



Review

Experimental design and methodological approaches used to assess the potential of essential oils in wound healing

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Abstract

Wound healing (WH) remains a complex clinical challenge, driving the search for effective therapeutic alternatives such as essential oils (EOs) derived from medicinal plants. However, the reliability and translational relevance of findings in this field are highly dependent on the methodological approaches used to assess EO efficacy. Diverse *in vitro* and *in vivo* models, alongside varying experimental protocols, have been employed, often leading to inconsistent or non-comparable outcomes. In this review, we critically examine these methodologies, highlighting their strengths, limitations, and impact on interpreting WH activity. By emphasizing the need for standardized and robust evaluation strategies, we aim to guide future research toward more reliable and reproducible assessments of EOs in wound healing. Furthermore, some details on the WH process and the chemistry of EOs are provided. To gather relevant data, several databases were utilized, including Google Scholar, ScienceDirect, Web of Science, Scopus, and PubMed. The literature revealed that many tests are used *in vivo* and *in vivo* to assess the WH effect of EOs, including Incision, excision, burns, scratch and microfluidic assay. Other tests such as human model are not usually employed for EOs but there are some on data on plants extracts. Interestingly, this review provides some significant discussing on the approaches used to evaluate the WH activity of EOs and can be taking as a strong scientific basic for the future research in this field.

Keywords: Wound healing; Essential oils; Methods; Approaches; *in vivo*; *in vitro*

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1. Introduction

The human skin acts as a protective shield, functioning as an external barrier against various environmental stresses. In addition to its protective role, it helps regulate temperature, defends against pathogens, and adapts to different climatic conditions. Human skin can experience a wide range of disorders, from simple dryness to complex issues like melanoma (Almoshari, 2022). The skin's surface epithelium plays a crucial role and function as armor, protecting the body from direct external damage. As the first line of defense, the epidermis is also the area most prone to frequent injuries (Biggs et al., 2020; Gurtner et al., 2008). Wound healing (WH) is very important when the skin suffers external injuries like irritation, cuts, or tears, which damage the underlying tissue's structure and function. This intricate process typically involves four phases: hemostasis, inflammation, proliferation, and tissue remodeling (Huang et al., 2024; Walter et al., 2023). Traditional practices in medicine have a long history in the earliest days of human civilization. It encompasses the written and oral knowledge passed down from our

ancestors, along with their experiences, beliefs, and insights for treating diseases in humans and animals. While various materials are significant in traditional practices, the use of plants is especially vital (Jabeen et al., 2024; Tiwari et al., 2004).

Although the significant expenses related to wound management, there is no single universally effective treatment for wounds. Clinical approaches are many employed, including surgical debridement of lesions, the application of dressings made with alginate, foams, or silver, and hyperbaric oxygen therapy (Alves et al., 2024). Essential oils (EOs) may provide an alternative and effective approach for WH, as they possess a variety of biological properties, including anti-inflammatory and antioxidant effects (Mssillou et al., 2020), antiviral, and antimicrobial activity (Avola et al., 2020a; Pérez-Recalde et al., 2018). Given these properties, EOs have gained much attention as potential alternative therapies for enhancing WH, neutralizing excess reactive oxygen species, and

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combating bacterial infections (Andjić et al., 2022; Meng et al., 2024).

Investigating the pathophysiology of WH and developing new methods and protocols for monitoring the healing process can significantly enhance treatment effectiveness and improve patient outcomes. Regular assessments of wounds will increase the likelihood of delivering more suitable care to patients (Masson-Meyers et al., 2020). Studying any physiological process relies on models, and the literature includes various *in vitro* and *in vivo* models for WH (Fronza et al., 2009; Galiano et al., 2004; Kandimalla et al., 2016). Researchers and scientists in the field of WH studies must provide key design issues such as study goals, wound type, sample characteristics, costs, timeframe, and available facilities. Utilizing multiple assessment methods enhances result reliability and deepens understanding of tissue repair mechanisms. Selecting the appropriate WH model at the outset is a critical determinant of the quality, efficiency, and translational value of the research. Methodological choices directly influence how accurately the biological effects of EOs are captured and interpreted. An optimal model—whether *in vitro*, *ex vivo*, or *in vivo*—should align with the specific research objective, such as evaluating anti-inflammatory activity, cell migration, angiogenesis, or tissue remodeling. Careful selection helps avoid redundant experiments, minimizes variability, and ensures that the data generated are both reproducible and comparable across studies (Hasatsri and Aramwit, 2017; Masson-Meyers et al., 2020; Zeng et al., 2018). Therefore, prioritizing robust methodological approaches from the beginning not only streamlines experimentation and reduces resource consumption but also strengthens the scientific validity of findings and accelerates the development of effective WH therapies.

