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Review

Plant-Derived Extracellular Vesicles in Cosmetics: Building a Framework for Safety, Efficacy, and Quality

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Abstract

Plant-derived extracellular vesicles (PDEVs) are rapidly gaining popularity in cosmetics and regenerative medicine due to their biocompatibility, natural origin and promising bioactive properties. Nevertheless, the absence of standardized guidelines for their characterization has resulted in an inconsistent, unregulated landscape. This compromises product reproducibility, consumer safety, and scientific credibility. Here, a comprehensive set of minimal characterization guidelines for PDEVs is proposed to include physical and chemical profiling, molecular marker identification, cargo analysis, and stability assessment under storage and formulation conditions. Functional validation through cellular uptake assays, activity tests, and advanced in vitro or ex vivo models that replicate realistic skin exposure scenarios is pivotal. Requirements for transparent labelling, reproducible sourcing, batch-to-batch consistency, and biological activity substantiation to support claims related to skin regeneration, anti-aging, and microbiome modulation are also required. By establishing a harmonized baseline for quality and efficacy evaluation, these guidelines aim to elevate the scientific standards and promote the safe, ethical, and effective use of PDEV-based ingredients in cosmetic and biomedical applications.

Keywords: plant-derived extracellular vesicles; cosmetics; proteomics; lipidomics; nanoparticle tracking analysis; tangential flow filtration; size-exclusion chromatography; Raman; quality control; calcium dynamics

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1. Introduction

Extracellular vesicles (EVs) are increasingly recognized as pivotal biological entities that mediate intercellular communication across diverse biological systems. These lipid bilayer-enclosed particles, naturally secreted by virtually all cell types, carry a complex molecular cargo composed of proteins, lipids, nucleic acids, and metabolites. Their intrinsic ability to transfer functional biomolecules across cellular barriers without eliciting significant immunogenicity or toxicity has attracted growing attention for their potential application as therapeutic vectors and active agents in various biomedical contexts [1].

In the past decade, the therapeutic relevance of EVs has been extensively investigated, particularly in the domains of regenerative medicine, dermatology, and cosmeceutical science [2–6]. In these fields, EVs are seen not only as delivery vehicles but also as modulators of inflammation, tissue repair, cellular differentiation, and extracellular matrix remodeling. Their nano-scale dimensions and natural origin make them highly biocompatible, while their capacity to encapsulate and protect labile bioactive components from degradation further enhances their value in both therapeutic and aesthetic formulations.

Substantial efforts have been devoted to the standardization of EVs of mammalian origin, culminating in the publication of the MISEV2023 guidelines by the International Society for Extracellular Vesicles (ISEV) [7]. These recommendations have established a robust framework for the isolation, characterization, and functional validation of EVs in preclinical and clinical research. However, despite the rapid proliferation of non-animal-derived EVs in commercial and experimental settings, particularly those obtained from plant sources, equivalent regulatory or scientific standards are currently lacking. This regulatory vacuum has left a significant gap in the quality control and scientific validation of these emerging vesicle classes.

Among non-animal EVs, plant-derived EVs (PDEVs) are gaining remarkable traction due to their natural abundance, environmental sustainability, and favorable safety profile. The cosmetic industry, in particular, has rapidly embraced PDEVs for incorporation into a wide range of topical products, often marketed with claims of anti-aging, antioxidant, barrier-enhancing, moisturizing, or microbiota-balancing properties [8–11]. Several cosmetic products, including serums and creams currently available on the market, incorporate PDEVs to improve skin appearance and function. For instance, the Exosome Hydro-Glow Complex Serum (Inkey List) is formulated with EVs derived from *Centella asiatica* to promote collagen synthesis, together with peptides, hyaluronic acid, and ectoine to enhance skin hydration and repair. The serum ExoBloom (Morganna's Alchemy) utilizes EVs from goji berry stem cells to support skin renewal and vitality. The Plant XO Youth Serum (Induction Therapies) is part of a professional skincare line based on PDEV, designed to promote rejuvenation and improve skin texture. The EXO | E Revitalizing Complex (Croma-Pharma) is a three-step system including a pre-treatment serum, an EV-based serum, and a post-treatment, featuring EVs obtained from the fermentation of *Ustilago cynodontis*, *Piper nigrum*, and *Withania somnifera*. Likewise, the Korean two-step treatment Vegan Exosome Skin & Scalp Treatment (BotanicExo) is a combination of lyophilized EV powder derived from botanical extracts with an activating solution for the skin and scalp. Their appeal lies in their plant-based origin, aligning with the growing consumer demand for clean-label, vegan, and naturally derived ingredients. However, in the absence of standardized scientific and regulatory guidance, the current market landscape is characterized by significant variability in product composition, naming conventions, and claimed functionality. Terms such as "phyto-exosomes", "botanical nanocarriers", or "natural liposomes" are frequently used interchangeably—often without biochemical verification or functional substantiation. This lack of nomenclatural precision and analytical rigor creates a confusing and unregulated environment in which products may be marketed with limited or no evidence of vesicular identity, purity, or biological efficacy. The consequences of this inconsistency are far-reaching. From a scientific perspective, the reproducibility of results obtained with such products is compromised. From a consumer safety standpoint, poorly characterized vesicles or contaminated preparations may lead to unanticipated biological effects. And from a commercial viewpoint, the absence of common reference standards undermines consumer trust, exposes manufacturers to liability, and complicates cross-market regulatory acceptance.

Given these challenges, there is an urgent need to establish a baseline of minimal yet rigorous characterization guidelines for PDEVs and other non-animal-derived EVs intended for cosmetic and regenerative applications. Such guidelines must balance practicality with scientific integrity, ensuring that both producers and users have access to reliable, validated, and reproducible materials. Only through such coordinated efforts can the full potential of these natural nanocarriers be safely and effectively harnessed. Here (Figure 1), a set of scientifically grounded minimal characterization guidelines for the use of non-animal-derived EVs in cosmetic and regenerative products is proposed. These guidelines are

intended to serve both producers, who are responsible for the isolation, processing, and labeling of EVs, and end users, such as cosmetic formulators, dermatologists, and product developers, who rely on reliable materials and data for effective application. The guidelines are intended to foster transparency, encourage regulatory harmonization, and support the scientific credibility of cosmetic products using natural vesicular ingredients.

