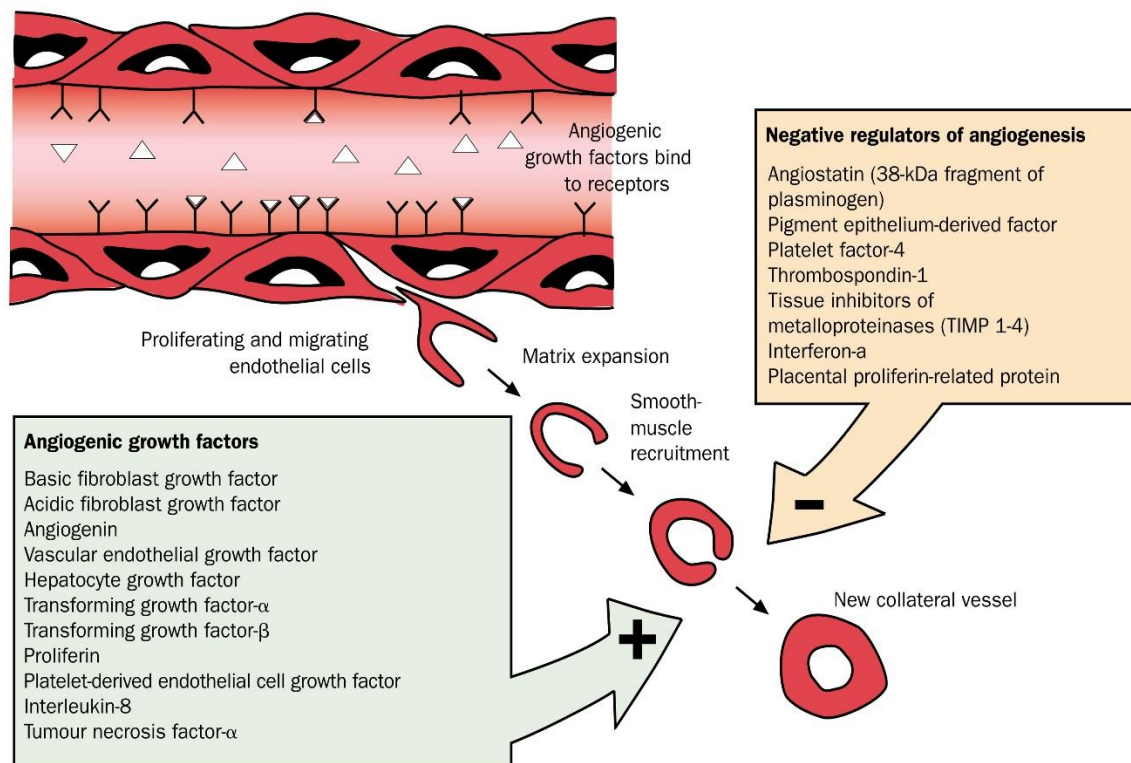


Figure 9. Formation of angiogenesis (135)

2.7.4. Remodelling

Wound healing is considered complete when epithelialization is complete. Remodelling begins with keratinocytes' differentiation and the dermis phase's formation (136). End of wound repair and the most prolonged phase, the maturation phase, granulation tissue is replaced by scar tissue release and the maturation of the ECM. In the maturation phase, the number of fibroblasts in the wound area decreases, homeostasis of collagen production is achieved, epithelialization is complete, the wound color is pale, the tensile strength increases, and the scar tissue shrinks in volume. During this phase, large amounts of cellular and vascular granulation tissue, which has a structure, slowly undergo structural and shape changes and rotate with scar tissue with less cellular and

vascular structure. Cellular reduction occurs by migration of cells out of the wound or by apoptosis. Fibroblasts multiply within the wound and build up an extra-cellular matrix (ECM), composing granulation tissue perfused with newly produced blood vessels. Simultaneously, the provisional matrix, mostly composed of type III collagen, fibrin, fibronectin, and hyaluronic acid, is regularly substituted with ECM, chiefly including collagen. The temporary matrix contains fibronectin and MMPs (114). MMPs are undetectable or in minimal amounts in healthy tissue, where they are found. In addition, macrophages have been newly recognized to polarize into a continuum of phenotypes, and the character of this polarization has a crucial influence on remodelling and scar formation (137).

The most important feature of this phase is the realization of collagen synthesis and the production of collagen synthesis regulation. Collagen is the connective tissue present in many tissues, such as skin, bone, and cartilage tissue. It is a structural protein (138). Collagen's most crucial task is to provide resistance against degradation and to ensure that the tissues enter a specific form. Collagen production continuously up to 4-5 weeks after injury continues. The first collagen produced after an injury is weaker than that in healthy skin. Over time, the first collagen threads are absorbed, and then thicker and regular collagen deposition occurs. For the situation to occur regularly, collagen synthesis and destruction are required. Remodelling may carry on to 2 years after wounding. This can clarify why closed wounds could rapidly endamage if attention is not paid to the first causal effects (124).

Wound tensile strength is the best indicator of morphological and chemical wound healing in terms of surgery. It is an important parameter to determine the strength of the wound in the remodelling phase. Tensile strength in wound healing occurs slowly. Wound tensile strength depends on the maturation of collagens. Wound tensile strength increases with the cross-linking of collagen fibers by changing their arrangement.

Wounds gain merely around 20% of their strength in the first three weeks. A wound has minimum fundamental strength in the first four days after injury. Throughout the second week, fibroblast production increased and built type I and III collagen. The deposition of propeptide type I collagen in the extracellular area throughout this phase brings a crucial elevation in tensile strength. Still, Type III collagen does not undergo fibril formation due to its amino acid structure; type III collagen still has a lower strength than type I. Despite the amount of type I collagen elevated during the second week, type

III collagen is still the primary component. Since the amount of type III collagen is still in the majority, wound strength is still less than 10 percent of uninjured strength. The wound's strength increases fast as type I fibrils are cross-linked and collect into large fibers. Four weeks after injury, the tensile strength recovery rate is over 50%. Until week 6, the intensity of firm collagen fibers starts to be similar to that of intact skin and the collagen matrix develops to more condensed and regularly aligned. During this process, wound disruption is not expected, considering the tensile strength of the wound is close to 80% of the undamaged skin. Still, wounds never gain the same tensile strength as uninjured skin (118,139).

2.7.5. *In Vitro* Models for Wound Healing

In vitro, wound models are mainly used to evaluate intercellular and intracellular signal transmission but cannot provide physiological conditions. *In vitro*, models include cell and tissue cultures and 3D matrices. The response of one or more cell types to certain stimuli can be recorded in the cell culture medium. Cell cultures include endothelial, fibroblast, and keratinocyte cells and cultures which are used in combination type and amount of collagen released from fibroblasts against growth factors, endothelial proliferation, and fibroblast contraction. Since the response they give alone to stimuli differs from the response they give when they are with other institutions, organ and tissue culture models, which are closer to *In vivo* conditions, are also used in wound healing research. On the other hand, 3D Matrices enable the evaluation of cell migration in the three-dimensional wound area *in vitro*.

In vitro wound recovery models examine the responses of a limited number of cell types to a limited number of stimuli. In contrast, *in vivo* models include components such as the ECM, cytokines, twist hunters, pH, evaporation, temperature, nutrition, general health, and cell types that play different roles in different cells of routine wound healing. In addition, it is possible to examine the consumption of tissue resources.

2.7.5.1. Extracellular Matrix Components

2.7.5.1.1. Collagenase Enzyme

Collagenases are the enzymes that break peptide bonds in collagen structure. Collagenase enzymes were first discovered in 1950. They aid in the degradation of extracellular substances in the infection of pathogens such as *Clostridium*. *Clostridium* species are clarified as the virulence factor that simplifies the distribution of gas gangrene

and frequently aims to connective tissue in muscle cells and other body parts. Collagen, a crucial substance of the animal ECM, is formed by the split of pro-collagen by collagenase enzyme; subsequently, it is secreted from the cell. In addition, to being secreted by some bacteria, the body can make collagenase part of the usual immune response (138,140). In addition, the Collagenase enzyme is used in treating Peyrone's disease (caused by excess scar formation) and gets FDA approval.

The production of collagenase in the human body is activated by cytokines that stimulate cells such as fibroblasts and osteoblasts and may lead to indirect tissue damage. The wound healing process has several steps, such as coagulation, formation of granulation tissue, reepithelization, and remodeling. Recent studies show that the only objective of the ECM is not only mechanical support for cells but also involved in tissue repair. Under normal conditions, the ECM is formed by collagen, the most plentiful and fundamental protein in the body. Collagen performs a vital role in all stages of the wound-healing. However, the inflammatory action stimulates the synthesis of skin enzymes, which leads to ECM destruction. MMP-1 is responsible for the degradation of the particular type I collagen. Recent studies claim that the degradation of collagen products delivered after collagenase breakdown stimulates the migration and action of essential inflammatory cells such as macrophages and has a crucial effect on wound healing (133,141).

Abnormal collagen deposition due to changes in collagen formation is observed in different pathological cases, such as hypertrophic scars and keloids. The injectable form of Collagenase is mainly used in wound treatment, especially in necrotic wounds. Necrotic tissues are significant obstacles to wound healing because the dead tissues develop suitable conditions for bacterial growth, which may cause chronic inflammation. Therefore, collagenase enzyme is injected into the necrotic tissue to perform enzymatic debridement. In addition, excessive collagenase activity is observed in rheumatoid arthritis, local tumor proliferation, extreme bone resorption after bone trauma, anastomotic leakage after intestinal surgery, and hyper granulation tissue after burns. Therefore, selective therapeutic improvement or inhibition of the collagen mechanism could be crucial to treat or prevent these conditions (140,142).

2.7.5.1.2. Elastase Enzyme

Elastase is a type of enzyme called protease that is primarily responsible for breaking down elastin, a protein found in connective tissues such as skin, ligaments, and blood vessels. It belongs to the family of serine proteases and is produced by various cells, including neutrophils, macrophages, and pancreatic acinar cells. There are two main types of elastase: pancreatic elastase and neutrophil elastase. Pancreatic elastase is produced in the pancreas and is involved in the breakdown of proteins in the digestive system. Neutrophil elastase is produced by neutrophils, a type of white blood cell, and plays a role in the immune response by breaking down bacteria and other foreign substances (143,144).

In addition to its role in breaking down elastin, elastase has been implicated in several disease processes, including chronic obstructive pulmonary disease, emphysema, and cystic fibrosis. In these conditions, elastase can lead to the destruction of lung tissue and other organs. Elastase inhibitors, which are compounds that block the activity of elastase, are being investigated as potential therapies for these and other diseases. Some elastase inhibitors are currently used in the treatment of conditions such as chronic obstructive pulmonary disease and alpha-1 antitrypsin deficiency, a genetic disorder that can lead to lung and liver disease (145).

2.7.5.1.3. Hyaluronidase Enzyme

Hyaluronidase is an enzyme that breaks down hyaluronic acid. This substance is naturally present in the body and plays a crucial role in maintaining the structure and hydration of connective tissues such as skin, cartilage, and joints. There are two main types of hyaluronidase: bacterial and mammalian. Certain types of bacteria produce bacterial hyaluronidase, often referred to as a spreading factor, as it facilitates the spread of bacteria by breaking down the ECM of tissues. Mammalian hyaluronidase is produced by the body and is involved in a variety of physiological processes, including tissue remodelling, wound healing, and embryonic development (146).

Hyaluronidase has several medical and cosmetic applications. In medicine, it is sometimes used as an adjuvant to enhance the absorption and distribution of drugs, particularly in local anesthesia, ophthalmology, and cancer treatment. It is also used in the treatment of certain medical conditions, such as lymphedema, which helps to break down excess fluid that accumulates in tissues. In cosmetic medicine, hyaluronidase is

used to dissolve hyaluronic acid-based fillers, which can help correct overfilling or asymmetry. However, Hyaluronidase can have side effects, mainly when used in high doses or inappropriately. These can include allergic reactions, swelling, bruising, and tissue damage. Therefore, it is essential to seek medical advice before using hyaluronidase and to only use it under the guidance of a qualified healthcare professional.

2.7.5.1.4. Effects of Collagenase, Elastase, Hyaluronidase Inhibition in Wound Healing Process

Collagenase, elastase, and hyaluronidase are enzymes involved in the degradation of ECM components, such as collagen, elastin, and hyaluronic acid, respectively. These enzymes play a role in tissue remodelling and turnover, but extreme activity can interrupt wound healing. Collagenase inhibition has been shown to improve wound healing by promoting collagen accumulation and reducing scar tissue formation. This is because collagen is a major ECM component, providing tissue strength and structure. In addition, inhibiting collagenase activity can promote the deposition of new collagen, which can help strengthen wound tensile and support wound healing (147).

Elastase inhibition has also been shown to promote wound healing by reducing the breakdown of elastin fibers in the ECM. Elastin is a protein that provides elasticity to tissues, and its degradation can impair tissue function and structure. By inhibiting elastase activity, tissue elasticity can be preserved, which can help promote wound closure and reduce scar formation. Hyaluronidase inhibition can also improve wound healing by preserving the integrity of the ECM. Hyaluronic acid is a vital component of the ECM, and its degradation can impair tissue function and structure. By inhibiting hyaluronidase activity, the integrity of the ECM can be preserved, promoting wound healing.

In conclusion, inhibiting the activity of collagenase, elastase, and hyaluronidase can improve wound healing. These enzymes play essential roles in tissue remodelling, but excessive activity can impair healing. Therefore, inhibiting their activity can effectively promote wound closure and reduce scar formation (142,148).

2.7.6. *In Vivo* Models for Wound Healing

In vivo, wound models are still the most preferred for examining wound healing because they provide complex conditions that can best mimic wound formation and tissue repair physiologically.

Although animals such as rodents, pigs, dogs, chickens, and sheep are used in animal models, histological differences are observed between species. Since the epidermis/dermis thickness is close to humans, the pig is the closest experimental animal to a human in examining wound healing. Although rodent skin is not closely related to human skin characteristics, small animal models are frequently preferred because they are practical to work with, easy to care for, and durable. It is possible to prefer genetically engineered mice, and there are many transgenic mouse models (149).

2.7.6.1. Incisional Wound Models

A frequently used wound model, known for many years, is the incisional wound healing model. It is the most commonly used method for examining wound tensile strength. Following the skin incision in the model, the wound is either left open or primarily sutured. At certain time intervals after the procedure, the block is removed from the incision site, and the force required to separate the wound edges is determined in a device designed to measure tension force. Histopathological samples can be taken, and the effects of systemic or topical wound healing agents can be measured quantitatively. Although primarily used for rodents, models that can be used in pigs or other animals have been defined (149).

2.7.6.2. Excisional Wound Models

It is the most commonly used and simplest wound model. An open wound is formed. Granulation formation, collagen deposition, reepithelialisation, and constriction can be investigated with this model. Wounds can be formed in different depths and sizes, full thickness (excisional) or partial thicknesses. The effects of different agents can be examined by opening several wounds in the same experimental animal. Digital photographs and some software follow wound surface area. Histological evaluation of wounds and molecular biological analyses (mRNA, protein, apoptosis) in excised tissues are also accessible. The most critical issue to note is that open wounds in some species heal more by contraction than by granulation and reepithelialisation (149).

2.7.6.3. Burn Models

Many burn models have been described that have been used to study wound healing and the systemic response to thermal injury. It is possible to create a complete- or partial-thickness burn. The thickness of the burn wound depends on the factors that determine the depth of the burn: temperature of the contact material, duration of contact,

skin thickness, and blood supply. If partial-thickness burns are desired, a low temperature should be applied to thicker skin for a short time. Methods such as a flame or metal placed in boiling water are used to create a contact burn. In addition to models such as scalding, flame, and hot object, burn models such as electrical burns and sepsis due to burn wounds have been described.

One of the significant disadvantages of burn models in rodents is that the response to burn-induced trauma is severe, and animals are lost before the end of the research. Other factors are that the body surface areas of different animal species are proportionally different from humans, and it cannot be predicted exactly whether a wound of a specific size in an animal produces the same systemic changes that occur in significant burns in humans (149).

2.8. Experimental Studies on the Pharmacological Activities of St. john's wort (*Hypericum perforatum*) Oleates

2.8.1. Bioactivity Studies of the St. John's wort Oleate in Olive (*Olea europaea*) Fixed Oil

Numerous pharmacological studies suggest that *H. perforatum* is used in traditional indications and HPO for treating abrasions, wounds, burns, and sunburns.

HPO loaded bilayer PEG-PCL polymeric membranes, then HPO was added to the PEG polymer solution by electrospraying and encapsulated. Membranes' time-dependent mass loss and controlled release of HPO from the membranes over time was studied under *in vitro* conditions. Gram-positive and gram-negative membrane antibacterial activity on bacterial strains has been observed by performing disc diffusion tests. Incubated with the L929 mouse fibroblast cell line, the *in vitro* biocompatibility of membranes (WST-1) and interactions with cells (cell adhesion, proliferation, and apoptosis-necrosis) were examined. As a result, the produced polymeric membranes are characterized by porosity, air permeability, moisture retention capacity, hydrophilicity, elasticity, antibacterial activity, and non-adherence to the wound; it is desired to be an ideal wound dressing in terms of features such as biocompatibility. Although it has optimum values, the HPO promotes wound healing through its controlled release from the membranes. *In vitro* studies have confirmed that it accelerates the process (150).

A study was conducted to investigate the effectiveness of HPO in pressure sores in intensive care patients. Standardized HPO was prepared by the sun maceration

technique with OO and applied to the pressure sore area of three intensive care unit patients thrice a day. Patients were evaluated by measuring wound diameters and histopathologic assessment at the start and end of treatment. As a result of this experiment, HPO is successful in treating pressure sores (151).

The topical application of HPO significantly enhanced wound healing in rats by promoting tissue regeneration and reducing inflammation (152). The wound-healing effect of *H. perforatum* is related to antimicrobial, anti-inflammatory, and fibroblastic activities and stimulates collagen synthesis. Süntar et al., HPO, showed that it accelerated wound healing in models applied to rats (153). In a study using HPO in treating burns in rats, at the end of 17 days, significant collagen deposition and anti-inflammatory effects were observed (154).

On the effect of HPO ointment in cesarean section injuries and hypertrophic scars, a randomized, double-blind clinical trial was conducted on 144 patients. Maceration in grape seed oil in formulation *H. perforatum*, which was left behind, was used as a treatment oil. Wound healing effect after the cesarean section was examined on the 10th day and evaluated with REEDA analysis (redness, edema, ecchymosis, discharge, and approximation). At day 40, including the degree of scarring, pigmentation, height, elasticity, and vascularity. It was evaluated using the Vancouver Scar scale. Significant differences in wound healing at day ten and REEDA scores were 0.19 (HPO), 0.75 (placebo), and 0.79 (control) ($p < 0.005$), respectively found. On the 40th postpartum day, scar formation was determined by the Vancouver scale as 3.3 (HPO), 5.0 (placebo), and 5.5 (control) ($p < 0.0001$) (treatment groups compared with placebo and control group). In addition, pain and itching in the cesarean section on the 40th day after delivery was reported to be significantly lower in the treatment group compared to the other groups (155).

Similarly, in a clinical study conducted on 24 patients who were delivered by cesarean section, the wound healing effect of *Calendula* sp. and HPO mixture (30:70) and wheat seed oil was compared as a placebo. After four weeks of administration, while 16% closure was observed in the surgical incision in patients treated with wheat placebo, the value was 38% in patients treated with a mixture of calendula and HPO (156).

Yeşilada et al.(157) conducted a study about the antiulcerogenic activity of the *H. perforatum* oil and its subfractions. Ethanol extract showed dose-dependent and vital

antiulcerogenic activity. (82.8 to 98.7% inhibition). Afterward, diethyl ether, ethyl acetate, and butanol subfractions of this extract were formed. All subfractions were found to be effective, but the effect of ether extract, which consists of liposoluble parts of plants and non-polar flavonoids, was found to be more noticeable (85.9% inhibition). Also, chloroform and butanol fractions were found moderately effective on *Helicobacter pylori* by agar dilution method.

The study aimed to treat endometriosis with an oil mixture of HPO and Sea buckthorn (*Hippophae rhamnoides* L.) oil. The activity was observed in surgically induced endometriosis in rats. A 15-mm piece of endometrium was sutured into the abdominal wall. Twenty-eight days later, a second laparotomy was performed to calculate the endometrial foci areas and to score intra-abdominal adhesions. The rats were treated with either vehicle, HPO, and *H. rhamnoides* oil mixture formulation or the reference (buserelin acetate). The tissue sections were analyzed via histopathological methods, and results show that there is a significant decrease in levels of TNF- α , VEGF, and IL-6, which are important markers of the etiology of endometriosis. According to the experiment results, HPO and *H. rhamnoides* oil mixture can be positioned in the treatment of endometriosis (158).

Anti-inflammatory and antibacterial activities are desired in treating atopic dermatitis, stimulation of keratinocyte differentiation, and relief of itching. *H. perforatum* extract seems quite suitable to meet these demands; however, many herbal extracts containing ingredients for atopic skin are more effective in sensitivity. Hyperforin is less than other components of *H. perforatum* and carries the risk of photosensitization. For this reason, in terms of flavonoids and hypericin efficacy, a poor but hyperforin-enriched cream for subacute atopic dermatitis was evaluated compared to a placebo for the treatment of observed cases. It showed a significant reduction in atopic dermatitis (45).