The use of protocols and methods to assess WH is complex, making it difficult to cover all options. This review aims to highlight commonly used *in vitro* and *in vivo* models commonly used in the evaluation of WH effects of EOs, along with various assessment methods like wound healing rate, image analysis, and biochemical assays. These insights would be helpful in developing effective experimental protocols using EOs for WH.

2. Research methodology

Data and information were collected from previous studies dealing with the methods and protocols used to assess WH effects of EOs. Multiple databases, such as PubMed, Scopus, Google Scholar, and Web of Science were used. The following terms were searched in combination: "Essential oils", "wound healing", "models", "methods", "approaches", "incision", "excision", "*in vitro*", "*in vivo*", "protocols", "medicinal plants".

3. Overview of the chemistry and bioactivities of EOs

EOs constitute complex combinations of naturally occurring compounds of moderate molecular masses, which are obtained from aromatic plants using distillation process and a variety of solvents (Raut and Karuppaiyil, 2014). These substances can be extracted from various parts of plants, including seeds, leaves, flowers, roots, fruits, herbs, barks, wood, and buds. These fragrant volatile oils possess a characteristic odors and colors, especially when fresh. They generally classified as secondary, plant metabolites,

produced in plants, but are not necessarily required for its development and growth for defense purposes. However, they are used to protect against pathogens, pests and environmental stress (Ninkuu et al., 2021; Silori et al., 2019). EOs are specifically synthesized by secretory tissues called glandular trichomes and certain other structures, which are dispersed mostly on surfaces of plant organs (Sharifi-Rad et al., 2017). Within the large number of plant-derived products, EOs merit particular attention, and represent one of the important products in the agricultural industry (Raut and Karuppaiyil, 2014). More than 3000 EOs have been isolated from species belonging to various plant families, including Rutaceae, Lamiaceae, Myrtaceae, Asteraceae and Zingiberaceae, of which 300 are important for commercial perspective, especially in the pharmaceutical and cosmetic industries. EOs are already among the most traded commodities, and by 2025, it is expected that their worldwide market would exceed \$15 billion (Yeshe and Wangchuk, 2022).

EOs are complex combinations of hydrocarbons and oxygenated derivatives in variable concentrations, which are primarily divided into two classes: terpenes and phenylpropenes (Agour et al., 2022). Terpenes arise from two different isoprenoid pathways (Ninkuu et al., 2021), and their structural variety is primarily controlled by the number of isoprene units (C_5H_8). Hemiterpenes (C_5) are constituted by 1 isoprene, whereas monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) consist of 2, 3 and 4 units, respectively (Bhavaniramya et al., 2019). In fact, terpenoids are considered as derivatives of terpenes with the presence of oxygenated part, and are mainly produced by the biochemical addition or removal of methyl groups (Pandey et al., 2017). Generally, the predominant compounds in EOs are monoterpenes (90%), followed by sesquiterpenes, while hemiterpenes and diterpenes represent minor constituents (Falleh et al., 2020). Monoterpenes are characterized by their diversity and are present in both monocotyledonous and dicotyledonous, angiosperms and gymnosperms, as well as in fungi and bacteria (Ninkuu et al., 2021). These molecules are present in monocyclic, bicyclic, and acyclic forms, and they have numerous cosmetic, pharmaceutical and food applications. For example, eugenol, linalool, carvacrol, carvone, thymol, and limonene, are active monoterpenes with numerous biological and industrial purposes (Masyita et al., 2022). The diversity of sesquiterpenes results from the gathering of 15 carbon structures, and the superposition of functional groups and substituents on this skeleton. Chemically, they exist either as hydrocarbons, such as farnesene and humulene, alcohols, including β -germacrenol, δ -elemanol, esters, like torilin and ejaonines, aldehydes, such as farnesal and lepidozenal, or as oxygenated, carbonylated or hydroxylated derivatives (Lorigooini et al., 2020). Diterpenes obtained from diverse resources have displayed inhibitory effects against pathogenic microbes (Ninkuu et al., 2021). Sesquiterpenes have been reported to exhibit antitumor, antifungal, anti-inflammatory and antimicrobial activities (Ninkuu et al., 2021). Diterpenes subclass, as non-volatile hydrocarbons, are also present in linear and cyclic forms.