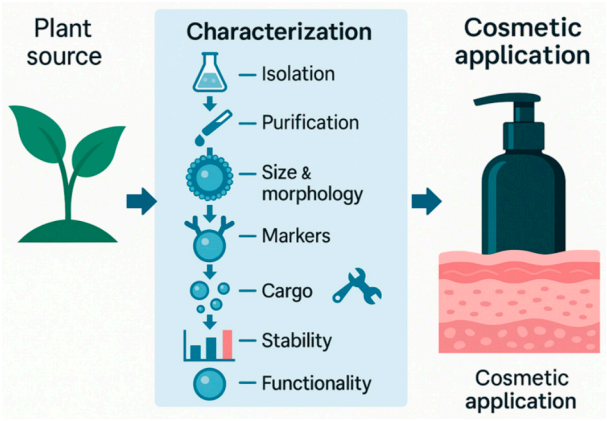

Figure 1. Scientifically grounded minimal characterization guidelines proposed for the use of non-animal-derived EVs in cosmetic and regenerative products.

2. Definition, Composition, and Functional Roles of EVs in Plants

EVs are nanoscale, lipid bilayer-enclosed particles secreted by virtually all domains of life, ranging from bacteria to higher eukaryotes. EVs are not passive by-products of cell metabolism but functional vectors of biological information, mediating intercellular and even inter-kingdom communication. Unlike apoptotic bodies or cellular debris, EVs are actively produced structures, released through highly regulated mechanisms such as endosomal trafficking (exosome-like vesicles), plasma membrane budding (microvesicles), or specialized secretory routes that are increasingly being elucidated in plants.

From a structural perspective, EVs are characterized by a phospholipid bilayer that stabilizes their architecture and protects their cargo from enzymatic degradation. Their molecular content is highly heterogeneous and reflects the physiology of the producing cell. De facto, EVs contain proteins, lipids, and nucleic acids. Proteins encompass tetraspanin-like markers, annexins, heat shock proteins, and enzymes associated with signaling and stress response. Lipids comprise phosphatidylserine, phosphatidylcholine, sphingolipids, sterols, and cutin-like molecules, which not only provide structural integrity but also act as bioactive mediators. Nucleic acids are small RNAs (miRNAs, siRNAs), mRNAs, and DNA fragments, enabling EVs to modulate gene expression in recipient cells. Secondary metabolites, especially in PDEVs, include polyphenols, carotenoids, and other antioxidants, which contribute to vesicle bioactivity [12].

Functionally, EVs serve as conserved communication modules, ensuring the targeted delivery of biological signals across short and long distances. In mammals, they regulate immunity, tissue repair, metabolism, and tumor progression. In plants, their role is increasingly recognized as pivotal for development, stress adaptation, and defense against pathogens. Specifically, PDEVs have been shown to mediate intercellular signaling during growth and differentiation, contributing to tissue patterning and organ development. They participate in stress adaptation, carrying antioxidants and stress-induced RNAs that modulate responses to drought, salinity, ultraviolet (UV) radiation, or temperature fluctuations. PDEVs contribute to plant immunity by delivering siRNAs to invading fungi or other pathogens, thereby silencing virulence genes in a process termed cross-kingdom RNA interference. They facilitate the remodeling of the plant extracellular matrix, trans-

porting enzymes and structural proteins that support cell wall dynamics. Additionally, they influence symbiotic interactions, for example, in plant-microbe or plant-mycorrhizal associations, where vesicles help modulate the biochemical dialogue at the interface [13,14].

Thus, in plants, EVs are not only intracellular "waste disposers" but sophisticated nanocarriers that orchestrate communication within and beyond the plant organism. They serve as adaptive messengers, integrating metabolic status and environmental cues into molecular signals that sustain survival, reproduction, and ecological interactions.

3. PDEV Versus Liposomes: Structure and Function Comparison

One of the most striking aspects of PDEVs is the intrinsic complexity of their architecture, which clearly distinguishes them from liposomes, the most widely used synthetic vesicular systems in pharmaceutical and cosmetic applications.

Liposomes are artificially engineered nanocarriers, consisting essentially of a phospholipid bilayer organized into spherical vesicles that can encapsulate hydrophilic or lipophilic compounds [15]. They were first developed in the 1960s as simplified biomimetic systems, designed to improve drug solubility, protect labile molecules, and enhance delivery through biological membranes. Their advantage lies in controllability: liposomes can be synthesized with defined lipid compositions, sizes, and surface modifications, making them versatile and scalable for industrial applications [16]. However, this very simplicity is also their limitation. Liposomes lack the biological complexity of natural vesicles, and their interaction with cells often remains nonspecific, leading to rapid clearance, limited targeting, and reduced functional integration with cellular pathways [17].

In contrast, PDEVs are naturally evolved nanostructures, secreted by plant cells as part of highly regulated physiological processes. Beyond their phospholipid bilayer, they carry a multilayered molecular cargo that includes proteins (enzymes, signaling mediators), nucleic acids (miRNAs, siRNAs, mRNAs), and secondary metabolites (polyphenols, carotenoids, terpenoids). This endogenous molecular fingerprint confers them not only structural stability but also functional intelligence, allowing them to participate actively in cellular communication, modulate gene expression in recipient cells, and trigger specific signaling cascades [18]. While liposomes are "empty shells" that must be loaded with exogenous compounds to acquire function, PDEVs are pre-loaded by nature with biologically relevant cargo that mirrors the adaptive strategies of the plant of origin.

Another fundamental difference lies in the interaction with biological systems. Liposomes typically enter cells through nonspecific endocytosis or membrane fusion, but lack the molecular recognition motifs that mediate targeted uptake. PDEVs, on the other hand, present surface proteins, lipids, and glycans that act as molecular addresses, facilitating selective recognition by recipient cells across kingdoms. This specificity underlies their unique ability to mediate cross-kingdom communication, a property that synthetic liposomes cannot replicate [19].

From a translational perspective, liposomes and PDEVs represent two complementary paradigms [20,21]. Liposomes provide manufacturing flexibility, reproducibility, and regulatory familiarity, but are constrained by their limited biofunctionality and need for active loading strategies. PDEVs, in contrast, are biologically authentic vesicles that carry an intrinsic repertoire of functional molecules with demonstrated roles in oxidative stress modulation, inflammation control, and barrier reinforcement—properties of direct relevance to cosmetic science. Their main limitations are linked not to bioactivity but to variability, scalability, and quality control, challenges that arise from their biological origin and that require advanced standardization frameworks.

