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MSc by Research Thesis
Material Sciences

From chemical composition to biological impact: an integrated in vitro and in vivo study of selected plant oils

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Declaration

This thesis has been composed by myself and the work submitted is my own, except where corresponding references or acknowledgements state otherwise. No portion of the work referred to in this thesis has been submitted in support of an application for another degree qualification for this or any other university or institute of leaning.

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Table of abbreviations

Abbreviations	Explanation
ANOVA	Analysis of Variance
BC	Before Christ
BHA	Butylated Hydroxyanisole
CAT	Catalase
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic Acid
EMA	European Medicines Agency
EOs	Essential Oils
EPSP	Excitatory Postsynaptic Potentials
ESCOP	European Scientific Cooperative on Phytotherapy
ET	Electron Transfer
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectroscopy
GHS	Globally Harmonized System
GLA	Gamma-Linolenic Acid
GPx	Glutathione Peroxidase
GSTs	Glutathione S-transferases
HAT	Hydrogen Atom Transfer
HMPC	Herbal Medicinal Products
HPLC	High Performance Liquid Chromatography
IC50	half-maximal inhibitory concentration
LC-MS	Liquid Chromatography-Mass Spectrometry
LLNA-EC3	Local Lymph Node Assay – Effective Concentration 3%
LS means	Least squares means
MANOVA	Multivariate Analysis of Variance
MS	Mass Spectroscopy
MUFA	Mono Unsaturated Fatty Acids

NIST	National Institute of Standards and Technology
P.B.S	phosphate-buffered saline
PAs	Pyrrolizidine Alkaloids
PUFA	Poly Unsaturated Fatty Acids
R.T.	Retention Time
ROS	Reactive Oxygen Species
SBS	Sea Buckthorn Seed Oil
SD	Standard Deviation
SDA	Stearidonic Acid
SET	Single Electron Transfer
SOD	Superoxide Dismutase
THC	Tetrahydrocannabinol
UAS	Upstream Activating Sequences
UFAs	Unsaturated Fatty Acids
UK	United Kingdom
UV	Ultra Violet
UVA	Ultra Violet A
UVB	Ultra Violet B
WHO	World Health Organization
CYPs	cytochrome P450 monooxygenases
GSTs	glutathione S-transferases

Abstract

Plant oils are widely used in cosmetic and pharmaceutical formulations due to their bioactive constituents and antioxidant properties; however, their safety and biological effects require systematic evaluation. This study aimed to conduct an integrated chemical, antioxidant and biological assessment of selected plant oils namely, calendula (*Calendula officinalis*), echium (*Echium plantagineum* or *Echium vulgare*), sea buckthorn (*Hippophae rhamnoides* L., *Elaeagnaceae*), and spelt (*Triticum spelta* L.) with potential cosmetic relevance.

Oil composition was characterized using GC–MS, antioxidant activity was assessed by DPPH radical scavenging, and in silico toxicity prediction was applied to major constituents and suggested that while several major constituents showed low predicted toxicity, certain compounds anywhere predicted to be safe (i, e., no risk profile). Biological effects were evaluated in two *Drosophila melanogaster* strains (Lancaster and wDah) using lifespan and negative geotaxis (climbing) assays.

GC-MS analysis confirmed the presence of antioxidant constituents in the oils. The DPPH radical scavenging assay experimentally verified their activity, showing that the antioxidant potency follows the order: Sea Buckthorn > Calendula > Echium > Spelt.

Survival analyses revealed pronounced strain-dependent responses. In the Lancaster strain, calendula and echium oils caused a clear reduction in survival at all tested concentrations, whereas sea buckthorn and spelt oils showed limited toxicity at low doses but induced reduced survival at higher concentrations. In contrast, none of the oils significantly affected survival in the wDah strain.

Locomotor analysis demonstrated oil-, dose-, and time-dependent effects. In the Lancaster strain, sea buckthorn oil preserved climbing performance at early time points, while prolonged exposure to all oils resulted in a marked decline in negative geotaxis performance. Spelt oil caused a dose-dependent reduction in climbing ability, whereas calendula and echium oils consistently impaired locomotor function. In the wDah strain, early climbing performance was largely maintained, with declines over time driven primarily by exposure duration rather than dose.

Overall, this study demonstrates that the biological effects of plant-based oils are strongly concentration-dependent and are modulated by both their chemical composition and the genetic background of *Drosophila melanogaster* flies. The integrated approach employed provides a comprehensive framework for evaluating both efficacy and safety of plant oils, supporting their informed application in cosmetic and related formulations.

Chapter 1: Introduction and Literature review

1 introduction

1.1 Background

In the 21st century, the cosmetic industry has experienced remarkable growth, marked by a 23% rise in the number of cosmetic products within the overall consumer market. As a result, the demand for premium-quality products, particularly those derived from plant-based raw materials, has been steadily increasing.[1] Natural bioactive compounds, particularly those derived from herbs, are widely utilized in alternative (green) medicine and cosmetic formulations. Research has mainly concentrated on medicinal plants abundant in secondary metabolites, bioactive constituents, or phytopharmaceuticals that demonstrate potent pharmacological effects such as anti-inflammatory, antimicrobial, antiparasitic, antimutagenic, antifungal, antioxidant, anti-aging, and anticancer activities.[1]

Secondary metabolites play a crucial role in the plant's chemical defense mechanisms and seemed to have appeared as a response of plants to the interactions with predators throughout the millions of years of co-evolution. These compounds are generally categorized into three main classes: terpenes, phenylpropanoids and N- and S-containing compounds. Among them, more than 3,000 essential oils (EOs) have been identified.[2] Essential oils have been utilized as natural antimicrobial agents in the pharmaceutical, food, and cosmetic industries for a considerable period.[3]

In addition, because they are considered more "natural" than most used medicines, their medical interest is increasing, and essential oils are slowly replacing drugs and medicines due to their reduced risk of side effects.[4] The increasing interest in essential oils may be partly attributed to consumer perception that "natural" products are inherently safer than synthetic medicines. [5, 6] This perception has been discussed in the context of chemophobia, a phenomenon describing heightened concern toward synthetic chemicals, despite limited correlation with actual toxicological risk.[7, 8]

EOs are basically substances that are extracted from different parts of plants, such as leaves, fruits, roots, or flowers, and are composed of a mixture of hydrocarbons, ethers, esters, alcohols, aldehydes, phenols, and terpenes.[9] EOs have different properties, such as anti-inflammatory, antioxidant, antibiotic, and antiviral activities. In many jurisdictions such as the European Union, essential oils used for cosmetic purposes are governed by Regulation (EC) No. 1223/2009 on cosmetic products.[10] This regulation defines permitted ingredients, required safety documentation, and maximum concentration limits for fragrance and allergenic substances in cosmetics.[11] Several constituents of essential oils are restricted in cosmetic applications unless present below specific thresholds or declared on the product label.[12]

Higher concentrations that may be necessary for antimicrobial or antifungal activity would likely move the product out of the cosmetic category and into a medicinal or therapeutic classification requiring additional regulatory approval and clinical validation.[12] Consequently, products on the market typically focus on antiaging, antioxidant and other relevant cosmetic properties, which align with market demands and compliance requirements.[9] On the other hand, their widespread use and potential benefits, there is still a significant lack of information regarding the safety and efficacy of essential oils. Reports of adverse effects such as neurotoxicity, hepatotoxicity and reproductive abnormalities show the need for further research in this area.[9]

To generate this knowledge, it is important to use animals as a live model organism. However, this issue has been widely debated within the scientific community, largely due to variations in ethical standards and regulatory frameworks across countries. So, this project aims to assess the antioxidant and antiaging potential and safety profile of plant-derived bioactive compounds through *Drosophila melanogaster*-based assays, providing insights for their possible cosmetic applications.[9] *Drosophila melanogaster* is a well-established model organism that has been extensively used to evaluate the toxicity of various compounds, including metals and plant extracts.[9] From a chemistry perspective, this project investigates the antioxidant activity of selected oils and their major constituents using the *1,1-diphenyl-2-picrylhydrazyl* (DPPH) (Figure 1) radical scavenging assay and Gas Chromatography–Mass Spectrometry (GC–MS).

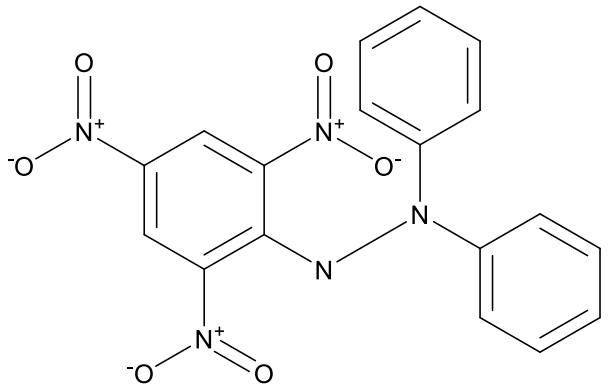


Figure 1. Structure of DPPH (drawn using Chem Draw).

Furthermore, in silico toxicity prediction is conducted to support our understanding of the toxicological properties of the major identified compounds, providing additional insight into their biological safety and potential mechanisms of action.

1.2 Motivation

The specific area of essential oils in the cosmetics industry was chosen as secondary metabolite and skin treatment both play a huge role in the development of new and existing products. Many traditional medicinal plants have been analyzed using advanced analytical methods (e.g., HPLC, MS, FTIR), leading to the discovery of various promising compounds for use in modern cosmetic formulations.[13] According to European Cosmetic Regulations and United States Food and Drug Administration (FDA) regulations, there is ban on using some essential oils such as Tea Tree Oil [14, 15] and Cannabinoids [16, 17] due to the toxicity, allergenic and tetrahydrocannabinol (THC) content of hemp oil (the psychoactive compound in cannabis) respectively. So, alternative oils were chosen which have received less attention despite their anti-aging and antioxidant properties. This review explores existing research on echium, calendula, sea buckthorn, and spelt oil to help the formulation of new cosmetic products with properties perhaps more elusive than anti-aging and antioxidant.

Given the vast number of EOs available, this study employs a targeted selection process, utilizing EOs produced by a local industry partner specializing in supercritical carbon dioxide (CO_2) extraction (CO_2 Extraction UK Ltd. In the Morecambe). The involvement of industry collaboration further refines the research direction by providing valuable insights and industry-specific perspectives. This collaborative approach ensures that the study remains both scientifically rigorous and commercially viable, ultimately contributing to the advancement of EOs applications in cosmetics.

1.3 Key Theme

This review begins with exploration of recent in vitro research on the oils and touching on the cosmetics industry's need and use of these oils in cosmetic formulation, and specific experiments on evaluating the final properties of products by assessing the applied active ingredients.

2 General Research Area

2.1 Essential oils

Throughout human history, the plant kingdom has served as a rich source of biologically active ingredients. Since ancient times, traditional medicinal plants have been recognized for their diverse biological properties and their role as an important source of bioactive compounds. For thousands of years, these plants have been used for health-care applications (e.g., remedy of disease). Advances in modern analytical techniques now allow for detailed qualitative and quantitative studies, enabling the isolation and evaluation of even trace amounts of compounds present in plant tissues. One particularly promising field of research involves exploring the potential of medicinal plant extracts to inhibit the growth and reduce the prevalence of serious pathogens.[18]

Based on their functional roles in cellular metabolism, organic compounds are broadly classified into two major categories: primary and secondary metabolites (Figure 2). Primary metabolites are defined as molecules directly involved in the biosynthetic and energy-generating pathways essential for cellular growth, development, and maintenance. These include amino acids, which form the building blocks of proteins; nucleotides, which constitute nucleic acids; simple sugars, which serve as energy sources and structural components of polysaccharides; and phospholipids, which are key constituents of cellular membranes. In contrast, secondary metabolites are not directly required for the basic metabolic processes of the organism and were historically regarded as non-essential or as mere by-products of primary metabolism. However, increasing evidence now highlights their crucial ecological and physiological functions, particularly in plant defense mechanisms and interactions with the environment.[19]

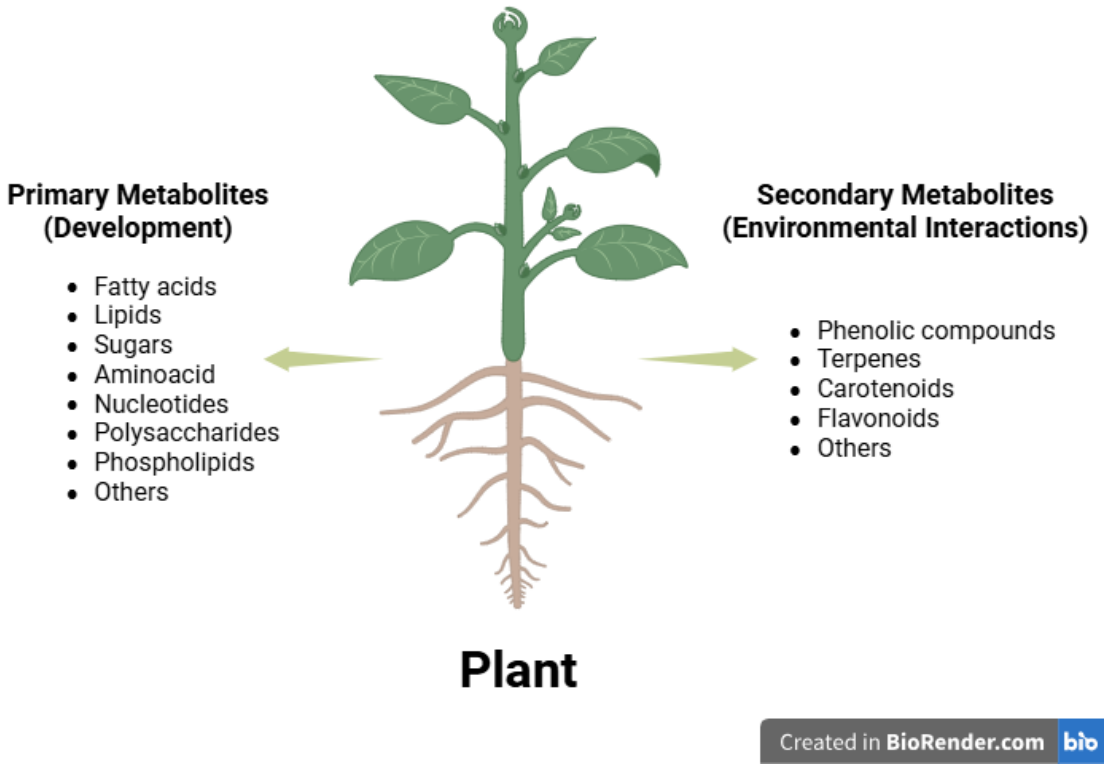


Figure 2. The difference between primary and secondary metabolites in a plant. (drawn using biorender)

It is widely accepted that the majority of the approximately 100,000 known secondary metabolites play essential roles in plant chemical defense systems, enabling plants to respond and adapt to biotic stresses such as herbivory and pathogen attack throughout millions of years of co-evolution. These secondary metabolites are generally classified into three major groups: terpenes, phenylpropanoids, and nitrogen- and sulfur-containing compounds. Among these, more than 3,000 EOs have been identified to date. [2] EOs have a long history of use as natural antimicrobial agents and are increasingly being incorporated into pharmaceutical, food, and cosmetic formulations.[3] Chemically, EOs are volatile, hydrophobic liquids produced by aromatic plants as secondary metabolites. They represent complex mixtures of low-molecular-weight compounds, typically characterized by a strong and distinctive odor. EOs can be obtained from various plant organs, including flowers, buds, leaves, stems, seeds, fruits, bark, and roots**,** through different extraction techniques.[20]

In addition, EOs comprise a wide range of molecular analogues that belong to various structural classes, including hydrocarbons, aldehydes, alcohols, ketones, and volatile or natural phenolic compounds.[21] The broad-spectrum biological activities of EOs are largely attributed to the complexity and variability of their chemical composition, which can significantly influence their mode of action.[20] Such compositional diversity is, in turn, affected by multiple factors, including local climatic conditions, seasonal variations, geographical origin, and experimental parameters involved in extraction and analysis.[22] Medicinal plants, therefore, represent an invaluable reservoir of natural bioactive compounds, offering vast potential for exploring multi-level interactions, among different constituents within a plant, between various plant organs or species, and even between plant-derived compounds and non-plant-based antimicrobials.[23]

Moreover, the combination of essential oils with other agents has been explored, as such combinations may result in enhanced efficacy through synergistic interaction.[22, 24] This review discusses several oils, namely calendula (*Calendula officinalis*), echium (*Echium plantagineum* or *Echium vulgare*), sea buckthorn (*Hippophae rhamnoides* L., *Elaeagnaceae*), and spelt (*Triticum spelta* L.) (Figure 3), which exhibit antioxidant, antiaging, antifungal, anti-inflammatory, antibacterial, anti-acne, and anticancer properties. It is important to note that although most literature refers to the EOs of these plants, in the present research the fixed oils were used, which also contain bioactive compounds responsible for similar beneficial effects. Moreover, there is limited research on the direct synergistic effects among these oils; however, based on their individual properties, they likely complement each other well, especially in skincare and hair care formulations.

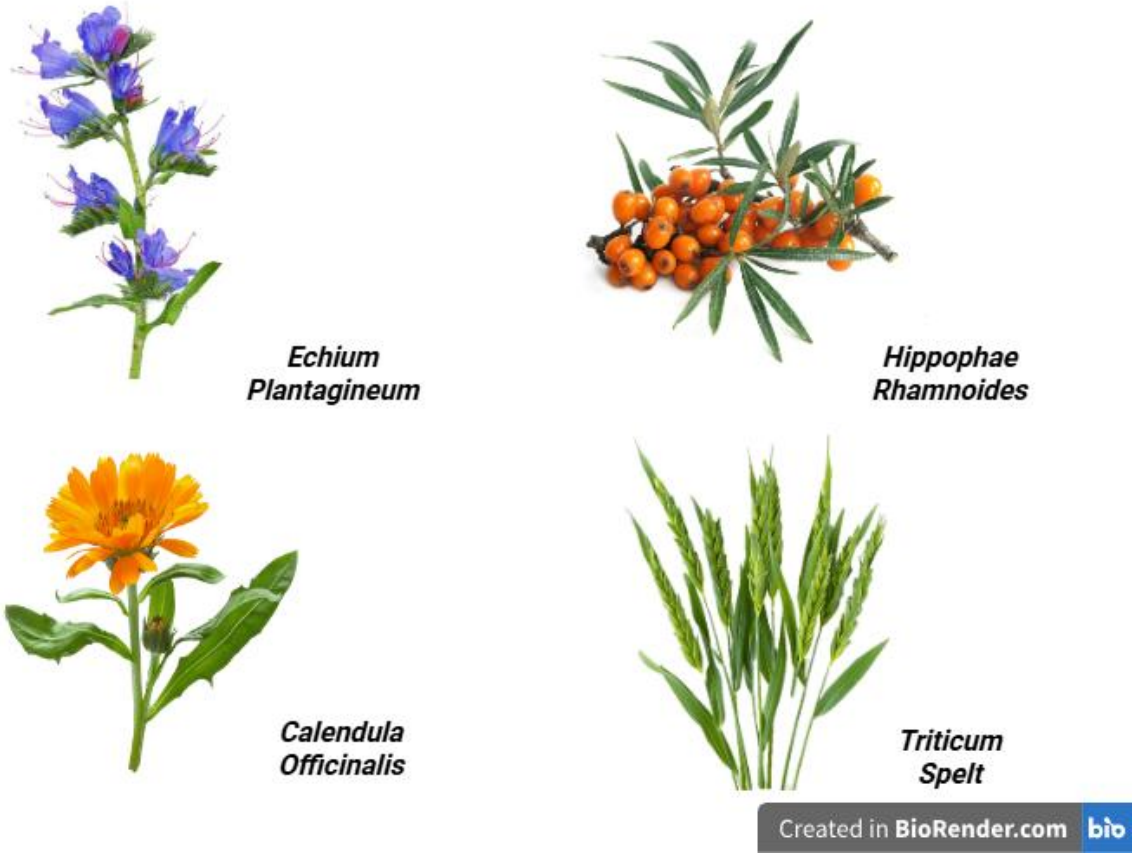


Figure 3. Representative plant sources used in this study: Calendula (Calendula officinalis), Echium (Echium plantagineum), Sea buckthorn (Hippophae rhamnoides L., Elaeagnaceae), and Spelt (Triticum spelta L.). (drawn using biorender)

2.1.1 Calendula (Calendula officinalis)

The *Calendula* genus (Figure 3), a member of the Asteraceae family, comprises between 10 and 25 species**,** among which *Calendula officinalis L.* is the most well-known and historically significant medicinal plant. These species, commonly referred to as pot marigolds, are widely utilized in pharmaceutical, cosmetic, and herbal medicine industries due to their anti-inflammatory, antimicrobial, and wound-healing properties. Other notable species within the genus include *Calendula arvensis* (field marigold), a wild variety often found in agricultural fields; *Calendula maritima* (sea marigold), an endangered species native to the Mediterranean coastal regions; *Calendula suffruticosa*, a perennial shrub-like plant adapted to arid environments; and *Calendula palaestina*, which is primarily distributed in the Middle East, although limited information is available about it.[25]

The Committee on Herbal Medicinal Products (HMPC) [26] of the European Medicines Agency (EMA) [26] recommends the use of *Calendula* flower preparations for skin inflammations, minor wounds, and inflammations of the mouth or throat based on traditional use. Although robust clinical evidence from large randomized controlled trials is lacking, *Calendula*-containing medicines have been safely used for at least 30 years. Available clinical data consist mainly of small-scale or non-randomized studies suggesting potential benefits in the management of certain skin condition.[27]

While various *Calendula* species share similar morphological features, *C. officinalis* is distinguished by its high concentration of bioactive compounds.[28] *C. officinalis* is an annual or biennial herbaceous plant, typically 30–60 cm in height, characterized by sessile, hispid, acute, and oblanceolate leaves. The leaves are hairy, alternate, petiolate, oblong, and spatulate, with entire or sparsely toothed margins. The lower leaves are oval with rounded tips, while the upper leaves are lanceolate with pointed ends. Leaf blades range from 2 to 4 inches in length. The plant develops a taproot with numerous secondary roots.[29]

The chemical composition of *Calendula* species has been extensively investigated.[28] They contain terpenoids, flavonoids, saponins, sterols, phenolic acids, coumarins, quinones, amino acids, essential oils, carotenoids, and other biologically active constituents responsible for anti-inflammatory, antioxidant, immunostimulant, antibacterial, antiviral, hepatoprotective, antispasmodic, and antitumor activities. Among these**,** triterpenoids, flavonoids, essential oils, and sesquiterpene**s** are considered the principal bioactive components of the extracts. Thus, *C. officinalis* synthesizes a wide range of compounds with diverse pharmacological actions that make it valuable for therapeutic applications. However, large-scale cultivation requires significant agricultural land and financial investment, and the quality of the plant material depends heavily on environmental factors such as temperature, rainfall, and soil contamination.[28]

Studies have demonstrated that *C. officinalis* oil exhibits significant therapeutic potential, including antifungal activity against various clinical fungal strains and protective effects against UV-B-induced oxidative damage in skin tissue.[30] Over the years, these pharmacological properties have been recognized by several authoritative organizations, including the European Scientific Cooperative on Phytotherapy (ESCOP), the European Medicines Agency (EMA), and the World Health Organization (WHO), which highlight the plant's wound-healing and anti-inflammatory activities.[31]

2.1.2 Echium (Echium vulgare)

The Echium genus is part of the Boraginaceae family, which includes around 148 genera and over 2,500 species, ranging from annuals and biennials to perennials, and occasionally shrubs, trees, or lianas.[32] These plants typically have stems and leaves densely covered with trichomes, both glandular and non-glandular types. Most species are native to Europe, Africa, and Asia (notably India and Iran), with some being endemic to limited regions such as Sardinia, Anatolia, and Uzbekistan.[33] Within this family, there are both rare and endangered species (for example, *Echium Russicum* in Poland and *Rindera Umbellata* in Moldova) as well as invasive species like *Anchusa officinalis*, *Echium vulgare*, and *E. plantagineum*, particularly in the Americas.[34]

Plants of the Boraginaceae family have been used in traditional medicine for over 2,000 years, both internally and externally, particularly in regions where they naturally grow and are easily accessible [34]. This family includes medicinally and cosmetically valuable plants that hold significance in pharmacology and cosmetology. Modern chemical analyses have validated many of their ethnopharmacological properties, leading to growing scientific and commercial interest in these species. This is largely due to their bioactive compounds, which can influence cellular functions and enhance skin appearance.[34] The therapeutic benefits of Boraginaceae plants are primarily attributed to the presence of various biologically active molecules, such as fatty acids, essential oils, phenolic acids, flavonoids, anthocyanins, tannins, naphthoquinones, saponins, allantoin, mucilage, pyrrolizidine alkaloids, and silica (SiO_2), which have been isolated from different plant parts of this family.[34-36]

Several clinical studies have confirmed the beneficial effects of Boraginaceae extracts in reducing inflammation and alleviating symptoms associated with various conditions, including gastrointestinal disorders, rheumatoid arthritis, atopic dermatitis, eczema, and psoriasis.[37, 38] Additionally, these extracts exhibit antimicrobial activity, being capable of destroying, inhibiting, or preventing the growth of microorganisms. Several anticancer compounds have also been identified within this plant family. Nevertheless, some toxic constituents have been reported, primarily linked to the presence of pyrrolizidine alkaloids (PAs), which are responsible for the potential toxicity observed in certain Boraginaceae species.[34]

The Boraginaceae family also comprises various herbaceous species, shrubs, and biennials. These plants are recognized for their bright, attractive flowers, their rich omega fatty acid content in certain species such as *Echium plantagineum* and *Echium vulgare*, and their ability to attract pollinators. Many members of this family are known to produce a diverse range of secondary metabolites, including alkaloids, naphthoquinones, polyphenols, phytosterols, and terpenoids. Among these, polyphenolic compounds, notably flavonoids and phenolic acids, exhibit a wide spectrum of pharmacological activities, such as antibacterial, anti-inflammatory, antiproliferative, antidepressant, antioxidant, antiparasitic, and antiviral effects.[1, 18, 39]

A review of the literatures reveal that Echium species have long been used in traditional medicine for their diuretic, diaphoretic, febrifuge, expectorant, analgesic, wound healing (vulnerary), sedative, and anxiolytic properties.[1] However, limited information is currently available regarding the phytochemical composition and biological activities of *Echium arenarium*. Other species, such as *E. plantagineum* (used for crop protection), *E. amoenum* (used as a tranquilizer and cardiac tonic), and *E. vulgare* (employed in ethnoveterinary medicine), have been found to contain various bioactive constituents, including shikonin derivatives, flavonoids, phenolic acids, pyrrolizidine alkaloids, fatty acids[40], as well as naphthoquinones[41], sterones[42], and phenol carboxylic acids[43]. These compounds are responsible for the diverse pharmacological effects attributed to the genus Echium.[1]

Echium oil is a complex mixture composed of aromatic and volatile compounds that belong to various chemical classes with diverse properties [43]. In members of the Boraginaceae family, these oils are primarily produced in glandular trichomes found on the flowers, leaves, fruits, and seeds.[44] Although the chemical composition of oils varies among species, certain compound groups, such as simple phenolics and terpenes, are largely responsible for their characteristic biological activities. The qualitative and quantitative profiles of these oils are species-dependent.[34]

Even within the same species, the composition and concentration of oil constituents can fluctuate due to factors such as geographical origin, plant age, cultivation conditions (e.g., soil moisture and nutrient availability), and environmental factors like light, humidity, and temperature.[45] Generally, Boraginaceae oils exhibit a highly complex composition, containing anywhere from a few to over a hundred individual compounds. The main bioactive constituents identified include simple phenolics (e.g., *thymol, carvacrol*), monoterpenes (*α-pinene, eucalyptol*, *α- and β-phellandrene*), diterpenes (*phytol*), sesquiterpenes (*α-bisabolol, α-humulene, trans-caryophyllene, alloaromadendrene, α-eudesmol, δ-cadinene, β-caryophyllene, β-gurjunene, β-ionene*), alkanes (*heptane, hentriacontane, eicosane*), esters (*di-isobutyl phthalate, methyl salicylate*), benzopyrones (*coumarins*), and aldehydes (*nonanal, benzene acetaldehyde = hyacinthine*).[34, 45-47]

2.1.3 Sea Buckthorn (Hippophae Rhamnoides L., Elaeagnaceae)

Sea buckthorn (*Hippophae rhamnoides L., Elaeagnaceae*) is native to Eurasia and grows both wild and cultivated across various countries, including Britain, China, Finland, France, Germany, India, Myanmar, Nepal, Pakistan, Romania, and Russia. Often referred to as the "wonder plant," it has wide-ranging applications in the food, medicinal, cosmetic, and pharmaceutical industries.[48] Research has shown that all parts of the plant, fruits, leaves, seeds, and roots, are rich in bioactive compounds such as carotenoids, flavonoids, tocopherols, sterols, triterpenols, isoprenols, organic acids, and vitamins[49-51], which contribute to its antibacterial, antioxidant, anti-inflammatory, antistress, and immunomodulatory activities.[49] The phytochemical composition of sea buckthorn varies depending on fruit maturity, species, size, geographic location, climate, and extraction method.[52]

Traditionally, sea buckthorn fruits, leaves, and seeds have been used in the treatment of inflammation, gastric ulcers, cardiovascular disorders, oxidative stress from radiation, hyperlipidemia, gingivitis, and various eye and skin conditions. Due to its skin-protective effects, sea buckthorn oil is widely employed in cosmetic products, including sunscreens.[49] Its therapeutic value arises from its phytochemical richness, which endows the plant with numerous biological and pharmacological activities. The fatty acids in sea buckthorn also nourish and restore skin, making it particularly effective for systemic skin disorders like atopic dermatitis. As a result, sea buckthorn extract is frequently incorporated into skin-softening and anti-aging formulations, helping to combat wrinkles, dryness, and other signs of premature skin aging.[49, 53]

Additionally, sea buckthorn oil provides exceptional support for women's health, helping to alleviate perimenopausal symptoms and promote overall well-being throughout life. Its numerous health-promoting effects include antioxidant, cardioprotective, anti-atherogenic, antibacterial, antiviral, immunomodulatory, anticarcinogenic, antitumor, and antiradiation properties. Notably, sea buckthorn seed oil (SBS) strongly absorbs in the UV-B range (290–320 nm), making it a natural sunscreen agent.[54]

The antioxidant activity of sea buckthorn extract was compared with vitamin C and Butylated hydroxyanisole (BHA) which are shown in Figure 4, they are typical antioxidant ingredients. The SBS extract exhibited antioxidant activity comparable to vitamin C and higher than BHA. After storage for 70 days at room temperature, the antioxidant activity of the SBS extract and BHA remained essentially unchanged, whereas vitamin C showed a significant reduction of approximately 30–40% in antioxidant activity. Furthermore, thermal stability tests demonstrated that the SBS extract retained its antioxidant activity up to 150 °C, compared with 100 °C for BHA and 200 °C for vitamin C, indicating favorable stability of the SBS extract under elevated temperature conditions [55]

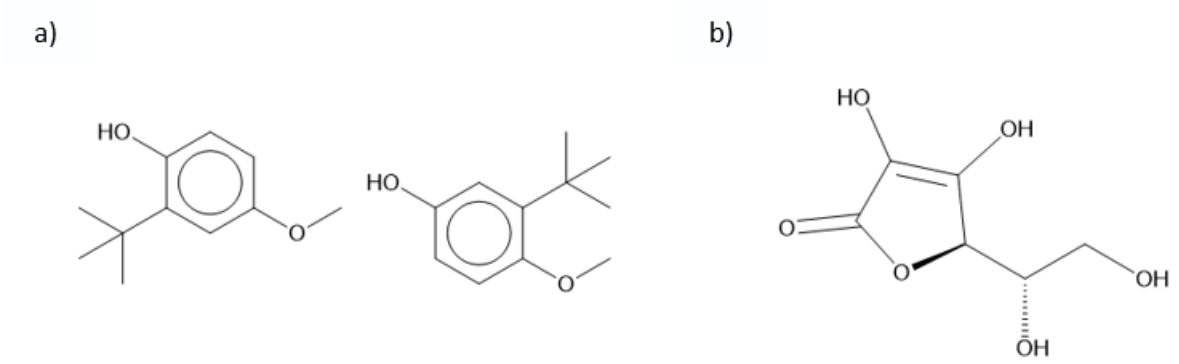


Figure 4. Structure of a) Butylated hydroxyanisol (BHA consists of a mixture of two isomeric organic compounds, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole. It is prepared from 4-methoxyphenol and isobutylene.) and b) Vitamin C (drawn using Chem Draw).