Phenylpropenes (C_9H_{10}), another type of biochemical components contained in EOs, are a class of volatile

phenylpropanoids, derived from the amino acid phenylalanine (Atkinson, 2018). Phenylpropanoids are metabolized via the shikimic acid pathway, through bonding of the aromatic ring of a phenol with cinnamic acid, especially the three-carbon propene part. This complex is oxygenated at the third, fourth, or fifth position and commonly contain a double bond between two carbon atoms (Dajic Stevanovic et al., 2020). The structural diversity of these molecules is related to the variety of substituents on the benzene ring and also the location of the double bond in the propenyl side chain (Atkinson, 2018). Estragole, anethole, chavicol and eugenol are examples of phenylpropenes that contribute to the aroma and flavor of numerous important spices and herbs. These bioactive substances have antifungal and antibacterial properties, moreover, they act as floral attractants for pollinators (Atkinson, 2018). Results of recent studies have shown that anethole exhibits an anticancer activity (Contant et al., 2021). In addition, safrole has been documented to exhibit various biological characteristics, and mainly possesses antimicrobial, antidiabetic, antifungal and analgesic activities (Eid and Hawash, 2021). Concentrations and numbers of molecules and stereochemical forms in the chemical profiles of EOs within the same species vary based on the environmental factors, biotic and abiotic conditions of soil, plant organs, vegetative stage and the extraction techniques (Ayaz et al., 2017).

Due to their biological properties, since antiquity, EOs have been employed in several traditional medical approaches across the world. Currently, EOs are becoming an integral part of daily living. Basically, they are integrated in a range of industries, including health care and cosmetics. Also, they are utilized in air fresheners, deodorizers, and various medical specialties, including homeopathy, balneology, massage, and pharmacy. Several researches have been conducted on biological properties of EOs, ranging from antimicrobial to anticancer activities, and have identified numerous bioactive compounds (Masyita et al., 2022). Various EOs, extracted from aromatic species, such as *Origanum compactum* Bent., *Cinnamomum zeylanicum* Nees and *Mentha viridis* L. have been reported to possess powerful antimicrobial activities against a variety of resistant fungus and both Gram (+) and Gram (-) bacteria (Bouyahya et al., 2020; El Abdali et al., 2023; Sihoglu Tepe and Ozaslan, 2020). Due to their bioactive compounds, EOs have been reported as strong antioxidants agent against free radicals, and have displayed a remarkable effect in attenuating the effects of oxidative damage (Bouyahya et al., 2020; El Abdali et al., 2023; Sihoglu Tepe and Ozaslan, 2020). Interestingly, Linalool, thymol, carvacrol, E-Cinnamaldehyde, carvone and 1,8-cineol are the most active EOs components implicated in the reported activities. The anti-diabetic activity of EOs extracted from *C. zeylanicum* (E-cinnamaldehyde, 81.39 %) and *M. viridis* (carvone, 37.26 %) was also proved (Bouyahya et al., 2020; Sihoglu Tepe and Ozaslan, 2020). The anti- Alzheimer activity was also reported for the EOs extracted from *C. zeylanicum* (E-Cinnamaldehyde, 81.39 %) and from three popular *Stachys* herbs (Bahadori et al., 2020; Sihoglu Tepe and Ozaslan, 2020). Other biological activities of

various EOs, including their effects against diabetes, inflammation and parasites, have been studied (Bahadori et al., 2020; Vega Gomez et al., 2021). Accordingly, their anti-inflammatory effect and their ability to neutralize free radicals, are the main reason why recent researches have demonstrated the significant implications of EOs in the treatment of cancer. Several mechanisms explicated the anticancer potential of EOs, including nuclear damage, ROS generation, cell cycle arrest, inhibition of metastasis and angiogenesis, induction of apoptosis and activation of DNA detoxification and repair systems (Gautam et al., 2014; Rajivgandhi et al., 2020). In this regard, EOs extracted from *Rosmarinus officinalis* L., *Origanum glandulosum* Desf., and *Mentha citrifolia* L., have all exhibited significant anti-cancer effects (Javed Ahamad et al., 2019; Rajivgandhi et al., 2020). The researchers attributed the studied activity to various anti-cancer compounds of EOs, such as verbenone, borneol, p-cymene, thymol, carvacrol, β -thujene and terpinolene.