In essence, liposomes were invented to mimic life, while PDEVs are products of life itself. The former offers technical advantages in formulation engineering, the latter

provides evolutionary advantages in molecular complexity and biological compatibility. For cosmetics, the two systems should not be seen as interchangeable: while liposomes remain valuable carriers, PDEVs embody a next-generation platform, offering authenticity, multi-functionality, and natural alignment with human biology, attributes that liposomes cannot fully reproduce (Table 1).

Table 1. Plant-derived extracellular vesicles vs. liposomes: comparative overview for cosmetic applications.

Attribute	Plant-Derived Extracellular Vesicles	Liposomes
Origin/ Biogenesis	Natural vesicles secreted by plant cells via regulated pathways (endosomal/exosome-like, microvesicle shedding); Exist in vivo in tissues and diet.	Artificially engineered phospholipid vesicles assembled in vitro (thin-film hydration, ethanol injection, microfluidics).
Structural architecture	Lipid bilayer with complex native composition (phospholipids, sphingolipids, sterols) plus embedded proteins/glycans; Heterogeneous nano-assemblies.	Primarily phospholipid bilayer(s); Composition defined by formulation; Typically protein-free unless functionalized.
Cargo	Endogenous multi-omic cargo: proteins/enzymes, small RNAs (miRNA/siRNA), mRNA, metabolites (polyphenols, carotenoids, lipid mediators); Pre-loaded by biology.	No intrinsic cargo; Requires exogenous loading of active pharmaceutical ingredient (hydrophilic in core, lipophilic in membrane).
Surface markers	Natural ligands (proteins, lipids, glycans) enabling selective uptake and cross-kingdom signaling; Potential tissue tropism.	Lack native recognition motifs; targeting achieved via synthetic ligands such as PEG, peptides, antibodies, if added.
Uptake/ Targeting	Multiple routes (endocytosis, membrane fusion) with context-specific specificity; Evidence for functional delivery of RNAs/metabolites.	Primarily nonspecific endocytosis/fusion; Targeting depends on engineered surface modifications.
Functional bioactivity	Inherent antioxidant, anti-inflammatory, barrier-reinforcing, and regenerative signals from native cargo.	Carrier itself generally inert (unless composition confers effects); Function determined by loaded actives.
Stability	Biological membranes with stabilizing proteins/lipids; Sensitive to storage/oxidation; Can be stabilized (lyophilization/cryoprotectants).	Stability tunable by composition (cholesterol, saturated lipids); Susceptible to leakage/aggregation without optimization.
Standardization/ reproducibility	Sensitive to source, season, and process (for raw-derived); In vitro-derived improves consistency but remains biologically variable.	Highly reproducible once process is fixed (defined lipids, controlled assembly, tight CQA).
Scalability/ Manufacturing	Raw: dependent on biomass; In vitro: bioreactors with TFF or SEC; Process complexity and QC burden higher.	Scalable, modular manufacturing; Well-established unit operations and supply chains.
Contaminant risk	Raw: agrochemical/microbial co-isolation risk (mitigable via organic sourcing/QC). In vitro: residual media components (PGRs, antibiotics) risk.	Low intrinsic contamination risk; Residual solvents/detergents from process must be controlled.

Table 1. *Cont.*

Attribute	Plant-Derived Extracellular Vesicles	Liposomes
Safety/ Biocompatibility	Evolutionary/dietary familiarity; Good tolerability reported; Must control contaminants and variability.	Generally safe carriers; Immunogenicity/irritation depends on lipids and surface chemistries (e.g., PEG).
Regulatory familiarity	Emerging category; Requires detailed characterization and provenance disclosure; Fewer precedents in cosmetics.	Well-known excipient class with established guidance; Easier to justify from CMC standpoint.
Customization/ Engineering	Limited direct engineering; Can modulate via source selection and elicitation; Post-isolation modification possible but delicate.	High tunability: size, charge, composition, surface ligands, stimuli-responsive designs.
Loading and release control	Primarily intrinsic cargo; Exogenous loading possible (electroporation, incubation) with variable efficiency.	Designed for controlled loading/release (remote loading, ion gradients, prodrug strategies).
Target product profile fit	Authentic, multifunctional bioactives; Ideal for 'natural/organic' lines and biologically rich claims.	Precise, standardized delivery vehicles; Ideal where strict uniformity and targeted delivery are required.
QC/ Characterization	Multi-omic fingerprints (proteomics, lipidomics, small RNAs), NTA or TRPS, functional potency assays; Broader CQA set.	Physicochemical QC (size, PDI, zeta potential), stability, release kinetics; Narrower, well-defined CQA set.
Key limitations	Variability (raw), contaminant control (raw/in vitro), complex analytics, scalability vs. authenticity trade-offs.	Limited intrinsic bioactivity; Potential rapid clearance and nonspecific uptake; Need for active loading and targeting.
Headline advantages	Nature-derived, preloaded, multimodal activity; cross-kingdom communication potential; consumer alignment with 'natural'.	Manufacturing control, reproducibility, regulatory familiarity, precise engineering and targeting options.
Cosmetic use cases	Antioxidant and anti-reactive oxygen metabolites serums, barrier-repair creams, soothing and anti-redness products, regenerative and anti-aging lines.	Targeted delivery of defined actives, photostability enhancement, controlled-release formulations, sensitive-skin minimal-ingredient lines.

CMC: chemistry, manufacturing, and control; NTA: nanoparticle tracking analysis; PDI: polydispersity index; PEG: polyethylene glycol; PGRs: plant growth regulators; QC: quality control; CQA: critical quality attributes; SEC: size-exclusion chromatography; TFF: tangential flow filtration; TRPS: tunable resistive pulse sensing.

4. Sources of PDEVs: Raw Biomass or In Vitro Cultures

PDEVs have recently attracted considerable interest as innovative ingredients in cosmetic formulations, yet the biological source from which they are obtained remains a fundamental determinant of their properties, reproducibility, and regulatory positioning. Broadly, PDEVs originate from two principal production routes: direct isolation from raw plant material or generation through in vitro plant cell or tissue cultures [22–25].