Sea buckthorn oil can be extracted from both the seeds and fruit pulp, which differ in chemical composition and bioactive content. Both oils are abundant in unsaturated fatty acids (UFAs), notably palmitoleic acid (C16:1), a rare component highly valued in cosmetology, as well as tocopherols, tocotrienols, and plant sterols. The pulp oil is particularly rich in carotenoids.[56] What distinguishes sea buckthorn fruit oil from other vegetable oils is its unique fatty acid profile, which includes omega-7 palmitoleic acid, a natural constituent of skin lipids that stimulates regeneration and accelerates wound healing. It also contains saturated fatty acids (palmitic and stearic acids) and a broad range of essential unsaturated fatty acids, such as alpha-linolenic (omega-3), gamma-linolenic (omega-6), linoleic (omega-6), oleic (omega-9), and eicosanoic (omega-9) acids.[56]

The gamma-linolenic acid (GLA) in sea buckthorn plays a key role in cell membrane integrity, epidermal nourishment, and oxygen supply to skin tissues. It can penetrate deeply into the skin and convert to prostaglandins, thereby protecting the skin from infections, reducing inflammation, easing allergic responses, and slowing the aging process.[56-58]

Furthermore, sea buckthorn oil contains a wide spectrum of vitamins, including A, C, E, F, P, and B complex. Vitamin A, present in the form of carotenoids (≈200 mg/100 g), contributes to the oil's regenerative and anti-wrinkle effects. Vitamin C, found at levels up to 15 times higher than in oranges (≈695 mg/100 g), provides potent antioxidant protection and defends against UVA and UVB radiation.[56, 59, 60]

Recent studies have also shown high concentrations of proanthocyanidins in sea buckthorn's seeds, roots, flowers, green berries, and stems.[61] These compounds exhibit multiple physiological and therapeutic effects, including anticancer and cardioprotective activities, retinal protection against photodamage, prevention of diabetic nephropathy, and regulation of body weight by modulating thermogenesis and gut microbiota.[62, 63] Proanthocyanidins also inhibit lipid peroxidation, platelet aggregation, and capillary hyperpermeability, with emerging evidence supporting their role in delaying skin aging.[64]

2.1.4 Spelt (Triticum spelta L.)

Spelt (*Triticum spelta* L.) is an ancient wheat species that has been traditionally cultivated in several Central European countries. Over the past few decades, it has attracted renewed interest as a healthier and more natural alternative to modern common wheat (*Triticum aestivum* L.), due to its lower degree of intensive breeding. Historical evidence suggests that spelt originated around 5000 BC and may be considered one of the ancestors of modern wheat. In response to the growing demand for organic and sustainable agricultural practices, spelt cultivation has gained importance once again, often being grown under organic conditions with the use of natural fertilizers and pesticides rather than synthetic chemicals.[65]

Spelt exhibits distinct physical, nutritional, and biological characteristics compared to common wheat. Its protective husk provides greater resistance to various environmental stresses, including frost, low temperatures, pests, and other adverse conditions. However, its longer stems make it more susceptible to lodging when exposed to fierce winds. In addition, spelt generally requires less fertilizer for cultivation than common wheat, reflecting its adaptability to low-input agricultural systems.[65-67]

With the expansion of commercial wheat production, spelt cultivation declined, and it was largely limited to small-scale production for livestock feed. Recently, however, spelt has regained attention in human diets due to its perceived superior health benefits compared to common wheat. It is considered easier to digest, making it suitable for individuals with gastrointestinal issues, as well as for children and the elderly. Although spelt contains gluten like wheat, its gluten has a different chemical structure that is more digestible, less harmful, and less likely to trigger allergic reactions. Consumption of spelt has been associated with a lower risk of various chronic diseases, including cancer, which is attributed to its content of polyphenolic compounds and their antioxidant properties.[65]

Phenolic acids and their antioxidant activity represent important components of wheat that have been extensively studied in recent years. The major simple phenolic acids found in wheat include ferulic, vanillic, syringic, sinapinic, caffeic, and p-coumaric acids, all of which have been identified as significant sources of dietary antioxidants. Historically, the phenolic content of whole grains was underestimated, but recent research has highlighted their substantial contribution to the nutritional and health-promoting properties of wheat.[68]

In contrast to spelt grains, which have been extensively characterized, the leaf extracts of young spelt grass have received considerably less attention that contain substantial amounts of essential nutrients and bioactive compounds, suggesting potential health-promoting effects for humans.[65]

Table 1 *Summary of the main bioactive compounds, skin benefits, safety considerations, and special characteristics of plant-derived oils used in this research.*

Oil	Key Bioactive Compounds	Main Skin Benefits	Safety (Toxicity Notes)	Special Characteristics	References
Echium Oil	**Z,** Z-8,10-Hexadecadien-1-ol, γ-linolenic acid (GLA), omega 3/6/9, I-(+)-Ascorbic acid 2,6-dihexadecanoate,	• Anti-inflammatory, • antimicrobial, • antioxidant, • antiproliferative, • antiviral, • wound healing.	Some species contain toxic pyrrolizidine alkaloids (PAs)	High SDA content; fast absorbing; suitable for sensitive skin	[1, 18, 39, 41, 44, 47]
Calendula Oil	8,11,14-Eicosatrienoic acid (Z, Z, Z-), I-(+)-Ascorbic acid 2,6-dihexadecanoate, 12-Octadecadienoic acid (Z, Z) (Linoleic acid)	• Anti-inflammatory, • Antimicrobial, • Wound healing, • Antioxidant, • Immunostimulant	Considered safe; widely approved by EMA, ESCOP, WHO	Potent anti-inflammatory profile; traditional medicinal use	[28-30]
Sea Buckthorn Oil	Palmitoleic acid (omega 7), Ascorbic acid 2,6-dihexadecanoate, vitamin C, 9,12-Octadecadienoic acid (Z, Z) (Linoleic acid)	• Antioxidant, • Anti-inflammatory, • antibacterial, • antiviral, • immunomodulatory, • cardioprotective, • anticarcinogenic, • UV-protective	Generally safe; rich in nutrients; widely used in food and cosmetics	Extremely rich in antioxidants; ideal for dry/damaged skin	[49, 53-56, 69]
Spelt Oil	9,12-Octadecadienoic acid (Z, Z) (Linoleic acid) 9-Octadecenoic acid (Oleic acid) Ascorbic acid 2,6-dihexadecanoate	• Moisturizing, • Antioxidant, • anti-cancer (polyphenols), • supports digestion	Contains gluten (more digestible form); generally safe	Unique sterol profile; beneficial for mature and dry skin	[65, 67, 68]

3 Uses in Cosmetics

A cosmetic product refers to any substance designed for application on the external parts of the human body, primarily to cleanse, protect, perfume, or modify its appearance. Cosmetic creams are semi-solid formulations with a viscosity high enough to prevent flow at room temperature, commonly existing as emulsions or gels. Depending on the type of dispersed phase, emulsions can be categorized as oil-in-water (o/w), water-in-oil (w/o), or a combination of both.[52]

The term “cosmeceuticals” refers to cosmetic products that exhibit pharmaceutical-like activity, often due to the inclusion of natural bioactive ingredients with beneficial effects. North America represents the largest market for natural and organic personal care products, followed by Europe and the Asia–Pacific region, with China and India playing key roles in the global herbal cosmetics industry. Among various categories, natural skincare products continue to dominate the global organic beauty market and were projected to become the most attractive segment, accounting for approximately 30.9% of the market share by 2024.[70] In 2023, the herbal beauty products market was valued at around US$ 73 billion, representing nearly 5.9% of the total beauty industry [71]. Overall, the global demand for plant-based cosmetics has experienced remarkable growth in recent years.[52]

For decades, numerous bioactive compounds obtained from plant sources have attracted scientific attention, not only as potential therapeutic agents but also as valuable constituents responsible for the desired effects of cosmetic formulations. The growing emphasis on health-conscious and eco-friendly lifestyles has further stimulated interest in natural products, particularly Phyto-cosmetics.[52]

Natural cosmetics are primarily preferred by individuals who value environmental sustainability, health, and beauty. Phytotherapy is generally regarded as a safe and often effective alternative to conventional treatments, particularly for certain skin disorders. However, the continuously increasing demand for natural ingredients presents a considerable challenge for cosmetic manufacturers.[52]

3.1 Challenges and Necessities of Production

Producing high-quality cosmetic products demands interdisciplinary cooperation among chemists, cosmetologists, botanists, toxicologists, and biologists. To remain competitive in a rapidly evolving market, it is essential to develop innovative formulations and continuously seek new natural ingredients that match or surpass the efficacy of conventional, often synthetic, compounds. Although sourcing natural raw materials from certified crops typically incurs higher costs than using synthetic alternatives, the investment is justified by the benefits, organic certifications, enhanced product appeal, and ultimately, greater consumer interest and trust.[72, 73]

This growing interest stems from the fact that the skin is a vital organ forming a protective barrier that covers the entire body, shielding it from harmful external factors. In addition to protecting against light, heat, and mechanical injury, the skin also serves as a defense against microorganisms, helps regulate body temperature, and enables sensory perception of touch, warmth, and cold. Increasing awareness of skin health and nourishment has consequently driven the demand for skincare cosmetics. As the body ages, the skin undergoes changes influenced by both intrinsic factors, such as cellular metabolism, genetics, and hormonal activity, and extrinsic factors, including prolonged exposure to pollution, ultraviolet radiation, ionizing radiation, toxins, and chemicals.[74]

3.2 Skin Structure and Integrity

The proper function and appearance of the skin relies on maintaining a delicate balance between the water content of the stratum corneum and the lipids present on the skin surface. This equilibrium can be disrupted by external factors such as humidity, ultraviolet radiation, temperature fluctuations, and hormonal changes. Structurally, the dermis consists predominantly of interwoven collagen fibers arranged in bundles.[75]

The collagen fibers in the papillary dermis are finer than those in the deeper reticular layer. When the skin is stretched, the collagen, due to its high tensile strength, prevents tearing, while the elastic fibers interlaced with it allow the skin to return to its original shape. With advancing age, numerous changes occur in the skin: wrinkles and pigmentation become more pronounced, moisture and lipid levels decrease, and the skin loses elasticity, leading to sagging.[75]

Assessing skin elasticity is crucial, as it is a less immediately visible indicator of aging compared to features like wrinkles. Research has shown that during the aging process, the elastic fibers within the papillary dermis gradually degenerate and lose their branched structure [76, 77]. The mechanical properties of the skin are extremely sensitive to epidermal hydration, which can be significantly improved using moisturizers. Non-invasive measurements of skin elasticity provide an objective and quantitative means to evaluate the complex effects of various dermatological and cosmetic formulations on the mechanical behavior and hydration of the epidermis.[75]

3.3 The Role of Emulsions in Skin Nutrition

Ultimately, skin nourishment through certain bioactive compounds can significantly influence the mechanical properties of the epidermis. One effective method of delivering such nourishment is through emulsions. Emulsions have shown enormous potential across the pharmaceutical, food, and cosmetic industries. Recently, they have gained renewed attention as efficient delivery systems for active ingredients, owing to their numerous advantages, including enhanced bioavailability of therapeutic agents. Within an emulsion, the therapeutic efficacy and spreadability of the active components are improved. Emulsions can take various forms, such as water-in-oil or oil-in-water systems, and are commonly used in creams, shampoos, gels, lotions, cleansing milks, serums, mousses, gel-creams, balms, fluids, and sticks.[75, 78]

These products can be classified as “dermato-cosmetics” or *cosmeceuticals*, which occupy the interface between medicine and cosmetics, a sector around which a highly advanced and rapidly growing industry has emerged.[79]

Regarding active ingredients, the challenges and stresses of modern life make compounds with antioxidant, anti-aging, anti-acne, anti-inflammatory, and antibacterial properties highly valuable in contemporary dermato-cosmetic formulations. Beyond their role in combating oxidative stress in the skin, these ingredients can provide additional benefits, such as promoting regeneration and repair, and improving minor skin imperfections like pigmentation spots or fine lines. Such bioactive compounds can be sourced from a variety of plants, including those previously discussed. These active ingredients can be extracted from different plants, such as those we discussed before.[72]

As previously mentioned, cosmeceutical emulsions are typically oil-in-water (O/W) formulations, created by blending active cosmetic ingredients with vegetable oils and functional substances, such as antioxidants, anti-aging agents, and vitamins or provitamins, at concentrations sufficient to provide the skin with a rich supply of nutrients and noticeable efficacy. Certain components can stimulate physiological processes, thereby enhancing the skin's natural regeneration. To minimize the risk of adverse effects, the levels of excipients, including preservatives, waxes, synthetic oils, and other artificial additives, should be kept to the lowest effective amounts.[72, 79]

The modern production of high-quality emulsions with long-term stability necessitates the use of emulsifiers, as oil–water mixtures are inherently thermodynamically unstable due to the immiscibility of the two phases. Emulsions, while thermodynamically unstable, can achieve kinetic stability through appropriate formulation and therefore require careful monitoring of parameters such as emulsifier concentration, droplet size distribution, zeta potential, and long-term stability.[80] The choice of emulsifier influences not only the functional properties and ease of emulsion formation but also the overall stability of the final product. Contemporary cosmetic formulations often employ amphiphilic polymers, such as phospholipids, proteins, polysaccharides, and other surface-active polymers, which stabilize emulsions by adsorbing at the oil–water interface and/or increasing the viscosity of the continuous phase.[72, 80]

Plants, being abundant sources of bioactive compounds, play a pivotal role in driving the cosmetics industry. Consequently, it is essential to continuously explore, identify, and incorporate these botanical ingredients into cosmetic formulations. Modern cosmetology heavily relies on well-known and valued plants, particularly those with a long history of use in traditional medicine. Rich information on the beneficial properties of numerous plants, both for treating various ailments and enhancing skin health, can be found in historical sources, including medieval herbals produced at the scientific centers of the time, as well as in prehistoric records and legends describing traditional skin care practices.[72]

3.4 The Importance of Plant-Based Oil

As previously discussed, plant-based oils are key bioactive ingredients extensively used in cosmetic formulations due to their emollient properties, ability to protect the skin barrier, and role in restoring cutaneous homeostasis.[79] These oils provide multiple benefits, including moisturizing, nourishing, and protecting both skin and hair.[56] They are increasingly preferred over mineral oils, reflecting consumers' growing interest in herbal products that have minimal side effects, low risk of allergic reactions, and reduced occlusive effects.[79]

Beyond their role as essential formulation components, vegetable oils are incorporated into cosmeceutical products for their beneficial effects on the skin, which arise from the fatty acid chain distribution in triglycerides and their associated compounds. The chemical composition of fatty acids, glycerol, and triglycerides enables vegetable oils to reinforce the lipid barrier, prevent trans epidermal water loss, soften and smooth the stratum corneum, and reduce skin inflammation. In contrast, mineral oils are generally avoided in skincare due to their potential occlusive effects. The proportion of vegetable oils in formulations varies and is determined based on their interaction with the primary active ingredients, including antioxidants.[79]

In summary, vegetable oils are valuable bioactive components in cosmeceutical formulations due to their emollient, protective, and anti-inflammatory properties. Their chemical composition, including fatty acids and triglycerides, allows them to reinforce the skin barrier, reduce water loss, and improve overall skin condition. Although practical formulation and in vivo or ex vivo testing on skin were beyond the scope of this study, the analysis of these oils provides valuable insights into their potential applications in skincare products as functional and antioxidant ingredients.

4 Chemical/Biological Assessments

Thousands of articles have been published on the use of active ingredients, including plant-based oils, in cosmetic products, but do each of these active ingredients have the same effect on the skin? What is important in the use of methods to investigate the effectiveness of these ingredients, which can vary depending on the properties of the ingredients. Since the focus of the research is on plant-based oils with antioxidant and anti-aging properties, we will review the in-vitro methods for investigating their effectiveness.

4.1 Chemical Assessment of Antioxidant Activity

Different radicals and analytical approaches are employed to study antioxidant activity and the products of oxidation. Among these, the 1,1-diphenyl-2-picrylhydrazil (DPPH•) radicals (Figure 5) are widely used as a model for assessing the free radical scavenging capacity of compounds, due to its stability under laboratory conditions and simplicity of measurement. Other radicals, such as hydrogen peroxide (H_2O_2), as well as methods involving heavy metal removal, are also applied in antioxidant research.[81-83]

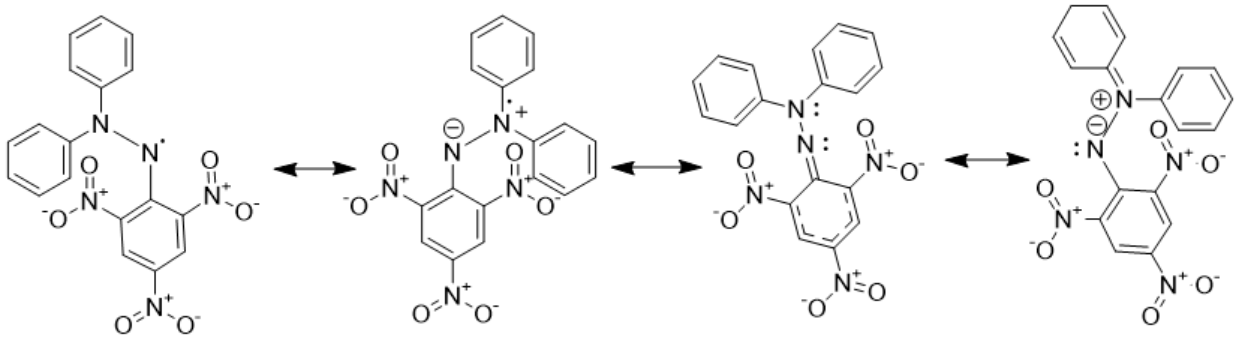


Figure 5. The chemical structures of a 1,1-diphenyl-2-picrylhydrazil radical (DPPH). (drawn using Chem Draw).

Oxidation processes are essential for cellular functions and survival. In aerobic organisms, energy is generated from organic molecules such as glucose through cellular respiration, but this process inevitably produces free radicals. Free radicals are species with an unpaired electron, which makes them highly reactive and potentially damaging to cellular components.[82]

While many free radicals are short-lived and highly reactive, some are stable under normal laboratory conditions, like DPPH• which allows their use in experimental studies of antioxidant activity. In biological systems, free radicals are strongly associated with oxidative stress, a concept increasingly recognized in medical and life sciences. Oxidative stress occurs when the production of reactive oxygen species (ROS) (Figure 6) exceeds the capacity of cellular antioxidant defenses, leading to molecular and cellular damage.[82, 84]

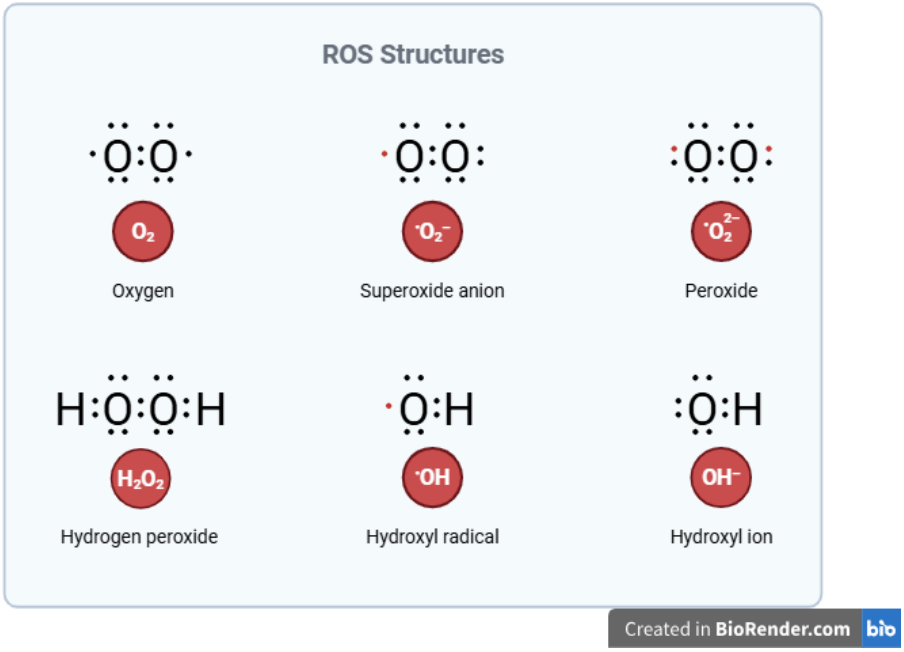


Figure 6. Overview of major reactive oxygen species (ROS), including Oxygen, Superoxide anion, Peroxide, Hydrogen peroxide, Hydroxyl radical, and Hydroxyl ion. Black dots represent paired electrons (lone pairs), while red dots indicate unpaired electrons (radicals). (drawn using biorender).

Free radicals are naturally produced in the body and play roles in normal metabolism, but excessive accumulation can contribute to degenerative conditions, including inflammation and aging. Environmental factors such as ultraviolet radiation and pollution can further exacerbate oxidative stress, highlighting its relevance to human health. Under normal physiological conditions, the formation of ROS is balanced by endogenous antioxidants, maintaining cellular homeostasis.[82]

When this balance is disrupted, excessive oxidative stress can lead to significant tissue damage, including lipid peroxidation in cellular membranes. Antioxidants act to mitigate these effects by neutralizing free radicals and interfering with oxidative processes at multiple stages.[30] The effectiveness of antioxidants depends on several factors, including their chemical structure, concentration, and interactions with other molecules.[82]

Reaction kinetics, thermodynamic properties, and molecular localization also influence antioxidant activity, determining how efficiently free radicals are scavenged, which contains the thermodynamics of the reaction between an antioxidant and a different oxidant, the reaction rate, and the antioxidant's ability to react. All these parameters should be considered when assessing the effectiveness of a specific antioxidant substance. So, there has been a parallel increase in methods used to estimate the efficacy of antioxidants in human metabolism.[82]

4.1.1 DPPH radical scavenging activity

Several bioanalytical methods are available to assess antioxidant activity. Among these, the 1,1-diphenyl-2-picrylhydrazil (DPPH) assay is the most widely used and reliable method for determining the radical-scavenging capacity of compounds.[81, 82]

This spectrophotometric method is simple, sensitive, fast, reproducible, and applicable to a wide range of samples, including beverages, pure substances, foods, and herbal extracts. This method is straightforward, sensitive, rapid, and reproducible and is therefore one of the most widely used and convenient radical scavenging assays for assessing the antioxidant capacity of compounds and herbal extracts. One of its key advantages is that it can be applied to compounds that are poorly soluble in water, making it suitable for a broader range of hydrophobic and lipophilic substances.[81]

4.1.2 Metal Chelating Assay by Ferrozine Reagent

Metal chelation involves the binding of metal ions by ligands to form stable ring-like complexes. Through this interaction, potentially harmful free metal ions are neutralized, preventing their participation in reactions that could disrupt cellular metabolism.[83]

Although trace metals such as Zn, Fe and Cu play essential roles as cofactors in numerous enzymatic processes, their accumulation beyond optimal levels can lead to toxicity. Excess free metal ions may catalyze the formation of reactive oxygen and nitrogen species (ROS and RNS), ultimately triggering oxidative damage to biomolecules, including lipid peroxidation in cellular membranes.[83]

Antioxidant activity based on metal chelation is commonly assessed using aqueous-based assays, as these methods rely on the interaction between hydrophilic chelating agents and metal ions in water-based systems. Although widely used for hydrophilic antioxidants, such aqueous-based methods are less suitable for non-polar samples, as limited solubility can interfere with the reliability of the measurements. In the case of non-polar matrices such as CO_2-extracted oils, poor solubility in aqueous media significantly compromises assay accuracy. Consequently, this method was deemed unsuitable for the oils investigated in this study, and alternative radical scavenging assays compatible with lipophilic compounds (e.g., DPPH) were selected.[85]

4.1.3 Chemical Characterization of Plant-based Oils and the Role of GC–MS

Chemical compositions of plant essential and carrier oils are inherently complex, typically containing fatty acids, terpenes, sterols, and antioxidant molecules. These constituents strongly influence their biological activities, and their detailed identification is considered the first step toward understanding functional properties and predicting potential bioactivity. Gas chromatography–mass spectrometry (GC–MS) is one of the most widely used analytical techniques for profiling volatile and semi-volatile compounds in natural oils due to its high sensitivity, selectivity, and ability to provide structural information. Through GC–MS, the major components of complex oil mixtures can be accurately identified and quantified.[86]

This type of characterization is particularly important for oils extracted by supercritical CO_2 methods, which preserve thermolabile and non-polar compounds contributing to antioxidant and other biological effects. Establishing a reliable chemical profile enables meaningful correlations between composition and outcomes observed in antioxidant or in vivo assays.[86]

The chemical composition of oils can vary depending on geographical origin, harvest season, and extraction technique. Although many plant oils may share similar classes of compounds, recent studies demonstrate that similarity in composition does not necessarily equate to similar biological efficacy; rather, the relative abundance of individual molecules plays a more critical role. Consequently, comparative analysis of oils under consistent extraction and analytical conditions has become a focus of current research.[86]

In this project, GC–MS was employed to identify the major constituents of Calendula, Echium, Spelt, and Sea Buckthorn oils, with the aim of confirming the presence of antioxidant-related molecules and supporting the results obtained from the DPPH radical scavenging assay.

4.2 Biological Assessment of Anti-aging Activity

4.2.1 Drosophila Melanogaster as a Model Organism

As discussed so far, plant-based oils have gained considerable attention across multiple industries, including food, cosmetics, and aromatherapy, due to their natural origin and bioactive properties. In fact, their perceived safety and lower risk of side effects compared to conventional medicines are driving increasing interest in medical and therapeutic applications. Furthermore, they have different properties including, antifungal, anti-inflammatory, anti-aging, antioxidant, antitumor, anticancer, etc. Approximately, all the plant-based oils exhibit at least four of these activities both in vitro and in vivo.

For example, Sea buckthorn oil exhibits multiple bioactivities including antioxidant [87], antimicrobial [87], anti-inflammatory [88] and protective effects against oxidative damage [89] in cell-based assays and animal models; species of Echium have been shown to possess antioxidant [90], anti-inflammatory [91], antimicrobial [1], and anticancer [92] effects in in vitro systems and selected in vivo studies; Calendula oil demonstrate antioxidant [93], anti-inflammatory [94], antimicrobial [95], and anticancer [95] activities in vitro and in vivo studies ; and phenolic-rich extracts from spelt seeds display significant antioxidant capacity [96] related to phenolic acids, supporting potential biological activity.[68, 97]

That is why they are interesting targets for drug discovery and diseases treatments. Although plant-based oils are using widely and they have lots of potential benefits, there is still a significant lack of information about the safety and efficacy of some oils. Reports of adverse effects, including neurotoxicity, hepatotoxicity, reproductive abnormalities, and delayed effects, underscore the necessity for further investigation in this field, while *Drosophila melanogaster* can be used as a model to do further investigation in this field of study.[98]

4.2.2 Why Drosophila Melanogaster?

Using animals as model organisms is crucial for generating such knowledge. Although there is ongoing scientific debate about the ethical and legislative differences among countries, data collected by the UK government in 2021 showed that more than three million procedures were conducted on animals, 57% of which were experimental procedures involving studies such as drug safety testing and the development of new treatments. The study of animal behavior is also important, and in many cases, animal testing cannot be completely avoided without compromising the quality of the results.[98, 99]

According to the "Three Rs"[100] guidelines, particularly the replacement principle established in 1959, the use of non-chordate animals (e.g., flies and worms) instead of chordates (e.g., mice, dogs, and primates) is one approach to conducting research in a more ethical manner, given that non-chordates are less sentient.[98]

Drosophila melanogaster is a widely used model organism for assessing the toxicity of diverse compounds, including metals and bioactive plant extracts, through endpoints such as mortality, behavior, development, reproduction, and oxidative stress biomarkers. For instance, exposure to lead (Pb) induces ROS-mediated iron dys-homeostasis, resulting in impaired development, reduced survival, decreased mobility, and lowered egg production[101]. Similarly, essential oil from *Rosmarinus officinalis* has been shown to increase mortality, impair locomotor behavior and developmental success, and induce oxidative damage by disrupting both enzymatic and non-enzymatic antioxidant systems in *D. melanogaster*. Some key advantages of using this species include its short life cycle (Figure 7), low maintenance cost in laboratory settings, and a genome that shares substantial homology with humans.[98, 102]

In addition to its genetic versatility, the fruit fly is an ideal model for behavioral studies due to the availability of well-established assays. These include the larval crawling assay, courtship and mating assays, and the adult climbing assay. Since the climbing test relies on the innate motor reflex of adult flies, it is widely used to detect impairments in locomotion and to assess conditions such as neurodegeneration that can compromise motor function.[9]

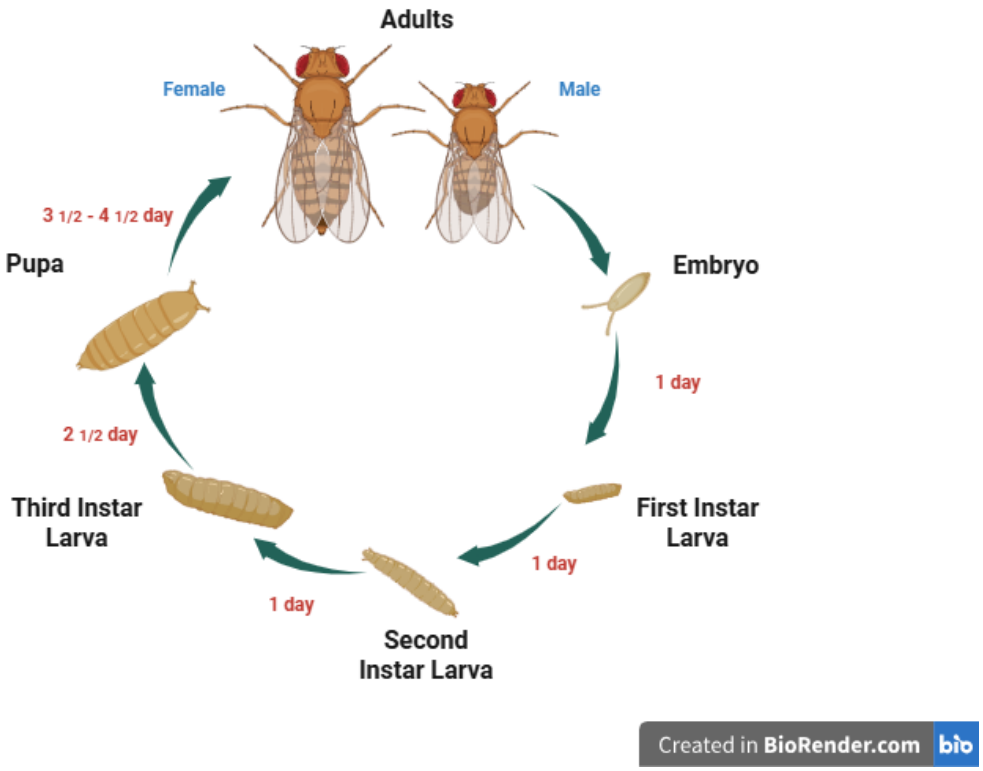


Figure 7. The whole life cycle of the fruit fly Drosophila is relatively rapid and takes only approximately 10-12 days at 25 C. The Drosophila development is divided into various stages: embryo, larva (first instar, second instar and third instar), pupa and adult. (drawn using biorender) [103]

4.2.2.1 Use of Other Non-Chordate Models for Essential Oil Assessment

In addition to *Drosophila melanogaster*, several other non-chordate organisms have been used to investigate the biological effects and toxicity of EOs. These models have mainly been employed for general toxicity screening, mechanistic studies, or evaluation of specific biological activities, rather than for direct dermal or cosmetic safety assessment.[104]

One of the most extensively used non-chordate models is *Caenorhabditis elegans*.[105] This nematode has been applied to assess the toxicity of EO constituents such as *thymol*, *carvacrol*, and *eugenol* by measuring endpoints including survival, locomotion, reproduction, and oxidative stress responses.[105] Previous studies have demonstrated that these compounds induce dose-dependent toxicity and modulate stress-response and detoxification pathways even at sublethal concentrations, highlighting the suitability of this model for mechanistic and physiological investigations rather than topical exposure modeling.[106, 107]

Another commonly used non-chordate system is *Artemia salina*, which is widely employed as a rapid and low-cost method for acute toxicity screening of EOs and plant extracts.[108] In this assay, larval mortality is recorded and LC_{50} values are calculated to estimate overall toxic potential.[108] This model is primarily used for preliminary hazard identification and compound prioritization, rather than for detailed biological or tissue-specific analyses.[109]

Importantly, with the exception of *Drosophila melanogaster*, these non-chordate models were not designed to evaluate dermal safety or skin-related endpoints. *C. elegans* lacks a stratified epidermis and a physical barrier comparable to vertebrate skin, limiting its relevance for topical or cosmetic applications.[110, 111]

Similarly, *Artemia salina* represents a highly simplified aquatic organism, and its application is largely restricted to acute lethality screening, providing minimal translational value for dermal exposure scenarios.[112]

In contrast, *Drosophila melanogaster* offers a higher level of biological complexity, including an epidermis, cuticle, differentiated epithelial tissues, and conserved regulatory pathways relevant to stress responses and toxicity. These characteristics allow the evaluation of contact-related effects, chronic exposure outcomes, behavioral alterations, and systemic toxicity within a single in vivo model.[113] As a result, *Drosophila* has been increasingly proposed as a bridge model between in vitro assays and vertebrate studies for toxicity and safety assessment of natural products, including essential oils.[9]

Although *Drosophila melanogaster* does not fully replicate human skin physiology, its combination of experimental tractability, genetic versatility, biological relevance, and ethical acceptability makes it the most informative non-chordate model currently available for integrated essential oil assessment when compared with other invertebrate systems.