The hyperforin contained in *H. perforatum* exerts an anti-inflammatory effect on T cells and stimulates keratinocyte differentiation. Ten patients with plaque-type psoriasis were treated with *H. perforatum* ointment in a case series. *Hypericum* ointment on one side of the body and placebo ointment on the other was applied twice daily for four weeks. As a result of *Hypericum* ointment, mild plaque PASI (Psoriasis Area Severity Index) scores in type psoriasis significantly decreased, but more extensive studies are needed to obtain a more conclusive result (159).

In another study, the wound-healing effects of a cream containing HPO in human subjects were investigated. The study found that the cream promoted faster wound healing and reduced scar tissue formation compared to a placebo cream. HPO is believed to promote wound healing through its anti-inflammatory, antioxidant, and antibacterial properties. It is also thought to stimulate the production of collagen, a protein essential for wound healing and tissue regeneration. However, it is important to note that more research is needed to fully understand the *In vivo* healing potential of HPO, as most of the studies conducted so far have been small and limited to animal or human subjects. Additionally, it is essential to consult with a healthcare professional before using HPO for any medical condition, as it can interact with certain medications and cause adverse effects in some individuals (42).

In the literature, it has been reported that HPO in normal wounds; although its positive effect has been shown, there is no study on its effect on problematic wounds. Diabetes has been chosen as an example of a nasty wound. Oral and topical application of HPO was significantly higher than the diabetic group ($p < 0.05$), topical OO was found to be close to a significant value ($p \leq 0.051$), and orally no significant effect of OO on breaking strength was found. Tissue hydroxyproline concentration in the oral and topical HPO groups was higher compared to the diabetic group ($p < 0.05$), topical OO had a significant value ($p \leq 0.053$), and oral OO did not have a significant effect. Regarding new vessel formation, on the seventh day, angiogenesis was higher in the oral and topical HPO groups compared to the placebo diabetic group, which was low at 21. days, but not statistically significant. It was shown that the inflammatory cell density on the third day was significantly higher in the oral HPO and topical OO groups compared to the diabetic group ($p < 0.05$), and the oral OO was relatively lower but higher than the diabetic group. HPO was found to have no effect. Findings of fibroblastic activity were significantly higher in the oral HPO and topical OO groups ($p < 0.001$). Collagen density was increased on the third day in oral HPO and topical OO groups and was shown to be significantly higher ($p < 0.05$). Epithelialization of wounds on the seventh day of HPO and topical OO groups compared to the diabetic group terms was found to be high but close to statistically significant ($p \leq 0.052$) (160).

HPO was found to be more effective than sulfonyl amide against *Staphylococcus aureus*. According to sources, other gram-positive and gram-negative bacteria: include *Proteus vulgaris*, *Escherichia coli*, and *Pseudomonas aeruginosa* (28,62,64).

On the other hand, Hyperforin showed no inhibitory effect against Gram-negative bacteria (*Escherichia coli*, *Salmonella* sp., *Klebsiella* sp., *Proteus vulgaris*, *Pseudomonas* sp., *Bacillus anthracis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Beta-hemolytic streptococci*) or *Candida albicans* (45,49).

A study showed that ozonated HPO improved wound healing in rats with excision wounds compared to the control group. The researchers suggested that this effect was due to the antimicrobial, antioxidant, and anti-inflammatory properties of ozonated HPO (161).

2.8.2. Bioactivity Studies of the St. John's wort Oleate in Sunflower (*Helianthus annuus*) Fixed Oil

The study investigated the anti-inflammatory and antibacterial activities of the three Oil-in-Water (O/W) creams, which were formulated containing 15% (w/v) *H. perforatum* oil extract as an active substance.

The macerates were prepared according to the traditional methods and macerated for 40 days; diverse fixed oils were used as vehicles; olive (*Olea europaea*), palm (*Elaeis guineensis*), and sunflower (*Helianthus annuus*) oil.

The researched O/W emulsions showed crucial anti-inflammatory activity in an *in vivo* sodium lauryl sulphate test. Both skin parameters evaluated in the experiment (electrical capacitance and erythema index) were brought back to the standard value after a seven-day treatment. After a seven-day treatment, all macerates reduced erythema to standard values; however, HPO and *H. perforatum* sunflower macerate performed crucially more potent activity than one prepared with palm oil on day 7. Almost all researched HPO extracts, and related creams show similar antimicrobial activity against most of the investigated pathogens with minimal inhibitory concentrations values of 1,280 µg/mL, 2,560 µg/mL, or >2,560 µg/mL (162).

In an experiment, three *H. perforatum* macerates were prepared according to the traditional methods. Fresh flowering parts were macerated in three different oils: HAO, OO, and palm oil, for 40 days with sunlight. This study focuses on the examine the gastroprotective activity of the gathered macerates regarding their quercetin content. HPLC analysis confirmed the presence of quercetin in all macerates investigated, with results as follows: 15.1, 5.8, and 21.7 µg/mL in HAO, OO, and palm oil, respectively. The gastroprotective activity was assessed by applying cold-restraint stress (CRS)

induced rat gastric mucosa lesions test. All the *H. perforatum* macerates perform the gastroprotective activity. In contrast to the lowest quercetin amount, HPO shows the highest protective effect on the (CRS) induced rat gastric mucosa lesions test. According to these results, quercetin is not the only component responsible for the gastroprotective activity; other components of the extracts also play a vital role in the gastroprotective action (163).

2.9. Experimental Studies on the Pharmacological Activities of Olive (*Olea europaea*) Fixed Oil

A study evaluated and prioritized potential alternative therapeutic options for wound healing in diabetic rats. The study compared the effects of honey, OO, pawpaw fruit extract, and iodine on wound healing in diabetic rats. The results showed that all the treatments significantly improved wound healing compared to the control group. However, honey and OO were the most effective treatments in promoting wound healing for diabetic rats, followed by pawpaw fruit extract and iodine. Honey, OO, and pawpaw fruit extract were found to promote wound healing by reducing inflammation, increasing collagen synthesis, and promoting angiogenesis. On the other hand, iodine was effective in reducing the bacterial load in the wound site but had limited effects on other wound healing parameters. This study suggests that honey and OO could be potential alternative therapeutic options for promoting wound healing in diabetic patients (164).

EVOO has been shown to have beneficial effects on skin wound healing. Studies have shown that topical application of EVOO can increase the rate of wound closure, reduce inflammation and oxidative stress, and improve the quality of newly formed skin tissue. One mechanism by which EVOO promotes skin wound healing is by activating the nuclear factor erythroid 2 (Nrf2) pathway. Nrf2 is a transcription factor that regulates gene expression in cellular defense against oxidative stress and inflammation. EVOO contains polyphenols and other bioactive compounds that activate the Nrf2 pathway and enhance skin cells' antioxidant and anti-inflammatory capacity. In addition to its effects on wound healing, EVOO has also been shown to promote the survival and migration of dermal fibroblasts. Fibroblasts play a critical role in skin tissue repair and regeneration. Studies have shown that EVOO can increase fibroblasts' proliferation, migration, and collagen production *in vitro*. In addition, the activation of the Nrf2 pathway also mediates these effects. These findings suggest EVOO has potential therapeutic applications in treating skin wounds and other skin disorders. However, further research is needed to

determine the optimal dose and frequency of EVOO application and to investigate its effects *in vivo* (87,165).

Nasiri et al. investigated the effect of topical OO on the healing of foot ulcers in patients with type 2 diabetes. The study was a randomized clinical trial that involved 60 patients with type 2 diabetes and foot ulcers. The results of the study showed that the patients in the treatment group had a statistically significant improvement in the healing of their foot ulcers compared to the control group. The researchers also found that the patients' topical OO was safe and well-tolerated. The study suggests that topical OO may be a valuable addition to standard wound care treatment for patients with type 2 diabetes and foot ulcers (166).

Karimi et al. investigated the impact of OO and honey on healing diabetic foot ulcers. The study was conducted on 90 patients with type 2 diabetes who had foot ulcers. The participants were randomly divided into three groups: group one received conventional treatment, group two received conventional treatment plus OO, and group three received conventional treatment plus a combination of OO and honey. The study found that combining OO and honey was more effective in promoting the healing of diabetic foot ulcers than conventional treatment alone or treatment with OO alone. The combination treatment group showed a significantly faster rate of wound healing, with an average healing time of 15 days compared to 25 days in the conventional treatment group and 20 days in the OO group. The researchers attributed the enhanced healing to OO and honey's anti-inflammatory and antioxidant properties. OO contains polyphenols and monounsaturated fatty acids, which are known to have anti-inflammatory effects. Honey contains natural antioxidants and has antibacterial properties, which can help prevent infection in the wound. The study suggests that combining OO and honey may be a helpful adjunct therapy for treating diabetic foot ulcers (88).

The researchers investigated the effects of the topical application of EVOO on the skin of atopic dermatitis patients. It was found that EVOO positively impacted skin hydration and barrier function and reduced inflammation and itching. However, it is essential to note that these studies were small and conducted in animals or a limited number of human subjects. Therefore, more research is needed to confirm these findings and determine EVOO's most effective dosage and application for wound healing in atopic dermatitis patients. It is also essential to consult with a healthcare professional before

using EVOO or any other natural remedy for wound healing, as some substances may cause allergic reactions(86,167).

In a study, researchers found that EVOO was effective against various strains of bacteria, including *Staphylococcus aureus* and *Escherichia coli*. To use EVOO for the treatment of traumatic ulcers is recommended to apply a small amount of the oil directly to the affected area and gently massage it in. This can be done once or twice a day, depending on the severity of the ulcer. It is important to note that EVOO should not be used as a substitute for medical treatment, and individuals with severe or persistent ulcers should seek medical attention (168).

Donato-Trancoso investigated the effects of OO on wound healing in mice with pressure ulcers. The results showed that mice treated with OO had significantly faster wound healing than the placebo group. The researchers also observed a significant reduction in oxidative damage and inflammation markers in the OO group, indicating that the treatment reduced tissue damage and promoted healing. The study suggests that OO may be a promising treatment for pressure ulcers and other wounds (169).

2.10. Experimental Studies on the Pharmacological Activities of Sesame (*Sesamum indicum*) Seed Fixed Oil

Several studies have evaluated the wound-healing potential of SO, both in animal models and humans. One study evaluated the wound-healing potential of SO in rats. The results showed that the rats treated with SO had significantly fast wound healing rates. The SO treatment also reduced inflammation and improved antioxidant enzyme levels, indicating its potential as an antioxidant agent. This study suggests that SO has the potential as a wound-healing agent, at least in rats (170).

Episiotomy is a surgical incision made in the perineum (the area between the vagina and anus) during childbirth to enlarge the vaginal opening. Episiotomies can cause discomfort, pain, and swelling in the area, and proper wound care is essential for healing.

Some evidence suggests that SO or *Sesamum indicum* ointment may effectively promote wound healing, including episiotomy wounds. For example, a randomized controlled trial found that women who used SO for their postpartum wound care had significantly less pain, inflammation, and redness than those who used a placebo ointment. It also found that SO effectively reduced pain and promoted healing in women with episiotomy wounds. While further research is needed to confirm the efficacy of SO

on wound healing, particularly on episiotomy wounds, the available evidence suggests that it may be a safe and effective option for promoting wound healing and reducing pain and inflammation. However, it is always recommended to consult with a healthcare professional before using any new treatment (171,172).

SO has been traditionally used in Ayurvedic medicine for its anti-inflammatory and antioxidant properties. It has been found to accelerate wound healing and reduce inflammation and pain in animal studies. SO can also reduce the formation of scar tissue. On the other hand, sulfadiazine is an antibiotic commonly used to treat burns and prevent infection. It works by inhibiting the growth of bacteria and preventing the spread of infection. A comparative study was conducted to evaluate the efficacy of SO and sulfadiazine in treating burns. The study included 100 patients with second-degree burns who were randomly assigned to receive either sesame oil or sulfadiazine. The results of the study showed that both treatments were effective in reducing pain and promoting wound healing. However, the group treated with SO had significantly better outcomes regarding reduced healing time and improved scar formation than those treated with sulfadiazine. In addition, the sesame oil group had lower infection rates than the sulfadiazine group. The study suggests that SO may be a viable alternative to sulfadiazine in the treatment of burns, especially for patients who may be allergic to sulfadiazine or have concerns about antibiotic resistance (173).

In a study, rats with excision wounds treated with ozonated SO showed a significant increase in wound closure rate compared to the control group, ozonated HPO group, and ozonated NSO oil group. The researchers attributed this effect to ozonated SO's anti-inflammatory and antioxidant properties (161).

2.11. Experimental Studies on the Pharmacological Activities of Black Seed (*Nigella sativa*) Fixed Oil

Ozonated oils have been studied for their potential effects on wound healing in rats. A study showed that ozonated NSO had a positive effect on wound healing in rats. The researchers found that the ozonated oil increased collagen deposition and angiogenesis and reduced inflammation in the wound area.

The available research suggests that ozonated oils, including SO, NSO, and HPO, may positively affect the wound-healing process in rats. These effects are thought to be due to these oils' anti-inflammatory, antimicrobial, and antioxidant properties. However,

further studies are needed to confirm these findings and to determine the optimal doses and treatment durations for these oils (161).

The wound closure activity of *N. sativa*, *Melastoma malabathricum*, *Pluchea indica*, and *Piper sarmentosum* extracts was evaluated on a scratched monolayer of human gingival fibroblasts. Results of the study showed that all four plant extracts demonstrated wound closure activity on a scratched monolayer of human gingival fibroblasts. Among the four plant extracts, *P. sarmentosum* showed the highest percentage of scratch closure at a concentration of 100 µg/mL, followed by *N. sativa*, *M. malabathricum*, and *P. indica*. The MTT assay showed that all four plant extracts were non-toxic to human gingival fibroblasts at the concentrations used in the study. The study suggests that these plant extracts have potential wound-healing properties and can be used in the development of natural wound-healing agents for oral tissue (174).

A study found that topical application of NSO to diabetic rats with skin wounds significantly increased wound closure and accelerated the healing process compared to a control group. Another study showed that thymoquinone promoted skin regeneration in mice with skin wounds by increasing the expression of growth factors and enhancing collagen production. In addition to its wound-healing properties, *N. sativa* has also been shown to have potential benefits for various skin conditions, including eczema, psoriasis, and acne (175).

Thymoquinone is the main active compound found in *N. sativa* seeds, which has been shown to have various biological activities, including anti-inflammatory, antioxidant and antimicrobial effects. In an *in vivo* animal model study, the effect of thymoquinone on wound healing was investigated. The results showed that thymoquinone significantly accelerated wound closure, reduced inflammation, and increased collagen deposition in the wound area compared to the control group. The mechanism behind the wound-healing effect of thymoquinone is thought to be due to its antioxidant and anti-inflammatory properties, which reduce oxidative stress and inflammation, leading to faster healing. This study suggests that *N. sativa* and its active compound, thymoquinone, may have potential therapeutic applications for wound healing in humans (94).

A study was conducted to investigate the effect of locally applied NSO on wound healing in rabbits. The results showed that the wounds in the NSO group healed faster

than those in the placebo group. The NSO group also had less inflammation and better epithelialization than the placebo group. The researchers concluded that the local application of NSO can promote wound healing in rabbits. While this study shows promising results for the use of NSO in wound healing, further research is needed to confirm these findings and determine the optimal dosage and duration of treatment. It is also important to note that while NSO is generally considered safe, individuals with allergies or medical conditions should consult with a healthcare professional before using it (176).

2.12. Experimental Studies on the Pharmacological Activities of Sunflower (*Helianthus annuus*) Fixed Oil

Several studies have been conducted on the effects of the topical application of HAO on open wound healing in lambs. The researchers found that the wounds in the HAO group had a significantly lower level of inflammation and a higher level of collagen deposition compared to the untreated group. The researchers concluded that the topical application of HAO may be a valuable treatment for accelerating the healing of open wounds in lambs (177).

A study investigated the effects of the topical application of HAO on experimentally induced wounds in horses. In the study, eight horses were divided into two groups of four. In each group, two circular full-thickness skin wounds were created on each horse's neck. One wound in each group was treated with HAO, while the other wound was left untreated as a control. The wounds were evaluated for wound contraction, wound closure time, and epithelialization time. The study results showed that the wounds treated with HAO had significantly faster wound contraction and closure times compared to the control wounds. The epithelialization time was also shorter in the wounds treated with HAO, although the difference was not statistically significant. The study concluded that the topical application of HAO can be beneficial in promoting wound healing in horses (178).

A histomorphological study on HAO showed that HAO promoted wound healing in turtles, with significant differences observed between the treated and the control groups. The histomorphological analysis revealed that HAO accelerated the healing process by enhancing collagen formation, increasing the number of fibroblasts, and promoting angiogenesis (179).

Studies have shown that HAO can positively affect the skin barrier in adults, which may have implications for neonatal skin care. A study found that HAO had positive effects on the skin barrier, with increased skin hydration and improved skin barrier function in people with atopic dermatitis. The study also found that HAO was safe and well-tolerated. HAO is considered safe for newborn skin, but it is important to note that each baby's skin is unique and may react differently to different products. Therefore, it is always a good idea to consult a healthcare professional before using new products on a baby's skin (180).





3. MATERIALS AND METHODS

3.1. Materials

The plant material *H. perforatum* was provided from a cultivation area in Balıkesir, Burhaniye (Turkey), in June 2021, and Ege Tarımsal Araştırmalar Enstitüsü, Tıbbi Bitkiler Şubesi Dr. Erdinç Oğur has confirmed the botanical identification. Voucher specimens were deposited at the Ege Tarımsal Araştırmalar Enstitüsü Herbarium İzmir/Menemen (ETAIEZ280621, sample no:01,01). All plant materials were dried in the shade at room temperature, protected from light, and stored in special cabinets.

The vehicle fixed oils were supplied by Nativital Doğal Sağlık Ürünleri, İstanbul/Başakşehir, and OO, NSO, SO, and HAO. Cas numbers are as follows: 8001-25-0, 90064-32-7, 8008-74-0, 8001-21-6.

3.1.1 Chemicals and Equipments List

Chemicals and Solvents

Material	Company
Acetic acid	Riedel de Haen; 30990
DMSO	Merck 67-68-5
Ethyl acetate	Merck Art. 864
Calcium chloride	Sigma-Aldrich 1043-52-4
Chloroform	Lab Scan
Methanol	Merck 67-56-1
N-(methoxy succinyl)-ala-ala-pro-val-4-nitroanilide (MAAPVN)	Sigma-Aldrich 70967-90-7
N-[3-(2-Furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA)	Sigma-Aldrich 78832-65-2
N-hexane	Merck Art. 4368
Oleorepin standard	Sigma-Aldrich 32619-42-4
p-Dimethylaminobenzaldehyde	Sigma-Aldrich 100-10-7
Sodium borate	Sigma-Aldrich 1303-96-4
Sodium hydroxide	Merck 1310-73-2

Sodium hyaluronate	Sigma-Aldrich B 1567
Tymoquinone standard	Sigma-Aldrich 490-91-5
Gill's Hematoxylin N.3,	Biooptica-05-M06015
Eosin-Y	Biooptica- 05-10003/L
Paraffin Histoplus	Thermo, 6774060

Equipments

Flat Slide	İsolab- I.075.05.004.050
Polylysine slide	Thermo Menzel Glaiser- 2800AMNZ
Microtome Blade	Thermo Mikrotome Bıçağı- 3053835
Lamella	Thermo lamel, 24X50 MM, 3.M-24501
Lid Paraffin Tray	Isolab- 07403001
Mounting Medium	Bioptica 05-BMHM508
U.V	Shimadzu UV-1280-VIS
LC-MS	Agilent, 1290 Infinity (LC)-6430 Tripquadropol (MS)
LC-MS C18 Colon	Zorbax SB-C18, HT 2.1x50 mm, 1.8 µm
HPLC (Balıkesir University)	Agilent Technologies 1260 Infinity
HPLC Colon	Inertsil ODS-4 5µm (4.6x250 mm (UP))
HPLC (Ege University)	Agilent 1100 Series
HPLC Colon	Agilent Zorbax C18(5mm* x4.6 x150mm)
Microscope	Leica CTR 6000
GC-FID (Anadolu University)	Agilent 7890B and Flame ionization detector
GC-MS (Anadolu University)	Agilent 7890B GC

3.2. Preparation of the Oleates

Four types of *H. perforatum* oleates in different fixed oils were prepared. All oleates were prepared following the traditional “Sun-maceration technique” described as follows: First, 125 g of the fresh flowering aerial parts of *H. perforatum* was macerated either in 500 g OO, 500 g SO, 500 g NSO, or 500 g HAO. In traditional folk medicine, people said that for the maceration process of *H. perforatum*, the drug should be exposed to the heat of the sun and the coolness of night. Therefore, the macerates were kept under the sunlight for 40 days and stored in a cool place overnight, and their shadows did not touch each other under the sunlight. Then the mixture was filtered to remove the solid residues to obtain oily extracts. Prepared oleates were stored in dark amber glass well-closed containers, protected from light. The oleates were kept at room temperature (15 °C to 25 °C).

3.3. Analysis of Oleates and Fixed Oils

3.3.1. Quantitative Analysis of Fixed Oils

Before analyzing the fixed oils, it was prepared an acceptance criteria (specifications) chart for base oils: OO, NSO, SO, and HAO, according to the European pharmacopeia and related standardization documents.