Although both approaches yield vesicular nanostructures with comparable morphology, the biological and industrial implications of these sources diverge markedly, and understanding this dichotomy is critical to assessing both the opportunities and limitations of PDEVs in the cosmetic field [26–28]. For the cosmetic industry, this distinction is far from academic: it determines how vesicles behave biologically, how consistently they can be produced, and how they should be evaluated for safety and efficacy. Yet in the

current marketplace, products labeled simply as "plant extracellular vesicles" may derive from either raw biomass or in vitro culture, with no disclosure of the origin. This lack of transparency obscures essential information for formulators, regulatory authorities, and ultimately consumers, who may equate the term "plant-derived" with natural authenticity, without realizing that two profoundly different technological pathways underlie the same label. For those working in cosmetics—whether in research and development, product formulation, or regulatory affairs -recognizing and interrogating the source of PDEVs is therefore indispensable to avoid misinterpretation of claims, to set realistic expectations of performance, and to ensure compliance with safety and quality standards.

4.1. PDEVs Derived from Raw Plant Material

PDEVs derived from raw plant material embody the intrinsic molecular complexity of intact botanical tissues—whether fruits, leaves, stems, roots, or seeds—capturing a snapshot of the plant's physiological and metabolic state at the moment of harvest. Their vesicular cargo is not a static entity, but a dynamic reflection of multiple variables: the developmental stage of the tissue, the metabolic fluxes sustained at that time, the local microenvironment, and the cumulative history of environmental exposures such as light intensity, water availability, soil composition, microbial interactions, or mechanical stress. In this sense, raw-material PDEVs represent a "molecular diary" of the plant's life cycle, encoding signals that are both constitutive and adaptive.

One of the most distinctive and scientifically valuable features of vesicles derived from raw plant biomass is their natural enrichment in stress-responsive molecular components, a complexity that is frequently diminished or entirely absent in vesicles obtained from in vitro culture systems [29]. These naturally occurring vesicles embody the plant's adaptive biology, serving as carriers of bioactive molecules that have evolved to mediate survival in fluctuating and often hostile environments. Their cargo includes a diverse spectrum of antioxidant metabolites such as flavonoids, carotenoids, and tocopherols; polyphenolic compounds with radical-scavenging and metal-chelating activity; lipid mediators that orchestrate membrane remodeling and signal transduction; and regulatory small RNAs capable of engaging in cross-kingdom communication with animal and microbial cells. Collectively, these molecules form a biochemical fingerprint of ecological resilience, reflecting the plant's innate strategies of defense, repair, and adaptation [30–32].

This fingerprint translates into functional properties of direct cosmetic relevance. By transferring stress-protective molecules to recipient human cells, raw-material PDEVs can mitigate oxidative stress in keratinocytes and dermal fibroblasts, strengthen epidermal barrier integrity, attenuate inflammatory cascades mediated by NF-κB and MAPK pathways, and potentially activate regenerative programs that restore tissue homeostasis [33,34]. Importantly, these vesicles do not merely supply exogenous antioxidants; they deliver complex, synergistic cargo packages that act in concert, reproducing aspects of the plant's natural defense systems within human skin. This ecological authenticity cannot be fully replicated in vitro, where plant cells, grown in nutrient-defined and stress-free environments, lack the environmental triggers that drive vesicle diversification and bioactive enrichment.

From a translational perspective, raw-material PDEVs represent the most "natural" form of these nanostructures, not only because they originate directly from intact tissues, but also because humans are already continuously exposed to them through diet. Fruits, vegetables, seeds, and leaves consumed as food all contain vesicles that transit through the gastrointestinal tract, engage with epithelial barriers, and influence systemic physiology. This dietary familiarity suggests a high degree of biocompatibility and safety, reinforcing the notion that vesicles from raw sources embody an authenticity and a continuity with

natural human exposure that is absent in vesicles generated artificially. In this sense, cosmetic formulations enriched with raw-material PDEVs can claim not only functional bioactivity but also a unique alignment with what is physiologically accepted by the human body, given that these vesicles are part of the molecules we have ingested for millennia.

At the same time, the complexity of raw-material PDEVs highlights their scientific uniqueness. By carrying molecules produced under real environmental stressors -UV radiation, temperature shifts, pathogen encounters—they encapsulate a biological memory of the plant's adaptive responses. This evolutionary imprint becomes a powerful asset when applied to skin biology: the same molecular defenses that protect a plant from oxidative damage, dehydration, or microbial attack may, when delivered in vesicular form, provide protective and restorative effects on human skin exposed to pollution, sunlight, or aging [35]. Thus, raw-material PDEVs can be considered as nature's own carriers of resilience, transferring strategies of adaptation across kingdoms.

However, their natural variability should not be misinterpreted as a weakness alone: it also represents a source of functional richness that distinguishes them from highly standardized but potentially impoverished vesicles of in vitro origin. For the cosmetic field, this distinction is pivotal. A vesicle derived from a fruit consumed daily carries not only molecules with demonstrable antioxidant or anti-inflammatory potential but also the cultural and biological legitimacy of being "edible" and "safe", qualities that resonate strongly with consumers and regulators alike. In other words, raw-material PDEVs are not merely cosmetic additives; they are an extension of the nutritional and ecological interface between plants and humans, now repurposed to reinforce skin health and beauty. Yet this richness has a double-edged nature. The same variables that endow raw-material PDEVs with functional diversity also render them highly susceptible to fluctuations that complicate standardization. Vesicle yield, size distribution, and cargo composition are influenced by factors as granular as soil mineral content, irrigation frequency, or day/night temperature variation. Seasonal shifts can reprogram metabolic pathways, altering the abundance of key antioxidants or signaling lipids; geographical origin introduces differences in secondary metabolite profiles linked to terroir-like effects; harvest timing—whether during early growth, full ripening, or senescence—imposes distinct molecular signatures. Post-harvest handling further adds layers of variability: storage conditions, mechanical processing, and extraction techniques all leave measurable imprints on vesicle integrity and cargo stability.

From a cosmetic science perspective, this inherent variability presents a formidable challenge. Two batches of PDEVs isolated from the same plant species, labeled under an identical cosmetic claim, may differ substantially in potency, stability, and biological activity. One preparation may exhibit robust antioxidant and anti-inflammatory properties, while another, derived from a different season or supply chain, may be comparatively inert. Such discrepancies directly impact not only the reproducibility of experimental data but also the reliability of marketed products, with consequences for both consumer trust and regulatory approval. In practice, this means that a label reading "plant extracellular vesicles from [species]" may conceal profound differences in functional performance, unless accompanied by rigorous characterization and transparent documentation of origin.