Alternatively, the ability to easily penetrate skin tissue, due to their lipophilic properties, made EOs potential and widely used agents to treat skin aggressions. Furthermore, their inhibitory effect on the enzymatic activity of certain enzymes involved in skin elasticity and aging, such as elastase and tyrosinase, explains their relevance. They also limit the proliferation of many dermatophyte fungi, which explains the marked trend in using essential oils and their derivatives in cosmetic and pharmaceutical products (Bouyahya et al., 2020). This is the case of the EOs extracted from *M. viridis* and *Eucalyptus smithii* R. T. Baker., which have exhibited dermato-protective and anti-dermatophyte properties (Bouyahya et al., 2020). In addition to this enzymatic and biochemical mechanisms, several works have reported the immunomodulatory effect of EOs obtained in particular from Lamiaceae, which possess a suppressive effect on the levels of IgE and on the IgE-mastocyte cross-linking, attenuating consequently the allergic reactions on the skin (Sim et al., 2019). Similarly, the study conducted on *Minthostachys verticillata* Epling., showed that the EOs of this plant and its major compounds, in particular pulegone, menthone and limonene inhibited the activation and degranulation of mast cells in the skin during passive cutaneous anaphylaxis tests, and demonstrated that limonene was the most active (Cariddi et al., 2011). Other EOs extracted from *R. officinalis* and some chamomile species were also mentioned, being active in the management of atopic dermatitis and eczema (Arora et al., 2022; Sarkic and Stappen, 2018). In parallel, and due to their antioxidant ability and inflammatory cytokine modulating effects, as well as their antimicrobial power, several EOs, notably those of lavender, oregano and salvia, have been effective in WH (Avola et al., 2020b; Farahpour et al., 2020; Samuelson et al., 2020). According to several studies, this healing effect of EOs is mainly related to monoterpenoids, such as carvacrol and thymol (Costa et al., 2019). Because of their benefits on the skin, their antimicrobial and anti-inflammatory effects, and due to their pleasant odor, the pharmaceutical industry has lately turned to the incorporation of EOs in cosmetics and perfumes. Currently, EOs and their isolated compounds are integrated in various cosmetic formulations such as

creams, gels, massage oils and ointments (Sarkic and Stappen, 2018). In addition to their preservative effect in cosmetic products, the EOs incorporated in these products ensure the hydration and elasticity of the skin, it is the case for example of EOs of rosemary tested on the skin of human subjects (Montenegro et al., 2017). Similarly, the EOs derived from *Helichrysum italicum* Roth., *Lavandula angustifolia* L., *Matricaria chamomilla* L., and *Citrus aurantium* var. *amara* L., are also used in multiples cosmetic preparations and perfumes (Sarkic and Stappen, 2018). Other EOs derived from *R. officinalis*, *Eucalyptus globulus* Labill., *Satureja montana* L., *L. angustifolia* and others, are used in cosmetics for the care of the scalp and the hair shaft. They are incorporated in shampoo, gel or lotion (Abelan et al., 2022). It has also been reported that terpenes ameliorate permeation through the *stratum corneum* of the skin (Oh et al., 2014). Nevertheless, there are limits to the use of certain EO's in cosmetic products due to certain side effects caused by certain EOs and their compounds, especially for allergic people.

Of all EOs components, terpenoids have been extensively investigated and described to play a crucial role in human health. In numerous *in vivo* and *in vitro* researches, monoterpenes and sesquiterpenes have been studied for their potential as anticancer, antimicrobial, anti-inflammatory, antioxidant, neuroprotective, antiaggregant, antiallergic, sedative, anticoagulant and analgesic agents (Masyita et al., 2022). As a complex mixture of volatiles, the effects and properties of EOs are a result of the bioactivity of these components and how they interact (El-Tarabily et al., 2021). Overall, EOs have been documented in several preclinical investigations using a range of animal and cell models, and their mode of action and pharmacological targets have also been clarified. However, further clinical investigations are required to demonstrate the EOs' ability as efficient and safe phyto-therapeutic agents on human health (Sharifi-Rad et al., 2017b).

4. A background on skin anatomy and WH process

The human skin is considered as the body's largest organ, responsible for synthesizing vitamin D, sensing external stimuli, and regulating body temperature. It consists of three layers: the epidermis, dermis, and subcutaneous tissue. The primary cell types in the epidermis are keratinocytes and dendritic cells, along with other important cell types such as Merkel cells, Langerhans

cells, and melanocytes. Basal cells in the epidermis undergo cycles of proliferation, enabling the outer layer to renew itself (Heidari et al., 2024; Mssillou et al., 2022; Nosrati et al., 2021; Yousef et al., 2024).