Fatty acid contents of the fixed oils were measured by GC-MS. The oil samples are derived with BF₃ (Boron trifluoride) according to the Transmethylation Method using BF₃. The samples converted into methyl esters of fatty acids found in the oil are injected into the system as 1 mL with a 40:1 split ratio has been done. The oils were analyzed by GC-MS using an Agilent 7890B GC 5977B- Mass Selective Detector System. Agilent HP-Innowax FSC column (60 m × 0.25 mm id with 0.25 µm film thickness, Agilent, USA) applied with helium as transporter gas (0.7 ml/min). GC oven condition was hold at 60 °C for 10 min and set up to 220 °C at a rate of 4 °C/min, then maintained stable at 220 °C for 10 min, then set up to 240 °C at a rate of 1 °C/min. Alkanes were managed as reference points in the calculation of relative retention indices (RRI). The Injector temperature was 250 °C. MS were taken at 70 eV. Mass range was 35–450 m/z. A library

search was carried out using the Wiley GC–MS Library and the TBAM Library of Essential Oil Constituents. Relative percentage amounts were calculated from TIC (Total Ion Chromatogram) by the computer (181,182).

The GC-FID analysis was carried out using an Agilent 7890 B GC system. The injector temperature was 250 °C, and the FID detector temperature was 250 °C. In order to obtain the same elution order with GC/MS, simultaneous injection was done by using the same column and appropriate operational conditions (181,182).

3.3.1.1. Olive (*Olea europaea*) Fixed Oil

In the official sources, European Pharmacopoeia used for prepare OO specifications, OO specifications are indicated as below (183–185);

Table 2. Specifications for olive (*Olea europaea*) fixed oil analysis

PROPERTIES	SPECIFICATIONS (183)
Appearance	Clear, transparent, yellow or greenish-yellow, liquid
Odour	Characteristic Odour
Acid Value (mgKOH/g)	Less than 2
Specific Gravity (g/mL)	0.913
Unsaponifiable Matter (%)	Less than 1.5
Peroxide Value (meq O ₂ /kg)	Less than 20
Solubility	Practically insoluble in ethanol (96 %), miscible with light petroleum (bp: 50-70 °C)
Oleorepin for Extra Virgin Olive Oil	No Value

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS
Aerobic Mesophilic Bacterial Count (184)	$\leq 1000\text{cfu/g}$	Eur.Ph. <2.6.12>
Yeast and Mould (184)	$\leq 100\text{cfu/g}$	Eur.Ph. <2.6.12>
<i>E. coli</i> (185)	Negative	Eur.Ph. <2.6.13>
<i>Salmonella</i> (185)	Negative	Eur.Ph. <2.6.13>

FATTY ACID	C-CHAIN	SPECIFICATIONS (%) (183)
Palmitic Acid	C16:0	7.50 – 20.00
Stearic Acid	C18:0	0.50 – 5.00
Oleic Acid	C18:1 (n-9)	56.00 – 85.00
Linoleic Acid	C18:2 (n-6)	3.50 – 20.00

Determination of Oleuropein

Sample preparation

5g of the oil sample was weighed into a falcon tube. 15 mL of MeOH was added to it and mixed in a vortex at 2000 rpm for 2 minutes. Afterward, the sample was subjected to a sound wave in an ultrasonic bath for 1 min. The sample was again subjected to the same conditions by vortexing. After the processes were finished, the sample was centrifuged at 5000 rpm for 5 minutes. After separating the supernatant, the same procedures were applied to the remaining OO sample 2 more times, and the obtained supernatants were combined. The methanol was removed by keeping the supernatant sample on the evaporator. After the remaining solid part was dissolved with 2.5 mL of methanol, it was passed through a 0.45 μm filter and prepared for HPLC analysis (186).

Methods

A previously developed and validated method was used during the analysis. Agilent Technologies 1260 Infinity was used as HPLC, and Inertsil ODS-4 5 μ m (4.6x250 mm (UP) was used as HPLC colon. It was carried out for 10 minutes at a flow rate of 1 mL/min, using the mobile phase composition: (A) 1% acetic acid/acetonitrile; (B) methanol (1/1) and flow chart set %55 A and %45 B ratios as mobile phase was used. The isocratic elution method was used. The capture time of the oleuropein standard was determined as 5.95 $\pm$ 0.1 minutes, and detection of the sample was done at 280 nm (186).

3.3.1.2. Sesame (*Sesamum indicum*) Seed Fixed Oil

SO, specifications indicated in the table below (184,185,187);

Table 3. Specifications for sesame (*Sesamum indicum*) seed fixed oil analysis

PROPERTIES	SPECIFICATIONS (187)
Appearance	Characteristic
Odour	Characteristic bland odour
Refractive Index	1.465 - 1.469
Relative density (g/mL)	0.915 - 0.924
Unsaponifiable Matter (g/kg)	Less than 20
Peroxide Value (meq O ₂ /kg)	Less than 15
Iodine Value (g I ₂ /100g)	104 - 120
Acid Value (mgKOH/g)	Less than 4.0
Sesamin	No value
Sesamol	No value

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS
Aerobic Mesophilic Bacterial Count (184)	≤ 1000 cfu/g	Eur.Ph. <2.6.12>
Yeast and Mould (184)	≤ 100 cfu/g	Eur.Ph. <2.6.12>
<i>E. coli</i> (185)	Negative	Eur.Ph. <2.6.13>
<i>Salmonella</i> (185)	Negative	Eur.Ph. <2.6.13>

FATTY ACID	C-CHAIN	SPECIFICATIONS (%) (187)
Palmitic Acid	C16:0	7.90 – 12.00
Stearic Acid	C18:0	4.50 – 6.70
Oleic Acid	C18:1 (n-9)	34.40 – 45.50
Linoleic Acid	C18:2 (n-6)	36.90 – 47.90

Determination of Lignan Content in SO

Device and materials: Spectrophotometer, Libra

Chemical Substances: Hexane UV, Chloroform UV

Solutions: Hexane: Chloroform (7:3, v/v)

Conducting the Analysis: According to the method described in Bhatnagar et al., oil samples (0.01 g) were dissolved in 10 ml of hexane: chloroform mixture (7:3, v/v) and read absorbance at 288 nm (188)

The spectrophotometer is reset with the solvent mixture.

Calculations: Lignan content was calculated using the following formula:

$$\% \text{ Lignans (as sesamol)} = [(A / W) \times (100/230.1)]$$

$$\% \text{ Lignans (as sesamin)} = [(A / W) \times (100/231.1)]$$

A: Absorbance of the sample

W: Weight of sample in gram /100 ml.

231.1 E^{1%}_{1cm} for sesamol

231.1 E^{1%}_{1cm} for sesamin

3.3.1.3. Black Seed (*Nigella sativa*) Fixed Oil Analysis

NSO specifications are indicated in the table below (184,185,189);

Table 4. Specifications for Black Seed (*Nigella sativa*) Fixed Oil Analysis

PROPERTIES	SPECIFICATIONS (189)
Appearance	Characteristic color
Odour	The characteristic odour
Refractive Index	1.4665 - 1.4695
Specific Gravity (g/mL)	0.917 - 0.9200
Acid Value (mgKOH/g)	Less than 6
Thymoquinone Content	No value

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS
Aerobic Mesophilic Bacterial Count(184)	≤1000cfu/g	Eur.Ph. <2.6.12>
Yeast and Mould(184)	≤100cfu/g	Eur.Ph. <2.6.12>
<i>E.coli</i> (185)	Negative	Eur.Ph. <2.6.13>
<i>Salmonella</i> (185)	Negative	Eur.Ph. <2.6.13>

FATTY ACID	C-CHAIN	SPECIFICATIONS (%) ⁽¹⁸⁹⁾
Palmitic Acid	C16:0	6 – 13
Stearic Acid	C18:0	2-12
Oleic Acid	C18:1 (n-9)	20 -50
Linoleic Acid	C18:2 (n-6)	35-62

3.3.1.4. Determination of Thymoquinone Content

Sample Preparation

The 0.100 g test sampled was weighed (± 0.001 g) in a 10 mL flask. It was filled up to 10 mL with hexane and vortexed. Approximately 1 mL of sample solution was transferred to the vial for analysis (190).

Methods

A previously developed and validated method was used during analysis on the HPLC system (Agilent 1100 Series) connected to a DAD detector, and the column used for the study is Agilent Zorbax C18 (5 μ m*4.6 x150 mm). The mobile phase composition water: 2-propanol: methanol (50:45:5 v/v/v) with a flow rate of 1 mL/min was used for the study. The injection volume (10 mL) and the detection of the sample were done at 254 nm. The isocratic elution method was used (190).

3.3.1.5. Sunflower (*Helianthus annuus*) Fixed Oil

HAO specifications are indicated in the table below; (184,185,187)

Table 5. Specifications for sunflower (*Helianthus annuus*) fixed oil analysis

PROPERTIES	SPECIFICATIONS (187)
Appearance	Characteristic color
Odour	Characteristic odor
Refractive Index	1.461 - 1.475
Specific Gravity (g/mL)	0.916 - 0.923

Unsaponifiable Matter (g/kg)	Less than 15
Peroxide Value (meq O ₂ /kg)	Less than 15
Iodine Value (g I ₂ /100g)	118 – 141
Acid Value (mgKOH/g)	Less than 4.0

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS
Aerobic Mesophilic Bacterial Count (184)	≤1000cfu/g	Eur.Ph. <2.6.12>
Yeast and Mould (184)	≤100cfu/g	Eur.Ph. <2.6.12>
<i>E. coli</i> (185)	Negative	Eur.Ph. <2.6.13>
<i>Salmonella</i> (185)	Negative	Eur.Ph. <2.6.13>

FATTY ACID	C-CHAIN	SPECIFICATIONS (%) (187)
Palmitic Acid	C16:0	5 – 7.60
Stearic Acid	C18:0	2.7 – 6.50
Oleic Acid	C18:1 (n-9)	14.00 – 43.00
Linoleic Acid	C18:2 (n-6)	45.4 – 74.00

3.4. Analysis of the Oleates Prepared

3.4.1. General Specifications of Hyperici Oleate and Determination of its Hypericin Content by UV Spectroscopy

Amount of Hypericin: Absorption spectrophotometry ultraviolet and visible region at least 0.005% German Monographs Commission E (191)

5 g of St. John's Wort oil and 10 g of refined corn oil are mixed. Absorption is measured using refined corn oil as a blank solution at a wavelength of 588 nm. Calculate the percentage of hypericin according to the formula below.

$$\text{Hypericin (\%)} = (\text{ABS} \times M_1 \times 4.5) / (M_2 \times 1000)$$

ABS: Sample absorption at 588 nm wavelength;

M1: St. John's Wort and corn oil total amount (g);

M2: Only the amount of St. John's Wort oil (g);

4.5: Spectroscopic factor.

System suitability: Sample absorption should be between 0.2 and 0.8. If it is over 0.8, St. John's Wort oil weighs down, and 10 g corn oil is added and analyzed as specified in the method. If the sample absorption is less than 0.2, the oil is unsuitable for the hypericin amount.

3.4.2. Determination of Hypericin, Hyperforin, and Pseudohypericin by LC-MS/MS

Extraction from oil samples was carried out with chloroform: methanol (4:6). For this purpose, 0.5 g oil sample was extracted with 4.5 mL solvent and injected into the LC-MS/MS device following the filtration process. In the analysis of the active ingredient composition of the samples, in the C18 column (Zorbax SB-C18, HT 2.1x50 mm, 1.8 μm), the column temperature was 35 °C, the mobile phase was water: methanol (A, 95:5, five mM ammonium formate, 0.01% formic acid) and methanol (B, 5mM ammonium formate, 0.01% formic acid) were used with a flow rate of 0.150mL/min. Active ingredients; pseudohypericin, hypericin, and hyperforin analysis conditions analysis conditions are given (192):

Duration (minutes)	A (%)	B (%)
0.00	95	5
0.50	95	5
2.00	80	20
10.00	50	50
20.00	10	90
40.00	10	90
41.00	50	50
42.00	80	20

3.5. Bioactivity Studies

3.5.1. *In vivo* Studies

3.5.1.1. Animals

BALBc-type mice were preferred for this experiment; YÜDETAM supplied animals, and approval was obtained from Yeditepe University Animal Experiments Local Ethics Committee and reference numbers are as follows 2022-26 and 2022-008. Experimental animals were accustomed to laboratory conditions for five days before the experiment.

3.5.1.2. *In vivo* Wound Healing Activity Model

The animals were randomly divided into three groups and placed in cages individually. One day before the experiment, the scapular areas of the animals were shaved with a shaver, and their weights were recorded. Before the experiment, animals were anesthetized by intraperitoneal injection of a combination of Xylazine (5 mg/kg) and Ketamine (80 mg/kg). After disinfection of the area where the wound was created with povidone-iodine and alcohol, four wounds were created, right and left, with the number 5 punch biopsy tool. Following the wound formation, only isotonic water was applied to the wounds of the animals in the negative control, Madecazol® for the wounds of the animals in the positive control group, and HPO, HP+SO (Sesame (*Sesamum indicum*) seed fixed oil macerate of *H. perforatum*), HP+NSO (Black seed (*Nigella sativa*))

fixed oil macerate of *H. perforatum*) , HP+HAO (Sunflower (*Helianthus annuus*) fixed oil macerate of *H. perforatum*) , OO, SO, NSO, HAO, were applied to the wounds of the animals. Next, fixed oils, oleates and reference drugs were applied so that the wound was closed entirely with the help of a spatula. Finally, bedding material was placed in the animals' cages to prevent foreign bodies from sticking to the wound area. The animals' awakening from anesthesia was followed by photographs taken on days 0, 4, 7, and 10, and the wound diameters were measured with the Image J program to evaluate the wound healing process.

Carprofen was administered subcutaneously as a pain reliever at a dose of 3 mg/kg twice a day for three days. Wound healing was monitored daily. The animals were euthanized by high-dose anesthesia after weighing them on the 10th day of the experiment. In histological examination, the angiogenesis, thick epithelial tissue, advanced type 1 collagen fibers, anti-inflammatory response, reepithelization and granulation tissue, hair follicles, and sebaceous glands were examined.

3.5.1.3. Histopathological Analysis

All the experimental tissue samples were fixed in 10% formalin for 6 hours at room temperature. After fixation, all the tissues were washed in tap water and then dehydrated in increasing ethanol concentrations as 70%, 80%, 90%, and 100%. Afterward, the clearing was performed in Xylene. Tissue samples were embedded in paraffin wax. Next, 5m thick serial sections of paraffin-embedded tissues were cut. All the sections were stained with hematoxylin and eosin staining (H&E) and then evaluated under a light microscope (Leica DM600).

Epidermal regeneration, epidermal thickness, granulation tissue, inflammatory cells, angiogenesis, fibroblast proliferation, collagen deposit, hair follicles, and sebaceous glands were evaluated under a light microscope after H&E-stained preparations. Semiquantitative evaluation of histological parameters for assessment of wound healing was evaluated using a scale: (-/+), almost no; (+), slight; (++), moderate; (+++), extensive; (-) absence.

3.5.1.4. Statistical Analysis

Quantitative data are summarized with means and standard deviations. Analysis of time-dependent repetitive measurements and group factors was performed with a mixed design analysis of variance (ANOVA). In these analyses, when the group×time

interaction effect was found to be significant, the groups were compared with the negative and positive control groups in each time period, and the results were evaluated with Bonferroni correction. In cases where the interaction effect was not significant, time effects were interpreted. 0.05 was accepted for the significance level. Statistical analyses were made with the SPSS (version 26) program.

3.5.2. *In Vitro* studies

3.5.2.1. Determination of Hyaluronidase Inhibitory Activity

The amount of N-acetylglucosamine released from sodium hyaluronate was measured according to the method described by Küpeli Akkol et al. (193–195). Bovine hyaluronidase was dissolved in 0.1M acetate buffer (pH 3.6) and mixed with different concentrations of the oils dissolved in 5% DMSO. For the control group, 5% DMSO was added instead of the extracts. After 20 minutes of incubation at 37°C, calcium chloride was added to the mixture, which was again incubated for 20 min at 37°C. Sodium hyaluronate was added and incubated for 40 min at 37 °C. After incubation, the mixture was treated with 0.4 M NaOH and 0.2 M sodium borate and then incubated for 3 min in a boiling water bath. The p-dimethylamino benzaldehyde solution was added to the reaction mixture after cooling to room temperature and was incubated at 37 °C for 20 min to develop color. The absorbance was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA).

3.5.2.2. Determination of Collagenase Inhibitory Activity

The samples were dissolved in DMSO. The sample solution and *Clostridium histolyticum* (ChC) were dissolved in 50 mM Tricine buffer and preincubated at 25 °C for 5 minutes. 2 mM N-[3-(2-Furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was prepared in the same buffer. Buffer, test sample, and enzyme were added to each well and incubated for 15 min. A substrate was added to the mixture to measure the optical density (OD) immediately decreased at 340 nm using a spectrometer (195).

3.5.2.3 Determination of Elastase Activity

The sample solution and human neutrophil elastase (HNE) enzyme were mixed in 0.1 M Tris-HCl buffer (pH 7.5) and then incubated at 25 °C for 5 min. N-Methoxysuccinyl-Ala-Ala-Pro-Val p-Nitroanilide (MAAPVN) was added to the mixture and incubated at 37 °C for 1 hour. The reaction was stopped by the addition of a soybean

trypsin inhibitor (1 mg/mL), and the optical density due to p-nitroaniline formation was measured directly at 405 nm. HNE inhibitory activity was measured similarly to CHC inhibitory activity (196). The enzyme inhibition activities were calculated according to the following formula:

Enzyme inhibition activity (%) = $(OD_{\text{Control}} - OD_{\text{Sample}}) \times 100 / OD_{\text{Control}}$ where OD_{control} and OD_{sample} represent the optical densities in the absence and presence of the sample, respectively (197).





4. RESULTS

4.1. Oil Analysis Results

4.1.1 Olive (*Olea europaea*) Fixed Oil

OO analysis is shown in the table below.

Table 6. Results of olive (*Olea europaea*) fixed oil analysis

PROPERTIES	SPECIFICATIONS	RESULTS
Appearance	Grassy green to bright yellow gold coloured liquid	CONFIRMS
Odour	Characteristic odour	CONFIRMS
Acid Value (mgKOH/g)	Less than 2	0.1
Specific Gravity (g/mL)	0.913	0.913
Unsaponifiable Matter (%)	Less than 1.5	0.2
Peroxide Value (meq O ₂ /kg)	Less than 2	0.3
Solubility	practically insoluble in ethanol (96 percent), miscible with light petroleum (bp: 50-70 °C)	CONFIRMS
Oleuropein	HPLC	155.44±10.06 µg/ ml

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS	RESULTS
Aerobic Mesophilic Bacterial Count	≤1000cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
Yeast and Mould	≤100cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
<i>E. coli</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS
<i>Salmonella</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS

FATTY ACID	C-CHAIN	SPECIFICATIONS (%)	RESULTS (%)
Palmitic Acid	C16:0	7.50 – 20.00	12.6
Stearic Acid	C18:0	0.50 – 5.00	2.9
Oleic Acid	C18:1 (n-9)	56.00 – 85.00	67.5
Linoleic Acid	C18:2 (n-6)	3.50 – 20.00	11.6

4.1.2. Black seed (*Nigella sativa*) Fixed Oil

NSO analysis is shown in the table below.

Table 7. Results of black seed (*Nigella sativa*) fixed oil analysis

PROPERTIES	SPECIFICATIONS	RESULTS
Appearance	Characteristic colour	CONFIRMS
Odour	Characteristic odour	CONFIRMS
Refractive Index	1.4665 - 1.4695	1.467
Specific Gravity (g/mL)	0.917 - 0.920	0.920
Saponification Value (mgKOH/g)	135-189	187
Acid Value (mgKOH/g)	Less than 6	5.20
Thymoquinone Content	HPLC-DAD	384. 5408±15.79 µg/g

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS	RESULTS
Aerobic Mesophilic Bacterial Count	≤1000cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
Yeast and Mould	≤100cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
<i>E. coli</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS
<i>Salmonella</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS

FATTY ACID	C-CHAIN	SPECIFICATIONS (%)	RESULTS (%)
Palmitic Acid	C16:0	6 – 13	12.5
Stearic Acid	C18:0	2 – 12	3.3
Oleic Acid	C18:1 (n-9)	20 – 50	23
Linoleic Acid	C18:2 (n-6)	35 – 62	55.6

4.1.3 Sunflower (*Helianthus annuus*) fixed oil

HAO analysis is shown in the table below.

Table 8. Results of sunflower (*Helianthus annuus*) fixed oil analysis

PROPERTIES	SPECIFICATIONS	RESULTS
Appearance	Characteristic colour	CONFIRMS
Odour	Characteristic odour	CONFIRMS
Refractive Index	1.461 - 1.475	1.465
Specific Gravity (g/mL)	0.916 - 0.923	0.922
Unsaponifiable Matter (g/kg)	Less than 15	3
Peroxide Value (meq O ₂ /kg)	Less than 15	1.5
Iodine Value (g I ₂ /100g)	118 - 141	130
Acid Value (mgKOH/g)	Less than 4.0	0.6

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS	RESULTS
Aerobic Mesophilic Bacterial Count	≤1000cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
Yeast and Mould	≤100cfu/g	Eur.Ph. <2.6.12>	CONFIRMS
<i>E. coli</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS
<i>Salmonella</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS

FATTY ACID	C-CHAIN	SPECIFICATIONS (%)	RESULTS (%)
Palmitic Acid	C16:0	5.00 – 7.60	6.10
Stearic Acid	C18:0	2.7 – 6.50	3.30
Oleic Acid	C18:1 (n-9)	14.00 – 43.00	33.20
Linoleic Acid	C18:2 (n-6)	45.4 – 74.00	55.00

4.1.4 Sesame (*Sesamum indicum*) seed fixed oil

SO analysis is shown in the table below.