The paradox of raw-material PDEVs, therefore, lies in their tension between richness and reproducibility. On one hand, they offer unparalleled access to the full spectrum of plant-derived biomolecules, many of which are stress-enhanced and potentially bioactive in human skin. On the other hand, they resist reduction to a uniform, industrially scalable product, challenging conventional frameworks of quality control. Addressing this paradox will require not only improved standardization in harvesting and isolation, but also the development of advanced analytical platforms capable of capturing and monitoring the subtle variations in vesicle composition that define their cosmetic relevance.

An additional and critically important dimension in the assessment of raw-material PDEVs is the risk of co-isolated agrochemical contaminants, most notably pesticides, herbicides, fungicides, and chemical fertilizers that are extensively used in conventional agriculture. By virtue of their lipid bilayer and nanoscale structure, EVs are not only natural carriers of endogenous bioactive cargo but also potential vectors for exogenous xenobiotics. Lipophilic compounds, in particular, demonstrate a strong propensity to integrate into the vesicular membrane or to be passively entrapped within the vesicle lumen. This phenomenon has been documented for polycyclic pesticides and organophosphates, whose chemical properties allow partitioning into lipid bilayers. For example, residues of chlorpyrifos, imidacloprid, or glyphosate—all widely employed in large-scale agriculture—could, in principle, co-purify with PDEVs derived from exposed tissues. Similarly, fungicides such as azoxystrobin or tebuconazole, frequently applied to fruit crops, may persist in plant tissues at sub-ppm levels and risk being concentrated in vesicular preparations.

The potential consequences for cosmetic applications are significant. Vesicles intended for topical delivery are designed to penetrate superficial skin layers, and their nanoscale dimensions may facilitate the transfer of both endogenous and exogenous cargo into keratinocytes, fibroblasts, and potentially systemic circulation through micro-abrasions or compromised barrier integrity. If residual agrochemicals are present, PDEVs could inadvertently act as Trojan horses, enhancing the dermal absorption of molecules that were never intended to reach human tissues in this form. Such an outcome would not only compromise the safety profile of PDEV-based products but also undermine consumer confidence in the entire sector, particularly given the increasing public sensitivity to issues of "clean beauty" and transparency in sourcing.

These considerations strongly argue for rigorous source selection, agricultural traceability, and chemical safety screening as integral components of PDEV characterization. From a scientific and industrial perspective, the most robust mitigation strategy is to prioritize certified organic cultivation systems, where the application of synthetic pesticides and fertilizers is either prohibited or strictly limited. For instance, apples, grapes, and tomatoes are among the most widely studied plant sources of EVs; yet these crops are also heavily treated in conventional agriculture. Using organic-certified apples or grape pomace as a starting material not only minimizes the risk of xenobiotic contamination but also aligns with consumer expectations of natural authenticity. Similarly, green tea (*Camellia sinensis*) and aloe vera—both of which are increasingly investigated for their vesicular fractions—should ideally be sourced from organic plantations to guarantee the integrity of their vesicle-derived actives [11,36–39].

Beyond agricultural practices, analytical verification must be mandatory. Techniques such as liquid chromatography–mass spectrometry (LC-MS/MS) or gas chromatography–mass spectrometry (GC-MS) should be systematically applied to detect and quantify potential residues in PDEV preparations, with detection thresholds benchmarked against both cosmetic and food safety standards. These analyses can reveal whether contaminants like DDT derivatives, carbamates, or neonicotinoids are present even at trace levels, allowing producers to exclude compromised batches before integration into formulations.

In the context of cosmetic science, therefore, sourcing PDEVs from raw plant material should not be considered a guarantee of safety per se. While such vesicles embody the most authentic and "natural" form of nanostructures—indeed, the same vesicles are routinely ingested through diet when consuming fruits and vegetables—their safety for topical application hinges on the purity of the agricultural inputs from which they are derived. A vesicle isolated from an organically cultivated apple not only carries the antioxidant polyphenols and stress-responsive RNAs characteristic of its biological origin, but also the assurance that it is free from pesticide residues that could otherwise compromise both

function and perception. Conversely, a vesicle extracted from conventionally farmed fruit may present an uncontrolled mixture of natural and anthropogenic molecules, confounding interpretation of efficacy and raising red flags for safety.

Thus, for the cosmetic industry, certification of agricultural practices, transparency of supply chains, and systematic chemical residue testing must become part of the minimal characterization criteria of PDEVs. Only by integrating these safeguards can manufacturers ensure that the functional promise of raw-material vesicles—skin protection, anti-inflammatory potential, regenerative capacity—is not overshadowed by the silent risk of xenobiotic contamination. In an era where consumers increasingly demand products that are both effective and authentically safe, the alignment of PDEVs with organic-certified sources should be considered not merely a marketing strategy but a scientific and regulatory imperative.

Crucially, a growing body of evidence demonstrates that the use of organically cultivated plants as a source of PDEVs does not result in reduced yield or compromised functional potential; on the contrary, it may favor the generation of vesicles with equal or superior bioactivity compared to those derived from conventionally farmed material. In their recent work, Trentini et al. [40] provided compelling data showing that PDEVs isolated from organically grown fruits maintained comparable vesicle concentration, size distribution, and marker expression (including canonical tetraspanins such as CD9- and HSP70-like proteins) relative to vesicles extracted from conventional crops. More importantly, when tested in in vitro models of oxidative stress, organically derived PDEVs exhibited a significantly enhanced capacity to reduce reactive oxygen metabolites (ROMs), a key determinant of oxidative damage in dermal and epidermal cells. This enhanced anti-ROM activity was paralleled by increased expression of antioxidant defense genes (e.g., Nrf2, HO-1) and by the suppression of pro-inflammatory mediators such as IL-6 and TNF-α in fibroblasts and keratinocytes exposed to inflammatory stimuli.

Mechanistically, the superiority of organic PDEVs is thought to derive from the adaptive responses triggered by cultivation under chemical-free and environmentally variable conditions. Unlike plants in conventional agriculture, which are often shielded from biotic and abiotic stress by synthetic pesticides and fertilizers, organically grown plants are subjected to a more diverse spectrum of ecological pressures, including microbial interactions, competition for nutrients, fluctuations in water availability, and natural pest challenges. These stressors activate endogenous defense pathways that promote the synthesis and vesicular loading of secondary metabolites—such as flavonoids, phenolic acids, and terpenoids—as well as stress-responsive small RNAs that modulate intercellular and cross-kingdom signaling. As a result, vesicles derived from organic sources embody a broader and more functionally potent cargo profile, with direct relevance to cosmetic applications in skin protection, anti-aging, and regenerative medicine.