4.2.3 Drosophila melanogaster as a Model Organism in Antioxidant and antiaging Studies

Although some earlier studies have investigated the larvicidal or insecticidal properties and biological activity of plant-derived extracts, EOs, plant-derived oil, or fruit-derived compounds, the aim of the present project is fundamentally different. Here, we focus on evaluating both the toxicity and the potential therapeutic effects of these oils using *Drosophila melanogaster* as an in vivo model, with the broader goal of supporting its use as an alternative to chordate animals. Accordingly, this study seeks to establish *D. melanogaster* as a viable and ethically favorable model organism for assessing the biological activity of plant-derived oils.[9, 114]

More specifically, we investigate the effects of Calendula, Echium, Spelt, and Sea Buckthorn oils on *D. melanogaster* lifespan and behavior, as well as their anti-ageing and antioxidant properties. By doing so, this work contributes to the scientific understanding of plant-based bioactive while adhering to the principles of humane animal research, particularly the reduction in the use of chordate species and their replacement, where possible, with non-chordate models.[9]

4.2.4 Lifespan Assay as a Biological Indicator of Antioxidant Potential

Aging is a complex and inevitable biological process characterized by a gradual decline in physiological functions at molecular, cellular, tissue, and organ levels. The most key features of aging include a decline in cellular function, compromised immune responses, reduced metabolic efficiency, progressive deterioration of organ structure, and the buildup of genetic damage. These changes collectively impair mobility, reproduction, and lifespan, and are largely driven by free radicals, reactive by-products of oxidative stress that contribute to both aging and the development of various diseases.[69]

With the rise of aging-related conditions, including neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, cardiovascular diseases, diabetes, and skin-related aging, there is an increasing global demand for anti-aging and antioxidant interventions, particularly in skincare products.[115]

The oxidative stress theory of aging posits that oxidative damage accumulates with age and represents a central mechanism underlying the aging process. Reactive oxygen species (ROS) are generated both endogenously, primarily due to mitochondrial activity, and exogenously through environmental stressors.[115]

Excessive ROS can damage key biological molecules, including proteins, lipids, and nucleic acids, and may also induce mutations in mitochondrial DNA. To counteract these harmful effects, organisms possess two complementary antioxidant defense systems. The first comprises endogenous antioxidants, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which neutralize ROS within cells and serve as the primary defense line. The second consists of exogenous antioxidants, such as vitamin C, carotenoids, and melatonin, which can scavenge free radicals, limit ROS formation, and provide an additional protective layer against oxidative damage.[115]

In this study, the fruit fly *Drosophila melanogaster* was selected as a model organism to investigate how the consumption of Calendula, Echium, Spelt, and Sea Buckthorn oils could influence fly lifespan in a dose-dependent manner. Antioxidants, by reducing ROS levels and supporting cellular homeostasis, are known to impact survival in various model organisms, including *Drosophila*. Lifespan assays do not focus on a single biochemical pathway; rather, they capture the cumulative physiological impact of a compound on metabolism, oxidative balance, stress resistance, and cellular maintenance.[115]

Consequently, these assays provide valuable complementary insight to in vitro antioxidant measurements, such as DPPH radical scavenging or metal-chelating assays, by determining whether chemical activity translates into meaningful biological effects within a living system. Utilizing lifespan analysis alongside chemical characterization enables a more comprehensive evaluation of plant oils, allowing assessment of whether oils enriched in specific bioactive compounds can modulate aging-related endpoints.[115]

Furthermore, the climbing assay was employed to determine whether dietary supplementation with these essential oils would alter locomotor performance and behavioral patterns in the flies, following the protocol described by Gargano et al., 2005. This assay is widely used in *Drosophila* research to assess age-related decline, neuromuscular integrity, and oxidative stress.[9] Because locomotor capacity decreases under conditions of cellular damage or mitochondrial dysfunction, these behavioral measurements provide complementary biological insights alongside lifespan analysis.[115]

Although primarily a methodological tool, such assays are instrumental in evaluating whether antioxidant-rich natural products can influence physiological function beyond mere survival. In the context of this project, the climbing assay also enabled investigation of the potential of certain oils to delay functional decline, an outcome that holds relevance for the development of cosmetic formulations such as creams, serums, and lotions.[115]

Building on the understanding of aging, oxidative stress, and the antioxidant potential of plant oils discussed in the previous sections, the following chapter describes the materials, experimental design, and analytical methods used in this study. It details the chemical characterization of Calendula, Echium, Spelt, and Sea Buckthorn oils using GC–MS to identify major bioactive constituents, as well as in vitro antioxidant evaluation through DPPH radical scavenging. In addition, in vivo assays, including lifespan and climbing assay in *Drosophila melanogaster*, were employed to assess the physiological and functional effects of these oils.

5 Conclusions based on the literature: rationale of the project and the strategy that was decided.

The scientific leap in this project is the integration of chemical analysis, antioxidant profiling, and in vivo biological assessment to characterize the effects of plant-derived oils, with a particular focus on spelt oil. Despite its unique biochemical composition, spelt oil has received little scientific attention and remains largely unexplored for potential skincare applications, making its systematic evaluation a novel contribution of this work.

This research investigates four selected oils based on their reported antioxidant and anti-aging properties. The project aims to evaluate their antioxidant capacity using both chemical assays and an in vivo model, *Drosophila melanogaster*.

To assess antioxidant activity, the DPPH radical-scavenging assay was employed, a method particularly suitable for analyzing lipophilic antioxidants, including oils extracted by supercritical CO_2. Absorbance measurements were recorded using a UV–VIS spectrophotometer to determine radical-scavenging efficiency across different concentrations. Only Hydrogen Atom Transfer (HAT) mechanism is considered within this analytical framework.[84]

GC-MS was used to identify the major constituents of each oil and to examine potential structural changes following exposure to DPPH. This approach is recommended for characterizing oils produced through supercritical CO_2 extraction due to their complex mixture of unsaponifiable components and thermally labile compounds.

In parallel with the chemical assays, biological investigations were conducted using *Drosophila melanogaster* to assess lifespan and locomotor performance (climbing assay). Two strains were used: *Dahomey* and the *Lancaster* strain (Figure 8). Male flies were chosen to reduce metabolic and behavioral variability associated with female reproductive cycles, thereby ensuring greater consistency and reproducibility within behavioral assays, which will be discussed more in chapter 5.

Figure 8. Lancaster strain of male (left) and female (right) Drosophila melanogaster a) Dahomey with red eyes. (Drawn using bio-Render)

The *wDah* and *Lancaster* strains of *Drosophila melanogaster* were selected to evaluate the physiological impact of plant-based oils and specific antioxidant constituents. The wDah strain is renowned for its high fecundity and exceptional longevity.[116] Because wDah retains the robust stress-resistance characteristics of wild populations, it is considered a gold-standard model for investigating how dietary interventions modulate the aging process.[117] Furthermore, wDah exhibits a highly plastic response to nutrient availability, making it particularly suitable for studying the life-extending properties of lipid-derived antioxidants.[118] In contrast, the *Lancaster* strain represents a standard laboratory lineage that often exhibits a shorter median lifespan and different reproductive kinetics compared to the *wDah* background.[119]

By utilizing both strains, this study accounts for genotype-by-diet interactions, which are crucial when administering polyunsaturated fatty acids (PUFAs) like linoleic acid. While these lipids can activate protective antioxidant signaling pathways, they are also highly susceptible to lipid peroxidation depending on the host's internal redox environment. Comparing the resilient *wDah* with the *Lancaster* strain allows for a rigorous assessment of whether the antioxidant benefits of vegetable oils are consistent across different genetic architectures or are dependent on the baseline metabolic efficiency of the organism.[119, 120]

5.1 Knowledge Gaps

Despite the growing interest in plant-derived oils for skincare applications, significant gaps remain in understanding their oxidative stability, synergistic behavior, and biological safety when used individually or in combination. The mechanisms underlying potential synergistic or antagonistic effects between carrier oils and bioactive oils remain largely unexplored, particularly in the context of antioxidant performance and in vivo biological outcomes. Moreover, except for well-known oils such as calendula, echium, and sea buckthorn, the scientific literature provides extremely limited data on spelt oil, its biochemical properties, and its compatibility with cosmetic formulations. These gaps highlight the need for systematic chemical, antioxidant, and in vivo investigations to better understand both the individual and combined behaviors of these oils and to establish their potential for safe and effective skincare formulations.[121-123]

Chapter 2: Materials and Methods

1 Materials

1.1 Reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH, free radical, 95%, from ThermoFisher); Iron (ll) chloride ($FeCl_2$, 98%, from Sigma Aldrich); and 4,4'-(3-(Pyridin-2-yi)-1,2,4-triazine-5,6-diyl) dibenzene sulfonic acid monosodium salt (Ferrozine, 97.21%, from BLDpharm).

1.2 Solvents

Dimethyl Sulfoxide (DMSO, for HPLC, 99.7%, from Sigma Aldrich); Methanol (Hyper grade for LC-MS, from Sigma Aldrich); Distilled water; and Hexane (≥95%, suitable for UV/Vis spectroscopy and HPLC, from Sigma Aldrich).

1.3 Standards and control materials

Quercetin (≥95%, for HPLC, from Sigma Aldrich); Na_2EDTA (dehydrate, from Apollo Scientific); Linoleic acid (analytical standard, standard for GC, from Sigma Aldrich); oleic acid (≥95%, for GC); (+)-α-Tocopherol (type V from vegetable, Type V, ~ 1000 IU/g, from Sigma Aldrich); L-Ascorbyl 2,6-dipalmitate (from Apollo Scientific); (z)-hexadec-9-enal (95%, from BLDpharm); and cis-8,11,14-eicosatrienoic acid (analytical standard, from Sigma Aldrich).

1.4 Test substances

The Spelt, Echium, Sea Buckthorn, and Calendula oils used in this study were kindly gifted for research purposes by CO_2 Extraction UK Ltd (Morecambe, England).

1.5 Biological materials

Regular food-grade granulated white sugar (sourced from a local supermarket); Brewer's Yeast (from MP Biomedical); Agar (supplied by BTP Drewitt); Water, methyl-4-hydroxybenzoate (≥ 99%, FCC); Propionic acid (~99%, bioreagent, suitable for insect cell culture, from Sigma Aldrich), Ethanol (from Sigma Aldrich).

1.6 Fly Stocks

Drosophila Melanogaster strain used were:

a. Dahomey
b. Lancaster

Dahomey is wild type strain collected in 1970 as a mass-bred strain from *Dahomey*, now Benin, West Africa and has been kept at medium-large population sizes in the lab since that time. White *Dahomey* (*wDah*) has had the w- allele, a loss-of-function mutation in the *white* gene that causes white eyes, crossed in. The resulting strain was backcrossed to the *Dahomey* wild-type background for several generations before being made homozygous for w- (w-/w-) again.

Lancaster is a wild-caught mass-bred strain originating from two locations in the city of Lancaster in October 2014.

2 Methods

2.1 Physical Characterization of Oils

The four oils differed in their physical properties. Densities were determined by weighing 1 mL of each oil: Calendula exhibited the highest density (1.05 $g.mL^{-1}$), followed by Sea Buckthorn (1.01 $g.mL^{-1}$), Spelt (0.99 $g.mL^{-1}$), and Echium (0.97 $g.mL^{-1}$). As Figure 9 shows, Color varied among the oils, with Calendula appearing dark orange, Echium medium orange, Sea Buckthorn light orange, and Spelt yellow. Viscosity and stickiness were assessed qualitatively by visual observation: Calendula was the most viscous and sticky, Echium moderately viscous, while Sea Buckthorn and Spelt appeared relatively thin and non-sticky.

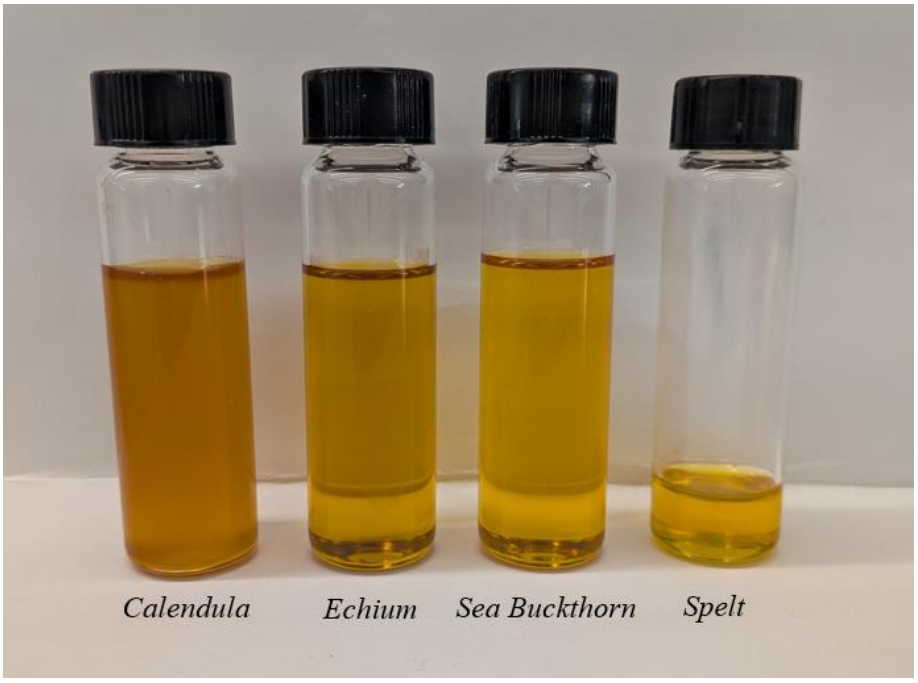


Figure 9. Plant-based oils used in this study. The amount of Spelt oil used corresponds to the remaining quantity available.

2.2 Chemical Method

2.2.1 DPPH Radical Scavenging Assay

The free radical-scavenging activity of the oils was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. For each oil, 1 mL of solution at concentrations ranging from 20 to 800 µL.mL^{-1}, prepared in 95:5 methanol: DMSO, was mixed with 2 mL of methanolic DPPH solution (100 µM). The mixtures were incubated in the dark at room temperature for 15 min, and the absorbance was recorded at 517 nm using quartz cuvettes (Figure 10).

Quercetin was used as a reference standard. It was prepared in the same manner as the oils, starting from an initial 1 µM quercetin solution, which was made by dissolving quercetin in 0.5 mL DMSO and 9.5 mL methanol. Dilutions to obtain final concentrations of 20–800 µM were then performed using a 95:5 methanol: DMSO solution. All measurements were performed in quartz cuvettes, and each experiment was repeated three times.[13, 124]

Figure 10. Schematic representation (left to right) of the DPPH radical scavenging assay employed in this study for evaluating the antioxidant activity of plant oils, from sample preparation to absorbance measurement (drawn using Chem Draw).

2.2.2 Metal chelating activity

To 1 ml of different concentrations (20-800 µL. mL^{-1}) of each oil that was diluted by 95:5 methanol: DMSO, were added 2.8 mL of distilled water and then mixed with 50 µL of 2 mM $FeCl_2$ and 150 µL of ferrozine (5 mM). The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. This formula $[(A_0- A_s)/A_s] \times 100$ was used to determine the inhibition percentage of ferrozine- Fe^{2+} complex formation, where A_0 represent the absorbance of the control, and A_s represent the absorbance of the extract/ standard. Na_2EDTA was used as positive control (Figure 11).[13, 83]

Figure 11. Schematic representation (left to right) of the metal chelating activity employed in this study for evaluating the antioxidant activity of plant oils, from sample preparation to absorbance measurement (drawn using Chem Draw).

2.3 Biological Method- Lifespan Assay

2.3.1 Egg collection

Egg collection was performed using a suspension method adapted from Moth and Barker.[125] This approach was selected to ensure uniform larval density and random sampling of eggs across the experimental population.[126, 127]

The suspension method, adapted from Moth and Barker and sharing elements with the approach described by Ralchev [128] is illustrated in Figure 12, which provides an overview of placing approximately 500 reproductively active flies (~1:1 female: male) into eleven petri dishes that serve as egg-laying cages (Figure 13.2), each containing 25 mL of food composed of 48 g sugar, 48 g yeast, and 14.4 g agar. This mixture is dissolved in 600 mL of tap water and heated in a microwave until it reaches boiling. Afterward, 18 mL Nipagin (10% w/v methyl-4-hydroxybenzoate in ethanol) and 3 mL propionic acid are added once the food has cooled to 60°C using cold water. When the medium solidifies, a mound of 1:1 yeast–water paste is placed at the center (Figure 13.1) and left for about 15 hours at 25°C under a black cover to prevent any light. During this time, eggs are laid on the agar surface or along the inner plate walls (Figure 12). The flies are then removed using CO_2 and a funnel, along with excess yeast (Figure 13.3).

Figure 12. Egg collection plate showing freshly laid Drosophila eggs on agar medium prior to suspension and transfer to experimental vials.

Next, the agar surface is covered with phosphate-buffered saline (PBS), and the eggs are gently released into the PBS using a small brush, then transferred into a 10 mL measuring falcon (Figure 13.4). In contrast to the original protocol by Moth & Barker, which used a sucrose solution for egg suspension, PBS was employed here. This substitution was made because PBS eased cleaner egg separation and reduced residual yeast contamination.

The plates are rinsed again, and the falcon is adjusted to 10 mL with PBS. After a few minutes, the eggs settle at the bottom while any remaining yeast paste dissolves or floats. Eggs are then collected from the bottom using a micropipette (Figure 13.5) and transferred into vials (16 µL) having 60 mL of food prepared using the sugar-yeast recipe (described in Section 2.2.3) (Figure 13.6). This procedure is specifically designed to minimize contamination of eggs with yeast.[127]

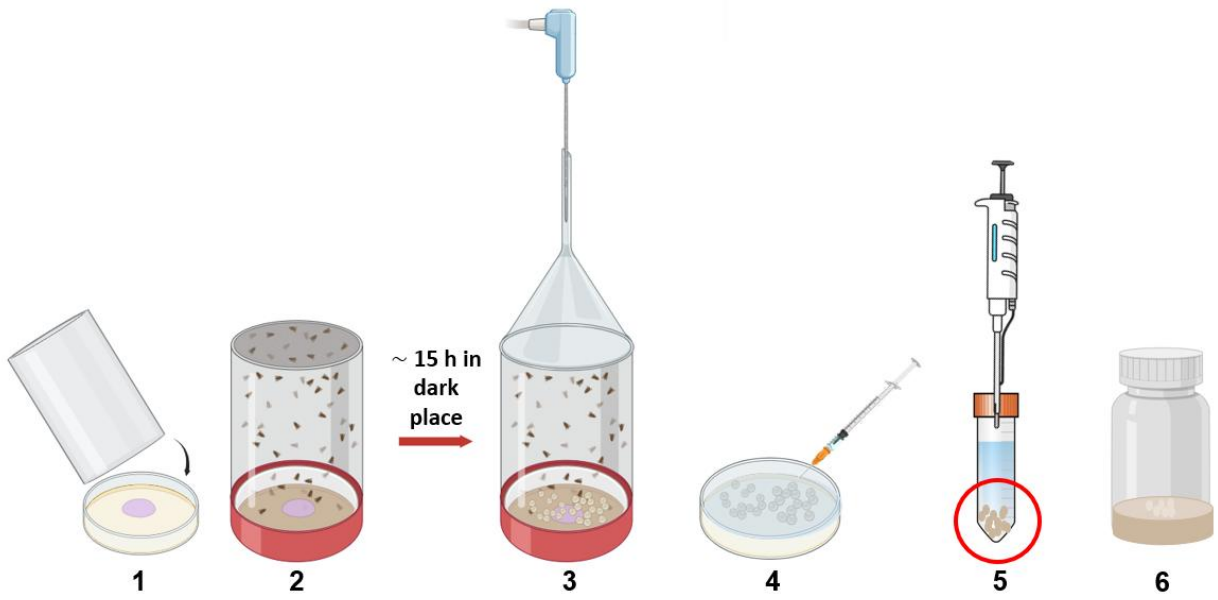


Figure 13. Schematic overview of the workflow of the suspension method for collecting Drosophila melanogaster eggs, adapted from Moth & Barker and Ralchev, including egg laying, fly removal, egg release in PBS, sedimentation, and transfer of purified eggs into food. (Drawn using bio-Render)

2.3.2 Mating and Larval Development

In the next stage of the experiment, the developmental cycle of *D. melanogaster* is checked after transferring the eggs into the bottles containing food. The whole life cycle of *Drosophila* is relatively rapid and takes only approximately 10–12 days at room temperature. The *Drosophila* development is divided into various stages: embryo, larva, pupa, and adult (Figure 7).[103]

Eggs are deposited on the food substrate, where embryonic development occurs within the egg. Within 24 h, first-instar larvae hatch and initiate feeding. This feeding and growth period lasts approximately four days, during which larval body weight increases by nearly 200-fold, primarily as a result of extensive endoreplication in larval tissues.[103]

However, the larval tissues will not be the part of the adult fly as these tissues are broken down during metamorphosis in the pupa stage. The imaginal discs, which are made up of diploid cells of undifferentiated epithelium, will eventually contribute to the development of adult fly structures.[103]

At the end of third instar stage, the larvae cease feeding and leave the food substrate to locate a suitable site for pupariation. During the pupa stage, metamorphosis occurs for four days, following which adult flies eclose. Adult female flies are normally larger than adult male flies with females weighing 1.4 mg and males 0.8 mg. Females are ready to mate in less than 24 h after eclosion and can lay up to 100 eggs per day. Adult flies live about two months after eclosion.[103]

2.3.3 Fly Maintenance and Lifespan Monitoring

To understand how consumption of the plant-based oils would alter the lifespan of the flies, a lifespan assay was performed. After the adult flies emerged, males and females were separated under a stereomicroscope using CO_2 anesthesia and a fine brush (Figure 14).

Although adult females are generally larger than males, sex separation was performed under a stereomicroscope based on abdominal shape, with males exhibiting a darker and more rounded posterior abdomen, whereas females showed a lighter and more elongated abdominal shape. For each vial, 20 males were transferred. The vials had been pre-filled with 5 mL of food prepared according to the sugar–yeast recipe.

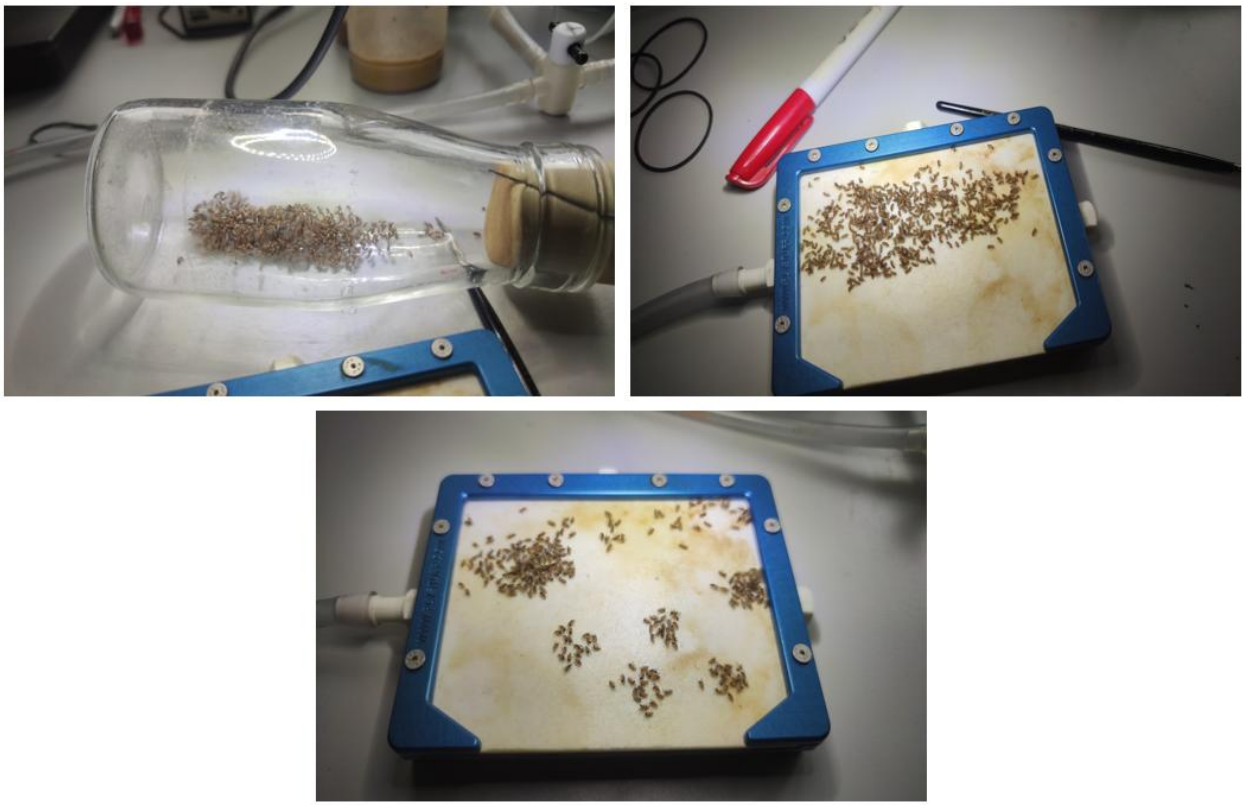


Figure 14. Process of sorting adult Drosophila melanogaster by sex. Flies were anaesthetized with CO_2, examined under a stereomicroscope, and separated using a fine brush before being distributed to treatment vials.

In this experiment, four plant-derived oils, Calendula, Echium, Spelt, and Sea Buckthorn, were evaluated at four different concentrations (0, 0.005, 0.015, and 0.05 %), each with five replicates. Two *D. melanogaster* species were used, and for each species, a control group consisting of 10 vials was included. Altogether, this needed the preparation of approximately 3 kg of food each week.

According to the Figure 15, to prepare the food, 3 L of water were added to a pot, and the electric stove was set to 240°C until the water reached boiling (Figure 15-1). Meanwhile, the dry ingredients were prepared by mixing 240 g sugar (Figure 15-2), 240 g yeast (Figure 15-3), and 48 g agar (Figure 15-4) using a handheld whisk. Once the water boiled, the dry mixture was gradually added while stirring continuously. The mixture was stirred gently until it boiled again, after which the temperature was reduced to 100–120°C. The mixture was allowed to simmer, being stirred every few minutes, until it reached boiling once more.

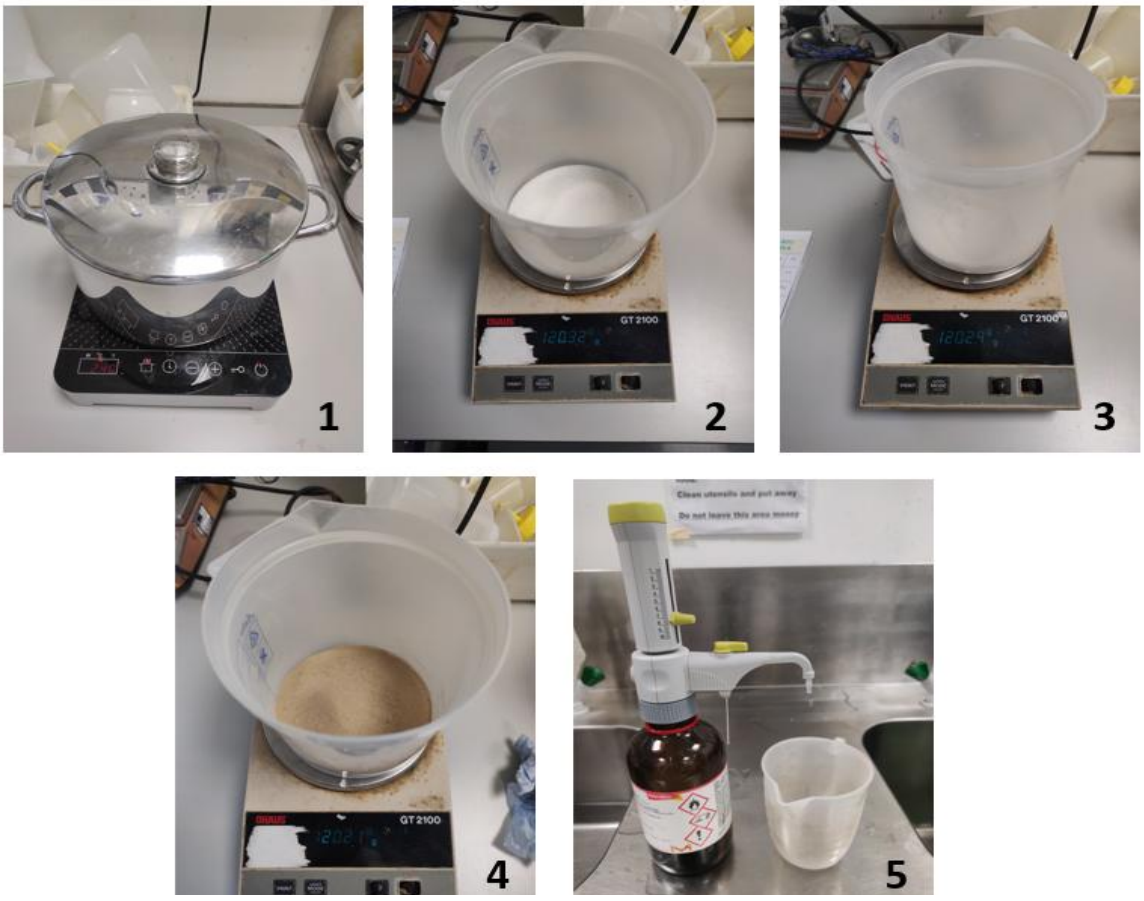


Figure 15. Preparation of the yeast–sugar food medium used for Drosophila assays. The mixture of sugar, yeast, and agar was brought to a boil, cooled to 60 °C, supplemented with Nipagin and propionic acid, before dispensed into vials for subsequent experiments.