Table 9. Results of sesame (*Sesamum indicum*) seed fixed oil analysis

PROPERTIES	SPECIFICATIONS	RESULTS
Appearance	Characteristic colour	CONFIRMS
Odour	Characteristic bland odour	CONFIRMS
Refractive Index	1.465 - 1.469	1.467
Specific Gravity (g/mL)	0.915 - 0.924	0.920
Unsaponifiable Matter (g/kg)	Less than 20	4
Peroxide Value (meq O ₂ /kg)	Less than 15	1.2
Iodine Value (g I ₂ /100g)	104 – 120	113
Acid Value (mgKOH/g)	Less than 4.0	0.5
Sesamin		%1.23
Sesamol		%1.22

MICROBIAL ANALYSIS	SPECIFICATIONS	STANDARDS	RESULTS
Aerobic Mesophilic Bacterial Count	$\leq 1000\text{cfu/g}$	Eur.Ph. <2.6.12>	CONFIRMS
Yeast and Mould	$\leq 100\text{cfu/g}$	Eur.Ph. <2.6.12>	CONFIRMS
<i>E. coli</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS
<i>Salmonella</i>	Negative	Eur.Ph. <2.6.13>	CONFIRMS

FATTY ACID	C-CHAIN	SPECIFICATIONS (%)	RESULTS (%)
Palmitic Acid	C16:0	7.90 – 12.00	9.80
Stearic Acid	C18:0	4.50 – 6.70	5.30
Oleic Acid	C18:1 (n-9)	34.40 – 45.50	39.00
Linoleic Acid	C18:2 (n-6)	36.90 – 47.90	43.50

4.2 Hypericin U.V Analysis Results

Hypericin U.V analysis results is shown in the table below.

Table 10. UV analysis of hypericin

Macerates	Hypericin %
HP+NSO	0.018
HPO	0.0062
HP+SO	0.0063
HP+HAO	0.011

4.3 Results of LC-MS-MS Analysis for Hypericin, Hyperforin, Pseudohypericin

Results of LC-MS-MS analysis for hypericin, hyperforin, pseudohypericin are shown in the table below.

Table 11. LC-MS analysis of hypericin, hyperforin, pseudohypericin

<i>H. perforatum</i> macerates	Pseudohypericin (µg/g)	Hypericin (µg/g)	Hyperforin (µg/g)
HPO	0.050	0.160	17.520
HP+NSO	0.120	0.687	33.460
HP+SO	0.080	0.338	17.460
HP+HAO	0.150	0.436	11.540

4.4. Results of Pharmacological Experiments

4.4.1. Results of the *In Vivo* Experiment

During the analysis of the measurement results, significant differences were found between the groups in terms of surface area measurements on the first day (baseline) (Table 13). For this reason, time-dependent group comparisons were made by calculating recovery (closure) percentages. The wounds in the upper right part of our experimental animals are defined as Right Up (RU), the wounds in the lower right part are called Right Down, (RD), the wounds in the upper left part are called Left Up (LU), the wounds in the lower left part are defined as Left Down (LD).

In our evaluation based on statistical wound closure percentages, our wound groups were grouped as RD, RU, LD, and LU when evaluated statistically;

According to the analysis results for R.U measurements, the group time interaction effect is not statistically significant; that is, the difference between the time-dependent changes (trend) in the groups is not statistically significant ($p=0.127$). Time and group effects were found to be substantial ($p<0.001$ and $p=0.001$, respectively). Since the time effect was found to be statistically significant overall (when the groups were ignored), pairwise comparisons were made to find the source of the difference. The differences between the general means (means obtained 21.3 ± 37.2 ; 79.6 ± 24 and 95.3 ± 8.6 , respectively) obtained at all time levels (day 4, day 7, and day 10) were statistically significant. It was found to be substantial.

Table 12. Group comparison of surface measurements at day 1 (baseline) at 4 wound sites

	RU 1. Day	RD 1. Day	LU 1. Day	LD 1. Day
HAO	10.68±2.29	9.04±3.82	7.99±3.24	7.78±4.39
NSO	19.98±4.24	17.92±2.82	14.41±4.57	28.8±24.24
NC	6.91±2.35	7.99±1.65	6.56±2.35	6.81±4.1
PC	9.61±5.84	10.64±3.66	13.87±4.05	13.71±4.38
H.P+HAO	10.42±2.73	12.78±3.56	11.34±4.94	10.38±4.54
HP+SO	13.56±6.91	13.03±4.41	14.29±6.31	15.59±4.97
HPO	12.15±4.01	13.74±4.15	15.95±4.24	13.02±3.31
HP+NSO	11.19±5.27	11.34±2.87	10.82±2.31	9.38±2.96
SO	16±2.33	14.88±6.47	18.22±12.24	19.81±11.66
OO	17.79±5.03	9.37±4.51	15.99±7.97	22.03±4.71
F	4.135	2.933	2.062	3.119
p	< .001	0.008	0.054	0.005

Table 13. Mixed design analysis of variance results for RU measurements

	Group×Time			Time	Group
	4. Day	7. Day	10. Day	F (p)	F (p)
HAO	20.9±23.3	73.5±30.6	93.8±6.5		
NSO	16.1±11.8	66.9±15.6	95.9±9.2		
NC	7.9±22.1	35±28	75.5±9.3		
PC	-4.6±65.1	83.6±18.2	91.3±9.8		
H.P+HAO	23±14.8	76.2±9.5	98.2±4.4	1.572	168.369
HP+SO	49.1±49.9	88.9±7.4	97.8±2.8	(0.127)	(<0.001)
HPO	51.8±33.7	99.5±1.2	100±0		(0.001)
HP+NSO	27.8±36.4	90.6±9.2	100±0		
SO	9.4±25.7	95.9±4.2	97.9±3.5		
OO	-1.3±35.4	78.6±32.3	100±0		
Total	21.3±37.2 ^a	79.6±24 ^b	95.3±8.6 ^c		

According to the results of the analysis for R.D. measurements, the group time interaction effect was found to be statistically significant; that is, the difference between the time-dependent changes (trend) in the groups was statistically significant (p=0.001).

In this case, the group means at each time level were compared with the negative and control groups. In the fourth day measurements, the mean closure rate for HP+SO (58.4 ± 20) and the mean closure rate for HPO (55±41.6) compared to the NC (-8.9±36.8) were statistically significant. A significant difference was found.

In the seventh-day measurements, the mean closure rates for HP+SO (90.2±10.9), HPO (90±13.7), and HP+NSO (91.9±9.6), and the mean closure rate for SO (88.6±12.9) compared to the NC (48.3±14.2) were statistically significant.

In the tenth-day measurements, the mean closure rate for NSO (99.3 ± 1.5), the mean closure rate for HP+HAO (97.8 ± 2.6), and the mean closure rate for HP+SO (98.4 ± 3.9), the mean closure rate for HPO (100 ± 0.0), mean closure rate for HP+NSO (100.0 ± 0) and SO (100.0 ± 0.0) for both the NC (81.2 ± 5.2) and the PC (83.6 ± 9.4). In addition, a statistically significant difference was found between the mean closure rate for OO (92.8 ± 9.9) and the NC (81.2 ± 5.2).

Table 14. Mixed design analysis of variance results for RD measurements

	Group×Time			Time	Group
	4. Day	7. Day	10. Day	F (p)	F (p)
HAO	25.9±17.7	66.1±22.3	92.8±7.6		
NSO	10.9±12	55.1±19.6	99.3±1.5 ^{*,†}		
NC	-8.9±36.8	48.3±14.2	81.2±5.2		
PC	44.2±18.3	72.2±7.6	83.6±9.4		
HP+HAO	21±12.9	74±7.8	97.8±2.6 ^{*,†}	3.418	264.608
HP+SO	58.4±20 [*]	90.2±10.9 [*]	98.4±3.9 ^{*,†}	(0,001)	(<0,001)
HPO	55±41.6 [*]	90±13.7 [*]	100±0 ^{*,†}		
HP+NSO	41.1±17.3	91.9±9.6 [*]	100±0 ^{*,†}		
SO	14.8±48.5	88.6±12.9 [*]	100±0 ^{*,†}		
OO	-9.2±26.4	76.6±17.7	92.8±9.9 [*]		
Total	26.7±34.3	76.1±19.5	94.9±8.1		

* Statistically significant difference was found with the negative control group

† A statistically significant difference was found with the positive control group

According to the analysis results for L.U. measurements, the group time interaction effect is not statistically significant; that is, the difference between the time-dependent changes (trend) in the groups is not statistically significant ($p=0.131$). However, time and group effects were found to be significant ($p<0.001$ and $p<0.001$, respectively). Since the time effect was found to be statistically significant overall (when the groups were ignored), pairwise comparisons were made to find the source of the difference. The differences between the overall means (15.6 ± 34.8 , 72.8 ± 31.3 , and 93 ± 14.2 , respectively) obtained at all time levels (day 4, day 7, and day 10) were statistically significant and were found to be significant.

Table 15. Mixed design analysis of variance results for LU measurements

	Group×Time			Time	Group	
	4. Day	7. Day	10. Day	F (p)	F (p)	F (p)
HAO	-7.1±55.6	63.6±25.8	96.6±5.7	1.514 (0.131)	196.695 (<0.001)	4.954 (<0.001)
NSO	- 24.4±17.8	39.4±42	95.7±3.1			
NC	-7±29.2	28.6±49.7	65.9±25.3			
PC	8.7±24	66±17	91.9±8.8			
H.P+HAO	25.1±29.2	79.6±9.9	93.9±6.7			
HP+SO	36.7±11.1	82.2±23.1	98.1±2.2			
HPO	33.4±39.5	99.2±2	100±0			
HP+NSO	37.2±27.9	89.8±10.8	99.2±2.1			
SO	18.1±9.7	84.4±15.3	97.3±2.7			
OO	21.5±40.2	82.9±26	87.8±25.8			
Total	15.6±34.8 ^a	72.8±31.3 ^b	93±14.2 ^c			

According to the analysis results for L.D. measurements, the group time interaction effect was found to be statistically significant; that is, the difference between the time-dependent changes (trend) in the groups was statistically significant ($p=0.004$).

In this case, the group means were compared with the NC and PC at each time level. In the fourth day measurements, the mean closure rate for HP+HAO (42.8 ± 24.2), the mean closure rate for HP+SO (62 ± 25.2), and the mean closure rate for HPO (56.1 ± 13),3) and the NC (-20 ± 34.6) statistically significant difference was found.

In the seventh day measurements, the mean closure rate for HP+HAO (90.1 ± 5.7), the mean closure rate for HP+SO (82 ± 19.8), and the mean closure rate for HPO (91 ± 8.7) and HP+NSO (82.6 ± 9.9), a statistically significant difference was found compared to the NC (-0.8 ± 100.1).

At the tenth-day measurements, a statistically significant difference was found between the mean closure rate calculated for all groups (92.8 ± 9.9) and compared to the NC. (61.7 ± 18.6).

Table 16. Mixed Design Analysis of Variance Results for LD Measurements

	Group×Time			Time	Group
	4. Day	7. Day	10. Day	F (p)	F (p)
HAO	3.7±18.2	55.5±38.8	88.4±18.1*		
NSO	-9.6±23.8	17.6±39.9	92.2±5.7*		
NC	-20±34.6	0.8±100.1	61.7±18.6		
PC	36.4±39.6	63.9±15.6	88.6±8.7*		
H.P+HAO	42.8±24.2*	90.1±5.7*	98.8±1.9*	2.498	131.47
HP+SO	62±25.2*	82±19.8*	96.9±3.8*	(0.004)	(<0.001)
HPO	56.1±13.3*	91±8.7*	99.5±1.2*		(<0.001)
HP+NSO	20.3±33.6	82.6±9.9*	93.1±8.7*		
SO	3.8±30.8	79.5±13.2	99.6±0.8*		
OO	25.6±20.9	63.6±28.8	90.9±7*		
Total	23.8±36.4	64.3±44.8	91.4±13.4		

* Statistically significant difference was found with the negative control group

† Statistically significant difference was found with the positive control group

4.4.2. Histopathological Assessment

In the negative control group (NC), skin samples exhibited an increased collagen deposit, granulation with highly inflamed cells, and minimal epidermal regeneration. The newly formed epithelium was much thicker than normal. Group 1 showed fewer capillaries than the other groups. Skin appendages (hair follicles with sebaceous glands) regeneration was not observed in group 1 skin sections.

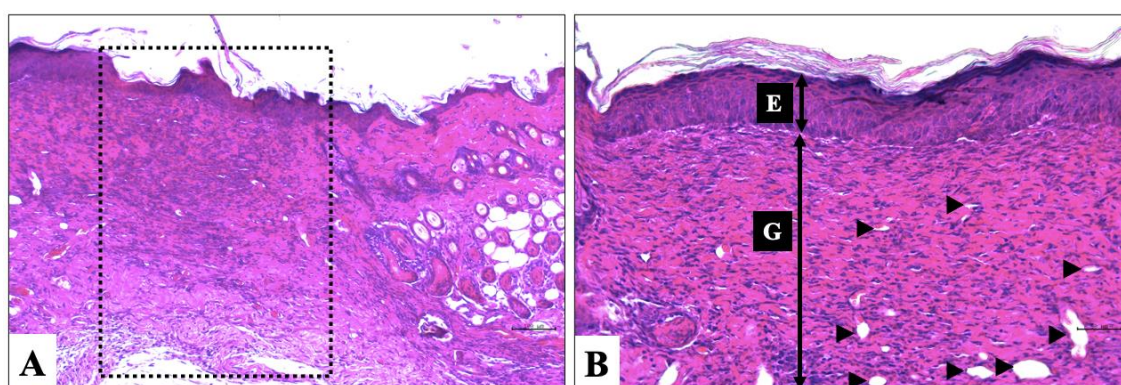


Figure 10. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 1 rat skin at post-injury day 10.

Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel.

In the positive control group (PC), re-epithelialization, generation, and deposition of collagen were observed. The regenerated skin tissue showed wound healing with no thickness of the epithelium, almost no granulation tissue, and inflammatory cells. Additionally, hair follicles with sebaceous glands in the regenerated area were observed. After the regeneration period, the skin gained normal thickness and morphology.

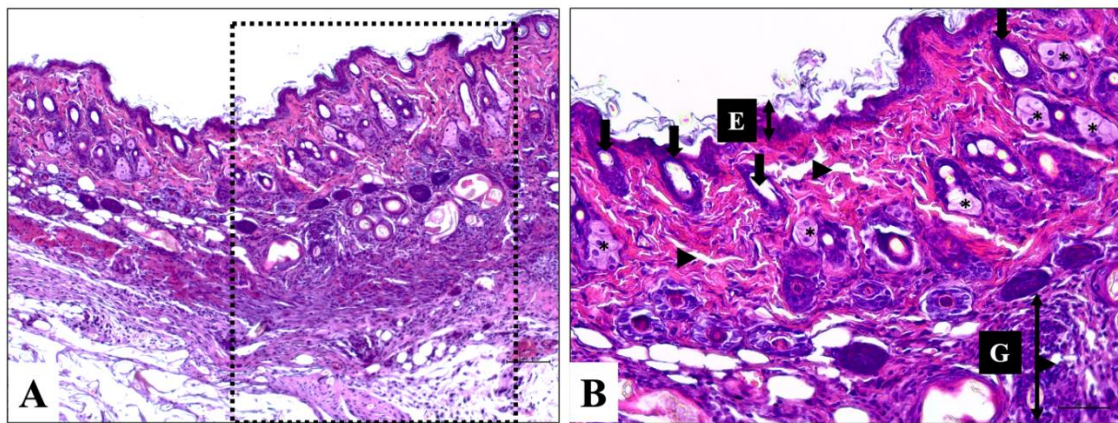


Figure 11. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 2 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel, thick arrow: hair follicle.

In the HPO group, good epithelial regeneration, increased granulation with inflammatory cells, increased fibroblast proliferation, and sufficient collagen deposition was observed during wound healing. Slight angiogenesis was observed, and slight skin appendages were detected on group 3 skin sections.

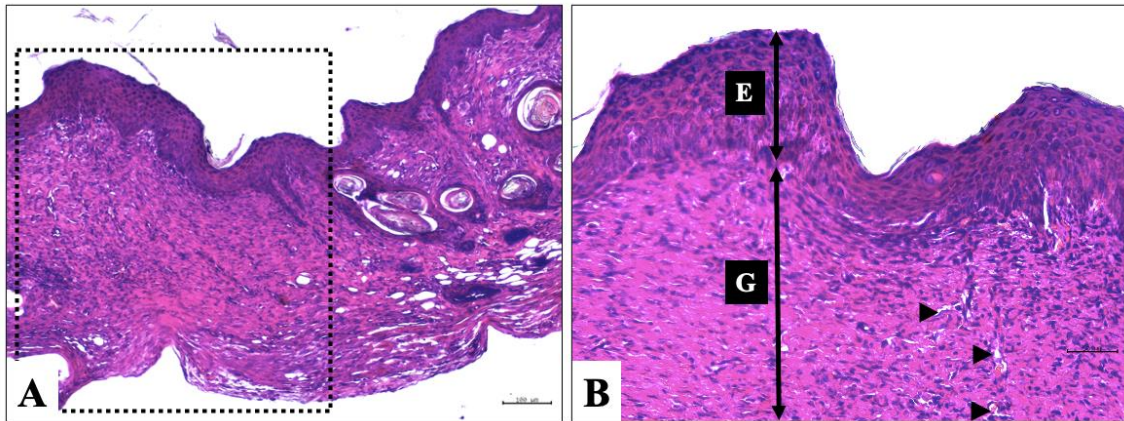


Figure 12. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 3 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel.

In HP+SO, re-epithelialization with slight epithelial thickness, moderate granulation in connective tissue with collagen deposit, and fibroblast proliferation was observed. The capillaries were more numerous in group 4 than in group 3. In addition, hair follicles were detected, but sebaceous glands were not observed in group 4 samples.

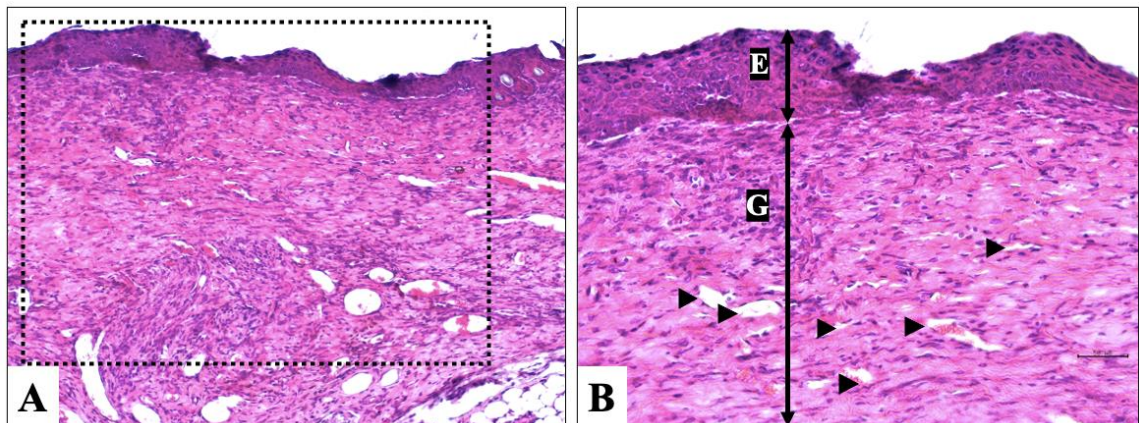


Figure 13. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 4 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel.

In HP+NSO, re-epithelialization with almost no epithelial thickness, slight granulation in connective tissue, normal collagen deposit, and moderate fibroblast proliferation was observed. After regeneration, the skin gained normal thickness, and angiogenesis was also normal. In addition, few hair follicles but no sebaceous gland were detected in group 5 skin sections.

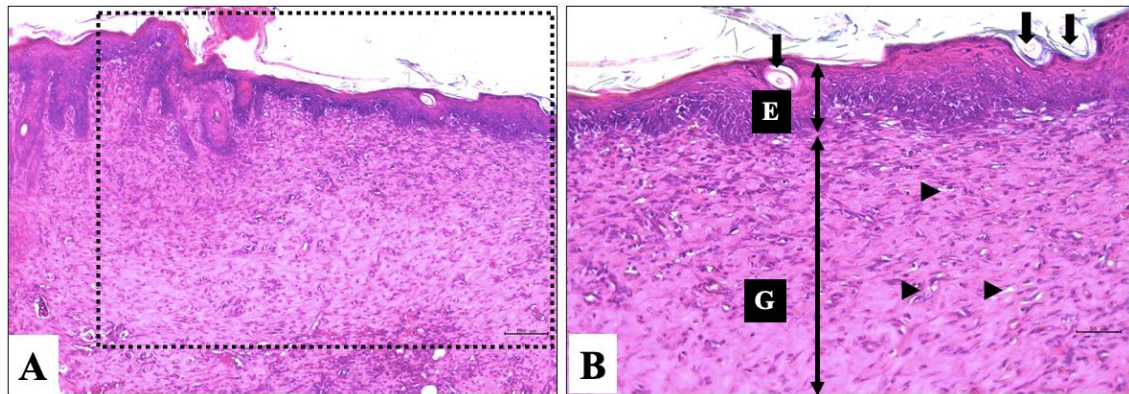


Figure 14. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 5 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel, thick arrow: hair follicle.

In HP+HAO, epithelial regeneration with moderate epithelial thickness, slight granulation, inflammatory cells in connective tissue, increased collagen deposit, and low fibroblast proliferation were observed. In addition, hair follicles and sebaceous glands were observed on skin samples in group 6.