From a translational perspective, this evidence carries profound implications (Table 2). It demonstrates that sourcing from organic agriculture not only mitigates the risks of pesticide and xenobiotic contamination, but also preserves or even enhances the functional properties most sought after in cosmetic formulations, particularly antioxidant and anti-inflammatory activities. By integrating organic sourcing into the production pipeline, cosmetic developers can therefore align three critical priorities: safety, efficacy, and consumer perception of natural authenticity. In light of the data by Trentini et al. and corroborating studies in plant physiology [40–44], it becomes clear that organic PDEVs should not be considered a compromise solution but rather the optimal standard for future cosmetic applications, combining ecological sustainability with measurable functional superiority.

Table 2. Plant-derived extracellular vesicles from raw material: labeling, advantages, limitations, and strategic approaches.

Origin	Recommended Labeling	Key Advantages	Main Limitations	Mitigation Strategies	Value Enhancement Strategies
Organic (certified) raw plant material	"Plant-Derived Extracellular Vesicles (PDEVs) from [botanical species] (fruit/leaf/root/seed), organically cultivated (certifying body and ID), geographical origin, harvest season/year, extraction method, batch/lot ID, pesticide panel (LC-MS/MS: not detected), microbiological compliance, vesicle QC profile (size distribution, EV markers, omics fingerprint)."	Highest natural authenticity and continuity with diet (vesicles ingested with fruits/vegetables). Stress-enriched cargo: polyphenols, carotenoids, lipid mediators, stress-responsive small RNAs. Strong consumer trust due to "organic" origin. Broad functional profile: antioxidant, anti-inflammatory, barrier-supporting, regenerative.	Batch-to-batch variability (seasonality, terroir, post-harvest handling). Stability issues (oxidation of lipids/polyphenols). Possible microbial burden from raw tissues. Co-extraction of unwanted molecules (e.g., polysaccharides, proteins).	Supply-chain standardization (cultivar selection, harvest timing). Strict post-harvest SOPs (temperature, atmosphere). Controlled blending of micro-batches. Advanced QC (multi-omics, Raman, TRPS). Stabilization via lyophilization or cryoprotectants. HACCP-based microbial controls.	Publish data dossiers on antioxidant/anti-inflammatory efficacy (ROM assays, cytokine modulation). Highlight barrier reinforcement (TEER, TJ proteins). Titrate active content (polyphenols, carotenoids, lipids). Provide QR code links to full CoA and methods. Support claims with ex vivo/clinical tolerability studies.
Conventional (non-organic) raw plant material	"Plant-Derived Extracellular Vesicles (PDEVs) from [species] (tissue), conventional cultivation; origin; harvest season; extraction method; lot ID; pesticide and agrochemical residue panel (LC-/GC-MS with compound list and thresholds); detoxification/clean-up steps applied; vesicle QC profile (NTA or TRPS, EV markers)."	Wide availability of raw material. Lower production cost. Bioactivity still present (depending on natural agronomic stress exposure).	Risk of pesticide/fungicide residues incorporated into vesicles (lipophilic molecules co-partitioning in bilayers). Marked variability between batches. Lower consumer perception of "naturalness". Potential regulatory hurdles due to xenobiotic residues.	Rigorous supplier selection (reduced pesticide input). Systematic residue screening (LC/GC-MS/MS for neonicotinoids, organophosphates, azoles, glyphosate/AMPA, carbamates, DDT derivatives). Preparative clean-up (solid-phase extraction, diafiltration). Strict acceptance thresholds. Batch-level traceability.	Demonstrate functional comparability to organic PDEVs with robust in vitro/ex vivo data. Highlight QC-driven standardization (controlled blending, validated acceptance criteria). Ensure full transparency with CoAs. Publish third-party validations and methodological white papers.

CoA: certificate of analysis; GC: gas chromatography; HACCP: hazard analysis and critical control points; LC: liquid chromatography; MS: mass spectrometry; NTA: nanoparticle tracking analysis; QC: quality control; SOPs: standard operating procedures; TEER: transepithelial electrical resistance; TRPS: tunable resistive pulse sensing.

4.2. Plant-Derived Extracellular Vesicles from In Vitro Cultures: Definition and Production

PDEVs obtained from in vitro plant cell and tissue cultures represent an alternative to vesicles isolated directly from raw botanical material. Unlike vesicles collected from intact fruits, leaves, roots, or seeds, these nanostructures are secreted by plant cells maintained under artificially controlled conditions, outside of their native tissue environment. In vitro culture systems encompass a spectrum of approaches, ranging from dedifferentiated callus tissues grown on solid nutrient media to suspension cultures in liquid media, and more advanced configurations such as organoid-like structures, hairy root cultures, and micropropagated shoots [45,46]. Each of these culture types provides a distinct cellular context for vesicle biogenesis, with profound implications for vesicle yield, cargo composition, and functional relevance. The process typically begins with the induction of callus, a mass of undifferentiated cells generated from small tissue explants (e.g., leaf discs, stem fragments, or root tips) plated onto nutrient-rich agar supplemented with plant growth regulators (PGRs). Auxins (e.g., 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid) and cytokinins (e.g., kinetin, benzylaminopurine) are commonly used to maintain cells in a proliferative, dedifferentiated state. Once callus formation is established, fragments of this tissue can be transferred into liquid medium to produce cell-suspension cultures, in which vesicles are released into the extracellular space and accumulate in the culture supernatant. In more specialized systems, hairy root cultures, generated by infection with Agrobacterium rhizogenes, provide a genetically stable source of rapidly proliferating root tissue that secretes vesicles enriched in secondary metabolites [47]. Similarly, organoid-like structures or shoot cultures can be engineered to more closely mimic specific plant tissues while retaining the scalability of in vitro methods. The culture media used in these systems typically consist of a basal mineral formulation (e.g., Murashige and Skoog medium), supplemented with vitamins, carbon sources (usually sucrose), and growth regulators. In some cases, complex supplements such as casein hydrolysate, yeast extract, or coconut water are added to stimulate cell proliferation. To avoid microbial contamination, antibiotics (kanamycin, streptomycin) and antifungal agents (amphotericin B) are frequently included during the establishment and maintenance of cultures. Under these conditions, plant cells proliferate indefinitely, continuously releasing EVs into the surrounding medium. Scaling up production requires transferring these cultures into bioreactors (e.g., shake flasks, stirred-tank, or wave bioreactors), where parameters such as pH, dissolved oxygen, light, and temperature can be tightly controlled [48]. In such systems, vesicle production can be optimized by adjusting nutrient ratios, growth regulator concentrations, or applying elicitors. For example, mild abiotic stresses (e.g., UVB pulses, osmotic stress, hypoxia) or chemical elicitors (e.g., jasmonic acid, salicylic acid) may be applied to increase vesicle yield or to enrich cargo with stress-responsive metabolites and RNAs [49,50]. Downstream, vesicles are harvested from the clarified culture supernatant using isolation methods similar to those applied to raw-material PDEVs, including tangential flow filtration (TFF), ultracentrifugation (UC), size-exclusion chromatography (SEC), or density-gradient separation, followed by characterization via nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), Raman spectroscopy, lipidomics, and small RNA profiling. The theoretical advantage of this approach lies in its reproducibility and scalability [51,52]. In vitro cultures are shielded from environmental variability such as climate, soil composition, or pathogen exposure, which makes the vesicle output more consistent from batch to batch. Furthermore, because no field pesticides or herbicides are applied, there is a lower likelihood of co-isolating agrochemical residues compared to raw-material PDEVs. In addition, the controlled environment facilitates GMP-like documentation, enabling producers to establish standard operating procedures (SOPs), process validation, and in-process controls, all of which are highly valued in cosmetic regulatory frameworks. However, it is important