The pot was then transferred to an ice-water bath in the sink and left to cool to 60°C, at which point 90 mL Nipagin, and 15 mL propionic acid (Figure 15-5) were added. The mixture was returned to the electric stove and kept at 60°C. At this stage, a solution of the oil and the cooked food is prepared according to the specified concentrations, and 5 mL of this mixture is added to each vial and transferred to a 17°C incubator.

As Figure 16 shows, every two days, the flies were transferred to fresh vials, and the number of dead flies was recorded in the Excel sheet shown in Table 2, that the D1 column is used to record the number of dead flies present in each vial before transferring the flies to a fresh vial. For each group of vials (e.g., group A), the number of dead flies in each vial is counted and entered the D1 column. After this, the flies are anesthetized with CO_2 and transferred into fresh vials.

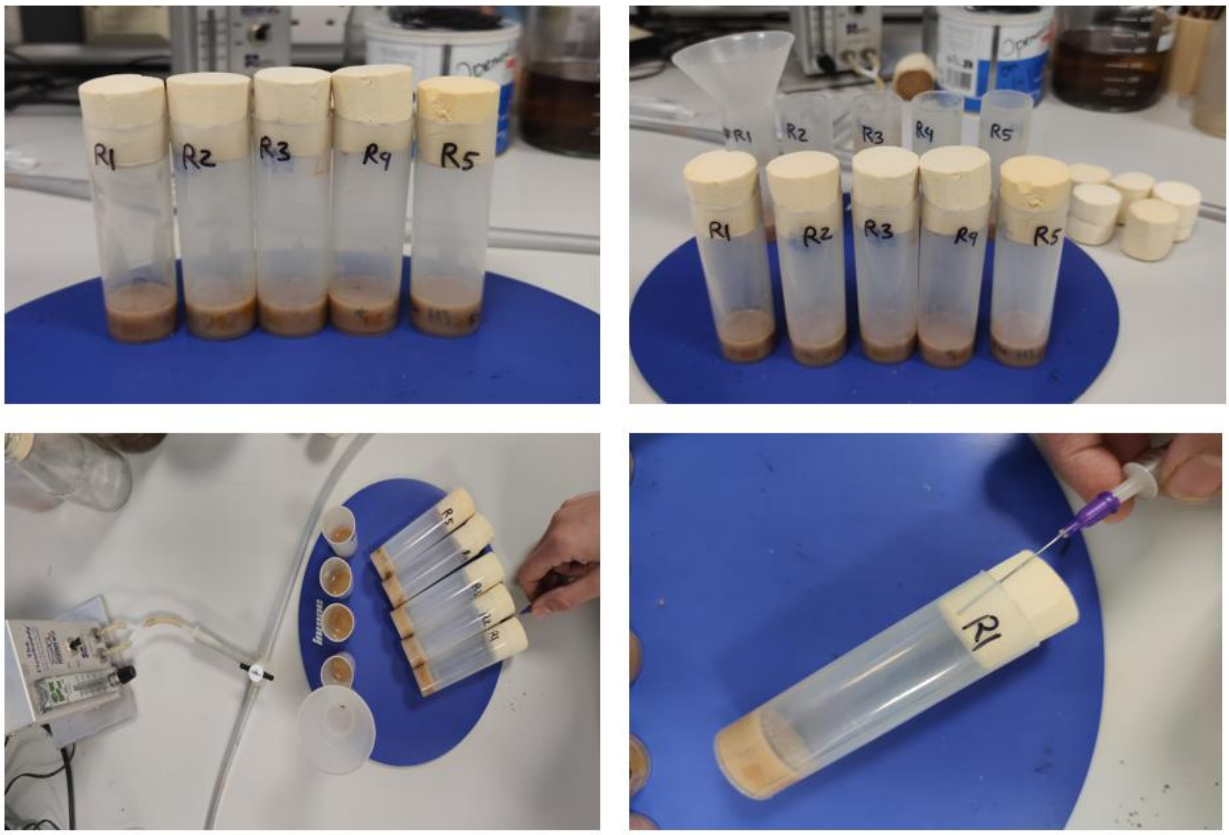


Figure 16. Two-day transfer cycle used in the lifespan assay. Flies were immobilized using CO_2, dead individuals were recorded, and the remaining adults were transferred to freshly prepared food vials.

Once the flies have been moved, the old vials are re-examined, and the remaining dead flies are counted again. If the number of dead flies left in the old vial is lower than the number initially recorded in D1, this shows that one or more dead flies were accidentally transferred into the fresh vial. Although the aim is to avoid transferring dead flies, this is sometimes unavoidable. In such cases, the number of dead flies unintentionally transferred should be recorded in the D2 column. This value corresponds to the difference between the number recorded in D1 and the number of dead flies staying in the old vial. Recording this information ensures that, during next scoring, it is possible to distinguish newly deceased flies from those carried over from the previous timepoint.

A useful internal check for accuracy is that any D1 value having deaths should be equal to or greater than the D2 value from the preceding transfer. A number appearing in the D2 column shows that dead flies were transferred to the subsequent vial; therefore, dead flies should be present in that vial when it is next scored.

Table 2. Recording of dead male Drosophila before (D1) and after (D2) transfer to fresh vials during lifespan assays.

Date	8/8/2025		10/8/2025		12/8/2025	
	D1	D2	D1	D2	D1	D2
A1						
A2	**1**	**1**	**1**			
A3						
A4	**2**		**2**	**1**	**3**	

2.3.4 Climbing Assay

2.3.4.1 Climbing assay apparatus and associated equipment

The climbing assay was performed using a custom-built wooden apparatus designed to hold 9 vertical tubes in parallel (Figure 17). The apparatus consists of a rectangular wooden frame where the bottom bar features numbered recesses (1–12) to align and support the base of the tubes. The top bar of the frame stabilizes the upper part of the tubes. All experiments were conducted inside a temperature-controlled incubator maintained at 17°C. Illumination was provided by the incubator's internal lighting system.[129]

For the assay, glass tubes (dimensions: e.g., 25 × 2.5 cm) were used as negative geotaxis vials. The tubes were sealed at the top with foam plugs to prevent fly escape. Images of the flies' vertical distribution were captured using a Canon EOS 6D digital camera equipped with a Canon EF 50mm f/1.8 lens ىmounted on a tripod. The camera was positioned approximately 150 cm away from the apparatus to capture the entire frame and was positioned to capture the entire frame, with settings adjusted to ensure clear visibility of the flies (f/4.0, 1/125 s, ISO 400).[129, 130]

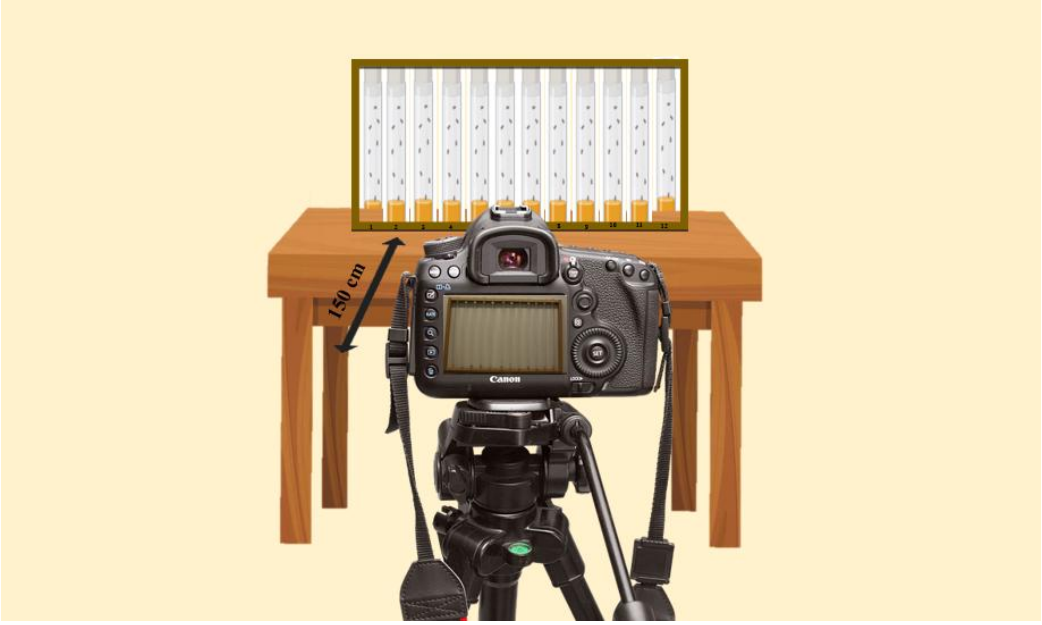


Figure 17. Experimental setup for the Negative Geotaxis (Climbing) Assay.

2.3.4.2 Climbing Assay

For the climbing (negative geotaxis) assays, male flies from the Lancaster and wDah strains were collected within one day of hatching. Flies were transferred to fresh food vials every two days, and the assay was performed on the day following their transfer to fresh medium at two different ages, 33 and 40 days post-eclosion which are shown in Figure 18 and 19, respectively. Each tube was tapped sharply three times in rapid succession to bring all flies to the bottom; this procedure induces negative geotaxis, a reflex in which flies move upward against the gravity vector when disturbed. After 15 seconds, a picture of the flies was recorded.

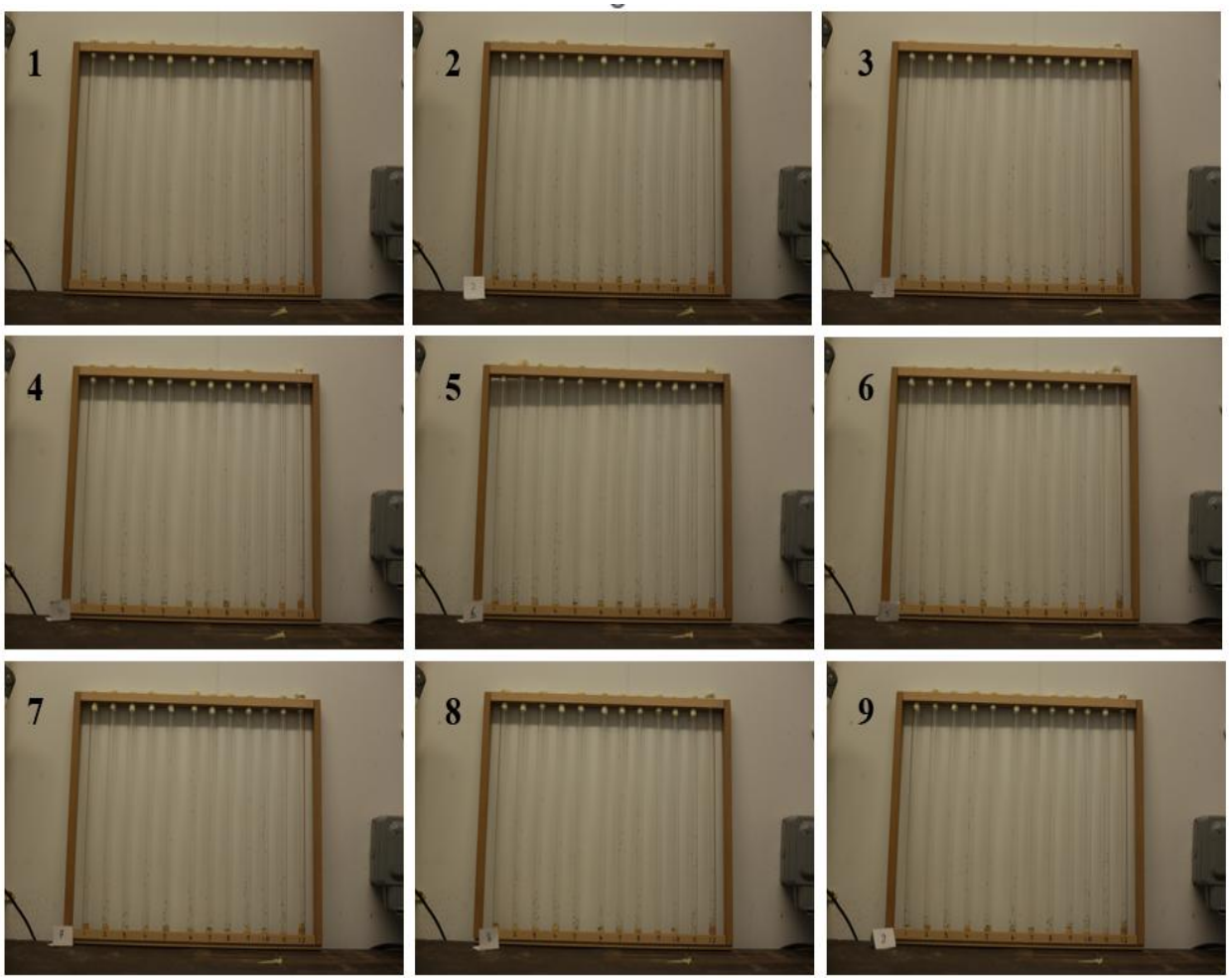


Figure 18. Image series of the Negative Geotaxis Assay at 33 days of age. Sequential panels (1–9) illustrate the image acquisition process used for recording the vertical position of Drosophila flies within the wooden-made frame. The panels show the flies at 33 days of age, proving the consistency and repeatability of the assay setup during the image capture phase of one trial. The number of flies was counted for each captured image.

As previously described, four different oils were evaluated, each at four concentrations (0, 0.005, 0.015, and 0.05%). For this assay, vials numbered 1 to 4 were selected and examined, meaning that four biological replicates were performed per dose. The resulting data were compiled in Excel spreadsheets and plotted based on genotype and age. Statistical analyses were performed using *STATISTICA* software (version 7.0, Stat Soft, Inc.). For handling, flies were briefly anesthetized with CO_2 (for a few seconds) and 10–20 flies transferred into glass tubes, which were after mounted on a wooden frame. Climbing assays were conducted at 17 °C and 50–60% relative humidity.[129-131]

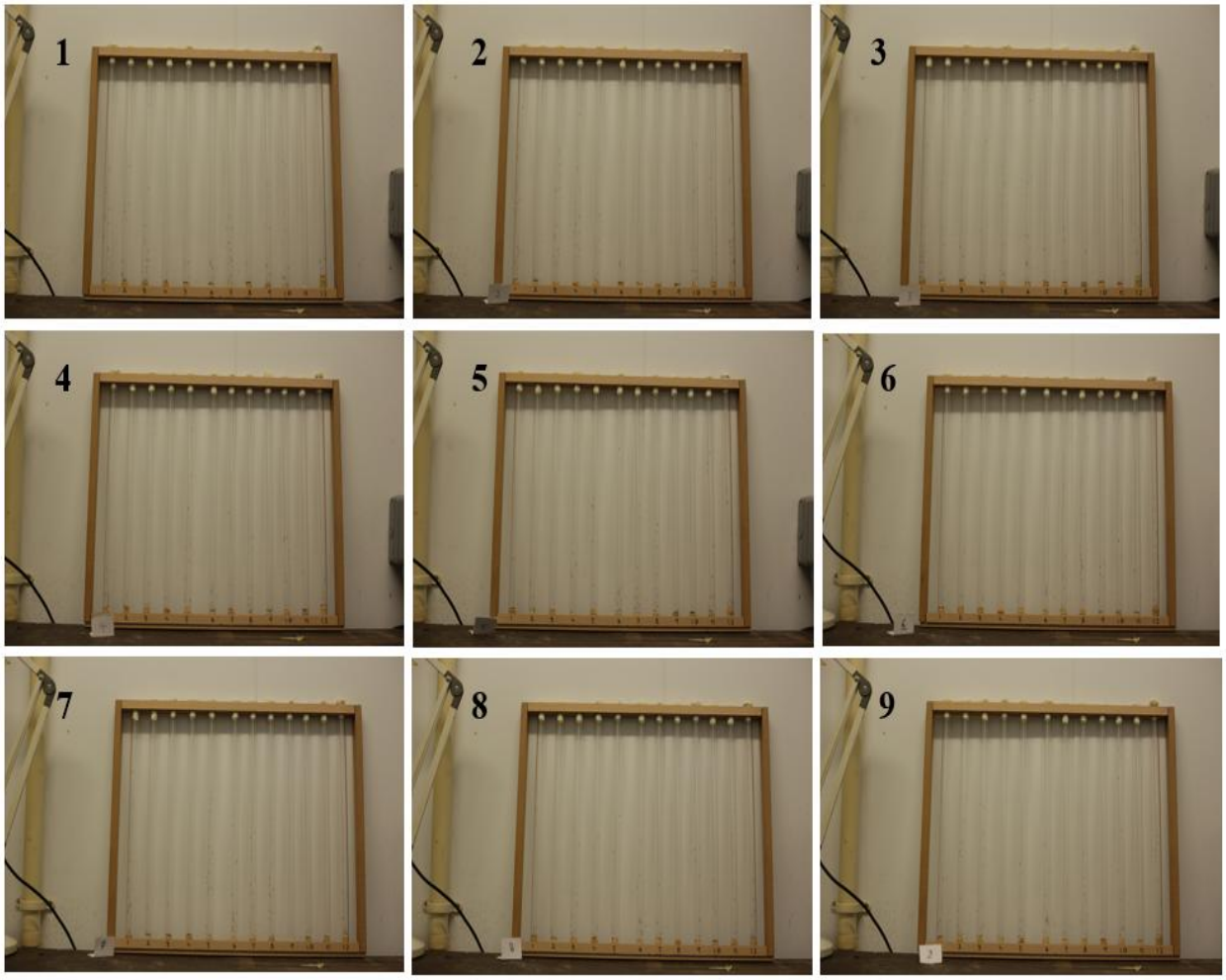


*Figure 19. Image series of the Negative Geotaxis Assay at 33 days of age. Sequential panels (1–9) illustrate the image acquisition process used for recording the vertical position of Drosophila flies within the wooden-made frame. The panels show the flies at **40 days of age**, showing the consistency and repeatability of the assay setup during the image capture phase of one trial. The number of flies was counted for each captured image.*

2.3.4.3 Data analyses and statistical tests

Digital images of the flies were transferred to a computer and analyzed using ImageJ (NIH, Bethesda, MD, USA). The climbing height of each fly was quantified based on pixel measurements, following standard thresholding and measurement procedures. The resulting data were exported to Microsoft Excel (Microsoft Corporation, Redmond, WA) and organized according to tube identity, with each tube representing a biological replicate.

Statistical analyses were performed using STATISTICA software (version 7.0). Climbing assay data were analyzed using two-way ANOVA, with oil type and oil concentration as independent variables. Analyses were conducted separately for each *Drosophila* strain, and interaction effects were included in the model. Flies were initially allocated at 20 individuals per vial, with variation in group size at the time of testing due to natural mortality. A p-value < 0.05 was considered statistically significant.

Survival data were analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank (Mantel–Cox) test. Analyses were performed separately for each oil, dose, and *Drosophila* strain. Sample sizes were selected based on previous *Drosophila* lifespan studies and were sufficient to detect biologically meaningful differences in survival.

3 Characterization

3.1 Gas Chromatography–Mass Spectrometry (GC–MS)

The chemical composition of the plant-based oil samples was analyzed using a Shimadzu GCMS-TQ8040 triple-quadrupole system (Shimadzu, Kyoto, Japan) equipped with an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent, USA).[86] Two sets of samples were prepared for analysis: (i) plant-based oils and analytical standards, diluted in n-hexane at a 1:10 (v/v) ratio, and (ii) reaction mixtures prepared for radical-scavenging assessment, consisting of the plant-based oils diluted in methanol and DMSO at a 95:5 (v/v) ratio and mixed with DPPH solution. Both types of samples were analyzed to enable comparison of the major volatile constituents before and after the DPPH reaction.

The GC oven temperature program was set as follows: initial temperature 60 °C (held for 2 min), increased to 140 °C at 15 °C/min (held for 2 min), then ramped to 180 °C at 5 °C/min (held for 3 min), and finally increased to 250 °C at 10 °C/min (held for 3 min). Helium was used as the carrier gas at 1.2 mL/min. The injector temperature was 250 °C, the split ratio was 20:1, and 1 µL of each sample was injected. The mass spectrometer was adjusted in electron ionization (EI) mode at an ionization energy of 70 eV.[86]

Identification of compounds was conducted by comparing mass spectra with the NIST Mass Spectral Library. Additionally, major constituents were verified using analytical standards, which were injected under the same GC–MS conditions. Retention times and fragmentation patterns of these standards were used to confirm the identities of key components. Comparative analysis between untreated samples and DPPH-treated mixtures enabled assessment of potential changes in the major volatile constituents following the radical-scavenging reaction.[86]

3.2 UV–Vis Spectrophotometry (DPPH Radical Scavenging Assay)

The antioxidant activity of the essential oil samples was evaluated using a DPPH radical scavenging assay, measured on an Agilent Cary 60 UV–VIS spectrophotometer. A 100 µM DPPH solution was prepared in methanol and kept protected from light prior to use. The plant-based oils were diluted in methanol/DMSO mixture for solubility and mixed with the DPPH solution to start the reaction. Reaction mixtures were incubated at room temperature for 20 minutes under the dark condition.

Absorbance measurements were recorded at 517 nm, corresponding to the characteristic absorption band of DPPH•. A methanol blank was used for baseline correction. The decrease in absorbance at 517 nm, compared to the DPPH control solution, was used to determine the radical scavenging capacity of each sample. All measurements were performed in triplicate, and results were expressed as percentage inhibition using the following equation [13]:

$$\%\ Inhibition = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

For selected samples, the reaction mixtures were after analyzed by GC–MS to compare the major volatile constituents before and after the DPPH reaction, enabling correlation between compositional changes and antioxidant behavior.

3.3 In silico Toxicity Assessment

In silico toxicity assessment was performed using the Nexus toxicity prediction software (version 6.2.0, Lhasa Limited) and its associated knowledge base to evaluate the potential toxicological profiles of the major constituents found by GC–MS analysis. This approach relied on **cross-**referencing established toxicity databases, rather than performing predictive modeling or molecular simulations, and was used to complement the in vivo toxicity, lifespan, and behavioral data obtained from *Drosophila melanogaster*.

Toxicity predictions were conducted by the Derek KB 2022 1.0 knowledge base by using mammalian models, including rodent (mouse and rat) and primate (human) systems, which are widely applied in regulatory toxicology and cosmetic safety assessment. Where relevant, bacterial models (*Escherichia coli* and *Salmonella* spp.) were included to supply preliminary indications of genotoxic potential.

Selected toxicity endpoints included genotoxicity, skin irritation, skin sensitization, neurotoxicity, and organ toxicity, based on their relevance to dermal exposure scenarios, cosmetic safety evaluation, and the behavioral assays employed in this study. The in-silico results were analyzed comparatively across species and integrated with experimental observations to support interpretation of potential toxicity mechanisms.

Chapter 3: Integrated Chemical Evaluation of plant-based Oils: GC–MS Characterization and Antioxidant Activity

Chapter 3 focuses on the chemical evaluation of the plant-based essential oils studied in this project. The main goals of this chapter are to find the major volatile constituents of the oils using GC–MS, assess their antioxidant activity via DPPH radical-scavenging assays measured by UV–VIS spectrophotometry, and in the following, evaluate their biological effects on *Drosophila melanogaster* through lifespan assays in Chapter 4. These complementary analyses supply a comprehensive understanding of the relationship between chemical composition, antioxidant potential, and biological activity. All experimental procedures are described in detail in Chapter 2.

1 GC–MS Profiling of Major Antioxidant Constituents in plant-based Oils

GC–MS analysis was performed as described in chapter 2, Section 3.1. The chromatographic profiles of the plant-based oils revealed the presence of several major volatile constituents, consistent with previously reported compositions of these botanical extracts. The dominant compounds identified included *9,12-Octadecadienoic acid (Z, Z)* (Linoleic acid), *9-Octadecenoic acid* (Oleic acid), *Ascorbic acid 2,6-dihexadecanoate*, *l-(+)-Ascorbic acid 2,6-dihexadecanoate*, *8,11,14-Eicosatrienoic acid (Z, Z, Z-)*, each confirmed through comparison with the NIST spectral database and verified by analytical standards injected under identical conditions.

Injection of pure standards enabled unambiguous confirmation of the major peaks, with retention times and fragmentation patterns matching those saw in the oil samples. This validation step strengthened the reliability of compound identification, particularly for high-abundance constituents.

To evaluate the impact of radical-scavenging reactions on the chemical composition, GC–MS profiles of the untreated oils were compared with those of the DPPH-reacted samples. In contrast to the untreated chromatograms, several major peaks corresponding to key constituents previously found in the oils were either markedly reduced in intensity or completely absent following the reaction. These changes show that these components participated actively in the radical-scavenging process, leading to their partial or total consumption. The disappearance or attenuation of these peaks suggests the formation of non-volatile reaction products that are not detectable under the GC–MS conditions applied.

Overall, the GC–MS results confirm that the oils have identifiable antioxidant-related compounds, some of which show measurable changes following the DPPH reaction. These findings supplied a chemical basis for interpreting the antioxidant performance (Section 3.2) and the later biological effects assessed in the lifespan assays (Section 3.3).

1.1 Chromatographic Profile of hexane-diluted Oils

The chromatographic analysis of the untreated plant-based oils supplied a baseline chemical fingerprint that enabled the identification of their major volatile constituents prior to any radical-scavenging reactions. The GC–MS chromatograms displayed well-resolved peaks corresponding to the dominant components naturally present in the oils, allowing direct comparison with reference standards and the NIST spectral library. This profile served as the foundation for next evaluations of chemical changes following the DPPH reaction.

1.1.1 Spelt oil (Triticum spelta L.)

The GC–MS chromatographic profile of the spelt oil which was diluted with hexane revealed a composition dominated by a small number of major volatile constituents. The principal peaks corresponded to fatty acids and vitamin-C–derived esters, which are characteristic components of botanical lipid extracts.

These compounds were identified based on retention times and mass spectral matching with the NIST library, and later verified through injection of analytical standards. As outlined in Table 3, the major constituents of the oil, together with their relative abundances, are presented in detail. The chemical structures of these key compounds are additionally shown in Figure 20.

Table 3. Major volatile compounds identified in hexane-diluted Spelt oil by GC–MS

RT (min)	Compound	Area %	Class
25.336	9,12-Octadecadienoic acid (Z, Z) (Linoleic acid)	50.39	Fatty acid (PUFA)
25.400	9-Octadecenoic acid (Oleic acid)	20.79	Fatty acid (MUFA)
22.585	Ascorbic acid 2,6-dihexadecanoate	18.30	Ester of vitamin C

Furthermore, three more constituents commonly recognized as characteristic markers of spelt oil *Hexadecenoic acid-methyl ester*, *Octadecanoic acid-methyl ester*, and *Heneicosane* were observed in trace to low levels. Despite their lower relative abundance, these compounds are of interest due to their reported antioxidant or bioactive properties, and their detection supports the authenticity and expected compositional profile of the oil.[132]

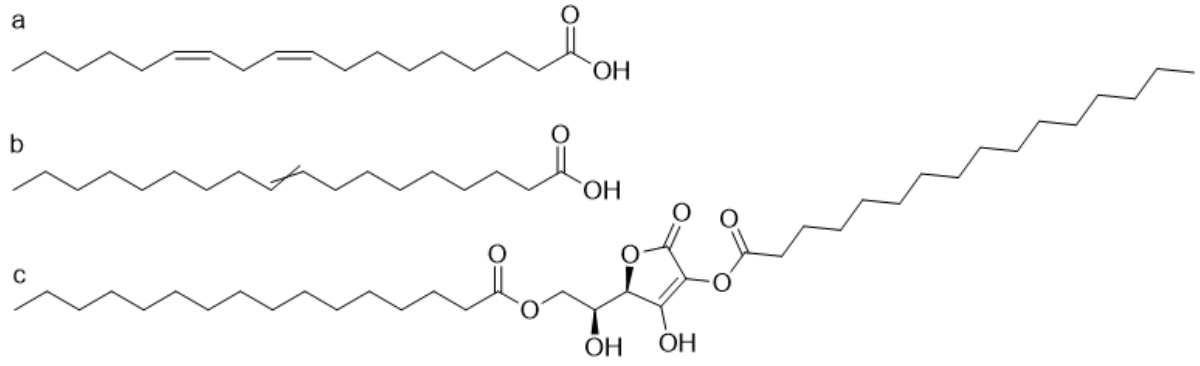


Figure 20. Chemical structures of the major spelt oil constituents, corresponding to the compounds reported in Table 3 and identified through GC–MS analysis, a) 9,12-Octadecadienoic acid (Z, Z) (Linoleic acid), b) 9-Octadecenoic acid (Oleic acid), and c) Ascorbic acid 2,6-dihexadecanoate (drawn using Chem Draw).

After summarizing the major constituents in Table 3, a detailed examination of the full chromatographic profile provides further insight into the chemical composition of the untreated oil. A representative chromatogram of the hexane-diluted spelt oil is shown in Figure 21. The major peaks correspond to the constituents listed in Table 3, with linoleic acid, oleic acid, and the ascorbyl ester displaying the highest peak intensities.

Minor peaks representing low-abundance constituents are also present, indicating the complex yet characteristic composition of the spelt oil. The chromatographic pattern is consistent with previously reported profiles of similar botanical oils, supporting the reliability of the analytical method. No additional unidentified high-intensity peaks were observed, indicating that the sample consisted primarily of well-defined lipid components.

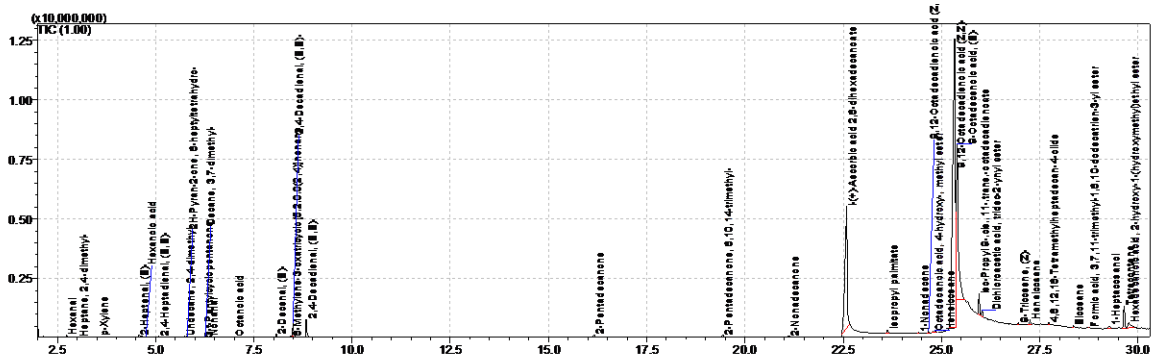


Figure 21. GC–MS chromatogram of the hexane-diluted spelt oil, showing major peaks corresponding to linoleic acid, oleic acid, and ascorbic acid esters.