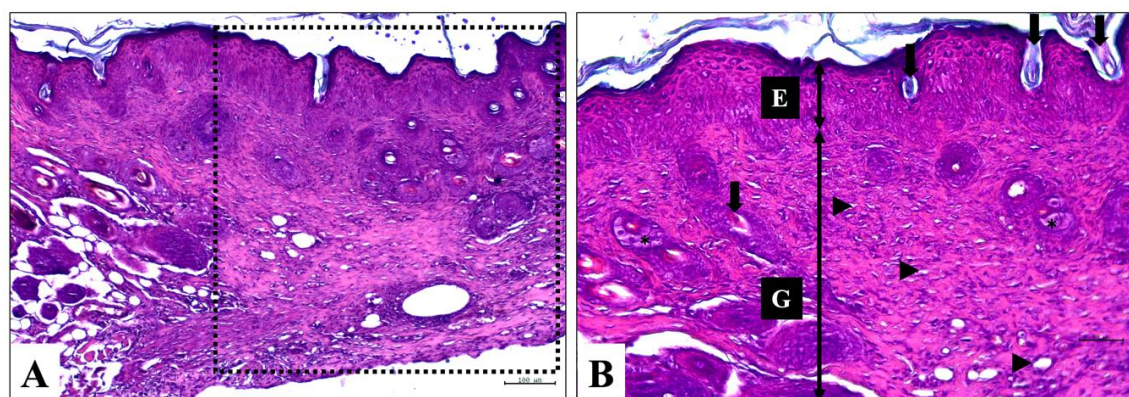


Figure 15. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 6 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel, thick arrow: hair follicle.

In the OO group, epithelial regeneration with almost no epithelial thickness, moderate granulation and inflammatory cells in connective tissue, and increased fibroblast proliferation and collagen deposit were observed. However, hair follicles or sebaceous glands were not observed.

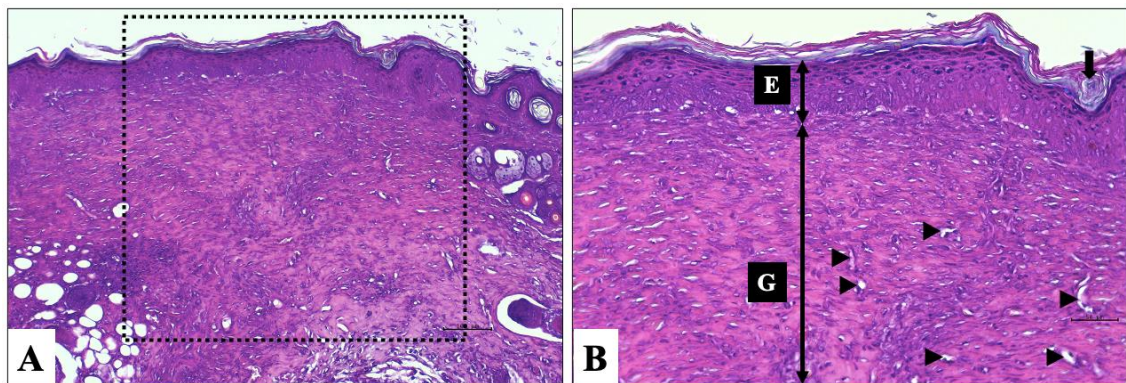


Figure 16. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining showing histological changes after the wound-healing process in group 7 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel, thick arrow: hair follicle.

In the SO group, epithelial regeneration without epithelial thickening, increased granulation and inflammatory cells in the connective tissue, and mild angiogenesis with increased proliferation of fibroblasts and collagen deposition was observed. In group 8, hair follicles or sebaceous glands in skin sections were not observed (Figure 8).

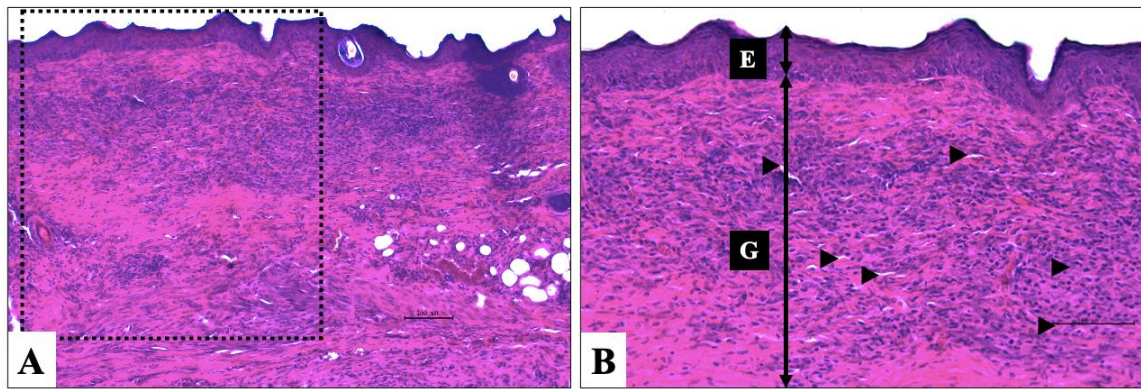


Figure 17. Histological aspects of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) show histological changes after the wound healing process in the skin of mice in group 8 on day 10 post-injury. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel.

In the NSO group, epithelial regeneration with moderate epithelial thickness, moderate granulation, and a low number of inflammatory cells in connective tissue, moderate angiogenesis, slight fibroblast proliferation, and collagen deposit was observed. In addition, many hair follicles with a low number of sebaceous glands were observed.

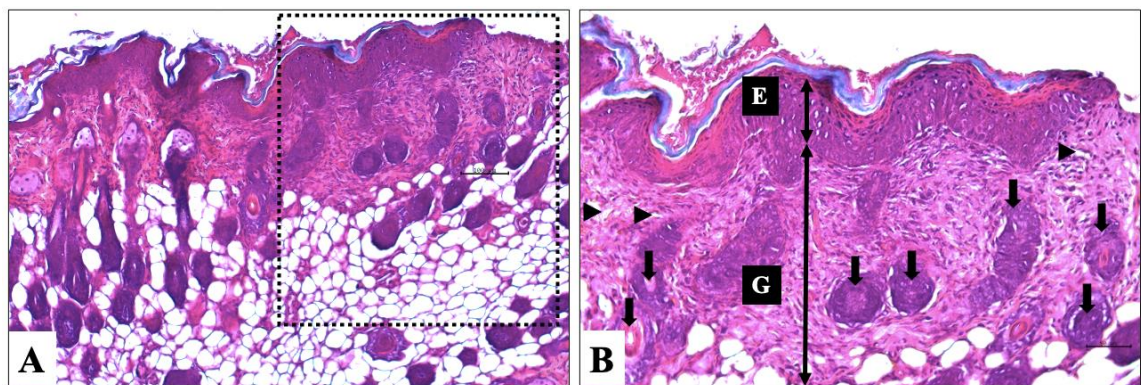


Figure 18. Histological features of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) staining show histological changes after wound-healing in group 9 rat skin at post-injury day 10. Scale bars are 100 μm (A) and 50 μm (B). E: Epithelium, G: Granulation tissue, arrowhead: vessel, thick arrow: hair follicle.

In the HAO group, epithelial regeneration with moderate epithelial thickness, slight granulation, inflammatory cells in connective tissue, increased collagen deposit,

and low fibroblast proliferation were observed. In addition, low hair follicles and sebaceous glands were detected on skin sections in group 10 (Figure 10).

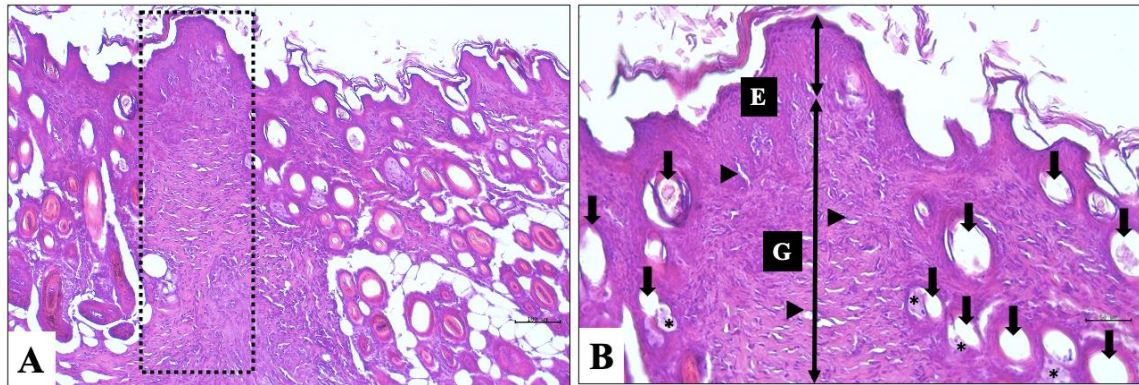


Figure 19. Histological aspects of wound healing in rats

Images of skin tissue sections stained with hematoxylin and eosin (H&E) show histological changes after the healing of cutaneous wounds of mice in group 10 on day 10 after injury. The scale bars are 100 μm (A) and 50 μm (B). E: epithelium, G: granulation tissue, arrowhead: vessel, thick arrow: hairy gland.

All skin samples were scored using a histological scoring system to assess wound healing. All skin samples from the experimental groups showed closed circulation and epithelial regeneration.

Table 17. Semiquantitative evaluation of histological parameters for assessment of wound healing

Histological parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
Epidermal regeneration	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Epidermal thickness	+++	-	++	+	+	++	-/+	-	++	++
Granulation tissue	+++	-/+	+++	++	+	+	++	+++	+	+
Inflammatory cells	+++	-/+	+++	+	++	+	+	+++	+	+
Angiogenesis	+	+++	+	+++	++	+++	++	+	++	++
Proliferation of fibroblast	+++	+	+++	++	++	++	+++	+++	+	+
Collagen deposit	+++	+	+++	++	+	+++	++	+++	+	++
Hair follicle	-	+++	+	++	+	++	-/+	-	+++	+
Sebaceous glands	-	+++	-	-	-	++	-	-	+	+

(-/+), almost no; (+), slight; (++) , moderate; (+++), extensive; (-) absence.

Group 1: Negativity control; Group 2: Positive control; Group 3: HPO; Group 4: HP+SO; Group 5: HP+NSO; Group 6: HP+HAO; Group 7: OO; Group 8: SO; Group 9: NSO; Group 10: HAO

It also compared skin tissue samples from all groups for epidermal thickness (Figure 20), granulation tissue (Figure 21), inflammatory cells (Figure 22), angiogenesis (Figure 23), the proliferation of fibroblasts (Figure 24), and collagen deposit (Figure 25).

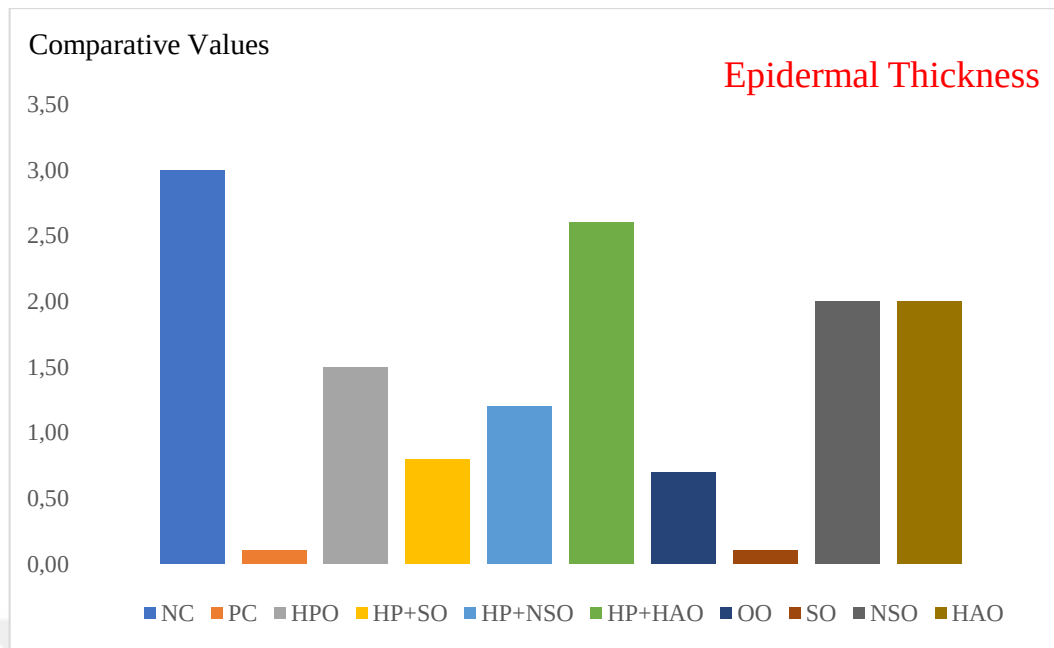


Figure 20. Comparison of skin tissue samples from all groups for epidermal thickness

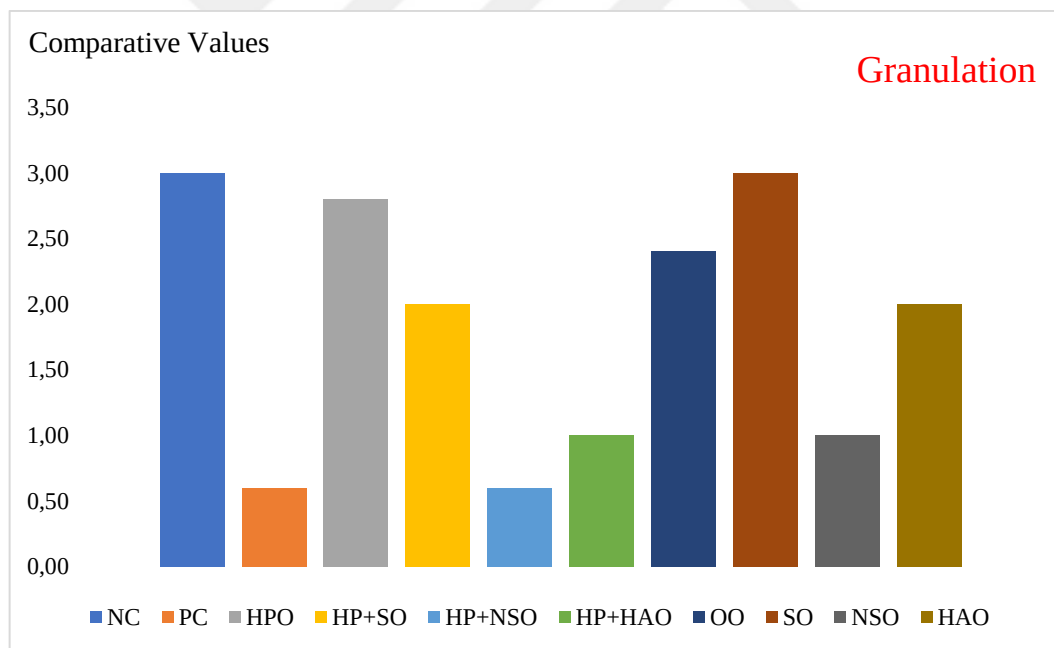


Figure 21. Comparison of skin tissue samples from all groups for granulation tissue

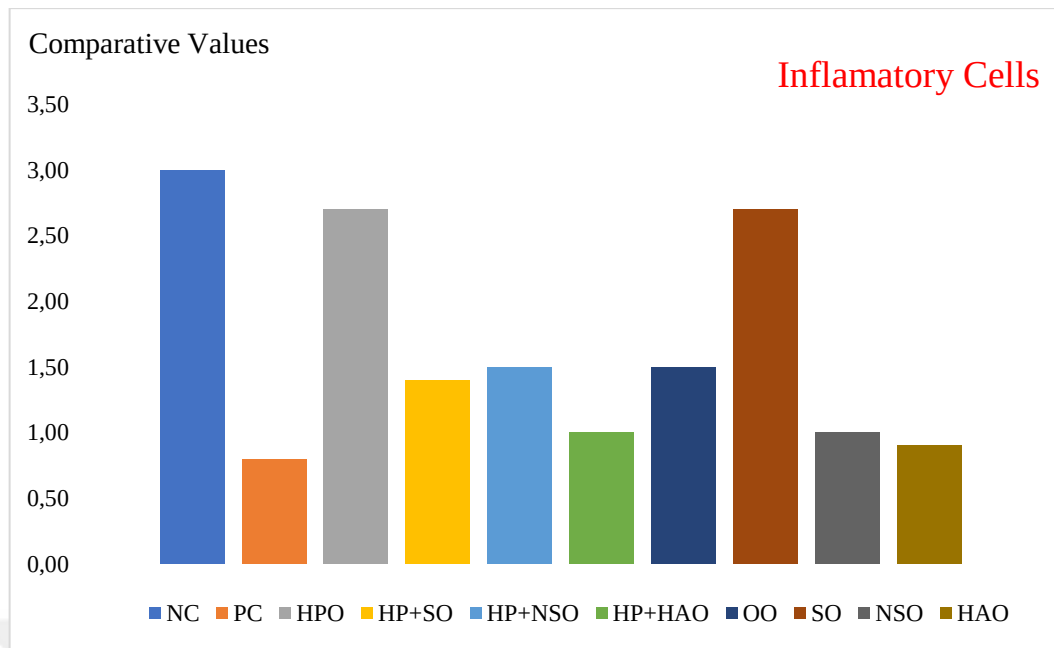


Figure 22. Comparison of skin tissue samples from all groups for inflammatory cells

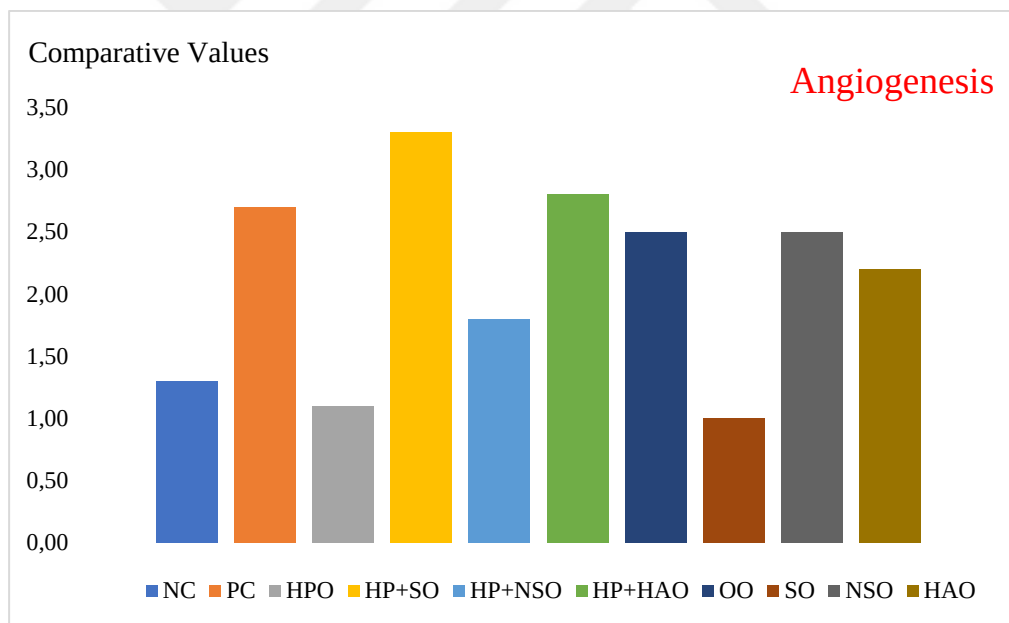


Figure 23. Comparison of skin tissue samples from all groups for angiogenesis

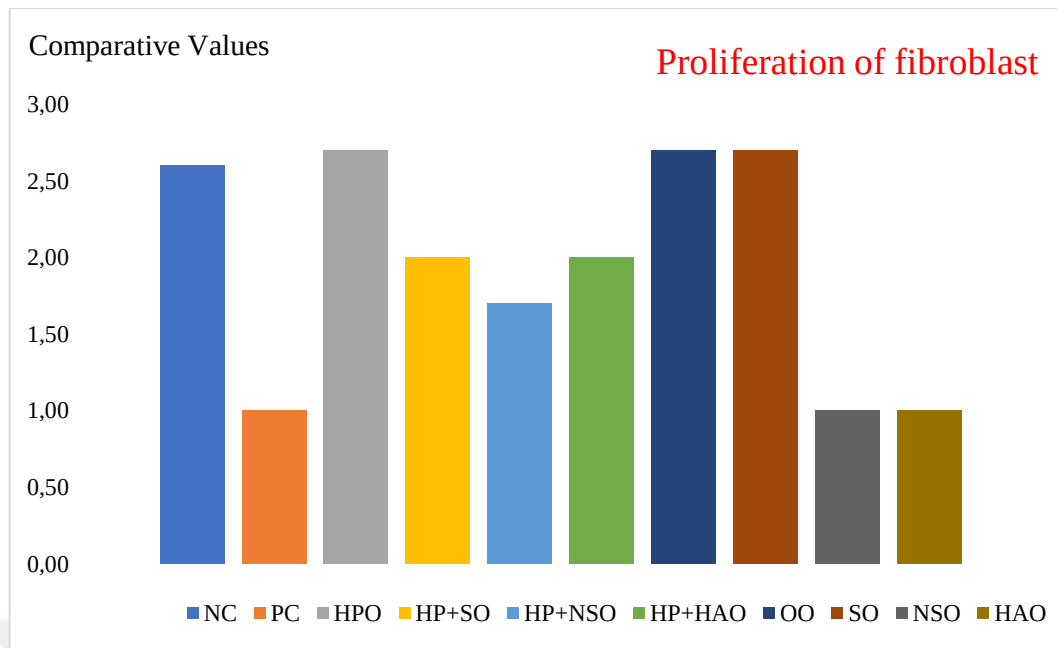


Figure 24. Comparison of skin tissue samples from all groups for the proliferation of fibroblast cells