to emphasize that vesicles obtained from in vitro cultures represent a biological system far removed from natural conditions. Dedifferentiated cells growing in synthetic media lack the tissue-specific architecture, developmental gradients, and environmental stress signals that shape vesicle biogenesis in intact plants. Consequently, their vesicular cargo may differ substantially: while in vitro PDEVs tend to be more uniform, they often show a reduced diversity of stress-induced molecules, such as polyphenols, carotenoids, and stress-responsive small RNAs, which are key contributors to the bioactivity of vesicles from raw biomass [53]. Moreover, the culture medium itself introduces novel risks: residual phytohormones, antibiotics, antifungals, and undefined supplements may persist in trace amounts and co-purify with vesicles. These substances are foreign to human physiology, raising questions about their compatibility and long-term safety, particularly when PDEVs are intended for topical application on compromised skin.

While PDEVs obtained from in vitro cell cultures offer the advantage of reproducibility and controlled manufacturing, a number of critical issues must be acknowledged when considering their integration into cosmetic formulations. First, the biological context of in vitro cultures is profoundly artificial and far removed from the ecological conditions in which plants have evolved and in which humans have coexisted with their vesicles through diet. In nature, humans ingest PDEVs embedded in fruits, vegetables, and seeds, vesicles that carry molecules shaped by stress exposure, development, and environmental interactions. By contrast, vesicles secreted by dedifferentiated callus or suspension cells proliferating in synthetic nutrient media do not mirror the complexity of intact tissues; they represent a biological entity that the human body has never naturally encountered. This raises fundamental questions about biological recognition, compatibility, and long-term safety.

Secondly, the culture environment itself introduces potential contaminants that may co-purify with PDEVs. In vitro plant cells are commonly maintained with antibiotics and antifungal agents to prevent microbial contamination, synthetic phytohormones such as auxins and cytokinins to sustain dedifferentiated proliferation, and often undefined supplements (e.g., yeast extract, casein hydrolysate) to promote growth. Residual traces of these compounds can remain in culture supernatants and be incorporated into vesicle preparations, posing risks of irritation, sensitization, or unintended biological activity when applied to human skin. For instance, residual kanamycin, streptomycin, or amphotericin B, widely used in plant tissue culture, could persist in trace amounts despite purification; synthetic hormones like 2,4-D or kinetin may also partition into lipid bilayers or interact with vesicular cargo. These xenobiotics are foreign to human physiology, and unlike food-derived PDEVs, the body has no evolutionary or dietary familiarity with them [54].

Another concern lies in the fundamental biology of in vitro cells. Callus and suspension cultures are highly dedifferentiated, lacking the tissue-specific architecture and intercellular communication that shape vesicle content in vivo. As a result, their vesicles may be compositionally skewed, enriched in proliferation-associated signals but depleted of stress-response molecules that are crucial for protective functions in skin. Moreover, culture conditions often involve growth factors and elicitors that further distort the molecular profile of vesicles, producing cargos not representative of native plant physiology. While these manipulations may increase productivity, they may also yield cargoes that are biologically ambiguous for cosmetic application. Finally, in vitro culture is an industrial system inherently divorced from nature: cells are kept in artificial media, under sterile conditions, often in the complete absence of environmental stressors, light cycles, or microbial symbiosis. This not only limits the functional repertoire of vesicles but also creates an unprecedented exposure scenario for the human organism, which is evolutionarily adapted to vesicles derived from whole plants but not to those secreted by artificially maintained cell lines. From a cosmetic science perspective, this raises critical issues of transparency,

risk assessment, and consumer acceptance. Without clear labeling and rigorous removal of media-derived contaminants, in vitro PDEVs may blur the line between naturally derived actives and engineered nanomaterials.

In summary (Table 3), while in vitro PDEVs present opportunities for standardization and scale-up, they also carry unique risks: (i) biological unfamiliarity for the human body, (ii) deviation from the natural molecular fingerprint of intact plants, (iii) incorporation of antibiotics, hormones, and growth factors used in culture, and (iv) potential consumer mistrust if their origin is not transparently declared. These critical aspects must be explicitly addressed through rigorous purification, comprehensive residue testing, and transparent disclosure of culture practices if in vitro PDEVs are to be credibly positioned within cosmetic innovation.

In conclusion, the comparison between PDEVs derived from raw plant material and those generated through in vitro cultures highlights a fundamental trade-off between ecological authenticity and industrial standardization (Table 4). Raw-material PDEVs embody the most natural form of vesicles, enriched with stress-responsive cargoes that mirror the plant's adaptive strategies and that humans already encounter through diet. Their functional richness, however, comes at the expense of variability, contamination risks, and the need for stringent quality controls. By contrast, in vitro-derived PDEVs offer reproducibility, scalability, and alignment with GMP-like documentation, but they remain biologically distant from native tissues, potentially carrying residual culture components unfamiliar to human physiology and lacking the complexity of naturally stressed vesicles. For the cosmetic sector, the future lies not in favoring one approach exclusively, but in transparent labeling, rigorous characterization, and evidence-driven positioning that make explicit the origin, advantages, and limitations of each source. Only through such clarity can PDEVs establish themselves as credible, safe, and innovative cosmetic ingredients, bridging consumer expectations of natural authenticity with the industry's demand for consistency and regulatory compliance.