1.1.2 Sea buckthorn (Hippophae rhamnoides L.)

The GC–MS analysis of the hexane-diluted Sea Buckthorn oil revealed a diverse profile of lipid-derived constituents. Dilution with hexane improved the volatility and chromatographic separation of the nonpolar components, enabling clear identification of major fatty acids, aldehydes, lipid esters, and long-chain hydrocarbons. As shown in Table 4, linoleic acid (*9,12-octadecadienoic acid*) and *cis-9-hexadecenal* were among the most abundant constituents, together accounting for nearly half of the total ion current.

Table 4. Major volatile compounds identified in hexane-diluted Sea Buckthorn oil by GC–MS.

RT (min)	Compound	Area %	Class
25.313	9,12-Octadecadienoic acid (Linoleic acid)	23.9	Fatty acid (PUFA)
25.393	cis-9-Hexadecenal	22.3	Unsaturated aldehyde
22.562	Ascorbic acid 2,6-dihexadecanoate	9.21	Ester of Vitamin C
26.031	Linolenic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (Z,Z,Z)-	3.17	Fatty acid ester (PUFA derivative)
26.958	9-Tricosene, (Z)-	6.28	Unsaturated hydrocarbon (Alkene)
27.255	Triacontane	5.45	Saturated hydrocarbon (Alkane)
29.284	1-Heptacosanol	4.29	Long-chain alcohol (Fatty alcohol)
29.650	Eicosane	5.28	Saturated hydrocarbon (Alkane)

The notably high abundance of *cis-9-hexadecenal* is consistent with previous reports describing the characteristic aldehydic aroma profile of Sea Buckthorn oil, which is known to contain various short-chain aldehydes and esters contributing to its unique scent. Studies have identified numerous volatile compounds, including specific ethyl esters and 3-methylbutyl esters, which contribute significantly to the overall aroma fingerprint detected by GC-MS analysis [133]. The chemical structures of these key compounds are additionally shown in Figure 22.

Furthermore, other identified compounds which are commonly recognized as characteristic markers of sea buckthorn oil *Z-9-tricosene*, *eicosane*, *heptacosanol*, and *caffeic acid* were observed in trace to low levels. They are a long-chain alkene commonly detected in plant surface lipids, long-chain alkanes, higher alcohols, fatty acid esters and phenolic derivatives that reflecting the wax-like and emollient characteristics associated with Sea Buckthorn-derived oils. Together, these findings demonstrate the multifaceted compositional profile of Sea Buckthorn oil as revealed by GC–MS.

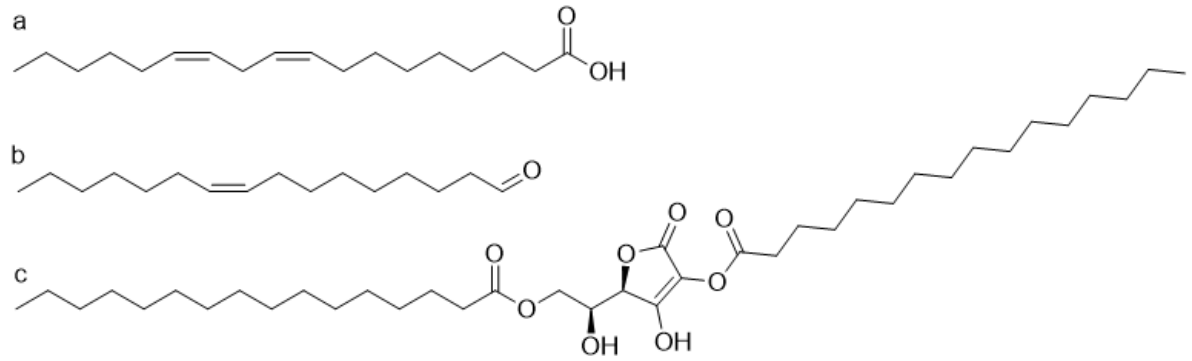


Figure 22.. Chemical structures of the major Sea Buckthorn oil constituents, corresponding to the compounds reported in Table 3 and identified through GC–MS analysis, a) 9,12-Octadecadienoic acid (Linoleic acid), b) cis-9-hexadecenal, and c) Ascorbic acid 2,6-dihexadecanoate (drawn using Chem Draw).

A representative chromatogram of the hexane-diluted Sea Buckthorn oil is shown in Figure 23. The major peaks correspond to the dominant constituents identified in Table 3, with linoleic acid and cis-9-hexadecenal exhibiting the highest peak intensities. Additional prominent signals include long-chain hydrocarbons and lipid esters, which collectively form the characteristic fingerprint of Sea Buckthorn oil under the applied GC–MS conditions.

Several minor peaks were also detected across the chromatographic trace, reflecting the presence of low-abundance lipid derivatives and accompanying volatiles. The overall chromatographic pattern is highly consistent with profiles previously reported for Sea Buckthorn berries and seed-derived oils, supporting both the reproducibility of the extraction approach and the robustness of the analytical method. No unexpected or high-intensity unidentified peaks were observed, indicating that the sample primarily comprised well-resolved, structurally recognizable lipid components.

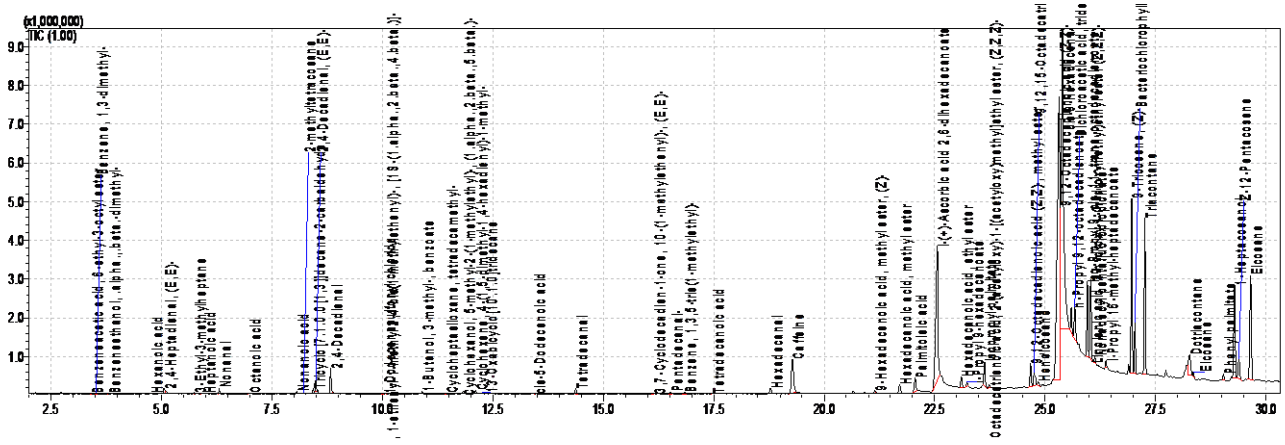


Figure 23. GC–MS chromatogram of the hexane-diluted Sea Buckthorn oil, showing major peaks corresponding to linoleic acid, and ascorbic acid esters.

1.1.3 Echium (Echium plantagineum)

The GC–MS analysis of the hexane-diluted Echium oil revealed a composition dominated by polyunsaturated fatty acid derivatives and antioxidant-related esters. Hexane dilution improved the volatility and chromatographic resolution of the nonpolar lipid fraction, resulting in clear peak separation and reliable identification of the major constituents. As shown in Table 5, *stearidonic acid* (SDA) represented the predominant compound, accounting for more than half of the total ion current and confirming the characteristic PUFA-rich profile expected for Echium oil.

Table 5. Major volatile compounds identified in hexane-diluted Echium oil by GC–MS.

RT (min)	Compound (Name)	Area %	Class
25.33	Stearidonic acid (SDA)	57.33	Long-chain unsaturated alcohol
31.45	l-(+)-Ascorbic acid 2,6-dihexadecanoate	16.45	Ascorbic acid ester (Vit. C derivative)
27.18	Methyl 5,11,14,17-eicosatetraenoate	3.55	PUFA ester (Eicosatetraenoic acid)
34.72	Vitamin E (α-tocopherol & isomers)	~4.4	Antioxidant vitamin
18.42	Isopropyl palmitate	1.01	Fatty acid ester
20.76	Linoleic acid ethyl ester	0.24	PUFA ester
21.15	9,12,15-Octadecatrienoic acid, methyl ester	0.61	Omega-3 fatty acid ester
19.37	Isopropyl linoleate	0.93	PUFA ester
28.64	8,11,14-Eicosatrienoic acid (Z, Z, Z)	1.08	PUFA

Other high-intensity peaks included *I-(+)-ascorbic acid 2,6-dihexadecanoate*, a lipophilic vitamin C ester, and *methyl 5,11,14,17-eicosatetraenoate*, a PUFA ester associated with eicosatetraenoic acid derivatives. Smaller but notable contributions from tocopherol-like compounds, long-chain alkanes (e.g., *dotriacontane*), and fatty acid esters such as *isopropyl palmitate* and *isopropyl linoleate* were also detected. Although several constituents appeared in relatively low abundance, they were retained in the table due to their recognized functional significance in Echium oil, particularly the minor PUFA esters and antioxidant-associated components. Their presence, even at low levels, contributes to the characteristic biochemical fingerprint of the oil. The chemical structures of these key compounds are additionally shown in Figure 24.

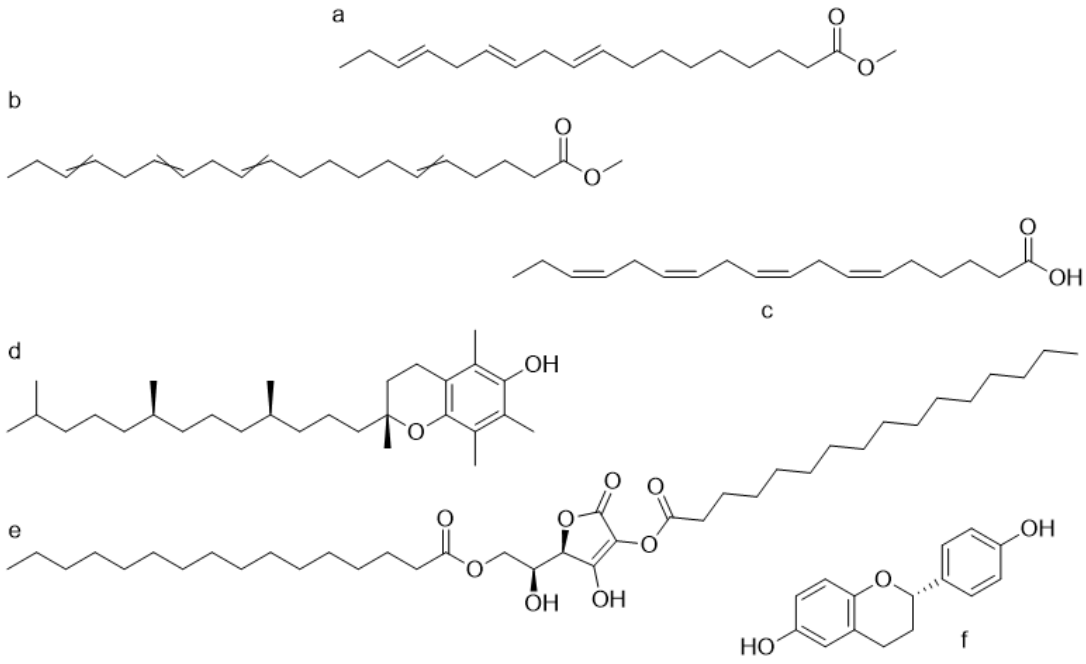


Figure 24. Chemical structures of the major Echium oil constituents, corresponding to the compounds reported in Table 4 and identified through GC–MS analysis, a) 9,12,15-Octadecatrienoic acid, methyl ester, b) Methyl 5,11,14,17-eicosatetraenoate, c) stearidonic acid (SDA), d) Vitamin E, e) l-(+)-Ascorbic acid 2,6-dihexadecanoate, and f) 2H-1-Benzopyran-6-ol derivatives. (Drawn using Chem Draw).

A representative chromatogram of the sample is shown in Figure 25. The major peaks correspond to the components listed in Table 5, with SDA and the ascorbyl ester displaying the highest peak intensities. The chromatographic pattern demonstrates a clean profile with no unexpected high-intensity unidentified peaks, supporting both the purity of the sample and the robustness of the analytical method.

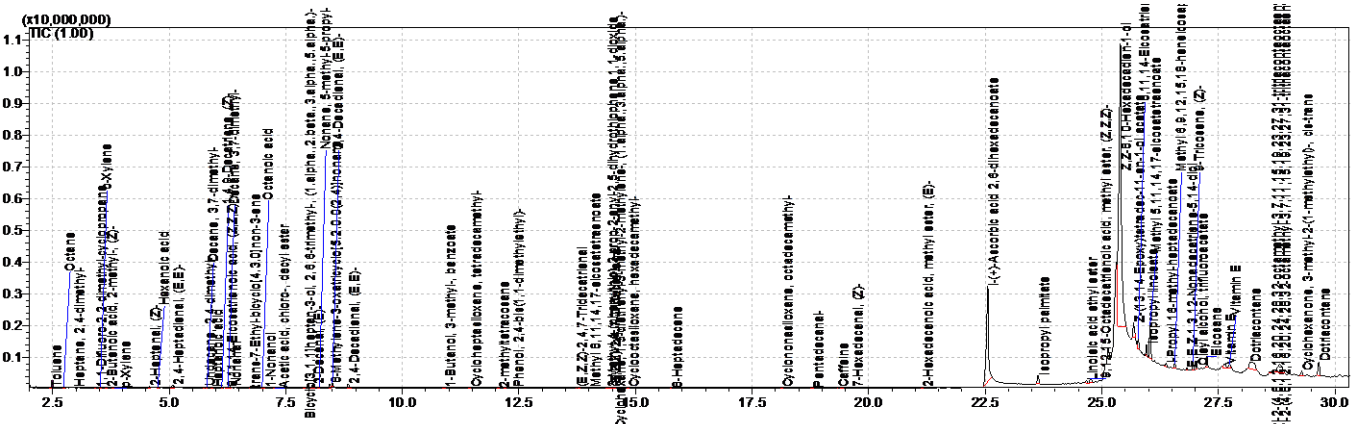


Figure 25. GC–MS chromatogram of the hexane-diluted Echium oil, showing major peaks corresponding to linoleic acid, and ascorbic acid esters.

1.1.4 Calendula (Calendula officinalis)

GC–MS analysis of the hexane-diluted Calendula oil produced a well-resolved chemical profile. Hexane dilution improved volatility and chromatographic separation of nonpolar constituents, allowing confident identification of the dominant components. As listed in Table *6, 9,12-octadecadienoic acid* (linoleic acid, Z, Z–) (37.5%) and *8,11,14-eicosatrienoic acid (Z, Z, Z–)* (21.3%) were the most abundant constituents, together accounting for most of the total ion current.

Table 6. Major volatile compounds identified in hexane-diluted Calendula oil by GC–MS.

RT (min)	Compound	Area %	Class
21.34	9,12-Octadecadienoic acid (Linoleic acid, Z, Z)	37.5%	PUFA (Polyunsaturated fatty acid)
24.82	8,11,14-Eicosatrienoic acid (Z, Z, Z-)	21.3%	PUFA (Omega-3/Omega-6 fatty acids)
18.92	I-(+)-Ascorbic acid 2,6-dihexadecanoate	8.0%	Vitamin C derivative
27.764	9-Octadecen-12-ynoic acid, methyl ester	3.76%	Fatty acid methyl ester (unsaturated, acetylenic)
22.74	Stearic acid (Octadecanoic acid)	1.1%	Saturated fatty acid
10.84	Isopropyl palmitate	0.7%	Fatty acid ester

I-(+)-ascorbic acid 2,6-dihexadecanoate was also detected at 8.0%, while *stearic acid* (1.1%) and *isopropyl palmitate* (0.7%) were present at low relative abundances; these minor constituents have been kept in the table for completeness. The chemical structures of these key compounds are additionally shown in Figure 26.

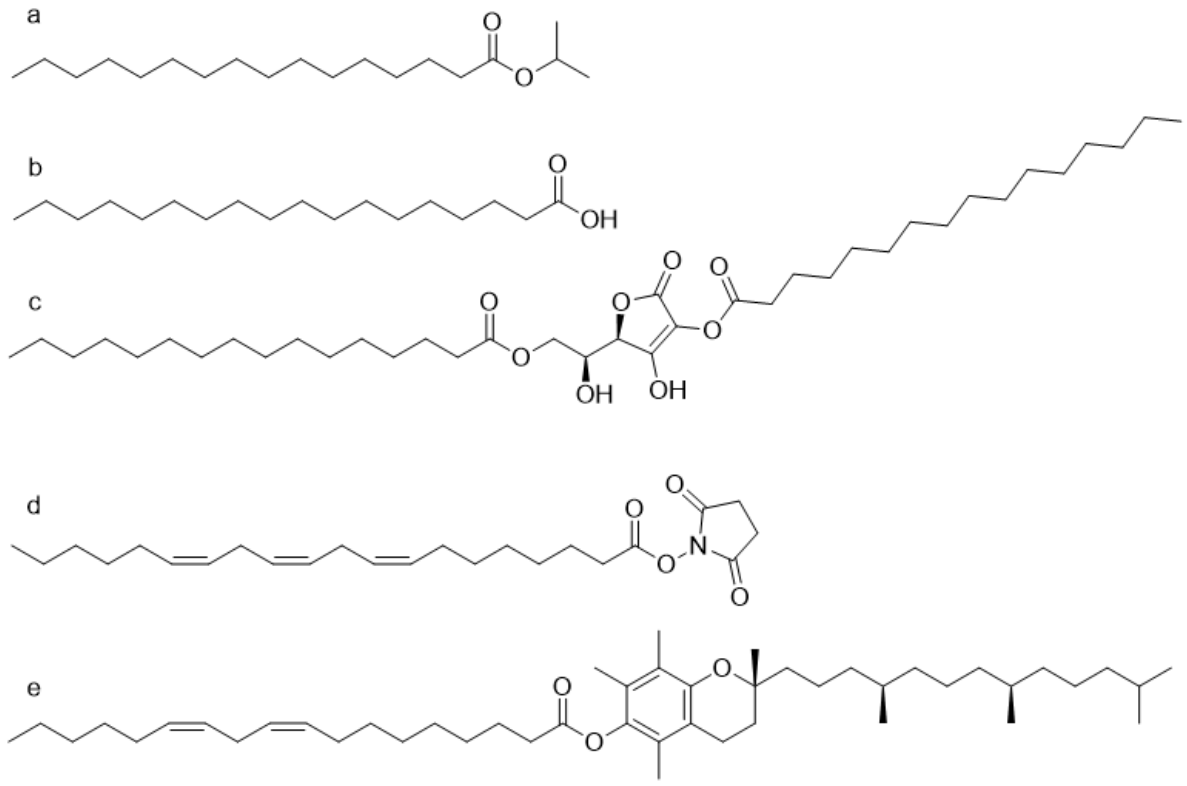


Figure 26. Chemical structures of the major Calendula oil constituents, corresponding to the compounds reported in Table 5 and identified through GC–MS analysis, a) isopropyl palmitate, *b)* stearic acid, *c)* I-(+)-ascorbic acid 2,6-dihexadecanoate, *d)* 8,11,14-eicosatrienoic acid (Z,Z,Z–), and e) 9,12-octadecadienoic acid *(linoleic acid, Z,Z–) (Drawn using Chem Draw).*

A representative chromatogram is shown in Figure 27; the chromatographic pattern is clean, with no unexpected high-intensity unidentified peaks.

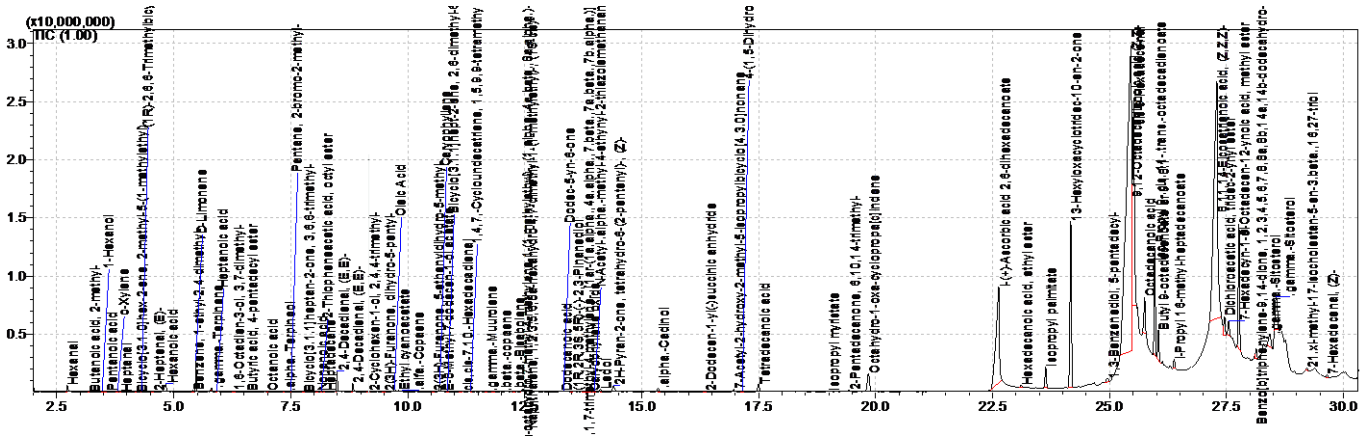


Figure 27. GC–MS chromatogram of the hexane-diluted Calendula oil, showing major peaks corresponding to linoleic acid, and ascorbic acid esters.

To confirm the identity of the key antioxidant constituents, GC–MS analyses were also performed on commercially available reference standards corresponding to the major compounds detected in the oils (e.g., Linoleic acid, oleic acid, and cis-9-hexsadecenal). Comparison of retention times confirmed the assignments presented in Table 7.

Table 7. Comparison of GC–MS identification parameters for selected compounds

Compound	RT in Spelt (min)	RT in Sea Buckthorn (min)	RT Standard (min)	ΔRT (Sample – Standard, min)	Confirmation
Oleic acid (9-Octadecenoic acid, (E)-)	25.4	25.393	25.45	Spelt: -0.05, Sea Buckthorn: -0.057	100% confirmed
Linoleic acid (9,12-Octadecadienoic acid (Z, Z))	25.4	25.393	25.385	Spelt: +0.015, Sea Buckthorn: +0.008	100% confirmed
cis-9-Hexadecenal (9-Hexadecen-1-ol, (Z))	–	25.393	26.781	Sea Buckthorn: -1.388	100% confirmed*

The table presents a comparison of retention times (RT) for three major compounds in Spelt and Sea Buckthorn oils with their respective pure standards. The RT differences between samples and standards (ΔRT) are calculated. Based on this comparison, the presence of Oleic acid, Linoleic acid, and cis-9-Hexadecenal in the samples is 100% confirmed. The asterisk (*) for cis-9-Hexadecenal indicates that this compound was detected only in the Sea Buckthorn sample and was not observed in Spelt. Among the identified major compounds, only those that could be reliably measured under the GC–MS conditions applied to the oils diluted in hexane were selected. This approach avoids more sample preparation or dilution steps, ensuring that the results are fully comparable across samples and standards. The GC-MS chromatogram of these standards are shown in Appendix 1.

2 Evaluation of Antioxidant Constituents of plant-based oils

To evaluate the radical-scavenging potential of the major antioxidant constituents identified in the plant-based oils, the DPPH assay was performed. This assay provides a quantitative measure of the ability of these compounds to neutralize free radicals. Following the assessment of DPPH activity, GC–MS analysis of post-reaction samples was conducted to investigate the specific compositional changes associated with radical quenching.

By comparing the chromatographic profiles before and after DPPH exposure, it was possible to identify which constituents actively participated in the antioxidant process, as well as any structural modifications, degradation, or formation of secondary products. The following sections present the DPPH results, along with the corresponding GC–MS observations, to provide a comprehensive view of both the functional and chemical responses of the oils.

2.1 DPPH Radical-Scavenging Assay and Metal-Chelating Activities

The radical-scavenging activity of the plant-based oils was assessed using the DPPH assay at concentrations ranging from 20 to 800 μL/mL (Figure 28). Among the tested oils, sea buckthorn exhibited the highest scavenging activity, reaching approximately 26% at the maximum concentration. Calendula showed moderate activity, whereas Echium and spelt displayed lower but concentration-dependent radical-scavenging effects.

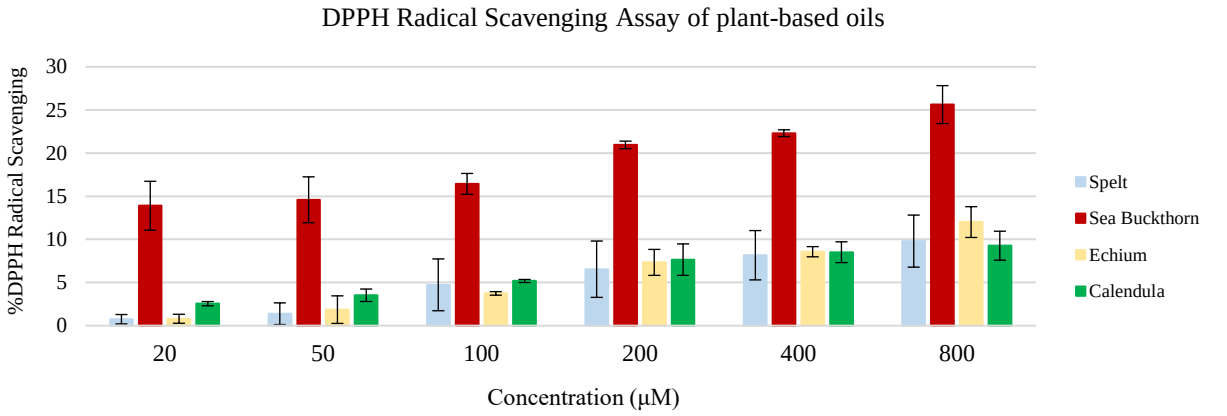


Figure 28.DPPH radical-scavenging activity of the CO_2-extracted plant-based oils at concentrations ranging from 20 to 800 µL/mL. Sea Buckthorn showed the highest activity, followed by, Calendula, Echium and spelt. Echium All showed concentration-dependent effects. Data are presented as mean ± SD of three independent replicates (n = 3). Error bars represent standard deviation.

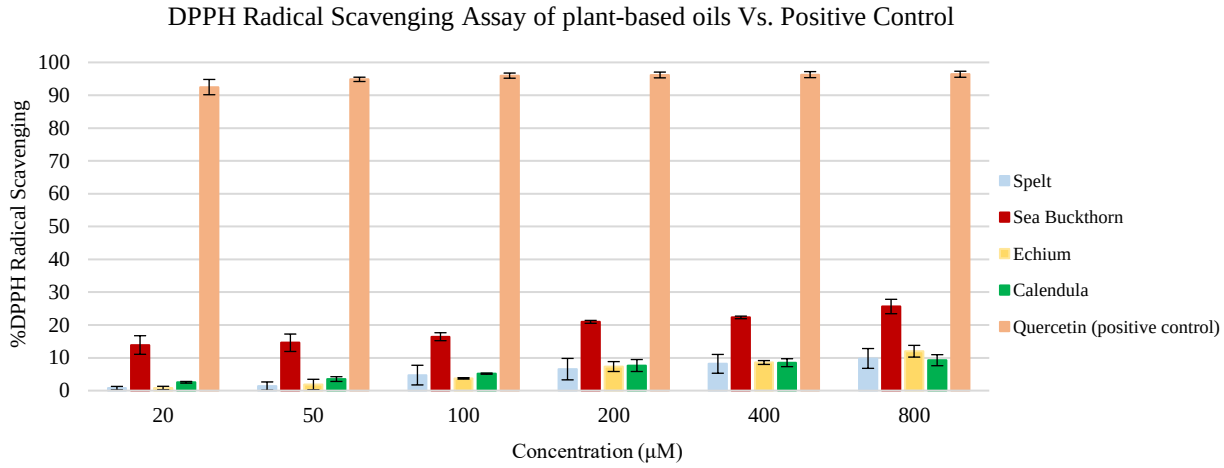


Figure 29. Radical-scavenging activity of plant-based oils determined by the DPPH assay (20–800 µL/mL). Quercetin (50 µg/mL) served as the positive control. Data are presented as mean ± SD of three independent replicates (n = 3). Error bars represent standard deviation.

The positive control, quercetin (1 μg/mL), achieved nearly complete DPPH neutralization across all concentrations, confirming the validity of the assay. Since none of the oils reached 50% DPPH scavenging even at the highest tested concentration, IC_{50} values could not be determined. Therefore, the results are reported as the percentage of radical scavenging at each concentration.

Comparison with the blank (containing only the solvent) indicates that the observed scavenging effects are primarily due to the antioxidant constituents of the oils rather than the solvents or baseline absorbance (Figure 29). These findings emphasize the contribution of the major antioxidant compounds identified by GC–MS to the overall radical-scavenging capacity of the oils. The corresponding quantitative data for all tested concentrations are summarized in Table 8, supporting the trends illustrated in Figure 29.

Table 8. Percentage DPPH radical-scavenging activity of the CO_2-extracted plant-based oils at concentrations ranging from 20 to 800 μL/mL. Values are presented as mean ± SD of three independent replicates (n = 3) of the scavenging percentage for each oil at each tested concentration vs. Quercetin. +/- represent standard deviation.

	%DPPH Radical Scavenging				
Concentration (μL/mL)	**Spelt**	**Echium**	**Calendula**	**Sea Buckthorn**	**Quercetin**
20	0.74 ± 0.547	0.47 ± 0.521	2.55 ± 0.250	13.90 ± 2.833	93.67 ± 2.327
50	1.38 ± 1.255	1.61 ± 1.597	3.52 ± 0.724	14.59 ± 2.662	95.82 ± 0.659
100	4.73 ± 3.003	3.50 ± 0.204	5.18 ± 0.178	16.43 ± 1.211	96.82 ± 0.789
200	6.55 ± 3.269	7.10 ± 1.504	7.65 ± 1.823	20.95 ± 0.438	97.00 ± 0.884
400	8.16 ± 2.862	8.34 ± 0.591	8.51 ± 1.206	22.31 ± 0.393	97.10 ± 0.918
800	9.05 ± 3.018	11.79 ± 1.786	9.27 ± 1.680	25.63 ± 2.197	97.19 ± 0.918

The DPPH solution is initially purple, and the addition of antioxidant-containing samples can induce decolorization. Figure 30 shows the color change for Sea Buckthorn oil, which shows relatively low antioxidant activity, resulting in minimal visible change, compared to quercetin, which caused a noticeable yellowing of the solution. While such visual changes may not always be readily clear, the quantitative absorbance measurements at different concentrations confirm the concentration-dependent radical-scavenging activity of the samples.