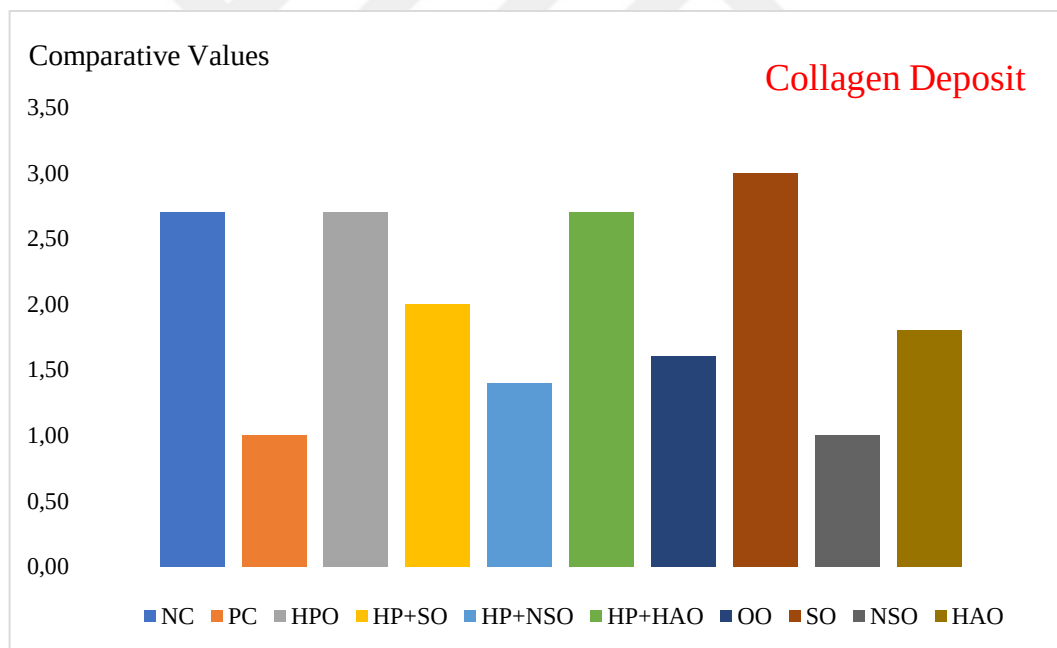


Figure 25. Comparison of skin tissue samples from all groups for collagen deposit

4.5. Results of *In vitro* Experiments

4.5.1 *In vitro* Wound Healing Activity

Table 18. Hyaluronidase enzyme inhibitory activity of the test materials

Material	Concentration ($\mu\text{g/ml}$)	Inhibition (%) $\pm$ S.E.M.
HAO	100	9.27 $\pm$ 1.09
NSO	100	7.08 $\pm$ 1.92
SO	100	14.11 $\pm$ 1.27
OO	100	15.21 $\pm$ 2.14
HP+HAO	100	21.05 $\pm$ 2.38
HP+NSO	100	19.25 $\pm$ 1.63
HP+SO	100	29.16$\pm$1.57*
HPO	100	41.33$\pm$1.87**
Tannic acid	100	82.08$\pm$1.14***

*: $p < 0.05$; **: $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

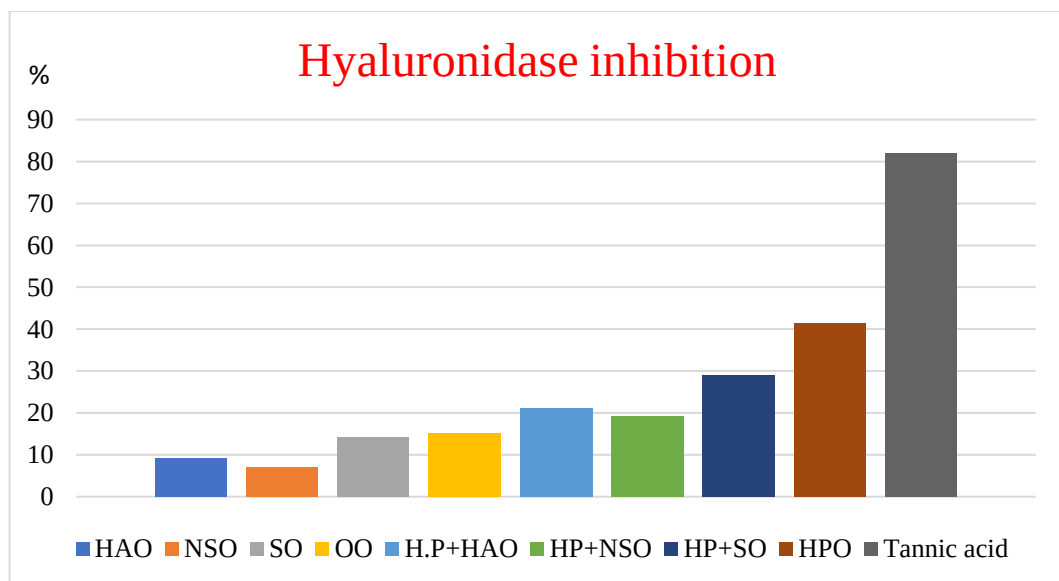


Figure 26. Comparison of hyaluronidase enzyme inhibitions of experimental groups

Table 19. Collagenase and elastase enzyme inhibitory activities of the test materials

Material	Concentration ($\mu\text{g/ml}$)	Collagenase inhibition (%) $\pm$ S.E.M.	Elastase inhibition (%) $\pm$ S.E.M.
HAO	100	7.06 $\pm$ 2.14	11.43 $\pm$ 1.70
NSO	100	9.24 $\pm$ 1.51	17.92 $\pm$ 1.84
SO	100	7.31 $\pm$ 1.62	22.08 $\pm$ 1.64
OO	100	11.78 $\pm$ 2.08	26.13$\pm$1.07*
HP+HAO	100	13.62 $\pm$ 1.57	17.05 $\pm$ 1.42
HP+NSO	100	15.26 $\pm$ 2.13	13.24 $\pm$ 1.16
HP+SO	100	13.04 $\pm$ 1.96	25.70$\pm$1.54*
HPO	100	16.13 $\pm$ 0.81	31.29$\pm$0.93**
Epigallocatechin gallate	100	38.09$\pm$0.74**	73.18$\pm$1.27***

*: $p < 0.05$; **: $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

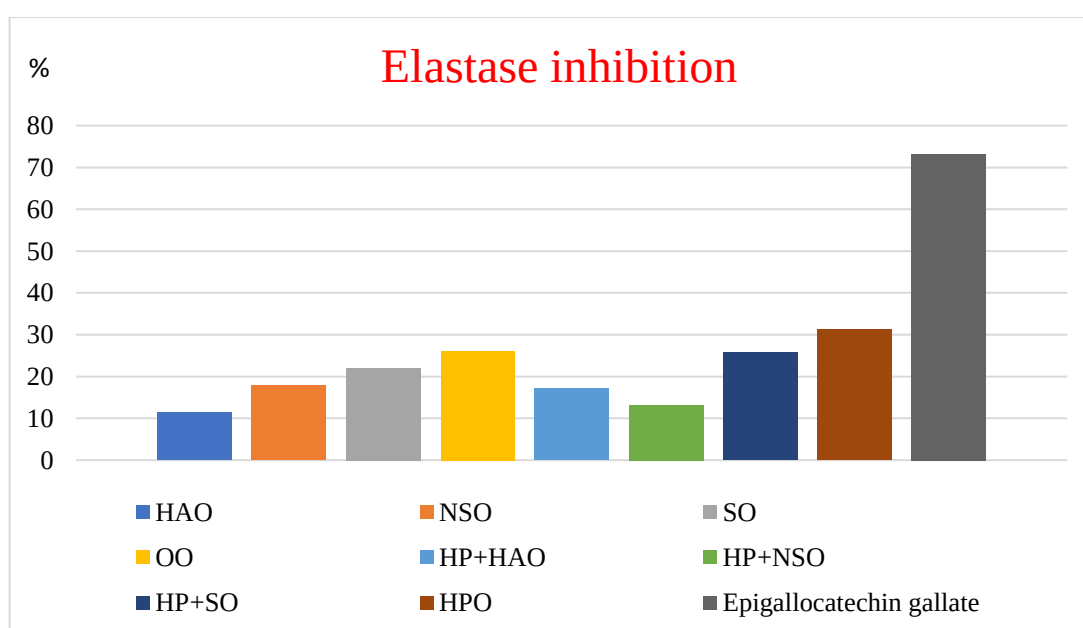


Figure 27. Comparison of elastase enzyme inhibitions of experimental groups

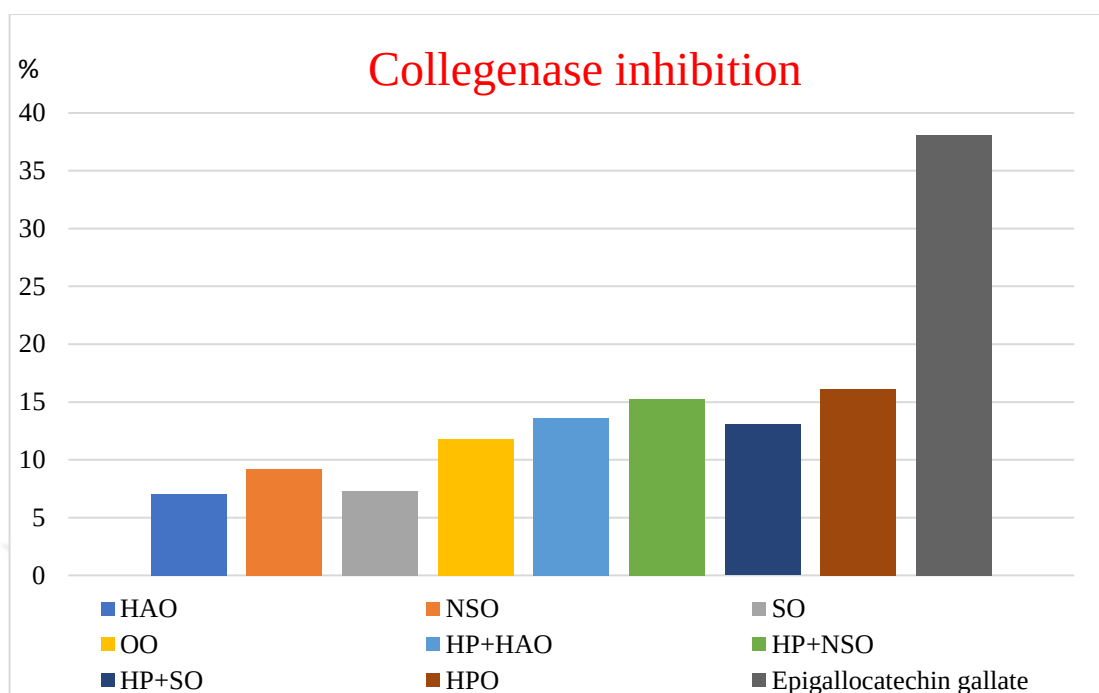


Figure 28. Comparison of collagenase enzyme inhibitions of experimental groups



5. DISCUSSION

Ancient Greek medicine has dated back to Aesculapius, who lived 1250 years ago, and continued with Hippocrates, who was born on the island of Cos/Kos in 460 BC. Temples called Asclepieia, which served as treatment centers, were built in the name of Aesculapius. The non-venomous snakes found in the temples helped the treatment by licking the eyes and wounds of the patients. Hippocrates was the first physician to apply antisepsis by using tar on wounds. Hippocrates described primary and secondary healing in wounds for the first time, used honey for cleaning dirty wounds, emphasized the need to treat wounds dry, gave information about wound healing, and reported signs of inflammation. It is known that in Homer's Epic of Ilias and Odyssey (1000 BC), surgeons took out the arrows, washed them with hot and boiling water, and cleaned them. He had mentioned military surgery and traumatology in 700 BC.

In ancient Roman medicine, Celsus (25 BC- 50 AD) left a work of eight books called "*De Re Medicina*"; in his fifth book, wound healing is mentioned, and among the drugs used for this purpose are gum, saltpeter, saffron, and sesame, and these are thought to be the first anesthetic drugs. In addition, Celsus defined the wound and suggested that newly formed wounds can be sutured after cleaning and removing foreign objects. The eighth book of the same work mentions fractures, luxation, and ruptures and describes the cardinal manifestations of inflammation.

The process of wound healing involves intricate and interconnected stages, namely inflammation, proliferation, and remodelling, which collectively address the damage to the skin. Inflammation serves as the initial response during the healing phase, acting as a protective mechanism for the tissue and aiding in the prevention of microbial infection (116). However, if the inflammatory phase is prolonged, the healing process is interrupted and might turn into chronic inflammation.

More than 400 *Hypericum* species are distributed in different geographies from Ecuador to Scandinavian countries (7). There are 107 taxa (96 natural species) and 49 endemics under 20 sections in Turkey (3). Among these species, *Hypericum perforatum* L. is the most common both in distribution and in medicinal popularity. The plant is cultivated in different parts of the world, especially in countries China, Russia, and Siberia (198). In addition, *H. perforatum* is distributed in Marmara, Black Sea, Mediterranean, Aegean, Eastern Anatolia, and partially Southeastern Anatolia regions in Turkey (8,199). *H. perforatum* is locally named as binbirdelikotu, kanotu, kılıçotu,

koyunkıran, kuzukıran, mayasilotu, püren, yaraotu and various others in Turkey. The English name of the plant is St. John's wort. It is thought to be named after this saint because of the golden-colored flowers blooming in June when John the Baptist was born.

Many clinical studies have proven the wound-healing effect of *H. perforatum*. It is thought that the activity might be due to enhancing the migration of fibroblasts migration and collagen deposition, as evidenced by increased tensile strength (126), and anti-inflammatory and antibacterial effects of hypericin, hyperforin, and pseudohypericin also contribute to the wound healing effect (200). It was reported that the lower fractions of hyperforin, whose amount changed, did not contribute positively or negatively to wound healing (152,201). Further *in vitro* evidence has demonstrated that the flavonoids (quercetin and amentoflavone), pseudo-hypericin, and hyperforin also contributed to anti-inflammatory activity. These components were demonstrated to inhibit the production of pro-inflammatory mediators such as PGE₂, TNF- α , and IL-10, but not enhance the inflammation-suppressing mediators. In addition, hyperforin was found to act as a dual inhibitor of COX-1 and 5-LOX (54).

Recent studies provide further evidence of the anti-inflammatory effect of *H. perforatum* by evaluating the inhibitory effects of the extracts and fractions on the acetic acid-induced increased vascular permeability (152). Nan Huang et al. (202) reported that pseudo-hypericin is the most crucial component in the anti-inflammatory effect. In addition, four constituents identified in the plant demonstrated that amentoflavone, chlorogenic acid, pseudohypericin, and quercetin effectively inhibited LPS-induced PGE₂ mainly with light activation and only when combined in the group of 2 or 3 chemicals if pseudo-hypericin was present. Moreover, the combinations of pseudo-hypericin with amentoflavone, quercetin, or all three substances were the most effective (202).

The *in vitro* and *in vivo* experimental studies have revealed that HPO and *H. perforatum* extracts provide the wound healing mechanism by triggering collagen production and fibroblast migration. However, it has been observed that it slows down the angiogenesis process by having an antiangiogenic effect on this process. (126,152). In a study, it has been reported that inhibition of angiogenesis by hyperforin inhibits tumor development in colon cancer (48). Furthermore, *H. perforatum* has been shown to have an anti-scarring effect on cesarean-section wounds (155). The skin barrier-repairing effect

of the HPO was also confirmed by experimental studies (45). At the same time, the effect of *H. perforatum* on scars formed after surgical cesarean sections were examined by Samadi et al. It was seen that it significantly reduced scar formation at the end of the 40th day of treatment (155) *H. perforatum* was also found to be equally effective on burns treated with sulfadiazine (45).

SO is known as a powerful fixed oil with antioxidant properties. SO exhibits a powerful antioxidant effect known to possess a wound-healing feature. Particularly sesamol was reported effective in wound healing. Sesame seed consists of 50-60% of high-quality oil rich in polyunsaturated fatty acids (PUFA) and natural antioxidants, such as sesamin, sesamolin, and tocopherol homologs. The antioxidant effects of sesame seeds have been used in wound healing, especially for burn treatments (106). Wound dressing selection is a significant factor for burn treatments, as wounds heal more rapidly in moist environments, and keeping such conditions would aid the healing process. In addition, controlling the moisture of the wound could contribute positively to wound healing. SO, which has intense moisturizing properties, is crucial for minimizing trans epidermal water loss and protecting the skin barrier. Tabatabaei et al. (203) investigated the influence of SO and calcium hydroxide on the non-surgical closure of third-degree burns in adult rats and reported that the wound closure timing had crucially reduced in patients receiving SO and calcium hydroxide. Therefore, it is vital to start using SO quickly before the maturation period is completed, in the remodelling phase to prevent scar formation in wound healing.

NSO is thought to have primary wound-healing properties due to its anti-inflammatory and antioxidant properties. Its active ingredient, thymoquinone, is believed to be successful in wound treatment due to its potent antimicrobial, anti-inflammatory, and antioxidant effects. In animal studies, NSO has proven to be dermatologically effective (204). In addition, the antioxidant and anti-inflammatory properties of NSO reduce the risk of developing chronic inflammation and hyper granulation (94,205), especially by shortening the inflammation stage from the wound stages (174).

HAO is another traditional medicine oil preferred in wound studies. The clinical research performed on horses showed that the HAO extract applied to the lumbar region provided successful results. At the end of 21 days, granulation tissue formation continued in the control group, while epidermal borders were formed in the HAO group (178).In

addition, anti-inflammatory action by HAO has been reported in a rat study where indomethacin given in HAO reduced paw edema induced by carrageenan by 79.5%. Furthermore, it was also found to enhance the levels of antioxidant defense systems in rat stomach tissues against oxidative damage (169).

Cutaneous inflammation is modulated by converting linoleic acid to arachidonic acid, a precursor to Prostaglandin E₂ (PGE₂). This polyunsaturated fatty acid (18:2n-6) is changed by desaturation and elongation of its molecule to arachidonic acid (20:4n-6), which is metabolized via the 5-LOX and COX pathways in leukotrienes (LTB₄, LTC₄, and LTD₄), prostaglandins (PGE₂, PGF₂, PGD₂, and PGI₂), and thromboxane A₂ (TXA₂) by polymorphonuclear cells. These substances have pro-inflammatory properties that can stimulate new vascularization locally, cell migration, proliferation, fibroblastic differentiation, and ECM synthesis (177). In infants with linoleic acid deficiency, the application of topical linoleic acid restored the skin barrier, as evaluated by the resolution of prior dermatitis. Therefore, it has been considered that topical application of the linoleic acid composition of HAO can strengthen the skin barrier function and reduce inflammation. Studies done so far; HAO has not been reported to cause contact dermatitis (206).

OO has been used in skincare and wound healing for many years. Oleic acid, present in olives, primarily comprises approximately 98-99% of fatty acids in the form of triacylglycerol. It also contains oleic acid esters (55-83%), palmitic acid (20-7.5%), linoleic acid (3.5%-21%), and other fatty acids like stearic acid (0.5-5%). In olives, the presence of oleic acid can replace linoleic acid, which is believed to primarily regulate inflammation and stimulate skin regeneration. Linoleic acid is thought to exert its main action through the modulation of inflammation and acts as a precursor to arachidonic acid. Arachidonic acid undergoes metabolism to form prostaglandins, prostacyclin, thromboxane, and leukotrienes, which facilitate local angiogenesis, fibroblast migration and differentiation, and extracellular matrix (ECM) regeneration. These processes collectively accelerate wound healing. Notably, the soluble components of olives, particularly phenolic acid compounds, encompass phenolic acids, phenolic alcohols (hydroxytyrosols and tyrosols), secoiridoids like oleuropein, hydroxytyrosol linked to the dialdehyde form of oleanolic acid, and flavonoids (80).

Phenolic compounds in OO have anti-inflammatory activity, and the polyphenols in OO are related to neuroprotective and antiaging effects, so they can promote the repair of epithelialized tissue that is effective in wound healing (207). Also, the squalene components in OO comprise vitamins K, D, and E, beta-carotene, and ubiquinol 10, which show antioxidant activity. Experiments have also clarified that, after being applied topically, OO causes angiogenesis by elevating VEGF levels (208) and, with omega-3 fatty acids, could prevent chronic inflammation (169). In addition, EVOO enhances cell vitality by improving the capacity of antioxidants and provides significantly higher mitochondrial membrane potential, which is crucial for protecting the mitochondrial function of keratinocytes. OO may promote wound healing by increasing epithelial regeneration.

Squalene in OO has an antioxidant and moisturizing effect, which may cure people who suffer from seborrheic dermatitis, acne, psoriasis, and atopic dermatitis. Panahi et al. studied the effect of a cream consisting of OO and Aloe vera jelly on atopic dermatitis compared to topical betamethasone application. Results presented that the recovery rate in the OO group was 64.5%, while 13.5% in the betamethasone, a considerable difference ($P < 0.001$) was detected (209).

Also, polyphenolic compounds in OO increase angiogenesis and blood flow to the wound area (210). Pure OO involves a vital substance called oleocanthal that shows an analgesic effect. The oleocanthal mechanism of action is similar to ibuprofen, which inhibits the production of prostaglandins (211) and helps relieve wound pain.