The dermis is a complex network of connective tissue, containing fibroblasts, mast cells, neurons, and blood vessels. It allows immune cells like lymphocytes and plasma cells to enter in response to various triggers. The dermis provides the skin with strength, flexibility, and elasticity, and it also contains sensory receptors, helps regulate body temperature, and protects against mechanical injury. Fibroblasts are the dominant cells in this layer, producing essential components of the extracellular matrix, including collagen and elastic fibers (Heidari et al., 2024; Mssillou et al., 2022).

When the skin is injured, its protective function is compromised, leading to wound formation. The healing process is complex and involves interactions among various cytokines, growth factors, chemokines, and components of the extracellular matrix. This process consists of four overlapping phases that work together to promote rapid wound healing (Annabi et al., 2014; Wang et al., 2021).

Hemostasis is the process that stops bleeding by forming a blood clot, occurring in two main stages. In the first stage, platelets adhere to the injury site and become activated, aggregating to create a platelet plug. In the second stage, thrombin is generated through both external and internal coagulation pathways, converting fibrinogen into fibrin. This fibrin combines with the platelet plug and blood cells to form a stable thrombus (Guo et al., 2021; Yang et al., 2024).

During the inflammatory phase, inflammatory cells differentiate into macrophages at the wound site. These macrophages release ROS with antibacterial effects and secrete proteases to help eliminate bacteria and damaged tissues. They also produce cytokines and growth factors that facilitate cell proliferation and migration, setting the stage for angiogenesis and tissue formation (Petkovic et al., 2020). In the proliferative stage, granulation tissue forms, neovascularization occurs, and the epidermis regenerates. The remodeling phase starts 2–3 weeks after the injury and can last for a year or longer, during which the new tissue is reshaped and the skin's mechanical properties are restored (Gurtner et al., 2008; Yang et al., 2024).

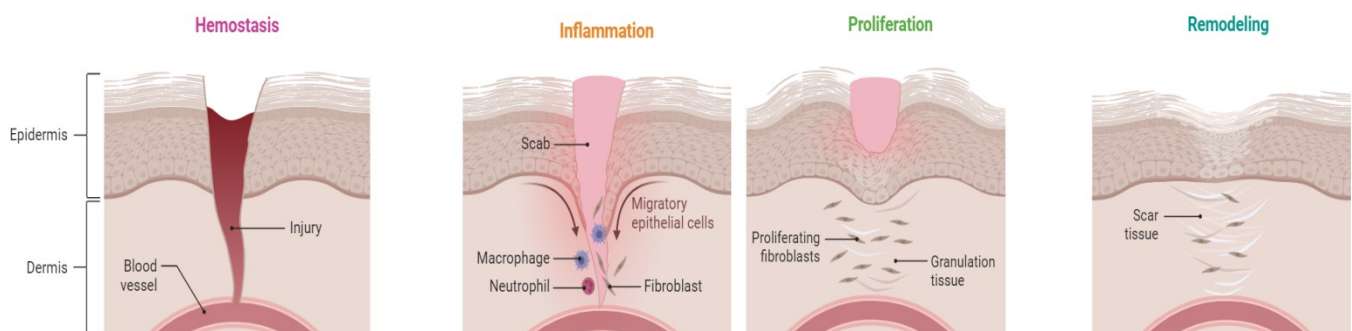


Figure 1. WH healing process including hemostasis, inflammation, proliferation and remodeling