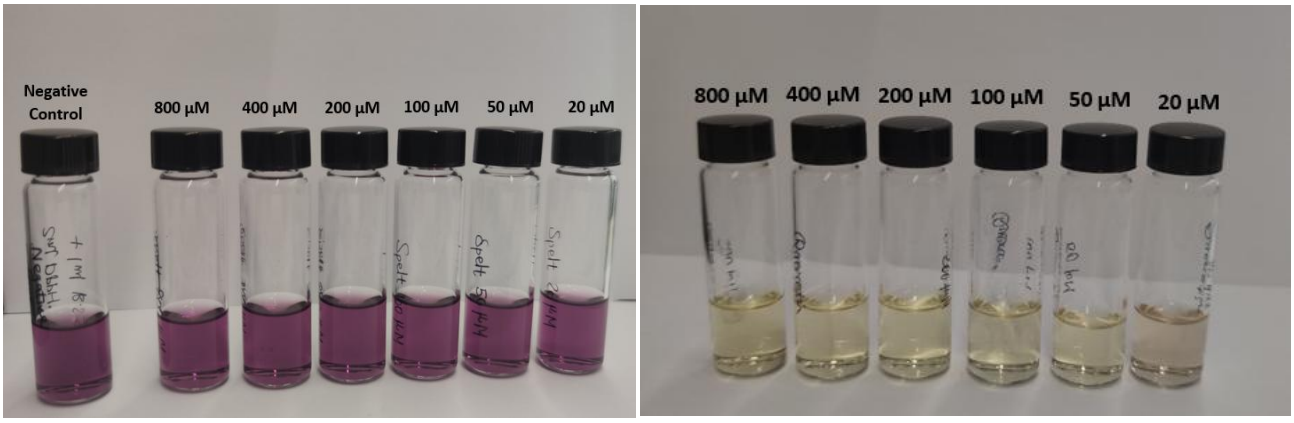


Figure 30. Visual representation of DPPH radical-scavenging activity. The left shows Sea Buckthorn oi + DPPH, exhibiting minimal color change due to relatively low antioxidant activity, while the right shows quercetin, the positive control, which caused a clear yellowing of the solution. Although color changes may not always be visually pronounced, quantitative absorbance measurements confirm concentration-dependent radical-scavenging effects.

Attempts to assess the metal-chelating activity of the plant-based oils were also conducted; however, the results were neither reproducible nor reliable. This limitation is primarily due to the aqueous nature of the assay, which is incompatible with oil-based samples. Since the oils were obtained via CO_2 extraction, they are enriched in lipophilic antioxidant compounds, which are poorly soluble in water. Consequently, the metal-chelating assay, which requires water-soluble antioxidants, was not suitable for evaluating the chelating activity of these lipophilic extracts.

3 Alterations in GC–MS Profiles Following DPPH Reaction

Following the identification of major antioxidant constituents in the plant-based oils and confirmation of their radical-scavenging activity through the DPPH assay, GC–MS analysis was conducted on post-reaction samples to investigate compositional changes specifically attributable to antioxidant–radical interactions.

To distinguish genuine reaction-induced alterations from solvent- or dilution-related effects, two parallel sample sets were prepared for each oil using a 5% DMSO / 95% MeOH (v/v) solvent system. Reaction mixtures contained the oil at a final concentration of 1 µL/mL together with 100 µM DPPH, whereas solvent controls were prepared identically but without DPPH. This comparative approach enabled the assessment of peak disappearance, reduction, or transformation in the GC–MS chromatograms, allowing the identification of constituents most susceptible to radical quenching.

Representative chromatograms of the solvent controls are shown in Figures 32a-d, and the corresponding DPPH-reacted samples are shown in Figures 30a-d. Comparison of the paired chromatograms for each oil (Figures 32a vs 31a for Spelt, 32b vs 31b for Calendula, 32c vs 31c for Sea Buckthorn, and 32d vs 31d for Echium) enabled the identification of constituents that decreased, disappeared, or showed minor shifts following interaction with DPPH. These qualitative alterations are attributed to the higher susceptibility of specific antioxidant components, particularly the lipophilic compounds enriched by CO_2 extraction, to radical quenching. Indeed, the GC–MS chromatograms of the oils after reaction with DPPH (Figures 31a-d) show a noticeable decrease in the intensity of several key peaks compared to the solvent control (Figures 32a-d). This reduction is consistent with the consumption of the major antioxidant constituents during radical scavenging, supporting their direct involvement in DPPH neutralization.

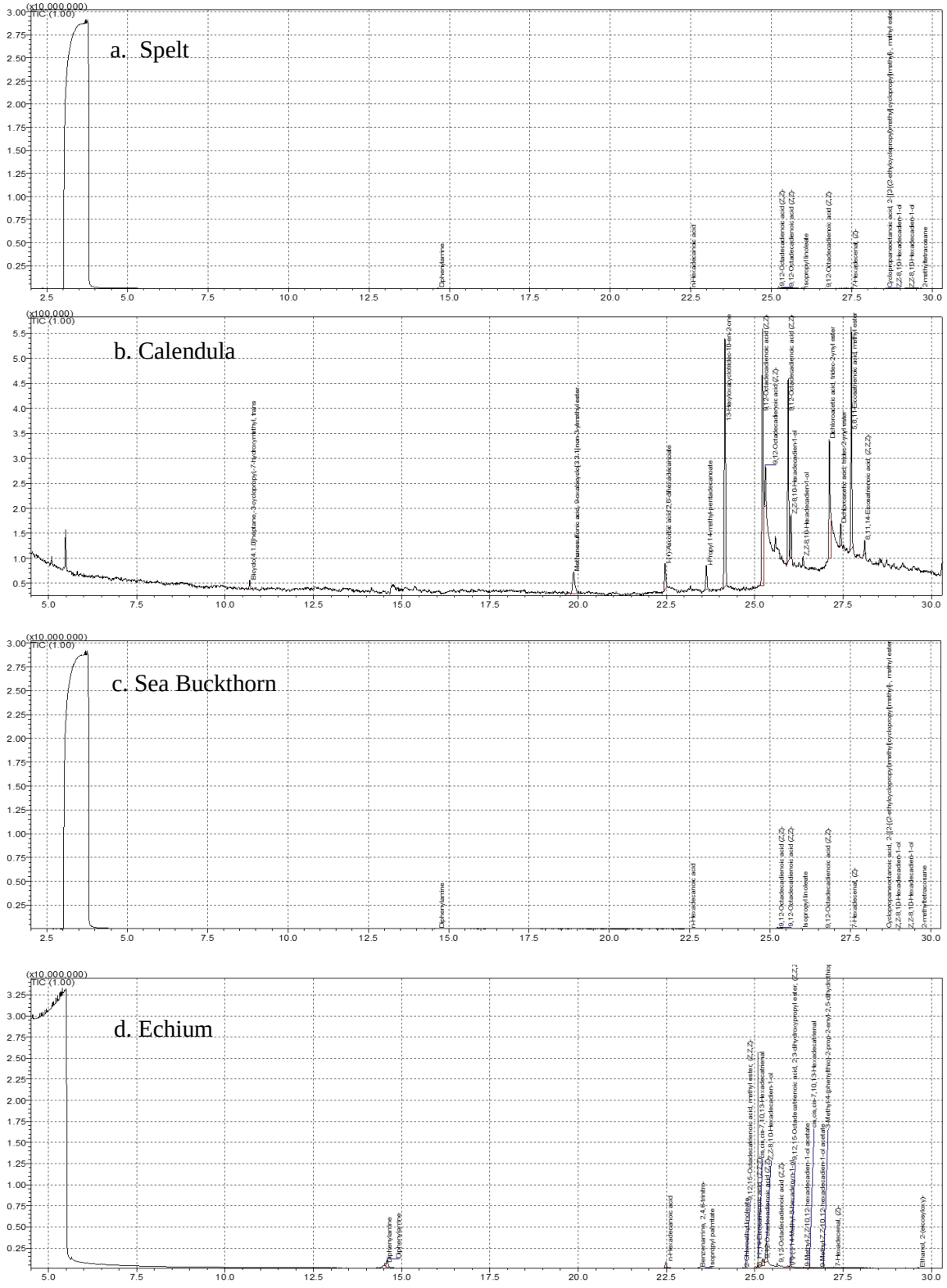


Figure 31. GC–MS chromatograms of plant-based oils (a. Spelt, b. Calendula, c. Sea buckthorn, and d. Echium) after reaction with DPPH (100 µM) in DMSO/MeOH (5:95 µL/mL). Comparative analysis with solvent controls highlights the reduction, disappearance, or transformation of major antioxidant constituents, indicating their involvement in radical scavenging.

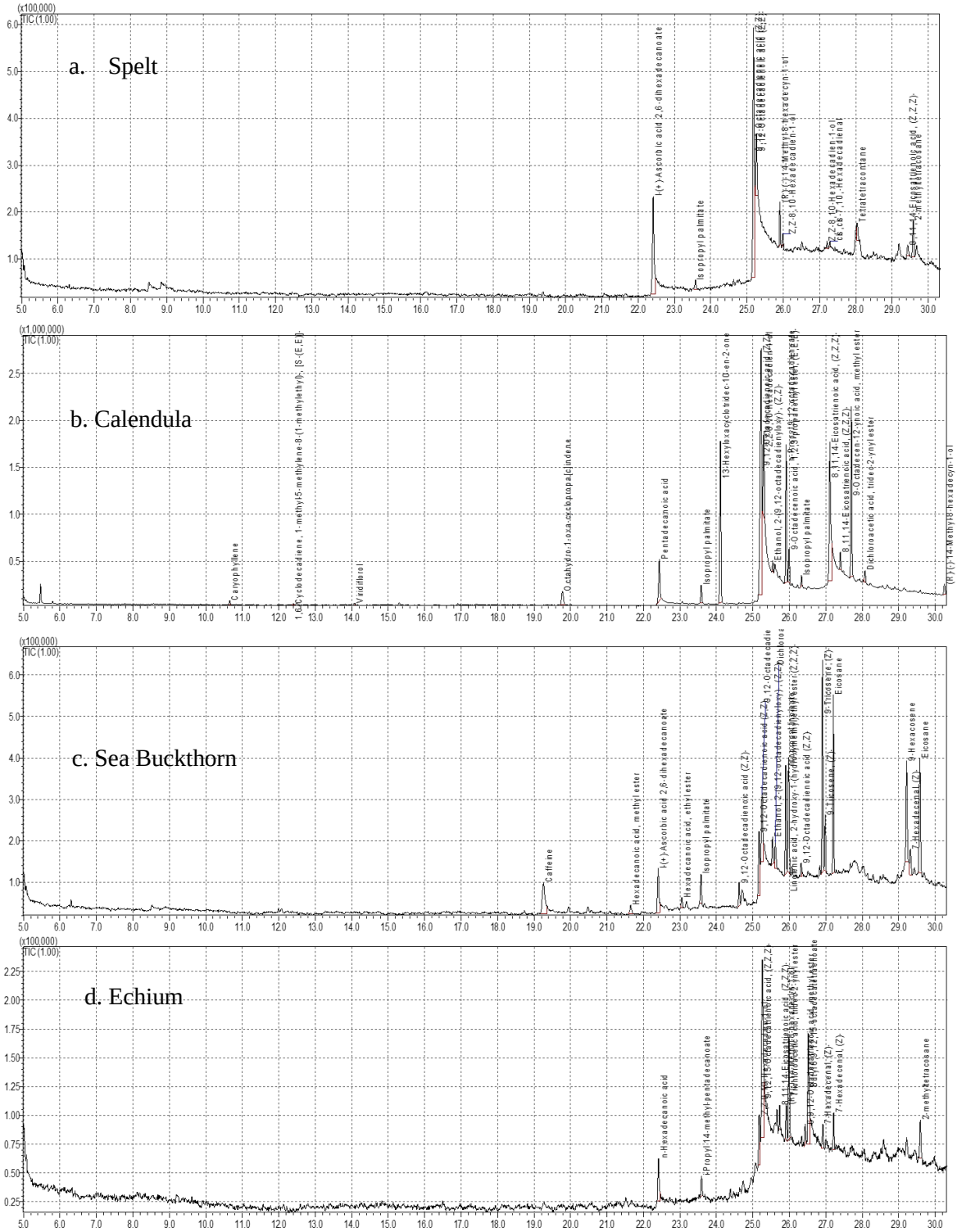


Figure 32. GC–MS chromatograms of plant-based oils (a. Spelt, b. Calendula, c. Sea buckthorn, and d. Echium) as a solvent control after diluting with DMSO/MeOH (5:95 µL/mL). Comparative analysis with plant-based oils GCMS chromatogram highlights the reduction, disappearance, or transformation of major antioxidant constituents, indicating their involvement in radical scavenging.

4 In silico toxicity prediction of selected compounds

In silico toxicity predictions for all selected compounds were performed using the Derek Nexus platform across the evaluated endpoints and mammalian species (human, mouse, and rat) (Chapter 2, section 3.3).

4.1 Linoleic acid

In silico toxicity prediction of linoleic acid shows no structural alerts associated with genotoxicity, skin irritation, skin sensitization, neurotoxicity, or organ toxicity were identified within the applicability domain of the model. Linoleic acid was predicted to be inactive in the bacterial in vitro mutagenicity (Ames) test, as no mutagenicity-related structural alerts or misclassified features were detected. These predictions are consistent with the simple aliphatic fatty acid structure of linoleic acid and the absence of known toxicophoric features, suggesting a low toxicological concern for the endpoints evaluated under the conditions modelled.[134]

4.2 Oleic acid

In silico toxicity prediction of oleic acid was consistently predicted to be a non-sensitizer for skin sensitization in all evaluated species, with no associated structural alerts identified within the applicability domain of the model. In addition, no alerts were triggered for genotoxicity or mutagenicity-related endpoints, including in vitro and in vivo mutagenicity.

Similarly, no structural alerts were found for irritation or major organ toxicity endpoints. These predictions are consistent with the simple aliphatic fatty acid structure of oleic acid and the absence of recognized toxicophoric features, indicating a low predicted toxicological concern for the endpoints assessed.[134]

4.3 Ascorbic acid derivative

In silico toxicity prediction of the ascorbic acid was predicted to be inactive in bacterial in vitro mutagenicity assays, with no structural alerts or misclassified features associated with genotoxicity identified within the applicability domain of the model. In contrast, a plausible skin sensitization potential was predicted across all evaluated species, including human, mouse, and rat.

This prediction was associated with the presence of a vinyl ester–type structural alert, which is known to act via a hapten-mediated mechanism involving electrophilic acylation. The predicted LLNA EC3 value (~24%) places the compound within GHS Category 1B, indicating a weak to moderate skin sensitization potential. These findings suggest that, while the compound is not predicted to be genotoxic, its structural features may contribute to skin sensitization risk, which should be considered in the context of dermal exposure.[134]

4.4 8,11,14-Eicosatrienoic acid (Z, Z, Z-)

According to the in-silico toxicity prediction of the polyunsaturated fatty acid (8,11,14-Eicosatrienoic acid (Z, Z, Z-)) no structural alerts were identified for any of the evaluated endpoints within the applicability domain of the model. The compound was predicted to be a non-sensitizer for skin sensitization across all species assessed, with no alerts associated with dermal irritation or corrosion.

Furthermore, no alerts were triggered for genotoxicity-related endpoints, including in vitro chromosomal damage, in vitro or in vivo mutagenicity, or non-specific genotoxicity. Similarly, no alerts were found for major organ toxicity endpoints, including hepatotoxicity and nephrotoxicity. Overall, the absence of structural alerts across a broad range of toxicity endpoints suggests a low predicted toxicological concern for this polyunsaturated fatty acid under the conditions modelled.[134]

4.5 cis-9-Hexadecenal (alkyl aldehyde compound)

In silico toxicity prediction of the alkyl aldehyde compound was predicted to be inactive in bacterial in vitro mutagenicity assays, indicating no associated structural alerts for Ames test, based genotoxicity. However, structure-based predictions suggested a plausible genotoxic potential in mammalian systems, including in vitro chromosomal damage and mutagenicity, which were attributed to the presence of an alkyl aldehyde functional group.

In addition to genotoxic endpoints, the compound was predicted to show plausible skin irritation and skin sensitization potential across the evaluated mammalian species. These predictions are consistent with the electrophilic nature of aldehyde groups, which are known to interact with nucleophilic sites in biological macromolecules such as proteins and DNA. Overall, the in-silico results indicate that, while the compound is not predicted to be mutagenic in bacterial systems, its structural features may confer an increased toxicological concern in mammalian cells, particularly with respect to genotoxic and dermal endpoints.[134]

4.6 Stearidonic acid (SDA)

As in silico toxicity prediction of Stearidonic acid (SDA) reveals, no structural alerts were identified for any of the evaluated endpoints within the applicability domain of the model. The compound was predicted to be a non-sensitizer for skin sensitization and showed no alerts for dermal irritation or corrosion.

Similarly, no alerts were triggered for genotoxicity-related endpoints, including in vitro and in vivo mutagenicity, chromosomal damage, or non-specific genotoxicity. No alerts were found for major organ toxicity endpoints such as hepatotoxicity, cardiotoxicity, or nephrotoxicity. Overall, the absence of structural alerts across a wide range of toxicity endpoints suggests a low predicted toxicological concern for Stearidonic acid under the conditions modelled.[134]

5 Summary of in silico toxicity profile of selected compounds

According to table 9, among the polyunsaturated fatty acids, including linoleic acid, oleic acid, Stearidonic acid (SDA), and 8,11,14-Eicosatrienoic acid (Z, Z, Z-), no structural alerts were found across the evaluated endpoints within the applicability domain of Derek Nexus. These compounds were consistently predicted to be non-sensitizers for skin sensitization, with no alerts for dermal irritation or corrosion. Similarly, no alerts were triggered for genotoxicity-related endpoints, including in vitro or in vivo mutagenicity, chromosomal damage, or non-specific genotoxicity. Major organ toxicity endpoints, such as hepatotoxicity and nephrotoxicity, also showed no predicted concern. Overall, these results show a low predicted toxicological concern for these fatty acids under the modelled conditions.

Ascorbic acid was predicted as a plausible skin sensitizer (LLNA EC3 = 24%, GHS 1B) for all mammalian species, while dermal irritation/corrosion showed no alerts. Genotoxicity-related endpoints in mammalian systems were predicted as plausible, while bacterial in vitro mutagenicity was inactive.

The alkyl aldehyde compound was inactive in bacterial in vitro mutagenicity assays; however, structure-based predictions indicated plausible genotoxic potential in mammalian systems, including in vitro chromosomal damage and mutagenicity. Plausible skin irritation and skin sensitization were also predicted, consistent with the electrophilic nature of the aldehyde group.

These findings suggest that, although polyunsaturated fatty acids exhibit minimal predicted toxicity, functionalized compounds such as ascorbic acid and alkyl aldehydes could pose an elevated toxicological concern, especially regarding genotoxic and dermal endpoints.

Table 9. Summary of in silico toxicity predictions for selected compounds.

Compound	Skin Sensitization	Dermal Irritation / Corrosion	Mutagenicity (in vitro/ in vivo)	Chromosomal Damage (in vitro)	Non-specific Genotoxicity	Hepatotoxicity	Nephrotoxicity
Linoleic acid	Non-sensitizer	No alerts	No alerts	No alerts	No alerts	No alerts	No alerts
Oleic acid	Non-sensitizer	No alerts	No alerts	No alerts	No alerts	No alerts	No alerts
Stearidonic acid (SDA)	Non-sensitizer	No alerts	No alerts	No alerts	No alerts	No alerts	No alerts
Ascorbic acid	Plausible	No alerts	Plausible (mammalian)	Plausible (mammalian)	Plausible	No alerts	No alerts
cis-9-Hexadecenal	Plausible	Plausible	Plausible (mammalian)	Plausible (mammalian)	Plausible	No alerts	No alerts
8,11,14-Eicosatrienoic acid (Z, Z, Z-)	Non-sensitizer	No alerts	No alerts	No alerts	No alerts	No alerts	No alerts

Having confirmed the antioxidant potential of the major constituents in the plant-based oils through DPPH assays and GC–MS analysis, we next aimed to investigate the biological relevance of these compounds in a living organism. The *Drosophila melanogaster* lifespan assay was employed as a well-established in vivo model to evaluate the effects of the oils on organismal longevity and overall health.

Chapter 4: Biological Impact of plant-based Oils in Drosophila melanogaster: A Combined Lifespan and Climbing Assay Approach

Drosophila melanogaster is a well-established in vivo model organism widely used for investigating the biological and toxicological effects of natural products, including plant-derived oils. Its short lifespan and well-defined behavioral responses provide a powerful platform for assessing whole-organism outcomes related to survival, physiological stress, and locomotor performance.[9] According to the chemical characterization and antioxidant profiling presented in the previous chapter, it is crucial to evaluate how differences in oil composition translate into measurable biological effects across treatments. Although the oils are a mixture of different compounds, it is impossible to fractionate the different components of the oils to evaluate each individually. In contrast, in silico testing of the main components may offer better insights about their exact effects.

In this study, two *Drosophila* bioassays were employed to answer this question. Lifespan analysis was used to quantify the effects of oil identity, dose, and strain background on survival and age-related decline. In parallel, the negative geotaxis (climbing) assay served as a functional measure of locomotor ability and neuromuscular integrity, enabling detection of sub-lethal or dose-dependent impairments. Together, these assays allow a systematic comparison of how each oil influences fly performance, whether oils differ significantly in their biological impact, and whether the responses are consistent or strain specific.

This chapter therefore examines the biological consequences of essential oil exposure by integrating lifespan and climbing outcomes across oils, doses, and strains. By linking these in vivo findings to the chemical and antioxidant data from Chapter 3, the aim is to develop a comprehensive understanding of how intrinsic oil composition drives variations in toxicity, physiological performance, and strain-dependent sensitivity.

1 Descriptive Overview

The biological effects of essential oils on *Drosophila melanogaster* were initially evaluated through a descriptive overview of lifespan and climbing performance across all treatments. For each oil and dose, five replicate vials containing twenty flies each were monitored. Following the descriptive overview of the experimental structure, the first level of analysis focused on lifespan patterns across all oils, doses, and strains.

2 Lifespan Assay Analysis

The lifespan assay was designed to quantitatively assess how variation in plant-based oils' identity, dose, and genetic background shapes survival outcomes in *Drosophila melanogaster*. In line with the overall aims of this chapter, this analysis focuses on determining: (i) What is the effect of oils, doses, and strains on the flies' survival profiles, (ii) How is the effect of each oil different from the other oils? and (iii) whether the two strains exhibit comparable or divergent sensitivity to the same treatments. By generating and comparing survival curves for every oil-dose-strain combination, this section provides a structured evaluation of how treatment-dependent and strain-dependent factors contribute to differences in longevity across the dataset.

2.1 Survival Patterns Across Oils, Doses, and Strains

The effect of plant-based oils was evaluated by exposing two strains of *Drosophila* flies, Lancaster and wDah, to food supplemented with oils at three different concentrations: 0.005%, 0.015%, and 0.05% v/v, beginning when the flies were 3 days old, over the experimental period. As previously described and is shown in Table 10, lifespan analysis was initiated with 20 flies per vial for each treatment group, with five independent vials per condition. Flies were transferred to fresh food and scored for survival every two days until all individuals had died. In addition, the median lifespan corresponds to the time point at which 50% of flies have died. Median lifespan is typically used to describe typical survival.

According to the Table 10, in the Lancaster strain, exposure to Calendula and Echium essential oils resulted in a statistically significant increase in mortality at all tested doses ($p < 0.05$), accompanied by reductions in median lifespan of up to 30% (Figure 33A), indicating that even the lowest concentration of these oils was sufficient to affect fly survival.

Sea Buckthorn oil demonstrated a dose-dependent toxic effect in Lancaster flies, with significant mortality observed at 0.015% and 0.05% concentrations ($p = 0.002$ and $p = 0.042$, respectively), which showed a reduction in median lifespan of 22% and 19%, respectively (Figure 33B-C), while the lowest dose (0.005%) did not produce a statistically significant effect but caused a reduction of 19% ($p=0.053$) (Figure 33A).

In the case of Spelt oil, only the intermediate dose of 0.015% led to a significant increase in mortality ($p = 0.01$) and a 14% decrease in median lifespan, whereas the lower and higher concentrations did not differ significantly from the control group.

Table 10. Pairwise comparisons of fly survival between essential oil-treated groups and control groups using the Log-Rank (Mantel-Cox) test. p-values indicate the statistical significance of differences in survival between each oil treatment and the corresponding control for Lancaster and wDah strains at three concentrations (0.005%, 0.015%, and 0.05% v/v). The percentage change in median lifespan relative to the control group is also reported. Values of $p < 0.05$ are considered statistically significant, indicating a higher mortality rate compared to control.

		Dose%	p value (Log Rank (Mantel-Cox)) vs. Control Group	Number of flies	median lifespan (%)
Calendula	Lancaster	0.005	0.009	20	-30%
		0.015	0.005	20	-30%
		0.05	0.004	20	-25%
Calendula	wDah	0.005	0.074	20	-17%
		0.015	0.23	20	-23%
		0.05	0.604	20	-20%
Echium	Lancaster	0.005	0.02	20	-19%
		0.015	0.029	20	-19%
		0.05	0.011	20	-25%
Echium	wDah	0.005	0.174	20	-17%
		0.015	0.198	20	-13%
		0.05	0.087	20	-17%
Sea Buckthorn	Lancaster	0.005	0.053	20	-19%
		0.015	0.002	20	-22%
		0.05	0.042	20	-19%
Sea Buckthorn	wDah	0.005	0.432	20	-13%
		0.015	0.161	20	-13%
		0.05	0.07	20	-17%
Spelt	Lancaster	0.005	0.51	20	-8%
		0.015	0.01	20	-14%
		0.05	0.08	20	-19%
Spelt	wDah	0.005	0.272	20	-10%
		0.015	0.415	20	-10%
		0.05	0.26	20	-17%

In contrast, none of the tested essential oils caused a statistically significant change in mortality in the wDah strain at any of the concentrations ($p > 0.05$), suggesting a strain-dependent difference in susceptibility to these oils (Figure 33E-F).

Overall, these results highlight that both the type of plant-based oils and the genetic background of the fly strain play important roles in determining toxicity, with Lancaster flies being more sensitive to the oils than wDah flies, and the toxic effect generally increasing with dose for those oils that showed significance.

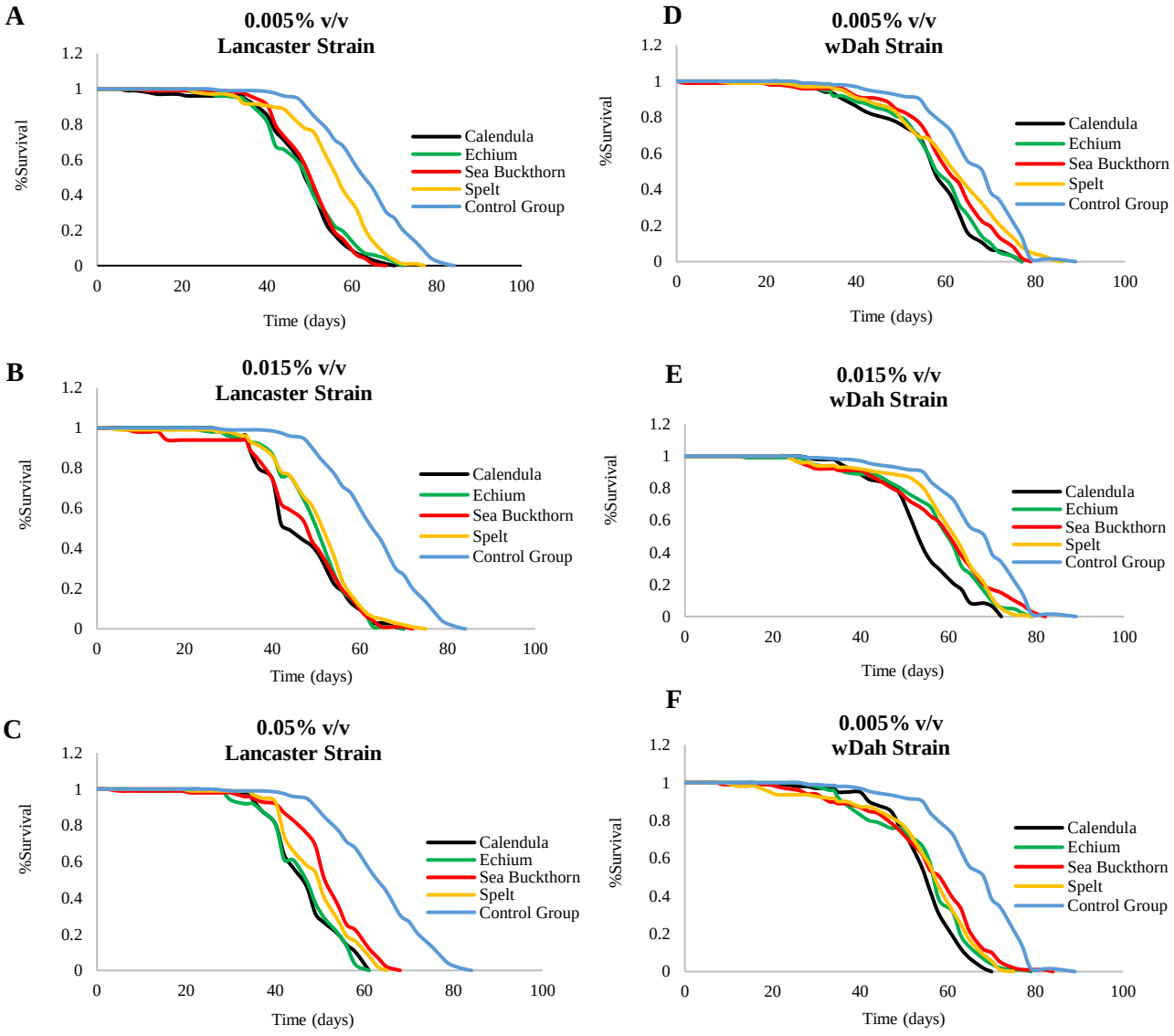


Figure 33. Line chart showing the effect of four essential oils (Calendula, Echium, Sea Buckthorn, and Spelt) on fly survival over the experimental period. Flies from Lancaster (A-C) and wDah (D-F) strains were exposed to three concentrations of each oil (0.005%, 0.015%, and 0.05% v/v). Lifespan analysis was initiated with 20 flies per vial for each treatment group, with five independent vials per condition. Each line represents the mean proportion of surviving flies at each time point for a given treatment, compared to the control group. Differences between treatments and controls were evaluated using the Log-Rank (Mantel-Cox) test, with $p < 0.05$ indicating statistically significant

To complement the day-by-day survival trajectories, mean lifespan values were calculated for each oil–dose combination to provide an overview of long-term survival performance. The mean lifespan represents the average lifespan of all individuals in a vial. This summary representation facilitates clearer comparison of dose-dependent effects within individual oils, independent of the temporal complexity captured in the survival curves. As shown in Figure 34B, the mean lifespan of Lancaster flies decreased progressively with increasing doses of Calendula and Echium oils, consistent with the significant mortality patterns observed in the survival analyses.

Sea Buckthorn oil also showed a clear dose-response trend, with a marked reduction in mean lifespan at 0.015% and 0.05%, whereas the 0.005% dose remained comparable to the control. In contrast, the mean lifespan profiles for Spelt oil revealed a non-linear pattern, with only the intermediate concentration (0.015%) producing a noticeable reduction. For the wDah strain (Figure 34A), mean lifespan values remained relatively stable across all oils and doses, reinforcing the absence of statistically significant effects identified earlier. Together, these bar-plot summaries provide an integrated visual metric that confirms the strain-dependent sensitivity of Lancaster flies and highlights the distinct dose-response characteristics associated with each plant-based oil.