In the present study, *in vivo* and *in vitro* wound healing effects of the macerates of the *H. perforatum* in OO, SO, NSO, and HAO and each of the fixed oils that make up the macerates were comparatively investigated. Firstly, the content analysis of macerates and fixed oils was carried out by LC-MS and UV analysis. The importance of making quantitative and qualitative analyses of the products and testing their compliance with pharmacopeia can be understood from the studies mentioned below. Han et al. (212) conducted a comparative study with NSO and HPO (from Hel Kimya), and NSO was found to be more effective. However, the content analysis of these oils, their hypericin, hyperforin, and pseudohypericin ratios were not determined. Also, there is another study that used HPO and NSO (Zade Vital) in their studies (213) NSO activity and wound healing properties were observed higher than HPO, but the efficiency of serum

physiologic was superior to both oils; however, since content analysis, standardization, and compliance with pharmacopeia were not specified, it became difficult for us to refer to this study.

In this study, hypericin content was measured by referring to German Commission E Monographs. According to the German Commission E Monographs, the standard hypericin rate should at least be 0.005% (191). As a result of the measurement, in HP+HAO was found to be 0.011%, in HP+NSO was 0.018%, in HP+SO was 0.0063%, and in HPO was 0.0062%. According to these results, the hypericin content in macerates were found above the set reference value. In addition, OO, SO, NSO, and HAO fixed oils were also analyzed following the reference oil standards and found appropriate. Simultaneously, the pseudohypericin, hypericin, and hyperforin amounts of each *H. perforatum* macerate was measured by LC-MS analysis. After the analysis process was completed, *in vivo* experiments were started.

The effect of our macerates and oils on the inhibition of collagenase, elastase, and hyaluronidase enzymes, which are of great importance in wound healing, were evaluated. The granulation tissue consists of an ECM, proteoglycans, hyaluronic acid, collagen, and elastin. Collagenase inhibition is necessary to support the formation of collagen fibers. Providing apoptosis and degradation of the ECM and immature type III collagen, the ultimate formation of mature type I collagen is critical for scar formation, integrity, and strength of wounds (133). This healing stage can continue for several months or even years. If there are failures during this phase, there may be excessive wound healing leading to hypertrophic or keloidal scarring or a chronic wound resulting in persistent granulation tissue. For this reason, over-stimulation of the collagenase enzyme may cause excessive collagen destruction and a decrease in the strength of the new ECM cells and granulation tissues formed, decrease the epidermal thickness, and decrease the durability of the tissue.

The elastase enzyme, a type of protease group of enzymes, is adjacent to the essential enzymes managed in granulation-conserved ones. However, overworking the elastase enzyme may destroy the newly formed ECM results, preventing the transition of the wound from the inflammation phase to the proliferation phase, and may cause chronic inflammation. Hyaluronidase enzyme inhibition is vital for the prevention of the breakdown the hyaluronic acid, which is necessary for wound healing, and preventing

transepidermal water loss to keep the scar tissue formation minimal. Hyaluronic acid is a significant component of early granulation tissue, and its degradation products seem proactive in wound healing (214). There is also variation in the levels of hyaluronic acid between the wound healing processes in fetal and adult, with persistently higher levels of hyaluronic acid present in fetal wounds that ultimately heal without scarring. It can be thought that excessive stimulation of the hyaluronidase enzyme will cause hyaluronic acid destruction, which results in the deterioration of the moisture balance of the wound and the formation of hypergranulation and hypertrophic scarring in the remodelling phase (215,216).

Based on the results stated in Table 18, the most significant hyaluronidase inhibitory activity (HI) was observed in HPO and HP+SO. And then, the result of the remaining group, highest to lowest; HP+HAO, HP+NSO, OO, SO, HAO, NSO.

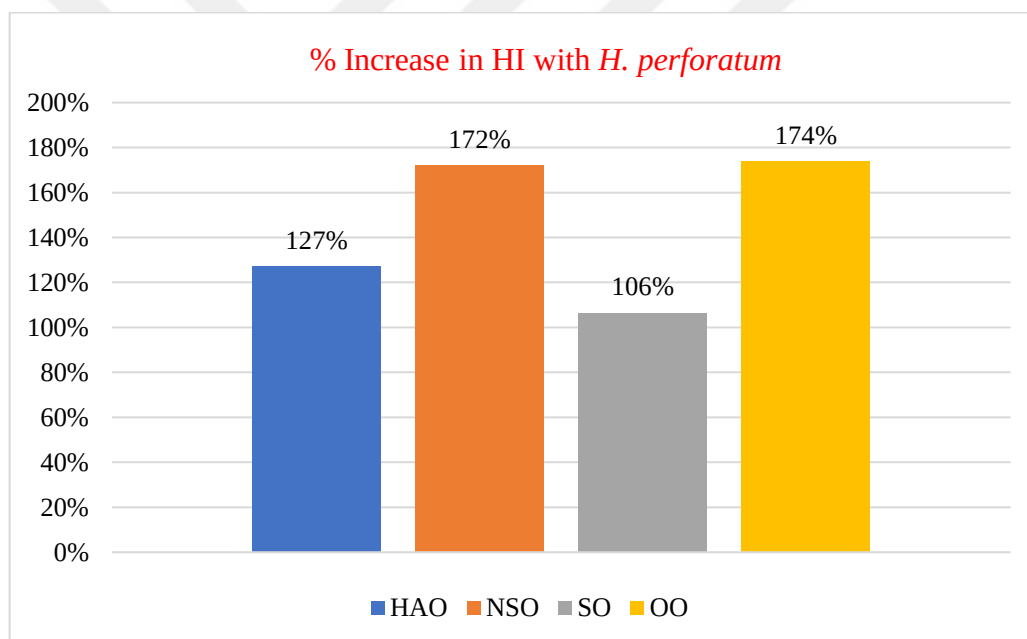


Figure 29. Changes in the hyaluronidase inhibitory effect (HI) after maceration with *H. perforatum*

According to this result, HI increased by 127% in HAO, 172% in NSO, 106% in SO, and 174% in OO after maceration with *H. perforatum*. As a result of this graphical analysis, the HI of fixed oils increased significantly when macerated with *H. perforatum*.

The elastase inhibitory activity (EI) results stated in Table 19 show that the EI of HPO, HP+SO, and OO is higher. The remaining groups' EI were as follows, highest to lowest: SO, NSO, HP+HAO, HP+NSO, and HAO.

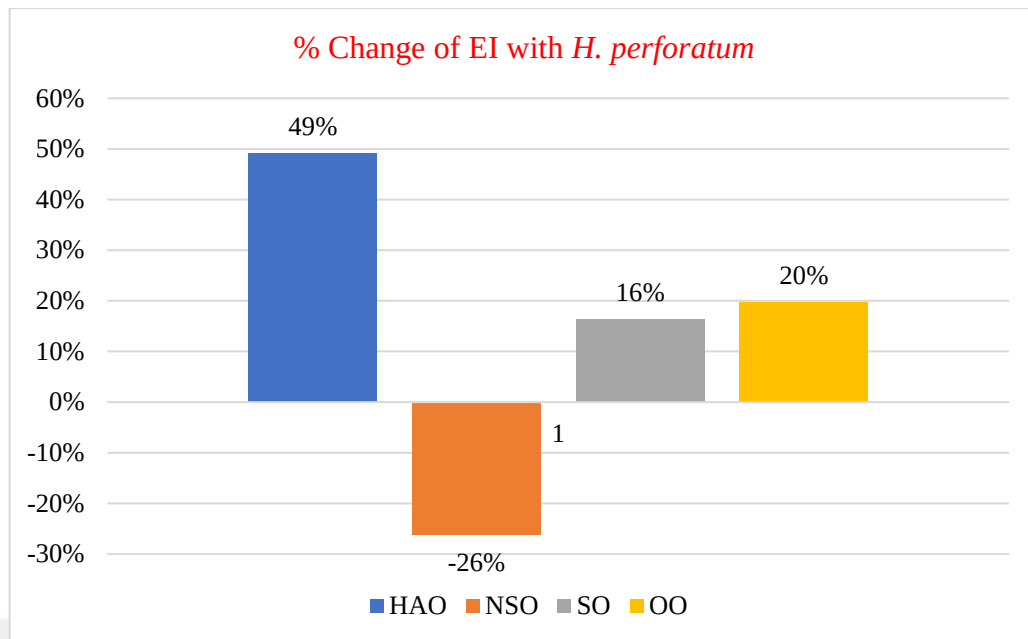


Figure 30. Changes in elastase inhibition (EI) after maceration with *H. perforatum*

When comparing the macerate forms of *H. perforatum* with the vehicle forms, the highest increase was seen in HAO by 49%. Increases of 20% and 16% were also observed in OO and SO, successively. However, in NSO, a 26% decrease in EI was observed compared to *H. perforatum* dissolved macerate. Therefore, except for the NSO experiment group, maceration with *H. perforatum*; increases the EI.

When the results of collagenase inhibitory activities were evaluated, the collagenase inhibitory activity (CI) results were not as evident and robust as the elastase and hyaluronidase results. However, it gives essential ideas regarding the comparison between the groups. The CI of the groups containing *H. perforatum* was close to each other, but the HPO group had the highest results. As indicated in Table 19, the results are in the order from highest to lowest; HP+NSO, HP+SO, HP+HAO, OO, NSO, SO, and HAO.

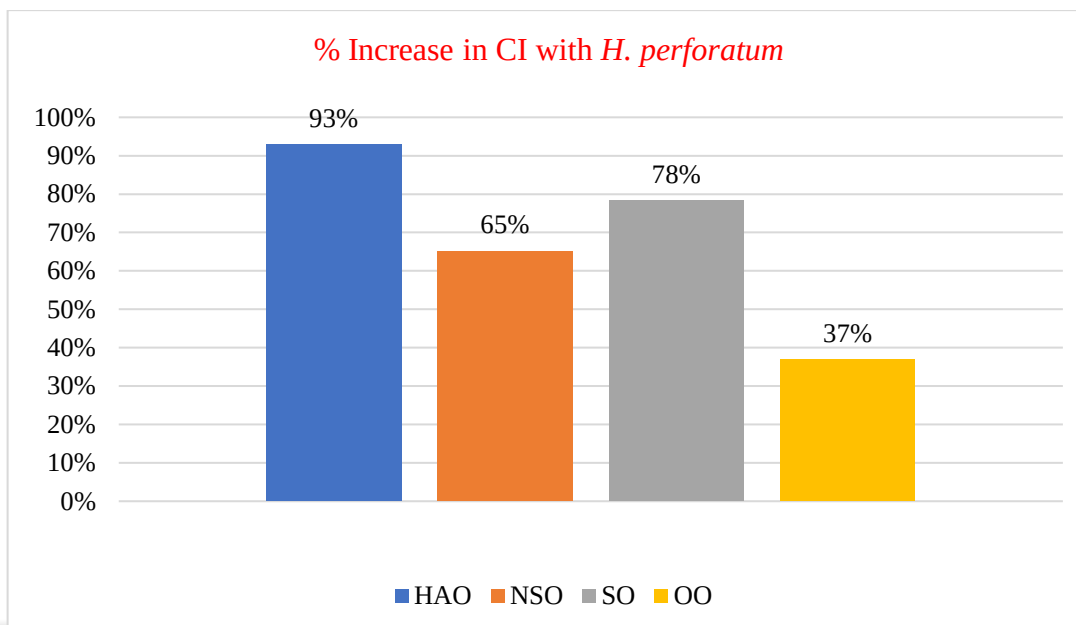


Figure 31. Changes in collagenase inhibition (CI) after maceration with *H. perforatum*

H. perforatum, whose results were examined, significantly increased the CI in each fixed oil in which it was deliquated. Relative to results, the increase of CI rates is higher in HAO, SO, and NSO macerates than in OO macerates.

After the evaluation of *in vivo* study results was completed, the histopathological results were evaluated as shown below in the graphics based on result shared Table 17;

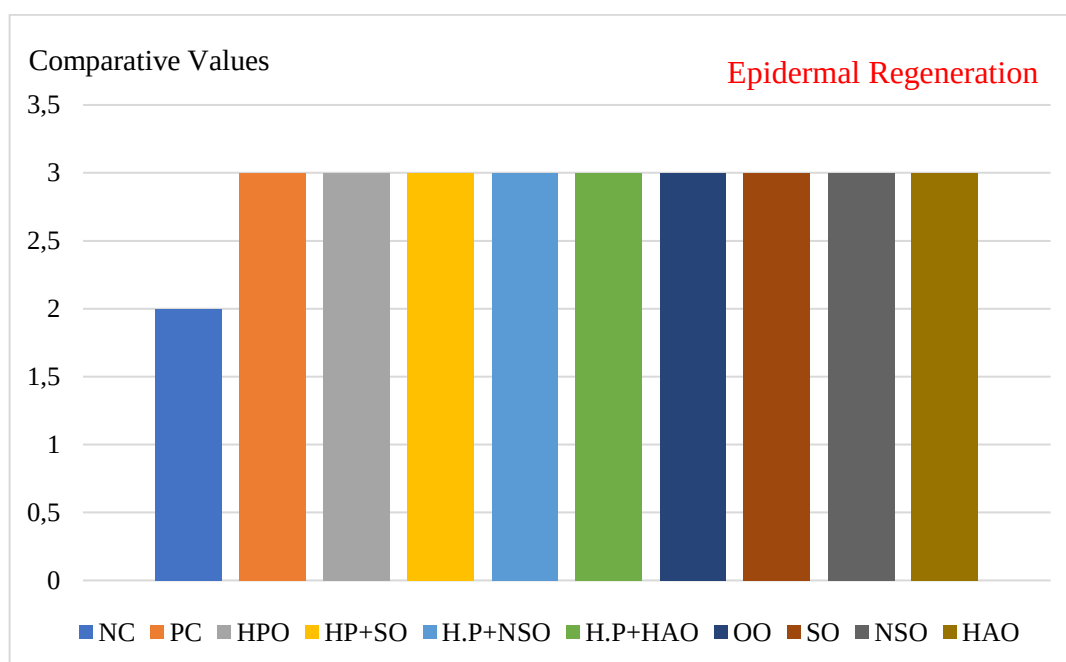


Figure 32. Comparison of epidermal regenerations of oleates and fixed oils.

Epidermal regeneration relies on an adequate balance between the proliferation and differentiation of epidermal stem cells and the removal of superficial dead cells (corneocytes) by desquamation to maintain or restore epidermal structure and functions (133). Therefore, when epidermal regenerations were compared, all experimental groups achieved a better epidermal repair than the NC. With this graph, the experimental groups achieved significant success compared to the control group, and the experiment was considered successful.

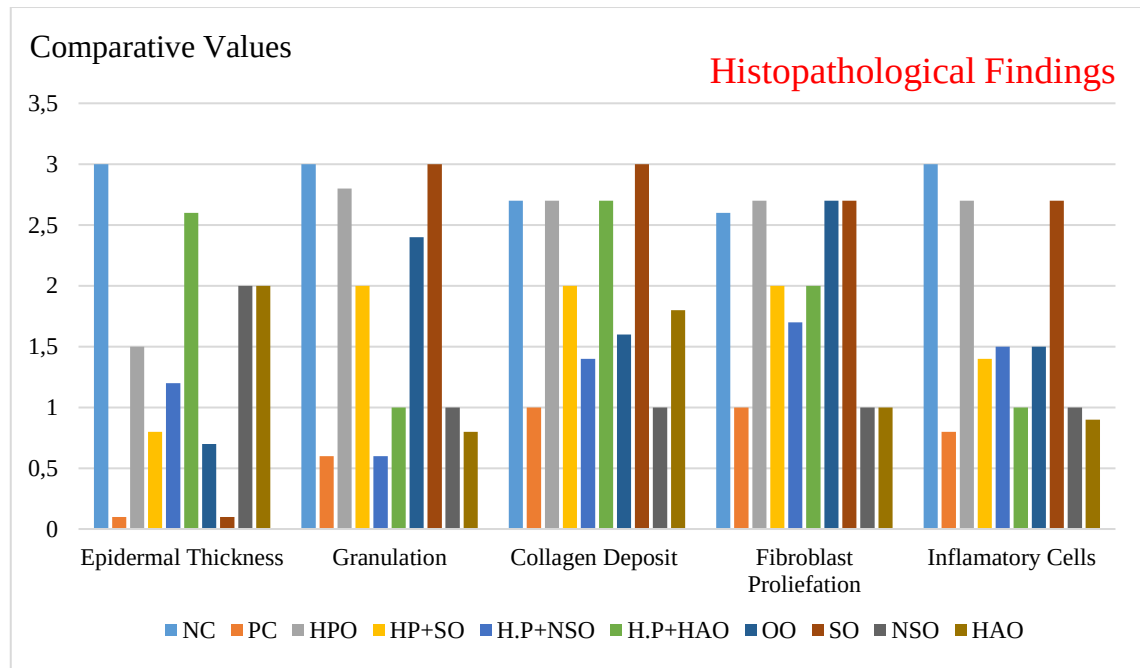


Figure 33. Comparison of histopathological findings of oleates and fixed oils.

Wound healing is a complicated process containing five phases, hemostasis, inflammation, proliferation, re-epithelialization, and remodelling. After the damage, the hemostasis phase is rapidly stimulated by the following reactions of a platelet plug and clot formation to stop bleeding. In the second step, impaired tissues and stimulated platelets trigger inflammatory responses by gathering immune cells such as neutrophils, leukocytes, and macrophages. When the inflammation is reduced, new blood vessels and connective tissues occur, and then the proliferation phase starts. In addition, the wound size also reduces due to wound contraction. The main action of proliferation is re-epithelialization, primarily achieved by fibroblast migration. After that, tissue maturation will indicate the remodeling phase, including regression of the neovasculature and restructuring of ECM (109). Disruptions to any of these cases could result in delayed wound healing to excessive scarring, comprising hypertrophic scars and keloid formation.

Excessive scar formation is specified by irregular and excessive accumulation of collagens (115).

When groups had compared each other regarding the epidermal thickness, indicated in Figure 33, the group with the highest thickness was the NC. NC epidermal thickness was thickest on the 10th day, and granulation tissue formation continued, which could be the beginning of hypergranulation and hypertrophic scar formation. PC group was the tissue sample with minor collagen formation. On examination of the PC group, almost no epidermal thickness was observed, granulation tissue formation is seen in minimal amounts, and fibroblast proliferation is at a low level. While this wound-healing model is acceptable for minor injuries (scratches, cuts, and abrasions), it is not preferred in chronic wound treatments (132). In chronic wound treatments, an average epidermal thickness is a significant finding; because a strengthened skin barrier is important in forming healthy epidermal tissue and especially in the prevention of dehiscence wounds, which is the biggest problem of chronic wound patients. However, uncontrolled collagen deposition causes scar formation, abnormal epidermal thickness, and causes weak fibrotic tissue formation, which is an undesirable situation. If the wound healing of the HPO was interpreted, the HPO group significantly increased fibroblast migration. This is one of the most significant parameters in new epithelial tissue. The histopathological examination and previous *in vivo* clinical studies (126) confirm its fibroblast proliferative effect; at the same time, when *in vivo* studies were examined, it was seen that the group which inhibited the collagenase enzyme the most was the HPO. One of the essential building blocks of granulation tissue is elastin and hyaluronic acid(148,217). HPO group had a high amount of granulation tissue as a result of the morphological examination, which is compatible with our *in vivo* EI and HI results, which are shared in Table 18 and Table 19.

The density of collagen tissue is crucial for the interpretation of wound healing parameters. Contrary to the fibrosis tissue that forms uncontrolled, healthy collagen tissue in the remodelling phase is very significant for the durability of the formed tissue. Collagen accumulation was higher in the HPO group, indicated in Figure 33, in addition to the high rate of epidermal regeneration compared to the NC, shown in Figure 32. This situation led to dense granulation tissue and fibroblast migration, and despite the intense collagen accumulation, the level of epidermal thickness remained at almost half the level of the NC. Hyaluronic acid can potentially normalize keloid fibroblast characteristic features such as hyperproliferation, growth factor production, ECM deposition, and

epidermal thickness, depending on the specific genotype of the keloid fibroblast cell line. Moreover, elastase inhibition effectively rescued angiopoietin-1 decrease and inhibited scar and keloid formation; thus, high levels of HI and EI may have contributed to the optimal level of epidermal thickness (214,218,219). As a result of these findings, while the healing parameters increase very positively, the epidermal thickness level does not increase in parallel, reducing the risk of hypertrophic scar and fibrotic tissue formation.

Comparing HPO and OO groups, shown in Figure 33, in the OO group, fibroblast proliferation and collagen accumulation are high; similar results were also observed in vivo studies shared Table 18 and Table 19, but when *H. perforatum* was macerated in OO, its contribution to the formation of granulation tissue and its effect on collagen production increased significantly. The number of inflammatory cells in the HPO group was lower when compared to the SO and NC groups, shared in Figure 33.; on the contrary, compared to other remaining oils in the group, the number of inflammatory cells is higher. Inflammation cells may not always harm the tissue, and when wounds are in the healing process, rapid suppression of inflammation may also increase the risk of scar formation (220). Furthermore, rapid reduction of leucocytes in the wound area may prevent the stimulation of a healing factor, especially since the presence of leucocytes is important in protecting the tissue from pathogens during the solid formation of the granulation tissue (221). In a study conducted by Yaşar et al. (222), the role of *H. perforatum* in the healing of perforated tympanic membranes (T.M.) was investigated, and it was found that leukocyte count, neovascularization, and fibroblast proliferation were significantly higher in the HPO group, and that fibrotic activity and collagen production increased wound healing at the perforation site. They reported that an increase in leukocyte counts enables MMPs to promote re-epithelization and remodelling and that an increase in fibroblasts promotes collagen synthesis and wound healing. Since the wounds in our experimental and control groups were mostly closed at the end of the 10th day, the histopathological samples were taken to see the cellular changes more clearly and to make more concrete comparisons with each other. On the other hand, studies were conducted for the anti-inflammatory effect of *H. perforatum* when the sample was taken after 21 days; obviously seen that inflammation cells were not observed (152). In addition, we can say that scar formation is significantly prevented in studies conducted with HPO for cesarean section scars compared with the control group at the end of 41st. While inflammation tissue is reduced significantly, inflammatory cells could continue to contribute to the prevention

of scar tissue formation at the end of the 41st day (155). How long and how much inflammatory cells (such as mast cells, macrophages, and leukocytes) should be suppressed is still debated today. Since each molecule's mechanism of action for wound healing may differ, it becomes more complex to interpret the inflammation processes (223).