5. Methods used *in vivo* and *in vitro* for WH assessment

Wounds are a serious therapeutic problem that affects patients, which results from any skin trauma, like surgical operations, accouchement, burns and diabetic ulcers. To confront this problem, researchers and medical scientists are very interested to potential alternative therapies that can promote the WH such as the use of natural plants-derived products (Boukhatem et al., 2021a). Natural compounds derived from medicinal plants have a wide range of potentialities (*i.e.*, antioxidant, anti-inflammatory, immunomodulatory, antibacterial effects...) Flavonoids, anthraquinones, shikonin, alkanin, and EOs, have powerful antioxidant properties by reducing ROS, blocking lipid peroxidation, and promoting angiogenesis, fibroblast cell proliferation and temporary ECM production. Indeed, some natural herbal remedies promote the expression of VEGF and TGF- β which are two important elements to accelerate angiogenesis, the process of granulation tissue development and collagen fiber deposition (Tiwari and Pathak, 2023). Unfortunately, Wound management is a complicated task, due to the WH process complexity and the wound environment, which includes the involvement of many cell-types like epithelial cells, inflammatory cells, and the symphony of repair phases, which includes inflammation, proliferation, re-epithelialization and remodeling (Masson-Meyers et al., 2020). In order to undertake research on WH, various models have been used. There are *in vivo*, *in vitro*, *in silico*, and purely clinical models (Wilhelm et al., 2017). *In vivo* (preclinical) models consist of wounding a laboratory animal and examining the closing of the wound as time progresses. Physical, chemical or biological modifications in the environment of the experimental animals are parameters used for experiments on skin wounds, including rodents, rats, rabbits, pigs and mice (Nauta et al., 2013; Stephens et al., 2013). Alternatively, it is possible to genetically transform some small mammals and create a new model that can resemble to defective human diseases including skin injury (Masson-Meyers et al., 2020). Furthermore, *In vivo* models are extensively used to analyze the WH activity, permitting a close resembling of the wound environment. Basically, these models involve several cell types, environmental cues and para-clinical interactions (Wong et al., 2011). Moreover, animals like rats and mice are the most commonly employed but the optimal model is one that mimics some physiological aspects of humans without the participation of human volunteers for the experiments (Geer et al., 2004). However, *in vitro* models are laboratory experiments that are routinely rapid, simple, and does not require complex ethical considerations compared to the manipulation of whole animals in paraclinical models, and allow the highlight of some important biochemical and physiological processes induced by the sample being tested (Agyare et al., 2016; Gottrup et al., 2000). Scratch model, skin explant culture, cell culture and proliferation are the essential models to evaluate the WH activity of pharmacological agents (Masson-Meyers et al., 2020).

5.1. *In vivo* WH models

Although its difference from human skin, animal skin has been used to investigate the complexness of WH process, especially on chronic injury. Models based on animal skin have been successful in evaluating and creating new treatments. Various scientific works have utilized the Wistar strain, and the Sprague-Dawley strain. The Lewis's strain, Dark Agouti strain, Brown ACI strain, and Nude/Hairless strain, have been used as well. Other animals like porcines and rabbits can also be used (Kumar et al., 2013; Sami et al., 2019).

5.1.1. Excision wound

Excisional wounds are considered the most widespread WH models in the literature (Nauta et al., 2013). These injuries are created by surgically removing all skin layers (epidermis, dermis, and subcutaneous fat) using tools such as scissors, lasers, or cauterization. Wound surface is then monitored through periodic photographs, and healing is assessed by measuring wound contraction relative to its original size (WH rate). In fact, the methodological and experimental variations cited in the literature differ in the number and type of animals, size of excision, and diameter (generally between 2 and 20 mm) and the instruments used to generate the wound. In addition, this model permits the study of hemorrhage, inflammation, granulation, skin regeneration, angiogenesis and remodeling during WH. For histological analyses, mice and rats can be locally anesthetized, while in some other animal models like rabbit ear, the animal is usually killed and then the biopsies are taken. Next, the sample taken is processed and examined for both sides of the epithelial edges of the wound. The tissue is also examined for granulation bed characteristics and collagen organization (Gottrup et al., 2000; Stephens et al., 2013; Wong et al., 2011). In excisional wound model, the wound site is easily accessible for administering topical agents (*e.g.*, pharmaceuticals, extracts, and oils from medicinal plants). For example, the WH ability of *Olea europaea* L. has been evaluated using excision wound (Elnahas et al., 2021). This species is a widespread tree in the Mediterranean countries offering vast benefits for human health; Elnahas et al., (2021) created a circular excision wound in diabetic and non-diabetic female rats divided into 8 batches according the treatment used.

5.1.2. Incision wound

This model is considered as the second most frequently mentioned model in literature, presenting greater homogeneity from one publication to another. Generally, wounds have a length of 10 to 15 mm, which are performed by a scalpel (Ansell et al., 2014). However, about one-third of the studies used sutures to reduce wound margins. This model allows the quantification of wound progression, determining the size, resistance, re-epithelialization, inflammatory response, determination of molecular and biochemical markers at the WH site (Ansell et al., 2014; Gál et al., 2009). Preferably, animals including mice, rats, rabbits and pigs are widely used to assess incisional WH. Studies have indicated different tests to study incisional WH, such as incisional wounds induced on the dorsal part of mice that allow the measurement of tensile strength by tensiometer (Masson-

Meyers et al., 2020; Stephens et al., 2013). Similarly, Januszyk et al., (2014) applied this model to estimate the stress of a surgical incision in the pig. A study performed by Ansell et al., (2014) examines the expression of growth factors in a rat model of WH. Rats were anesthetized using ketamine (45 mg/kg) and xylazine (5 mg/kg). The application of incision (1 cm) was made with a scalpel blade n° 21 on the dorsal part, for a period of 5 days, the rats received a local application of lavender oil with a dose of 0.5mL once a day which showed no sign of edema, discharge, or local infection thanks to the anti-inflammatory and antibacterial properties of lavender.