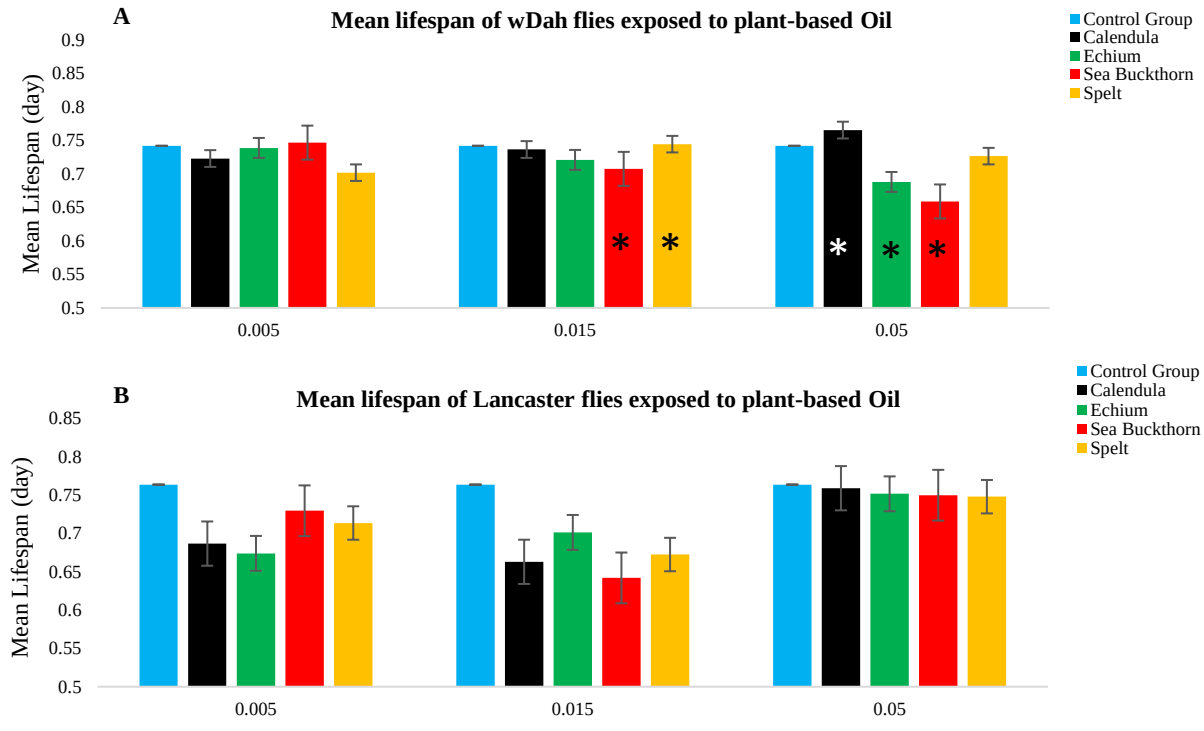


*Figure 34. Mean lifespan of Lancaster and wDah flies exposed to three doses of plant-based oils. Data are presented as mean ± SD. * indicates $p < 0.05$ compared to the blank, determined by ANOVA. Mean lifespan represents the average lifespan of all flies in each group.*

Detailed lifespan graphs for the four oil concentrations, categorized by oil type, are included in the Appendix 2.

While survival analyses supply essential information on the lethal effects of oil exposure, they do not capture potential sub-lethal impairments that may precede mortality. Locomotor performance, particularly negative geotaxis behavior, is a sensitive indicator of neuromuscular function and physiological stress in *Drosophila melanogaster*. Therefore, to evaluate whether plant-based oils affected functional performance beyond survival outcomes, climbing assays were conducted across oils, doses, and strains.

3 Effects of Plant-Based Oils on Negative Geotaxis Performance or Climbing Assay

3.1 Overview of locomotor performance across oils, doses, and strains

To understand how the animal model used would respond to an essential oil diet, the group performed the climbing assay using three sets of plant-based concentrations of four oils, namely: Calendula (*Calendula officinalis*), Echium (*Echium vulgare*), Sea buckthorn (*Hippophae rhamnoides L., Elaeagnaceae*), and Spelt (*Triticum spelta L.*).

Overall, exposure to plant-based oils produced distinct, strain- and dose-dependent effects on locomotor performance. Lancaster flies showed a pronounced reduction in climbing ability in response to several oils, whereas the wDah strain showed comparatively stable performance across treatments. The size and direction of the effects varied depending on oil type and concentration, indicating differential susceptibility and non-uniform dose–response relationships.

To quantitatively assess these effects, climbing performance data were analyzed using two-way ANOVA, with oil type and dose as fixed factors, and time (day 33 and day 40) treated as a repeated measure. Analyses were conducted separately for each strain to account for inherent genetic differences in baseline locomotor activity.

3.2 Statistical approach for climbing assay analysis

Climbing performance in the negative geotaxis assay was quantified using mean climbing height as the primary response variable. Data were analyzed to assess the effects of dietary exposure to plant-based oils across different concentrations and time points. Oil type and dose were treated as fixed between-subject factors, while time (day thirty-three and day 40) was included to capture potential age-dependent changes in locomotor performance.

To account for inherent genetic and physiological differences between fly lines, statistical analyses were conducted separately for the Lancaster and wDah strains. This strain-specific approach allowed for a clearer evaluation of treatment effects without confounding baseline differences in locomotor activity. Where proper, multivariate analysis of variance (MANOVA) was applied to simultaneously evaluate the combined influence of dose and time on climbing performance, followed by univariate two-way ANOVA to resolve factor-specific effects and interactions.

The inclusion of interaction terms enabled assessment of whether the size and direction of dose effects varied over time. Where significant main effects or interactions were detected, pairwise comparisons were conducted to determine differences between treatment levels.

3.3 Climbing assay in Lancaster strain

3.3.1 Effect of oil and dose on climbing ability in Lancaster strain

The results of the climbing assay conducted in *Drosophila melanogaster* (Lancaster strain) demonstrated that neuromotor performance was influenced by the type of plant-based oil, the applied concentration, and the duration of exposure. Comparison of responses at two experimental time points (Day 33 and Day 40) indicates that the effects of essential oils are primarily dose-dependent, with certain oils additionally exhibiting time-dependent modulation of their effects.

According to Figure 35A, clear differences were observed among treatment groups on Day 33. At this time point, Sea Buckthorn oil at a concentration of 0.05% exhibited the highest mean climbing height (~349 mm), exceeding that of the control group (~322 mm). This observation shows that, within this exposure period, this treatment did not exert an inhibitory effect on locomotor performance and was associated with preserved climbing ability. In contrast, Spelt oil showed a significant dose-dependent reduction in climbing performance ($p = 0.0009$), with increasing concentrations resulting in a marked decline (≥50%) in mean climbing height relative to the control.

For Calendula oil, statistical analysis revealed a significant main effect of dose on climbing performance ($p = 0.0037$), while neither the main effect of time ($p = 0.8411$) nor the time × dose interaction ($p = 0.2150$) reached statistical significance. These results show that the effect of Calendula oil on locomotor performance was primarily dose-dependent and remained consistent across the experimental time points.

Echium oil induced a pronounced impairment of locomotor performance. A highly significant main effect of dose was detected ($p = 0.0002$), together with a considerable time × dose interaction ($p = 0.0034$), indicating that the magnitude of the dose-dependent effect varied over time and became more pronounced following prolonged exposure.

Sea Buckthorn oil also showed a significant dose-dependent effect on climbing performance ($p = 0.0003$), accompanied by a considerable time × dose interaction ($p = 0.0009$), suggesting that the impact of this oil on neuromotor function was modulated by exposure duration.

With continued exposure until Day 40, as shown in Figure 35B, the response pattern changed substantially. At this later time point, the control group displayed a pronounced increase in mean climbing height (~527 mm), likely reflecting age-related physiological changes. In contrast, all oil-treated groups exhibited markedly lower climbing performance relative to the control, indicating cumulative adverse effects of oil exposure on neuromotor function (~61-93% reduction).

Among the treatments, Echium oil resulted in the lowest mean climbing heights across all concentrations on Day 40, being the greatest reduction (up to 92%) in locomotor performance under prolonged exposure conditions. Conversely, Calendula oil at 0.05% kept a higher level of climbing performance relative to the other treated groups at Day 40 (~67% reduction compared to the control group), although this value remained substantially lower than that of the control. These findings show that while chronic exposure to plant-based oils generally leads to impaired locomotor performance, the severity and temporal progression of this effect depend on oil type and concentration.

Overall, these results highlight the importance of assessing the effects of plant-based oils on neuromotor performance across multiple time points, as early responses do not necessarily predict outcomes following prolonged exposure. The observed differences among oils are consistent with distinct dose-dependent and time-modulated response profiles and should be considered when interpreting their biological effects.

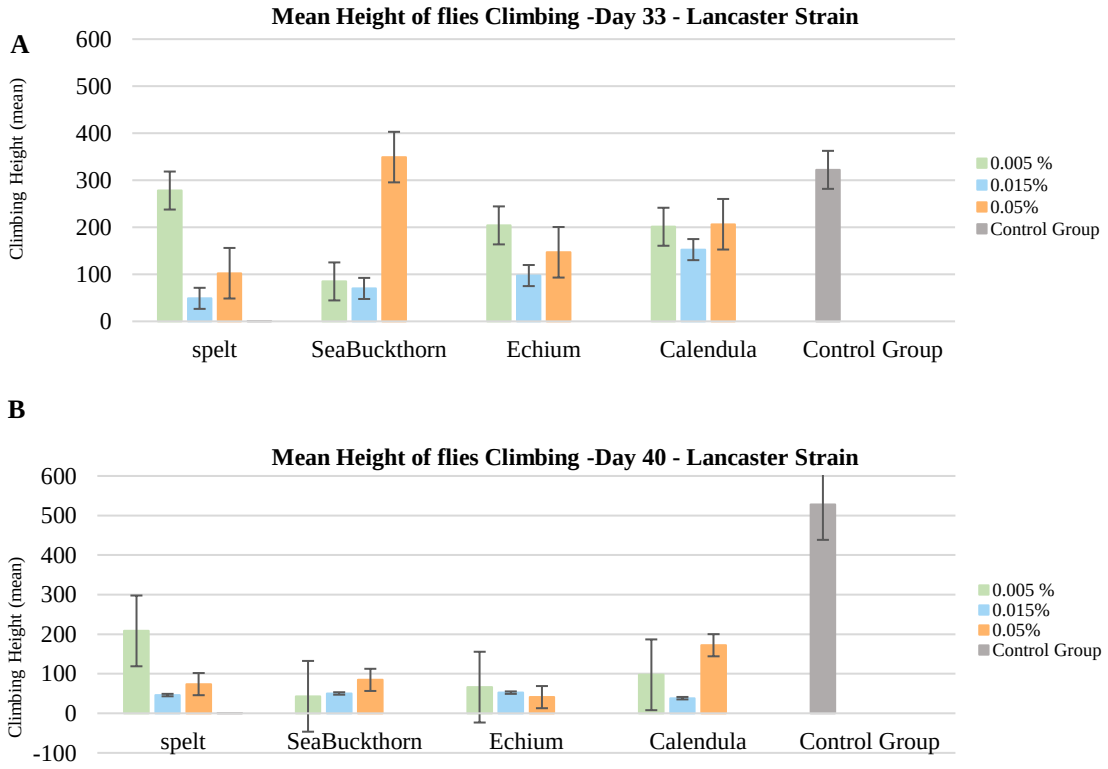


Figure 35. Effect of plant-based oil type, concentration, and exposure time on climbing performance in wDah strain Drosophila melanogaster. Mean climbing height was measured on Day 33 (A) and Day 40 (B) following dietary exposure to Spelt, Sea Buckthorn, Echium, and Calendula essential oils at the indicated concentrations. *Each concentration was tested using four independent vials, with 10-20 flies per vial (approximately 80 flies per treatment group). Data represent mean ± Std Err calculated from vial means.* Statistical analysis was performed using two-way ANOVA to evaluate the effects of essential oil, concentration, exposure time, and their interactions. Significance was accepted at $p < 0.05$.

3.4 Climbing assay in wDah strain

3.4.1 Effect of oil and dose on climbing ability in wDah strain

On the other hand, the climbing assay conducted in the wDah strain of *Drosophila melanogaster* revealed a response pattern distinct from that saw in the Lancaster strain, showing strain-dependent variability in neuromotor sensitivity to essential oil exposure. In this strain, locomotor performance was primarily influenced by exposure duration and interaction effects rather than by dose alone, highlighting a different response profile compared with the Lancaster strain.

As shown in Figure 36A, on Day 33 the control group showed a mean climbing height of ~254 mm. In comparison, most essential oil-treated groups displayed mean climbing heights that were equal to or higher than the control. Sea Buckthorn essential oil at 0.015% and Calendula essential oil at 0.05% produced the highest mean climbing heights (~411 mm and ~404 mm, respectively). Treatments with Echium and Spelt essential oils also kept climbing performance at or above control levels across most concentrations. Consistent with these observations, statistical analysis showed that Calendula oil did not produce a significant main effect of dose on climbing performance in the wDah strain ($p = 0.0700$).

Following prolonged exposure to Day 40 (Figure 36B), the control group showed a substantial increase in climbing performance (~649 mm), consistent with age-related physiological changes. Although all oil-treated groups showed lower climbing heights than the control at this time point (21-73%), several treatments kept relatively elevated levels of locomotor performance. The highest mean climbing heights among treated groups were seen for Echium at 0.015% (~510 mm) and Sea Buckthorn at 0.05% (~496 mm).

For Calendula essential oil, a considerable time × dose interaction was detected ($p = 0.0149$), indicating that the effect of dose on climbing performance differed between Day 33 and Day 40, despite the absence of a significant main effect of dose. In contrast, Echium oil did not exert a significant dose-dependent effect in the wDah strain ($p = 0.8794$), nor was a considerable time × dose interaction observed. However, a significant main effect of time was detected for Echium ($p = 0.006$), suggesting that changes in climbing performance were primarily driven by exposure duration rather than oil doses.

Similarly, Spelt and Sea Buckthorn oils did not show significant main effects of dose in the wDah strain; however, significant effects of time were detected for both oils (Spelt: $p = 0.0032$; Sea Buckthorn: $p = 0.0034$). Additionally, Sea Buckthorn showed a considerable time × dose interaction ($p = 0.0314$), indicating that the dose-dependent response varied across experimental time points.

Overall, these results indicate that the wDah strain shows a response profile distinct from that of the Lancaster strain. While early exposure to essential oils did not result in overt impairment of locomotor performance, prolonged exposure was associated with reduced climbing ability relative to the control, predominantly driven by time-dependent effects rather than dose alone. The presence of considerable time and interaction effects, particularly for Calendula and Sea Buckthorn oils, underscores the importance of considering both exposure duration and genetic background when evaluating neuromotor responses to plant-based oil supplementation.

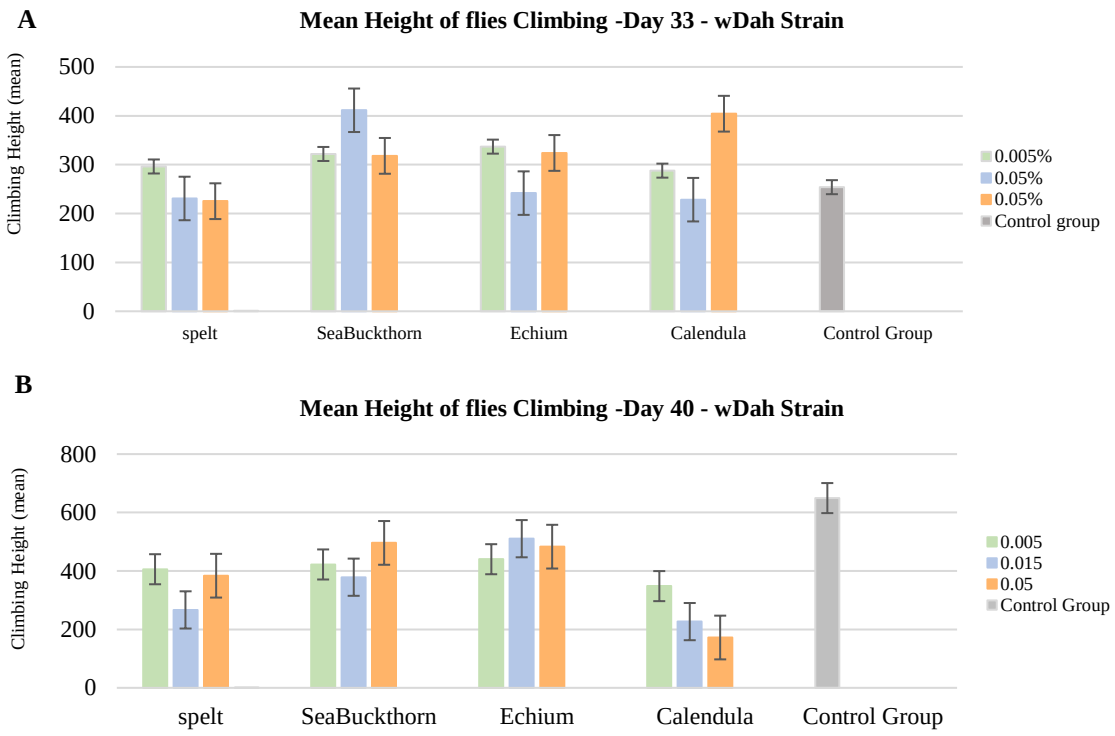


Figure 36. Effect of plant-based oil type, concentration, and exposure time on climbing performance in Lancaster strain Drosophila melanogaster. Mean climbing height was measured on Day 33 (A) and Day 40 (B) following dietary exposure to Spelt, Sea Buckthorn, Echium, and Calendula essential oils at the indicated concentrations. *Each concentration was tested using four independent vials, with 10-20 flies per vial (approximately 80 flies per treatment group). Data represent mean ± Std Err calculated from vial means.* Statistical analysis was performed using two-way ANOVA to evaluate the effects of essential oil, concentration, exposure time, and their interactions. Significance was accepted at $p < 0.05$.

3.5 Summary of climbing assay findings

To evaluate strain-dependent variability in neuromotor responses to dietary essential oils, the climbing performance of the Lancaster and wDah strains of *Drosophila melanogaster* was directly compared across the same treatments and time points. The results indicate notable differences in sensitivity and temporal dynamics between the two strains.

In the Lancaster strain, several oils induced statistically significant dose-dependent effects on locomotor performance. Specifically, Calendula oil reduced climbing ability in a dose-dependent manner ($p = 0.0037$), whereas neither time nor the time × dose interaction reached significance ($p = 0.8411$ and $p = 0.2150$, respectively). Echium oil exerted a strong dose-dependent reduction in climbing performance ($p = 0.0002$), accompanied by a considerable time × dose interaction ($p = 0.0034$), indicating progressive impairment over the exposure period.

By contrast, in the wDah strain, the main effect of Calendula oil dose was not statistically significant ($p = 0.0700$), though a considerable time × dose interaction was detected ($p = 0.0149$), demonstrating that the effect of dose varied across experimental time points. No statistically significant effects of Echium oil were seen in the wDah strain under the experimental conditions evaluated. Other oils, including Spelt and Sea Buckthorn, generally produced smaller reductions in climbing performance in wDah compared to Lancaster.

Overall, these comparisons highlight that the wDah strain exhibits greater resilience to essential oil exposure, particularly during the early phase of exposure, whereas the Lancaster strain is more susceptible to dose-dependent impairment. The presence of considerable time × dose interactions in wDah (for Calendula) and in Lancaster (for Echium) further underscores that the temporal dynamics of neuromotor responses are strain specific. These findings emphasize the importance of considering genetic background when evaluating the neurobehavioral effects of plant-based oils, as responses observed in one strain may not directly extrapolate to another.

Chapter 5: Results and Discussion

This study aimed to investigate the biological effects of selected plant-based oils on *Drosophila melanogaster*, integrating chemical characterization, antioxidant potential, and functional assays. The oils were first analyzed by GC-MS to identify their main bioactive components, and their radical scavenging activity was evaluated using DPPH assays.

Subsequently, in silico analyses provided insights into potential molecular interactions and bioactivity of these compounds. Finally, in vivo experiments, including lifespan and climbing assays, were conducted to assess the functional consequences of oil supplementation in two *Drosophila* strains (Lancaster and wDah). Together, these complementary approaches allowed us to link chemical composition and predicted bioactivity with observed physiological outcomes, highlighting both dose- and strain-dependent effects.

1 Dose-dependent effects and concentration trends

1.1 Radical scavenging vs oils' doses

According to the Figure 37, the DPPH radical scavenging assay demonstrated a clear concentration-dependent increase in antioxidant activity for all tested oils. Calendula exhibited the highest scavenging activity, reaching approximately 25–26% at the highest concentration, whereas Spelt, Sea Buckthorn, and Echium showed more modest increases, plateauing around 9–12%. Notably, while radical scavenging generally rose with concentration, the curves for Spelt, Sea Buckthorn, and Echium showed a slower, near-linear increase, suggesting a limited capacity to scavenge radicals compared to Calendula. These results indicate that although all oils have antioxidant potential, their effectiveness varies substantially, likely reflecting differences in the composition and abundance of bioactive constituents such as unsaturated fatty acids and terpenoids.

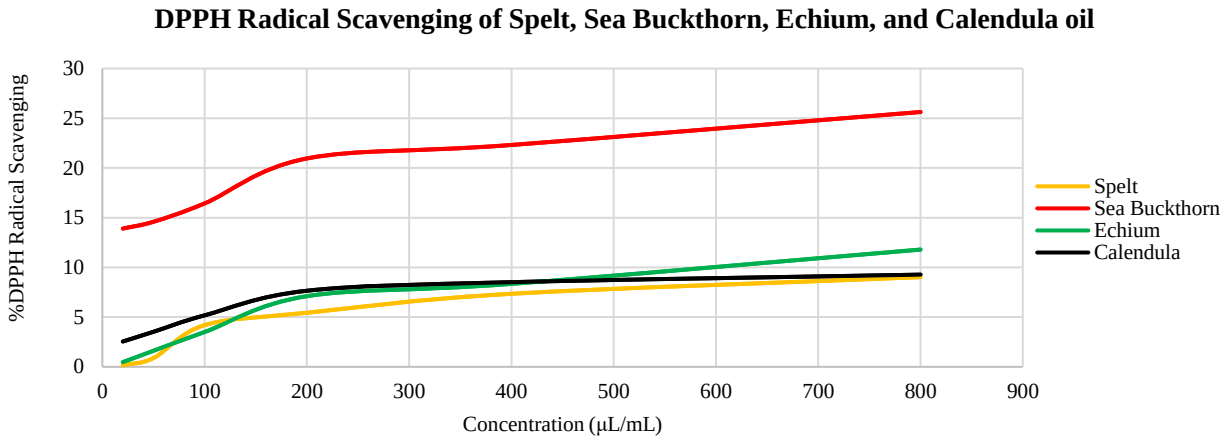


Figure 37. Percentage of radical scavenging activity of the tested plant oils at different concentrations. Data are presented as mean ± SEM of three independent experiments.

Importantly, the in vitro radical scavenging trends do not always correspond directly to in vivo [135] effects on lifespan and locomotor performance. In the Lancaster strain, exposure to Calendula and Echium oils led to dose-dependent reductions in mean lifespan, consistent with the significant mortality observed in these groups. Sea Buckthorn and Spelt oils showed more variable effects: while some doses resulted in modest reductions, other concentrations did not produce statistically significant changes compared to the blank. In contrast, in the wDah strain, none of the oils caused statistically significant reductions in mean lifespan, highlighting the strain-dependent sensitivity of flies. These findings emphasize that the relationship between antioxidant capacity measured in vitro and physiological outcomes in vivo is complex, and can be influenced by both genetic background and dose-specific responses.

This discrepancy may reflect the influence of endogenous antioxidant defenses, metabolic transformation of bioactive compounds, or hormetic effects, where low to moderate doses activate protective cellular pathways, while higher concentrations may induce oxidative stress or neuromuscular impairment. Thus, in vitro radical scavenging provides a useful but partial predictor of biological outcomes, emphasizing the importance of considering both chemical composition and organismal responses when evaluating functional effects of plant-derived oils.[136]

1.2 DPPH radical scavenging mechanism

Some compounds can react with DPPH radicals either by donating a hydrogen atom or by transferring an electron. Phenolic compounds are among the most active substances in this reaction because they readily reduce DPPH radicals. Hydrogen atom abstraction may involve coupled electron and proton transfer steps and is therefore generally considered a hydrogen atom transfer (HAT) process. As a result, the DPPH assay reflects the overall reducing ability of plant extracts present in the solution.[84]

1.2.1 Interactions of DPPH· with H-Atom Donors

1.2.1.1 Quercetin

The antioxidant capabilities of H-atom doner compounds (ArOH) are quantified by the following reaction:

$$ArOH + ROO\cdot \rightarrow ArO\cdot + ROOH$$

Of all the compounds with antioxidant properties, quercetin was chosen as the DPPH test standard, which is a good example of this situation that shows the DPPH radical interaction of quercetin as an H-donor phenolic compound. In the present radical scavenging system (Figure 38), the antioxidant activity of quercetin proceeds primarily through a hydrogen atom transfer (HAT) mechanism. In the first step, quercetin donates a hydrogen atom from its phenolic hydroxyl group to the DPPH• radical, resulting in the formation of the reduced DPPH–H species and a quercetin-derived phenoxyl radical. Subsequently, this radical undergoes a rapid electron transfer (ET) process, leading to further stabilization of the quercetin radical through extensive resonance delocalization.[84]

The stabilized radical species is then capable of reacting with an additional DPPH• molecule, contributing to the overall radical scavenging capacity observed in the assay. This reaction scheme illustrates the overlap between HAT and SET pathways, emphasizing that these mechanisms may operate sequentially and cannot always be clearly distinguished in phenolic antioxidant systems.[84]

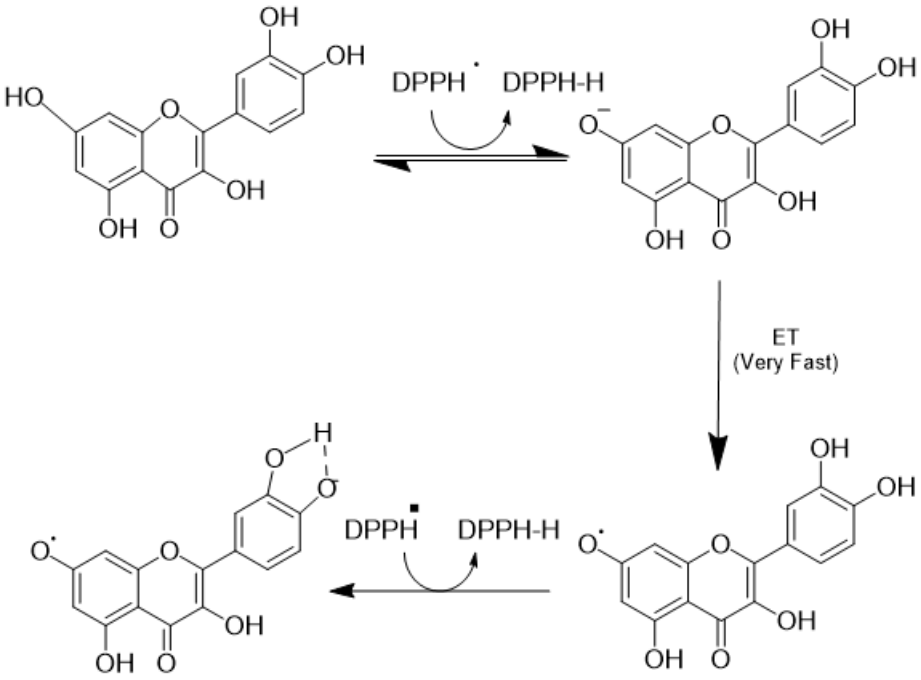


Figure 38. The reaction between DPPH free radicals and a quercetin molecule. (Drawn using chemdraw)

2 Strain-specific sensitivity analysis: Lancaster and wDah

The present study demonstrates a clear and consistent strain-dependent sensitivity to plant-based oils between the Lancaster and wDah strains of *Drosophila melanogaster*. Lancaster flies showed significantly higher mortality and reduced locomotor performance when exposed to *Calendula* and *Echium* oils at all concentrations, while *Sea Buckthorn* exhibited dose-dependent toxicity. *Spelt* affected only the intermediate dose. In contrast, the wDah strain displayed no significant changes in lifespan across all oils and doses, and climbing ability was largely maintained, with only minor reductions observed over time. This pattern indicates that genetic background strongly influences susceptibility to plant-derived compounds, with Lancaster flies being more sensitive than wDah.

The inclusion of least squares means (LS Means) and interaction plots for both strains (Day 33 vs Day 40) provides quantitative support for these observations. In Lancaster (Figure 39), the LS Means highlight a pronounced dose-dependent reduction in climbing ability for Spelt, Echium, and Calendula oils, with cumulative effects over time, whereas Sea Buckthorn showed milder impairment at lower doses but significant reduction at 0.05%. In contrast, LS Means for wDah (Figure 40) flies remained relatively stable across doses and oils, confirming relative resistance, though subtle reductions were observed following prolonged exposure.

Such strain-dependent differences have been reported previously, particularly in response to oxidative stress and environmental toxins, where genetic variability among *Drosophila* lines leads to wide-ranging differences in oxidative stress resistance, ultimately affecting lifespan and neuromuscular function. Together, these findings suggest that differences in genetic background, potentially involving detoxification capacity and oxidative stress response pathways, underlie the observed strain-specific susceptibility.[137, 138]

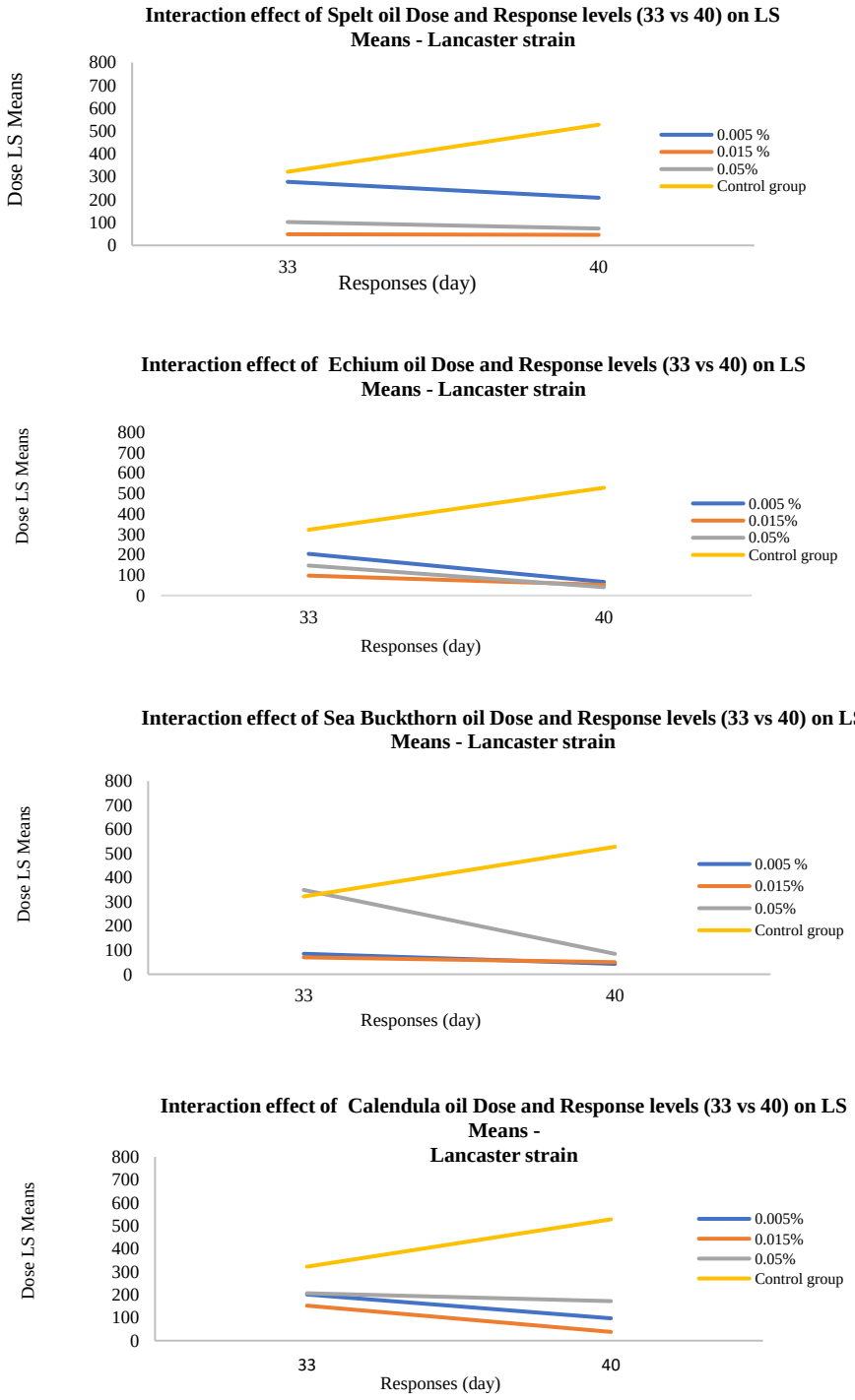


Figure 39. Least squares means (LS Means) and interaction plots illustrating the effects of dietary supplementation with Spelt, Sea Buckthorn, Echium, and Calendula oils on climbing performance in Drosophila melanogaster (Lancaster strain). Mean climbing heights are shown for three oil concentrations (0.005%, 0.015%, and 0.05% v/v) and the control group at Day 33 and Day 40. Interaction plots depict dose–time relationships, while LS Means plots summarize overall dose-dependent effects on locomotor performance. These results highlight oil-specific and dose-dependent alterations in neuromotor function, with more pronounced impairments observed following prolonged exposure. The experimental design and sample size were identical to those described in Figure 34.