The highest collagen accumulation and fibroblast stimulation were observed in SO preparation after the HPO group, shown in Figure 33. SO significantly increased collagen accumulation and proliferation of fibroblasts and supported the formation of new granulation tissue; however, it may not form any epidermal thickness; this type of wound healing model can only be supported in the treatment of acute wounds (224). Acute wounds, defined as those that have damaged the epidermis or have not reached the dermis, such as post-traumatic wounds or first-degree burns, healing in a short time (3-4 weeks) and minimal scar formation on the healing process can be evaluated positively (225). Since the wound reaches the dermis and hypodermis in the treatment of chronic wounds (wounds that have not closed at the end of 6-8 weeks), after a successful granulation tissue formation process, it is important to create an optimal level of epidermal thickness, to form a strong skin barrier, and to prevent dehiscence of the wound and fibrotic tissue formation (132).

When the morphological wound healing performance of HP+SO was evaluated, the rate of fibroblast proliferation and the amount of collagen formation were significantly close to those of the group in which the HPO group and SO were used alone, shared in Figure 33. This result coincides with the successful results of the HP+SO group obtained *in vivo* experiments shared in Tables 18 and 19. Furthermore, its suppressive effect on inflammatory cells was significantly higher than the preparation in which SO was used alone. It has a strong supporting effect on new granulation tissue. Unlike the preparation in which SO is used alone, it creates epidermal thickness and contributes more to the strength of the skin barrier.

When evaluating the NSO group, it is known that NSO has powerful antibacterial and anti-inflammatory effects (226). Based on the low number of inflammatory cells that were observed histopathologically at the end of the 10th day of the experiment, indicated in Figure 33, a high rate of the anti-inflammatory response could be mentioned. It is thought that the primary effect mechanism of NSO in wound healing is based on its

antioxidant and anti-inflammatory activity by inhibiting nitric oxide and cytokines and antibacterial activity mainly due to its thymoquinone content (174). In this study, NSO does not significantly increase collagen production and fibroblast proliferation. Also, in the experiment conducted by Ab Rahman et al. (174), any collagen-increasing effect of NSO was not observed. Sallehuddin et al.(227) investigated the efficacy of thymoquinone in petroleum jelly in an alloxan-induced diabetic wound. Through the gross morphology observation, wound contractions in the thymoquinone group were significantly greater than that in the control group on day three but significantly lower than the control group on days 7 and 14. This study suggests that the effect of thymoquinone was on the early stages of wound healing, which can prove the moderate collagen stimulation by NSO on day 10th. In addition, the NSO group contributes to the skin barrier by increasing epidermal thickness, and the angiogenic activity might contribute to this process (228).

On the other hand, HP+NSO increased collagen production and fibroblast proliferation following the *in vivo* experiment results. Although the inflammation response rate is very similar to the preparation in which NSO is used alone, a slight decrease in epidermal thickness has been observed, as stated in Figure 33. This morphological observation shows that when *H. perforatum* is macerated in NSO, it increases its collagen-stimulating and fibroblast migration-triggering properties. When all these parameters are evaluated, HP+NSO supports collagen and fibroblast production and shows a rapid anti-inflammatory response. Considering the antibacterial and antioxidant activity of NSO, HP+NSO could be positioned as an infection-control agent in the treatment of chronic wounds, especially if the side effects of silver preparations and topical antibiotics are considered (229).

HAO preparation shows that, although collagen production increased slightly, it keeps behind other groups in fibroblast production and granulation tissue formation. The suppression rate of inflammatory cells was high, as in the NSO preparation. It is known that the anti-inflammatory agents synthesized by the intense linoleic acid in HAO contribute to wound healing and the repair of the skin barrier (230). In the study by Odabaşoğlu et al. (85), the effect of HAO on the anti-inflammatory response rate in the carrageenan test at the end of 4 hours was 49%, while for OO was 39.8 %. The histopathological examination results in this study showed that HAO anti-inflammatory response rate was slightly higher than OO. Linoleic acid is an agonist at peroxisome proliferator-activated receptor-alpha (PPAR- α), which enhances keratinocyte

proliferation and lipid synthesis, and skin barrier repair, affecting the secondary healing intention of the HAO primarily. When the results of HP+HAO were examined, a significant increase was observed in collagen accumulation and fibroblast proliferation compatible with the *in vivo* results. The anti-inflammatory response rate remained similar to the preparation in which HAO was used alone, and the rate of granulation tissue formation increased.

When the result of HAO and HP+HAO on epidermal thickness is assessed, as demonstrated in Figure 33, the epidermal thickness increased significantly compared to the HAO used alone. Still, since the rate of granulation tissue formation did not increase at the same rate as epidermal thickness formation, the possibility of hypergranulation risk and formation of hypertrophic scar and fibrotic tissue is considered low (231), since the HP+HAO group increases collagen and fibroblast production and significantly increases epidermal thickness. It has been demonstrated that it strengthens the skin barrier significantly (232). It is also well known that HAO contributes to skin hydration by reducing trans-epidermal water loss and moisturizing the skin (233). Considering the skin barrier reinforcing effect and moisturizing effect of HAO, HP+HAO could meet the expected characteristics of suitable barrier support to be used for protective purposes in treating chronic wounds.

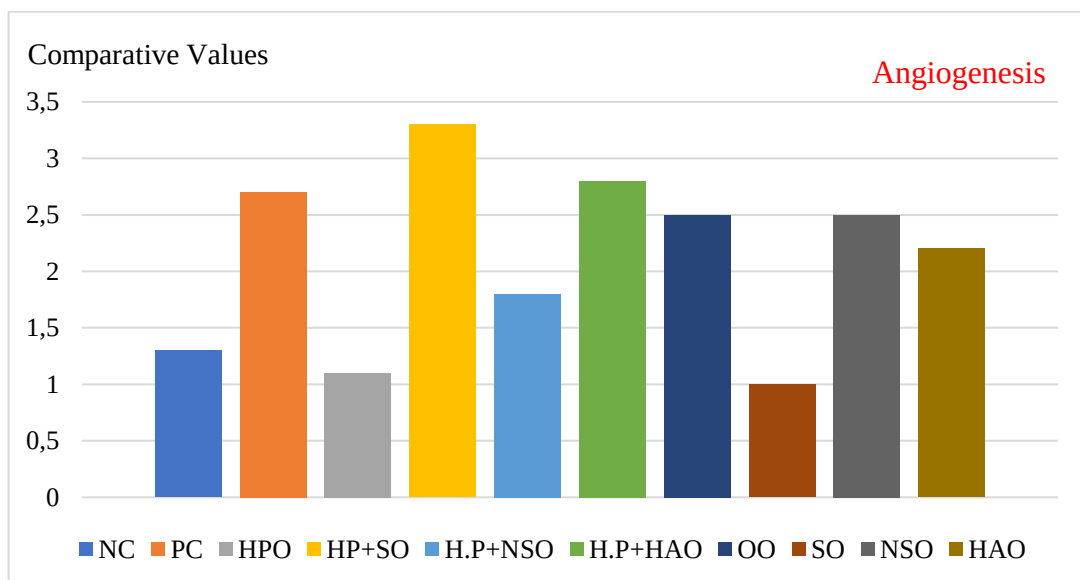


Figure 34. Comparison of angiogenesis induced by the oleates and fixed oils.

Angiogenesis is an essential parameter in wound healing. Newly formed wound vascularity is significant, especially in feeding the newly formed granulation tissue. It

provides oxygen support to the newly formed tissue and helps the tissue be nourished. Physiological angiogenesis in adults appears under limited circumstances: the female reproductive cycle, organ growth, and wound healing. Angiogenesis is also an important factor in pathological development (124). It is crucial in diseases like tumor growth, endometriosis, psoriasis, and rheumatoid arthritis. In the granulation phase, an elevated angiogenic factor is released, causing a robust angiogenic response and providing vascularization (137). This neovasculature provides a channel for nutrition and oxygenation of tissue and cell trafficking to aid wound healing. Disruption of angiogenesis results in delayed or disrupted wound healing, which may cause chronic wound development. Deficient vessel formation is a crucial factor in chronic wounds. The development of chronic wounds due to insufficient vascularization is apparent in patients with diabetes and arterial and venous ulcers. Recent clinical studies shows that reducing the angiogenic response may support normal wound healing (234).

The angiogenic activity assessment of the experiment and control groups showed that the highest level of angiogenesis was observed in the HP+SO group, as demonstrated in Figure 34. In comparison, the lowest level was monitored in HPO. In previous studies with HPO, it was observed that the macerate showed an antiangiogenic effect (235). Also, (126) observed in the study conducted on chicken embryos by *H. perforatum* state that it did not increase angiogenesis but increased collagen and fibroblast production. It would not be right to evaluate this result as unfavorable in wound healing parameters because using antiangiogenic agents at low doses reduced the level of vessel formation but did not eliminate angiogenesis, then routine wound healing continued. This observation highlights that under physiological conditions in the oral mucosa, wounds in the mucosa are known to heal rapidly and exhibit a less angiogenic response than the skin (236). In addition, scarless wound healing has been found to have a reduced angiogenic response. HPO shows a low level of angiogenic effect. However, it accelerates fibroblast migration at a high rate. Therefore, it can provide a significant advantage for wound types that are important to heal without hypertrophic scar formation (such as surgical wounds) (237). In addition, a high angiogenic effect is not desired in oncological wounds due to the increased vascularity feeding the tumoral tissue; therefore, HPO can be suitable in these types of wounds. Angiogenesis is essential in some wound models, especially with damaged vascular structure; it is crucial to provide vascularization in the wound area.

Especially in wound models with impaired vascular structure (Venous ulcers, arterial ulcers, diabetic wounds)(120,124,238) ; since the amount of oxygen reaching the tissue would decrease, it may lead to disrupted tissue nutrition, endangered the formation of granulation tissue and the risk of necrosis would be increased and the possibility of the wound turning into a chronic wound (137) . Thus, the use of HP+SO preparation in such types of wound models can significantly contribute to treatment. The angiogenic effects of the other groups were ordered from the highest to the lowest as follows; HP+HAO, PC, OO, NSO, HAO, HP+NSO, NC, HPO, and SO, as indicated in Figure 34. It is also proven that OO (129), SO (105) and HAO (239) have angiogenesis-promoting properties. Although there is no certainty about the angiogenesis-stimulating effect of NSO, it has been observed that the active ingredient TQ selectively reduces vascularity in the tumor tissue. On the contrary, it does not reduce the vascularization of the normal tissue (240). When the experiment results are examined, after maceration of *H. perforatum* in the OO and NSO, a slight decrease is observed in the angiogenic properties. On the other hand, the angiogenic effect of SO and HAO increased significantly.

While evaluating the histopathological wound parameters, other parameters, such as hair follicles and skin appendage formations, were also assessed. A study conducted by Ito et al. 2008, has proven that hair follicle formation is vital in wound healing and follows a similar mechanism in the epidermis but is not necessary for routine wound healing and has no direct relation to being detected between wound healing and hair follicle formation (241).

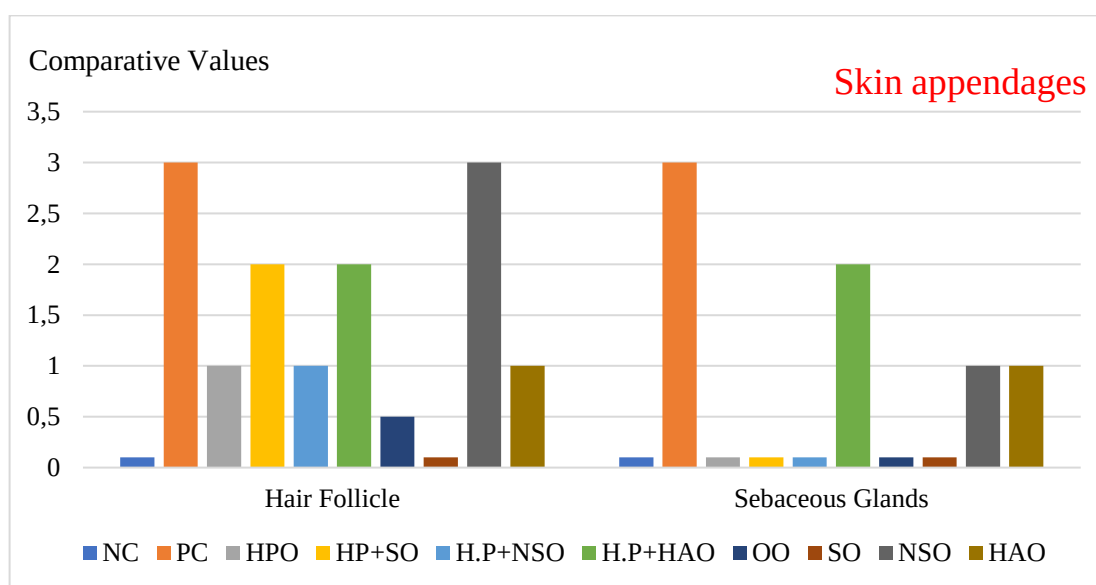


Figure 35. Comparison of hair follicles and sebaceous glands of oleates and fixed oils.

Based on the results in Figure 35, the PC group is the most enthusiastic group in hair follicle formation. It has been named that Madecasol™, the trademark name of PC, has had a significant effect on hair follicle formation in previous studies (242). Based on the results in Figure 35, NSO significantly supports the formation of hair follicles when used alone, confirming previous study results (243). While no significant hair root stimulating effect was observed in the experimental groups in which SO and HAO were used alone, the hair root stimulating effect increased significantly in their macerates HP+HAO and HP+SO, and also similar effect was observed in the increase of their angiogenic effects. Corresponding to the experimental results, an increase in angiogenic activity significantly affects the formation of hair follicles. This phenomenon has been reported in previous studies, and this relationship can be considered an essential parameter in interpreting the relationship between hair follicles and wound healing mechanisms (130).

According to the LC-MS and UV analysis results shared in Table 10 and Table 11, the increase in hypericin and hyperforin ratios does not directly affect the wound healing parameters when we evaluate HP+SO, HPO, HP+HAO, and HP+NSO macerates. In previous studies, no direct effect of change in the amount of hyperforin on wound healing was found (152). When we examine the pseudohypericin ratios, the highest pseudohypericin amount was found in the HP+HAO group, and the lowest amount was in the HPO group. In previous studies, it was observed that pseudohypericin is one of the essential components in the anti-inflammatory effect of HPO (202). In this study, when amounts of pseudohypericin were compared, the highest value was found in HP+HAO preparation, the anti-inflammatory response rate was high, and the lowest level was in HPO preparation, the anti-inflammatory was lower at the end of the 10th day compared to HP+HAO preparation. However, since many molecules in herbal preparations can interact synergistically, it may be challenging to relate wound parameters directly through the change of these molecules.



6. CONCLUSION

H. perforatum is a crucial herb traditionally used in wound treatment for centuries. This study evaluated the wound healing potentials of its traditional oleates form comparatively in different fixed oil bases, *i.e.*, HPO, HP+SO, HP+HAO, and HP+NSO, and each of the fixed oil bases individually. When *in vivo* results were evaluated, the traditional form HPO and HP+SO have demonstrated the best synergetic results. When *in vivo* and *in vitro* results are evaluated together, different forms of macerate can be more effective according to the wound types. Tissue management, infection or inflammation, moisture balance, and edge of wound (TIME) algorithm is essential for evaluating wound healing. The TIME rule can be summarized as follows: (244)

- Tissue: The examination and removal of nonviable or foreign substances (such as necrotic tissue, slough, exudate, and debris) present on the wound's surface through debridement.
- Infection/inflammation: Evaluating the cause of each wound, determining the necessity of using topical antiseptics or systemic antibiotics to control infection, and managing non-infectious inflammation appropriately.
- Moisture imbalance: The assessment of the origin or cause of wound exudate and its subsequent management.
- Edge of wound: The evaluation of non-progressing or undermined wound edges, along with the condition of the surrounding skin.

In wound treatments, a treatment strategy is created according to the T.I.M.E. algorithm by considering the above parameters. It is imperative to nourish the tissue in wounds requiring debridement at the tissue stage. SO has been used successfully in necrotic wounds (245). When *H. perforatum* is macerated in SO, its angiogenic properties increase significantly, as shared in Table 17, which is a critical parameter for treating necrotic tissue; while an increase in epidermal thickness is not desired in the treatment of necrotic wounds (245). When evaluated in this respect, the HP+SO preparation will significantly contribute to the success of the treatment. Especially in diabetic wounds, since the vessels are damaged, oxygenation and nutrition of the tissue would be deteriorated; in these wounds, HP+SO macerate can contribute significantly to wound treatment by supporting tissue blood circulation with its angiogenic effect and at the same time, it can contribute to tissue repair with its collagen stimulating and fibroblast migration triggering effects. Chronic wounds are formed in arterial and venous ulcers as

tissue blood supply is impaired due to vascular damage. HP+SO macerate can also be preferred in treating this type of chronic wound (105,238,246).

The angiogenic effect is undesirable in oncological wounds. The increase in the vessels feeding the tumor may help the growth of the tumor and cause the cancer to spread (48). HPO may be much more appropriate in critical wounds where this antiangiogenic effect is expected. At the same time, HPO, which has a low angiogenic effect and increases collagen production and fibroblast migration, can be preferred in cases where minimal scarring is desired, such as plastic surgery operations (124,234). HPO can be chosen to treat psoriasis because of its antiangiogenic effect (45).

Infection control is a crucial parameter in wound care. After the debridement process, the symptoms of infection can be controlled with these parameters; change in odor, the existence of purulent secretions, and exudates. Then, a culture is taken from the wound to detect pathogen type, and disinfectants or topical antibiotics are used to prevent the wound from harmful microorganisms to prevent acute microbial colonization and contamination (247). However, these preparations generally do not have a selective effect, and some antiseptics may also damage the healthy tissue flora (248). Therefore, HP+NSO can be preferred in infection control. The antibacterial property of *N. sativa* is well-known, and NSO has already been used in infection control, especially in Middle East societies (249). According to the result of this experiment; HP+NSO; showed a moderate anti-inflammatory action; and there was an increase in the rate of collagen synthesis and fibroblast production compared to NSO used alone, as shown in Table 17. Thus, HP+NSO could be an essential alternative to NSO for wound treatment.

Preventing wound dehiscence after healing is an essential parameter as well. Most of the patients who receive chronic wound treatment are bedridden, undergoing dialysis treatment, or suffering from chronic diseases such as diabetes and blood pressure control. Hence, the possibility of wound dehiscence is high. In such patients, it is vital to protect the skin barrier after the wound is treated and apply the daily preparations that support the skin barrier (250). HP+HAO can have a very successful effect in this indication. Based on histopathologic results in this experiment shared in Table 17, HP+HAO strengthens the skin barrier by increasing the epidermal thickness and enhancing the skin barrier with its angiogenic and collagen stimulation effects. Patients who are bedridden for a long time suffer from pressure ulcers and incontinence-related dermatitis. Therefore, HP+HAO

may topically be applied to patients' risk areas to avoid incontinence dermatitis and pressure ulcers. Also, HP+HAO could apply to dialysis patients whose skin has thinned due to sodium and potassium loss to increase epidermal thickness and prevent dehiscence after healed wounds.

To summarize this study, the *H. perforatum* plant, when macerated in SO, HAO, OO, and NSO preparations, contributes positively to their wound healing effects and improves the different wound healing parameters of these fixed oils. HPO and HP+SO preparations showed the highest synergistic effect in *in vitro* studies, as shown in Table 18 and Table 19. HPO is the mainly used traditional maceration form of St. John's Wort for wound healing, and previous clinical studies, and this study, have proven its effectiveness. On the other hand, HP+SO maceration should be tested in more clinical studies; especially in wound types that require debridement treatment and need strong angiogenic effects, such as 2nd and 3rd-level burns, diabetic wounds, and arterial and venous ulcers. Since the treatment process of the chronic wound and the protection of peri-wound skin is long and arduous, the best solution is to prevent wound dehiscence. Therefore, more studies can support the positive results of HP+HAO use in barrier formulations. Furthermore, it can be used in geriatric patients, increasing patient comfort and reducing the economic cost of wound dehiscence to patients' relatives.

The infected wounds are treated mainly with antiseptics, topical antibiotics, and silver products. However, these products have some side effects and difficulties in use, such as; antiseptics delaying wound healing by disrupting fibroblast and keratinocytes proliferation, antibiotic resistance is another significant problem restricted to repetitive use of antibiotics, and silver products having cytotoxicity (251–253). Therefore, more clinical trials should be done for HP+NSO utilization in infection control because HP+NSO does not interrupt the proliferation of fibroblast and keratinocytes; on the contrary, it supports the fibroblast and collagen production based on the histopathological examination in this experiment, and bacteria are unable to develop resistance due to multiple chemically complex phytochemicals present in the plant extract (254).

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