5.1.3. Skin burn

Burns are among the most common skin injuries in humans, with high medical costs and significant physical, psychological and social impacts, prolonged or eternal (Amrati et al., 2023). The healing patterns of burns can be determined by the application of hot water to a specific area of the skin. Another technique is used to induce burns by thermal shock, which involves the direct use of a hot metal plate to the skin (Walter et al., 2023). Thermal burns can be useful to measure re-epithelialization, assess granulation tissue production, angiogenesis, retraction, WH, and wound tissue biochemistry, according to burn depth (Walter et al., 2023). Using animal models presents some limitations for burn research due to the absence of appropriate models to treat the endpoint of hypertrophic scars. Moreover, there are some limitations concerning the studies duration where some species like rodents, which heal by contracture are used. However, some other species like pigs, resemble to humans in WH process, unfortunately, the healing time is longer, and in addition to their high cost, the quantification of scar hypertrophy presents some difficulties in managing them as subjects (Friedrich et al., 2017). Caliari-Oliveira et al., (2016) showed the use of an extensive, acute rat burn model, similar to human third-degree burns, to measure the

potency of xenogeneic mesenchymal stromal cells as a perspective treatment for severe burns and to improve the WH. Deep burns (2 cm x 3 cm) on all three levels of the skin were created by a gentle pulse from a metal plate heated to 200°C and applied on dorsal area for 25 seconds. WH was evaluated by calculation the contraction from digital images of the wounds during the test and at the end of it. Histopathological examinations (vascularization, tissue granulation, total inflammatory polymorphonuclear cells, and collagen fibers), and myeloperoxidase (MPO) assays were also evaluated.

5.1.4. Human models of WH

It makes sense to use humans as a WH model, as the pathology and physiology of WH are identical to those occurred in the patient. Consequently, several wound models are available and can be applied to a human volunteer. For example, the Partial Thickness Wound is characterized by a thin wound that is applied to the epidermis and superficial dermis, without causing damage to the blood vessels (Dykes et al., 2001; Wilhelm et al., 2017). The Blister Wound Model can be utilized to examine the effects of various drugs and chemicals and the epidermal regeneration in humans. Several devices are employed to produce uniform, small blisters on the skin throughout suction, avoiding direct transdermal invasion. Normally, many blisters will be found in the same part or in numerous places of the body. Furthermore, these blisters can be performed in the midvolar forearms by applying a vacuum of 20 cm Hg. In these conditions, normal human skin will start to blister after 35 to 55 minutes (Kumar et al., 2013). When it comes to evaluating new medications in clinical trials, human models are very beneficial. However, getting human specimens is not always simple. Patients with chronic wounds do not present similar wound types or microbial compositions, and finding volunteers can be challenging (Sami et al., 2019).



Figure 2. Wound healing methods used *in vivo* and *in vitro* for EOs

Table 1. Examples of *in vivo* tests used to assess the WH effects of some EOs

Methods / tests	Duration	Animals used	Parameters	Essential oils	Country	References
Incision	10 days	Male CD-1 strain mice	Efficacy / Contraction / Histology	<i>Bursera morelensis</i> Ramirez.	Mexico	(Salas-Oropeza et al., 2020)
Burns	20 days	Swiss albino mice	Efficacy / Histology	<i>Cleistocalyx operculatus</i> (Roxb.) Merr. et Perry	Vietnam	(Tran et al., 2018)
Excision	16 days	Wistar rats	Efficacy / Histology	<i>Lavandula stoechas</i> L.	Algeria	(Boukhatem et al., 2021b)
Excision	9 days	Swiss mice	Clinical aspect / Morphology	<i>Melaleuca alternifolia</i> Cheel.	Brazil	(de Assis et al., 2020)
Incision	21 days	Wistar rats	Contraction/ Epithelialization / Histology / Biochemical analysis of blood	<i>Helichrysum italicum</i> (Roth) G. Don.	Serbia	(Andjić et al., 2021)
Excision	16 days	BALB/c mice	Wound closure / Histology / Immunofluorescent /	<i>Cinnamon verum</i> J. Presl.	Iran	(Seyed Ahmadi et al., 2019)
Burns	21 days	Sprague Dawley rats	Wound closure / Histology / Cytokine Levels	<i>Artemisia judaica</i> L.	Saudi Arabia	(Mohammed et al., 2022)
Excision	14 days	Sprague Dawley rats	Contraction / Epithelialization / Histology / Collagen Density	<i>Murraya koenigii</i> (L.) Spreng.	Malaysia	(Nagappan et al., 2012)