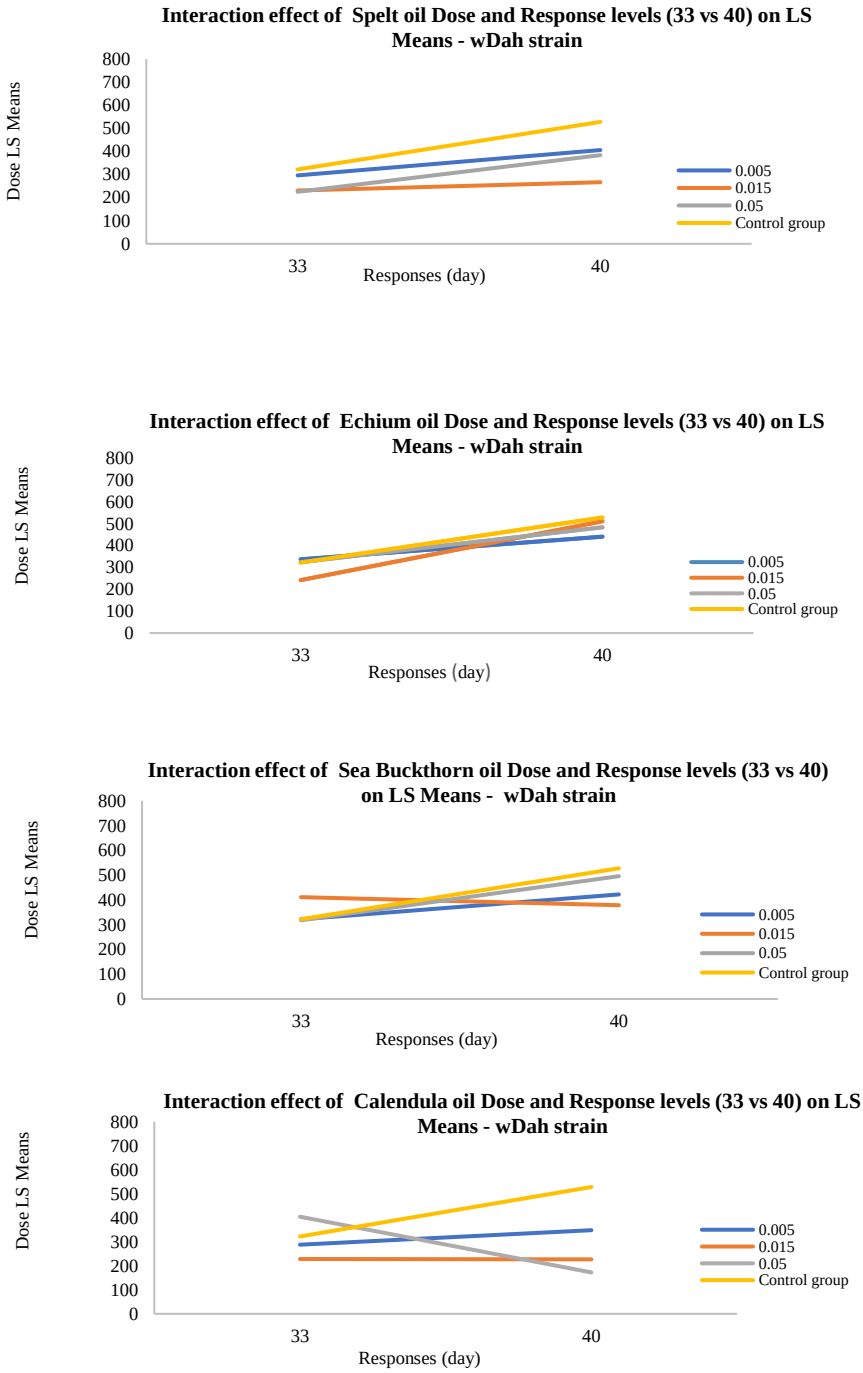


Figure 40. Least squares means (LS Means) and interaction plots illustrating the effects of dietary supplementation with Spelt, Sea Buckthorn, Echium, and Calendula oils on climbing performance in Drosophila melanogaster (wDah strain). Mean climbing heights are shown for three oil concentrations (0.005%, 0.015%, and 0.05% v/v) and the control group at Day 33 and Day 40. Interaction plots depict dose–time relationships, while LS Means plots summarize overall dose-dependent effects on locomotor performance. These results highlight oil-specific and dose-dependent alterations in neuromotor function, with more pronounced impairments observed following prolonged exposure. The experimental design and sample size were identical to those described in Figure 35.

2.1 Potential Biological Mechanisms Underlying Differences

To interpret the mechanistic basis of strain-specific sensitivity in *Drosophila melanogaster*, it is useful to consider the interplay between metabolic detoxification, oxidative stress regulation, and neuromuscular integrity. These pathways have all been implicated in responses to xenobiotics and behavioral performance under toxic stress in insects.[139-142]

2.1.1 Metabolic Detoxification Pathways

Inter-strain variation in sensitivity to xenobiotics has often been linked to differences in the expression and regulation of phase I and phase II detoxification enzymes in *Drosophila melanogaster*.[139-141] These enzymes include cytochrome P450 monooxygenases (CYPs) and glutathione S-transferases (GSTs), which are involved in oxidative metabolism and conjugation of xenobiotic compounds, respectively.[142, 143]

Studies in *Drosophila melanogaster* have shown that exposure to xenobiotics can induce the expression of specific CYP and GST genes, suggesting these gene families contribute to detoxification processes in the fly model.[139] For example, a subset of CYP genes (e.g., Cyp6a2, Cyp6a8, Cyp12d1) and several GST genes have been reported to increase in expression after treatment with phenobarbital or other xenobiotics, consistent with an adaptive response to chemical stress.[144]

Genetic variation affecting the baseline or inducible expression of these enzymes may influence the detoxification capacity of different strains, potentially explaining differences in survival and behavioral endpoints under oil exposure.[139, 141, 144]

2.1.2 Oxidative Stress Response

Many plant-derived compounds have been associated with pro-oxidant effects in *Drosophila*, including increased production of reactive oxygen species and perturbation of antioxidant defenses, which can manifest as elevated mortality and impaired locomotion.[102]

The intracellular antioxidant defense system in flies includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and GST, which modulate ROS levels and protect against oxidative damage.[102] For example, exposure to certain essential oils has been shown to disrupt the balance of antioxidant activities in *Drosophila melanogaster*, consistent with a reduction in redox homeostasis and increased oxidative stress markers.[102]

Furthermore, components of conserved signaling pathways such as the Nrf2 ortholog (CncC) have been implicated in coordinating detoxification and stress response gene expression in *Drosophila*, suggesting a genetic basis for variation in oxidative stress resistance between strains.[102, 145]

2.1.3 Genetic Control of Behavior and Neuromuscular Sensitivity

Negative geotaxis (climbing behavior) in fruit flies is widely used as a measure of neuromuscular performance and can be affected by toxic exposures that perturb neuronal or muscular function.[131] Neurotoxic mechanisms of certain secondary metabolites (e.g., terpenoids and phenolics) have been associated with changes in geotaxis behavior in *Drosophila*, potentially through modulation of neurotransmitter systems or mitochondrial function.[145]

Mitochondrial dysfunction, often linked to oxidative imbalance, has also been reported in *Drosophila melanogaster* exposed to plant essential oils, suggesting that energy metabolism disruptions may contribute to impaired locomotion.[146] Such physiological effects may differ between genetic backgrounds, as strains with more robust antioxidant defenses or efficient detoxification may better preserve neuromuscular function under stress.[146]

2.2 Implications for Interpretation of Plant Oil Effects

Overall, the data indicate that both genetic background and oil type/dose must be considered when evaluating toxic and sub-lethal effects in *Drosophila*. LS Means and interaction plots quantitatively confirm that Lancaster flies are more sensitive, showing cumulative dose- and time-dependent reductions in locomotor performance and lifespan, whereas wDah flies demonstrate a more resistant phenotype, with effects primarily influenced by exposure duration rather than dose. These findings underscore the importance of strain selection in toxicological and pharmacological studies of plant-based compounds and suggest avenues for mechanistic studies targeting detoxification and antioxidant pathways.

3 Effects of Major GC-MS Identified Compounds on Lifespan and Locomotor Performance

To elucidate the mechanistic basis of the observed effects of plant-based oils on *Drosophila* lifespan and locomotor activity, the major compounds identified by GC-MS in four oils were analyzed. These included polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), vitamin C derivatives, vitamin E, and other fatty acid esters. Given their known roles in antioxidant defense and detoxification pathways, their potential contribution to lifespan and neuromuscular performance was evaluated.[147]

3.1 Spelt Oil

GC–MS analysis revealed that the oil was predominantly composed of 9,12-octadecadienoic acid (linoleic acid, PUFA; 50.39%), followed by 9-octadecenoic acid (oleic acid, MUFA; 20.79%) and ascorbic acid 2,6-dihexadecanoate (18.3%). Linoleic acid, as a major polyunsaturated fatty acid, plays a critical role in maintaining membrane integrity and supporting antioxidant defense mechanisms; however, previous studies suggest that its biological effects may be dose-dependent, with potential non-linear outcomes at higher concentrations due to lipid accumulation.[148]

Oleic acid, a monounsaturated fatty acid, is generally associated with protective effects, contributing to membrane fluidity and metabolic homeostasis.[149] In addition, the presence of ascorbic acid 2,6-dihexadecanoate indicates a substantial antioxidant capacity, potentially enabling effective scavenging of reactive oxygen species and preservation of locomotor function. Consistent with these observations, in-silico toxicity predictions classified both linoleic and oleic acids as non-toxic and non-mutagenic, while the ascorbic acid derivative exhibited a high antioxidant potential, supporting the experimentally observed biological effects.

3.2 Sea Buckthorn Oil

GC–MS profiling indicated that linoleic acid (23.9%) and cis-9-hexadecenal (22.3%) were the predominant constituents of the oil. Linoleic acid is well recognized for its antioxidant and anti-inflammatory properties, contributing to membrane stability and modulation of oxidative balance. Cis-9-hexadecenal, an unsaturated aldehyde, may also exert bioactive effects; however, aldehydic compounds are known to display increased chemical reactivity, which can induce mild oxidative stress at elevated concentrations.[54]

In addition, an ascorbic acid derivative (9.21%) was detected, suggesting a supportive role in reactive oxygen species scavenging and potential neuroprotective effects.[56] Consistent with these chemical characteristics, in-silico toxicity assessment predicted linoleic acid to have low toxicity, cis-9-hexadecenal to exhibit moderate reactivity primarily at higher doses, and the ascorbic acid derivative to possess strong antioxidant potential. Together, these findings indicate a balance between protective antioxidant activity and dose-dependent reactivity that may underlie the observed biological responses.

3.3 Echium Oil

GC–MS analysis revealed that stearidonic acid (SDA) was the major component of the oil (57.33%), alongside other long-chain PUFAs and EPA-related esters. These polyunsaturated fatty acids are widely associated with antioxidant and anti-inflammatory activities and play important roles in maintaining membrane structure and cellular signaling.[1]

However, previous evidence suggests that excessive PUFA exposure may impose metabolic or neuromuscular stress, particularly through enhanced lipid peroxidation at higher doses.[150] In addition, vitamin E (α-tocopherol) was detected at approximately 4.4%, consistent with its well-established role as a lipid-soluble antioxidant that protects membrane integrity and supports neuromuscular function.

Notably, in the Echium oil analysis, lesser amounts of pyrrolizidine alkaloids were also detected, which have been reported as toxic compounds and may explain why this oil was classified among the toxic oils in this study. Nevertheless, their use in cosmetic or health products is considered safe if prepared as a standardized extract, in which these compounds are removed, or their levels are carefully controlled.[151-153] Consistent with these biochemical properties, in-silico toxicity predictions classified SDA and PUFA esters as non-toxic with moderate-to-high antioxidant activity, while α-tocopherol exhibited strong antioxidant potential.

Overall, this compositional profile suggests a predominantly protective antioxidant effect, which may be modulated by dose-dependent PUFA-related stress mechanisms, and highlights the importance of monitoring potentially toxic compounds, such as pyrrolizidine alkaloids, in the safe application of plant-derived oils.

3.4 Calendula Oil

GC–MS analysis indicated that the oil was primarily composed of linoleic acid (37.5%) and 8,11,14-eicosatrienoic acid (21.3%), both polyunsaturated fatty acids (PUFAs, omega-3/6) essential for neuronal health and antioxidant defense. While these PUFAs confer protective effects, excessive exposure may lead to mild toxicity due to lipid peroxidation.[30, 154] In addition, an ascorbic acid derivative (8%) and stearic acid (1.1%) were detected, with the former contributing to reactive oxygen species scavenging and partial antioxidant protection, whereas stearic acid is largely biologically inert. In-silico predictions aligned with these observations, classifying the PUFAs as non-toxic with antioxidant potential, the ascorbic acid derivative as antioxidant-active, and stearic acid as exhibiting extremely low toxicity. Overall, the compositional profile suggests a predominance of bioactive antioxidant compounds with minimal inherent toxicity.

Overall, the major compounds identified across the four plant-based oils, particularly polyunsaturated fatty acids (PUFAs) and vitamin C derivatives, demonstrated substantial antioxidant potential, which is consistent with the observed neuroprotective effects on locomotor performance. However, certain long-chain PUFA derivatives and unsaturated aldehydes appeared to induce mild dose-dependent toxicity in Lancaster flies, aligning with the reductions in both lifespan and climbing ability observed experimentally. The in-silico predictions support these experimental findings, highlighting that the effects of these compounds are largely dose- and strain-dependent, driven primarily by their PUFA composition and antioxidant content. In contrast, the wDah strain exhibited relative resistance to these effects, which is likely attributable to enhanced detoxification and antioxidant pathways.[148]

4 Comparison with Previous Studies

The results of this study demonstrated a clear strain-dependent response to the tested plant oils. In the *Drosophila melanogaster* Lancaster strain, *Calendula* and *Echium* oils significantly reduced lifespan and locomotor activity, *Sea Buckthorn* showed a dose-dependent effect, and *Spelt* was effective only at intermediate concentrations. In contrast, the wDah strain exhibited no significant changes in lifespan or locomotor activity across all oils and concentrations, indicating a lower sensitivity to these plant-derived compounds.

These findings are partially consistent with previous studies on *Drosophila*, which have reported dose-dependent effects of plant extracts and essential oils on lifespan and locomotor behavior.[9, 102] Similarly, the reduction in lifespan and locomotor performance observed in Lancaster flies aligns with studies on *Rosmarinus officinalis* essential oil, which increased mortality and impaired geotaxis behavior.[102]

Regarding neurophysiological mechanisms, research on *Lippia alba* essential oil indicated that its main components, more specifically Citral, can reduce synaptic transmission, leading to impaired neural function and decreased locomotor activity in *Drosophila*. Citral significantly decreases excitatory postsynaptic potentials (EPSPs) and inhibits Ca^{2+} influx at nerve terminals, which may partially explain the reduced behavioral performance observed in Lancaster flies.[155]

Overall, these results suggest that the effects of plant oils on *Drosophila* are strain-specific, with Lancaster flies showing higher sensitivity than wDah. This strain-dependent pattern highlights the importance of genetic background in modulating responses to plant-derived compounds and provides a framework for future studies investigating underlying molecular mechanisms.

5 Research Limitations and Future Directions

This study has several limitations that should be acknowledged, although none of them represent critical flaws in the experimental design or interpretation of the findings. One limitation relates to the varying population sizes across experimental groups over time, particularly in lifespan and climbing assays. As mortality increased during the course of the experiment, the effective sample size decreased, which may have reduced statistical power and influenced P values at later time points. Consequently, some biologically relevant trends may not have reached statistical significance. Future studies incorporating larger initial cohort sizes and additional biological replicates would improve statistical robustness and increase power to detect subtle strain- and dose-dependent effects.

Another limitation concerns the statistical approach employed for lifespan and locomotor analyses, which primarily captures cumulative effects over defined time points and may not be sensitive to early mortality events. This is particularly relevant for the *wDah* strain, where early or transient mortality effects may have occurred without being fully reflected in later survival or performance measures. More refined survival modeling approaches, including time-to-event or hazard-based analyses, could help resolve early-stage effects in future investigations.[156]

The present study was also conducted exclusively using male *Drosophila melanogaster*. While this approach reduces variability associated with sex-specific differences in metabolism, lifespan, and behavior, it limits the generalizability of the findings. Sexual dimorphism in stress resistance, detoxification capacity, and aging phenotypes has been well documented in *Drosophila*, and females often exhibit distinct responses to dietary and toxicological interventions. Therefore, inclusion of both sexes in future studies would provide a more comprehensive understanding of sex-dependent susceptibility to plant-based oils and their bioactive components.[156, 157]

An additional methodological limitation arises from dosing via food intake. Administration of plant oils through the diet does not guarantee uniform exposure across individuals, as feeding behavior can vary between flies, strains, and over time. As a result, actual ingested doses may differ among individuals within the same treatment group, potentially contributing to variability in lifespan and locomotor outcomes. Future studies could incorporate complementary exposure strategies, such as controlled micro-feeding or measurement of food consumption, to better quantify individual dosing.

Finally, although strain-dependent differences in susceptibility were clearly observed, the underlying mechanisms were not directly assessed. The greater sensitivity of the *Lancaster* strain relative to the *wDah* strain is likely attributable to genetic differences affecting detoxification pathways, oxidative stress responses, and neuromuscular regulation, as discussed earlier. However, direct measurement of antioxidant enzyme activity, detoxification gene expression, or lipid peroxidation markers would strengthen mechanistic interpretation. Future studies integrating molecular and biochemical analyses alongside expanded sample sizes and additional strains would provide greater resolution and further enhance the interpretative power of this model system.

6 Conclusions

This thesis aimed to provide an integrated evaluation of selected plant-derived oils with potential cosmetic relevance by combining chemical characterization, in vitro antioxidant assessment, and in vivo biological analysis. GC–MS profiling confirmed the presence of several fatty acids and antioxidant-related constituents within *calendula*, *echium*, *sea buckthorn*, and *spelt* oils. The DPPH radical scavenging assay demonstrated measurable antioxidant activity across all oils, with *sea buckthorn* oil showing the highest activity, followed by *calendula*, *echium*, and *spelt*.

However, the biological experiments in *Drosophila melanogaster* revealed dose-dependent and clear strain-dependent responses. In the Lancaster strain, several oils reduced survival and locomotor performance at certain concentrations, whereas the wDah strain showed greater tolerance and limited physiological impact. These findings indicate that the biological effects of plant oils are influenced not only by their chemical composition but also by exposure conditions and genetic background.

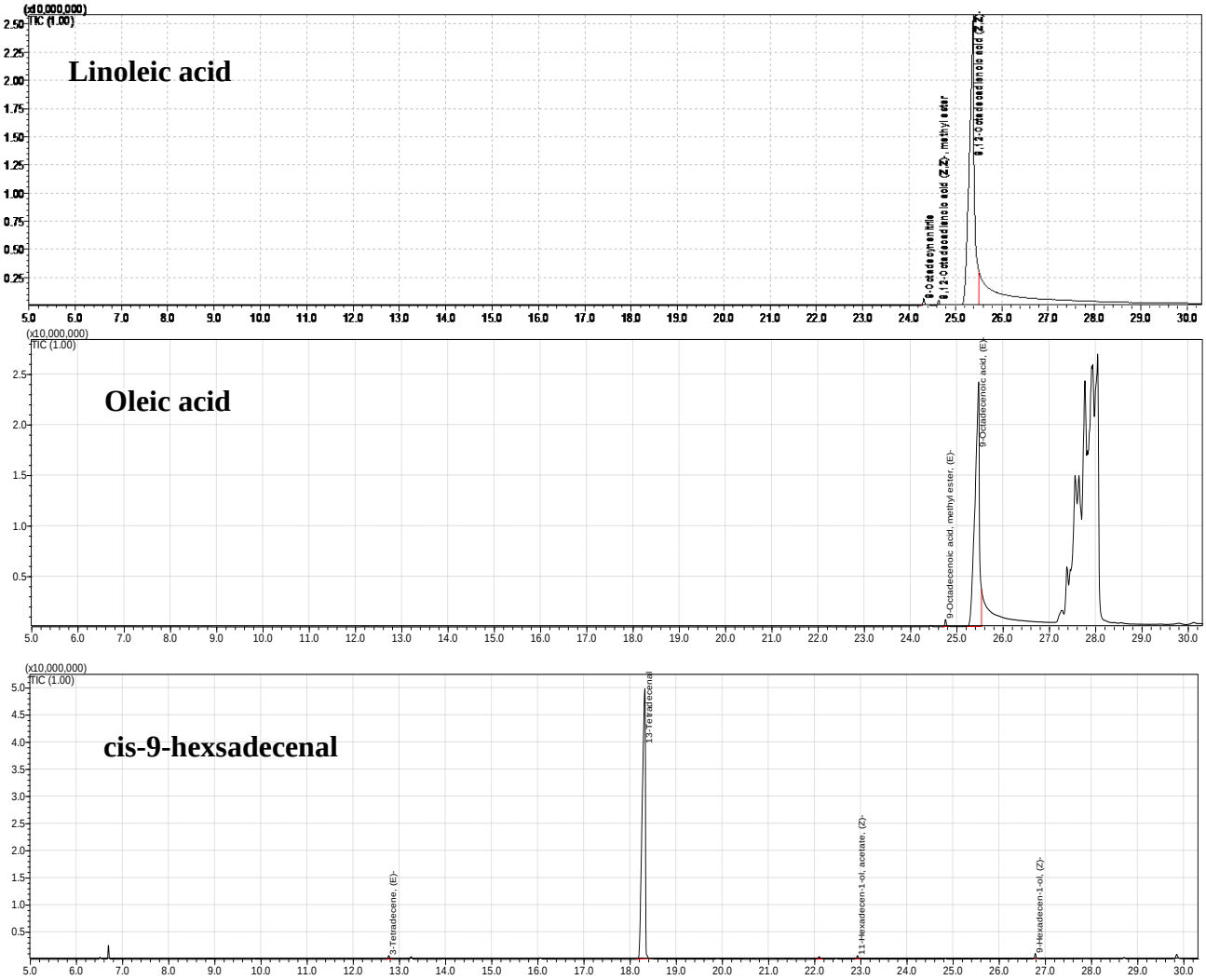
Importantly, the comparison between chemical antioxidant assays and in vivo outcomes highlights that antioxidant capacity measured in vitro does not necessarily translate into beneficial biological effects. Factors such as bioavailability, metabolic transformation, and organism-specific responses can significantly influence biological outcomes. [82, 115]

From a cosmetic science perspective, these results emphasize that the incorporation of plant-derived oils into formulations should not rely solely on chemical antioxidant measurements. In addition to complying with regulatory requirements such as Regulation (EC) No. 1223/2009 governing cosmetic ingredients, formulators should also consider potential dose-dependent, cumulative, or delayed biological effects that may not be evident in short-term chemical assays. [10-12] Consequently, integrating chemical profiling, computational prediction, and biological testing provides a more reliable framework for evaluating the safety and functional potential of plant oils intended for cosmetic applications.

Chapter 6: Appendices

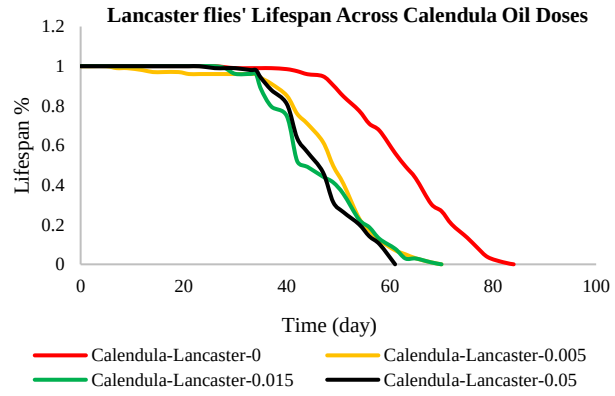
Appendix 1: GC–MS chromatograms of the standards

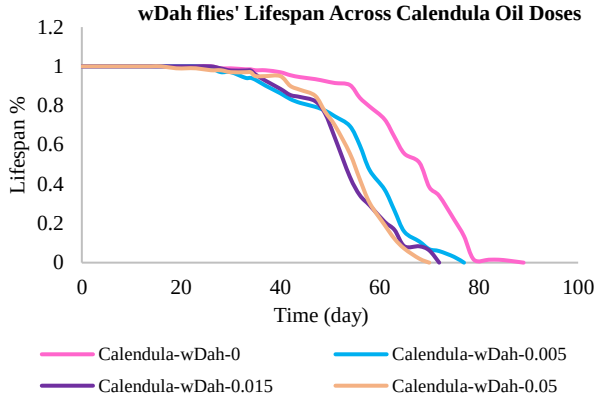
Figures show the GC–MS chromatograms reference standards corresponding to the major compounds detected in the oils (e.g., Linoleic acid, oleic acid, and cis-9-hexsadecenal). Comparison of retention times confirmed the assignments presented in Table 6, Chapter 3.

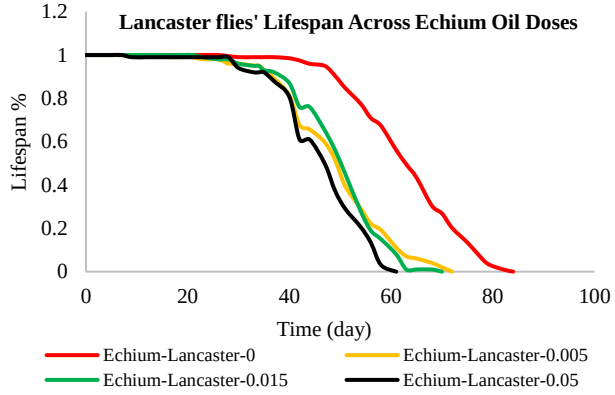


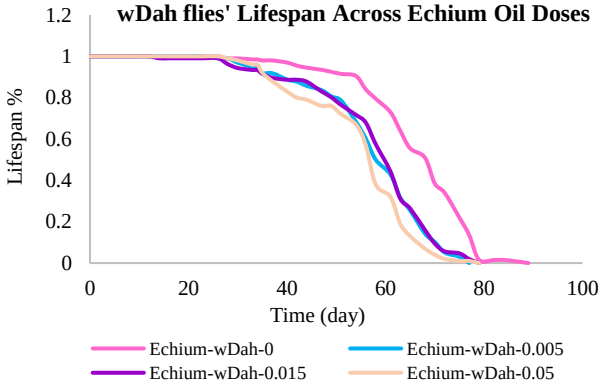
Appendix 2: Supplementary lifespan plots for different oil concentrations

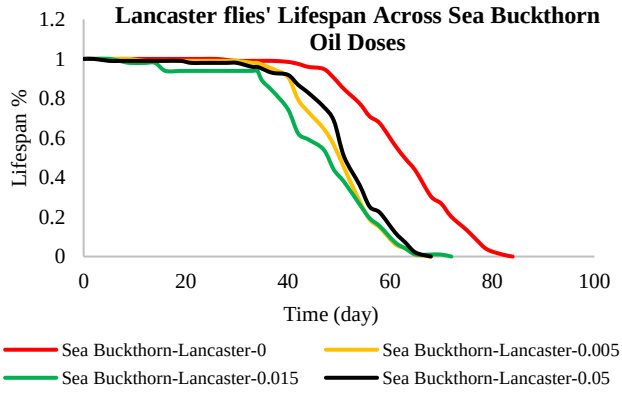
This appendix presents the lifespan curves for *Drosophila melanogaster* exposed to four different doses of each tested oil. Data are shown separately for each oil type to allow direct comparison of dose-dependent effects.

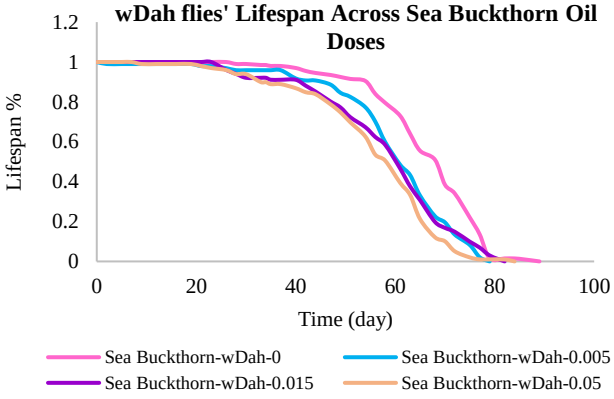


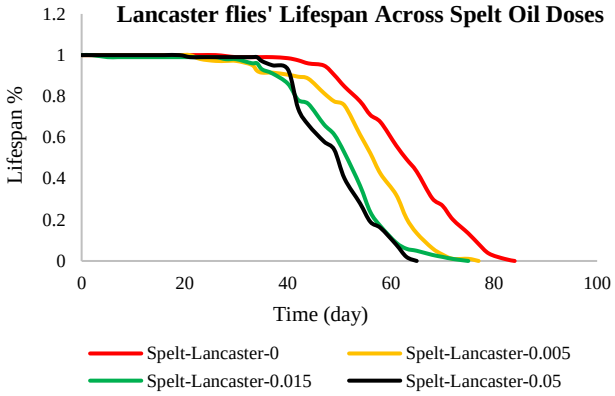


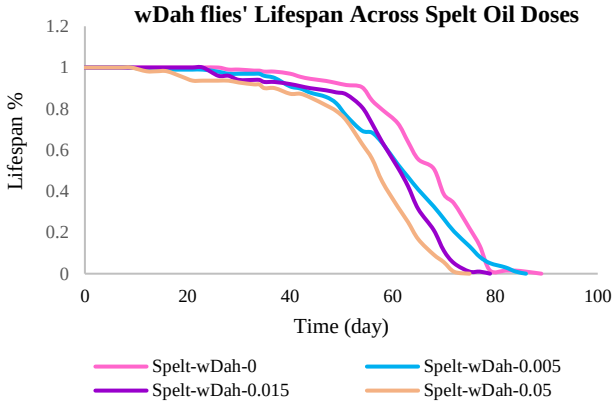









Lancaster flies' Lifespan Across Spelt Oil Doses
Lifespan %
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time (day)
Spelt-Lancaster-0
Spelt-Lancaster-0.005
Spelt-Lancaster-0.015
Spelt-Lancaster-0.05


wDah flies' Lifespan Across Spelt Oil Doses
Lifespan %
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Time (day)
Spelt-wDah-0
Spelt-wDah-0.005
Spelt-wDah-0.015
Spelt-wDah-0.05

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