5.2. *In vitro* models

In vitro models are relevant alternatives to confront the ethical issue in animals and most suitable for high throughput screening allowing the elucidation of mechanisms at the molecular level to devolve the processes of WH (Low et al., 2021a). *In vitro* tests are performed on different types of cells like fibroblasts, and epithelial ones (keratinocytes) (Stamm et al., 2016). *In vitro* WH tests are favored due to many reasons such as flexibility, ease and cost effectiveness (Molinie and Gautreau, 2018). The most common *in vitro* WH methods are the scratch test and the microfluidic assay (Schagen et al., 2018; Wang et al., 2019).

5.2.1. Scratch test

One the relevant characteristics of the scratch test is because it is considered as an easy, economical, effective protocol to evaluate the effect of phytochemicals on WH by making a "scratch" in a cell monolayer. In general, this test consists of taking images between the beginning of the scratch and at a defined time intervals and comparing the images to evaluate the rate of cell migration (Liang et al., 2007).

The scratch test offers myriad benefits, including cell migration that mimics *in vivo* WH process as the wound release's cytokines, capturing the movement of living cells, and monitoring events that occur inside cells. This test is also considered ideal for studying the migration of different types of adherent monolayer cells, and it allows simultaneous microscopic analysis of protein distribution and morphology (Goetsch and Niesler, 2011; Low et al., 2021a).

However, this test has numerous limits; it takes much time than other tests, large amounts of cells are needed. Moreover, the scratch assay is impractical for specialized primary cells, due to the difficulty of collecting them in

large amounts, the existence of different widths of stripes, makes comparison difficult, culturing cells in a static and open environment does not precisely simulate the WH process *in vivo* (Low et al., 2021b).

5.2.2. Microfluidics assay

The microfluidics test is generally consisting on the migration of cells towards wounds and it is feasible to study complex cellular behavior. The culture of cells can be performed using microfluidic devices in an *in vivo*-like microenvironment. Furthermore, the application of external stimuli is controlled and precise. In this assay, the induction of wounds is conducted by enzymatic depletion, cell exclusion or depletion. Trypsin is the main solution employed to separate the cells, resembling to a wound that occurs *in vivo* (Halldorsson et al., 2015; Low et al., 2021b).

This test provides a lot of advantages such as: the use of a reduced volume of culture media, no direct contact with cells or the medium, and presents reliable results with controlled assays. The whole procedure is integrated, including the ability to change the speed of fluid flow in response to the mechanical stimulation's effects on WH and the chemical impacts (Halldorsson et al., 2015; Low et al., 2021b).

However, there are some challenging issues with using microfluidic devices. Obviously, this technique requires some expertise and a daily replacement of the medium. Furthermore, the control of the humidity in the incubator and the formation of cell clumps is necessary. In addition to these managements, the control of air bubbles is required and the observation of the microfluidic device solution to prevent its leakage is mandatory (Low et al., 2021b).

6. Conclusion

Recently, Eos have attracted growing scientific interest

due to their diverse health-promoting properties, among which their WH potential represents a particularly promising area of research. However, despite the large volume of published studies, the considerable heterogeneity in experimental designs and evaluation methods makes it difficult to draw firm and comparable conclusions regarding their true therapeutic value. In this review, we systematically summarized and critically analyzed the main *in vitro* and *in vivo* methods used to assess the WH activity of EOs, highlighting their respective strengths, limitations, and suitability for different stages of research.

Overall, our analysis underscores that methodological design is a decisive factor in determining the reliability, reproducibility, and translational relevance of findings in this field. Future research should therefore move toward more standardized, validated, and purpose-driven experimental frameworks, ensuring better comparability across studies. Importantly, advancing this field will require multidisciplinary strategies that integrate clinical research to confirm efficacy in human-relevant conditions, biochemical and molecular approaches to elucidate mechanisms of action, and pharmaceutical formulation to optimize delivery systems, stability, and bioavailability of EOs. Such an integrated approach will not only strengthen the scientific foundation of EO-based WH research but also accelerate their translation from experimental models to safe and effective clinical applications.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

Data will be available upon request from the corresponding author.

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