Table 3. Plant-derived extracellular vesicles from in vitro cultures: labeling, advantages, limitations, and strategic approaches.

Origin	Recommended Labeling	Key Advantages	Main Limitations/Critical Issues	Mitigation Strategies	Value Enhancement Strategies
PDEVs from in vitro plant cell/tissue cultures (callus, suspensions, organoid-like tissues, hairy roots)	"Plant-Derived Extracellular Vesicles (PDEVs) from in vitro plant cell culture (species; cell line/callus/hairy root ID; tissue of derivation), culture mode (solid vs. suspension; bioreactor type), media class (chemically defined vs. complex), PGRs/elicitors used (names/grades), antibiotics/antifungals policy, process scale (batch/fed-batch/continuous), purification train (e.g., TFF, SEC, gradient), lot ID, residuals panel (LC/GC-MS: PGRs, antibiotics, solvents; LOQ/LOR), microbiological specs (TAMC/TYMC, endotoxin), vesicle QC (mode size, P/particle, lipid/particle, markers, omics fingerprint)."	High reproducibility and batch consistency (decoupled from season/terroir). Scalable (stirred-tank/wave bioreactors; DoE optimization). Lower risk of agrochemical residues vs. raw materials. GMP-like documentation feasible (SOPs, validation).	Biological distance from native tissues: cargo may lack stress-enriched complexity (polyphenols, carotenoids, stress-miRNAs). Residual culture components (PGRs like 2,4-D/BAP; antibiotics/antifungals; complex supplements) may co-purify. Compositional skew from dedifferentiated cells: potential shear-induced artifacts in reactors. Consumer perception: less "natural/authentic".	Use chemically defined, antibiotic-free media and qualify suppliers (pharma-grade inputs). Implement residuals control (LC/GC-MS/MS for PGRs, antibiotics, solvents; strict acceptance <LOQ). Orthogonal purification (diafiltration and SEC and SPE for lipophiles). Control shear/oxygen/light; PAT for pH/DO; CFD-informed bioreactor settings. Identity–purity–potency matrices (TRPS/NTA; markers; Raman/lipidomics; small-RNA profiling).	Elicitation programs (JA/SA pulses, UVB/blue light, hypoxia, nutrient shifts) to re-introduce stress-enriched cargo while retaining batch control. Publish comparability data vs. raw-derived PDEVs (anti-ROM, cytokine modulation, barrier assays). Transparency narrative (cleaner xenobiotic profile; full CoA data). Develop fit-for-purpose release assays (TEER, TJ proteins, irritation/ sensitization surrogates).

2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; CFD: computational fluid dynamics; CoA: certificate of analysis; DO: dissolved oxygen; DoE: design of experiments; GC: gas chromatography; JA: jasmonic acid; LC: liquid chromatography; LOQ: limit of quantification; LOR: limit of reporting; MS: mass spectrometry; NTA: nanoparticle tracking analysis; PAT: process analytical technology; PGRs: plant growth regulators; QC: quality control; ROM: reactive oxygen metabolite; SA: salicylic acid; SEC: size-exclusion chromatography; SOPs: standard operating procedures; SPE: solid-phase extraction; TAMC: total aerobic microbial count; TEER: transepithelial electrical resistance; TFF: tangential flow filtration; TJ: tight junction; TRPS: tunable resistive pulse sensing; TYMC: total yeast and mold count.

Table 4. Plant-derived extracellular vesicles comparison: from raw material versus in vitro culture.

Aspect	Raw-Material: Advantages	Raw-Material: Disadvantages	In Vitro Culture: Advantages	In Vitro–Culture: Disadvantages
Source and definition	Isolated directly from intact plant tissues (fruit/leaf/root/seed); captures in vivo biology and stress-driven signaling.	Subject to agronomic and environmental variability; Heterogeneous tissue inputs.	Secreted by plant cells grown under controlled conditions (callus/suspensions/hairy roots/organoid-like) with defined media and PGRs.	Artificial, extra-organismic system far from native tissue context; Dedifferentiated biology.
Bioactive cargo profile	Stress-enriched fingerprint (polyphenols, carotenoids, lipid mediators, stress-responsive small RNAs); Dietary familiarity.	Cargo fluctuates with season/terroir/post-harvest; Potential co-extraction of undesired macromolecules.	Tunable via elicitation (JA/SA, light, hypoxia) while maintaining control; More uniform cargo distribution.	Baseline cargo may lack ecological complexity and stress signatures seen in field tissues.
Efficacy (cosmetic relevance)	Robust antioxidant/anti-ROM, anti-inflammatory, barrier-reinforcing and regenerative signals (when well-sourced).	Batch-to-batch potency differences; Efficacy claims harder to generalize without extensive QC.	Batch-consistent functional readouts; Amenable to fit-for-purpose release assays (TEER, cytokine modulation).	May show narrower activity spectrum unless elicited; Biological equivalence to raw not guaranteed.
Contaminants risk	When organic: minimal agrochemical residues; Aligns with 'clean beauty'.	Conventional crops: risk of pesticides/fungicides (lipophilic) co-partitioning into vesicles; Microbial burden from biomass.	Lower risk of field agrochemicals; Cleaner xenobiotic profile possible.	Risk of residual media components: PGRs (2,4-D/BAP), antibiotics/antifungals, complex supplements; Shear-induced fragments.
Standardization and reproducibility	Authenticity and ecological legitimacy; Can be standardized with strict supply-chain control and blending.	High intrinsic variability from biology and supply chain; More demanding QC to release.	High batch-to-batch reproducibility; DoE-driven optimization; GMP-like documentation feasible.	Requires stringent control to remove culture-derived residuals; Identity may diverge from native vesicles.
Scalability and cost	Abundant biomass for some crops; Upcycling of by-products (peels/pomace).	Seasonal availability; Logistics/post-harvest constraints; Cost of rigorous QC and decontamination.	Continuous production in bioreactors; Predictable supply; Efficient upstream/ downstream workflows.	CapEx/OpEx for bioreactors and sterile operations; Media costs; Process complexity.

Table 4. *Cont.*

Aspect	Raw-Material: Advantages	Raw-Material: Disadvantages	In Vitro Culture: Advantages	In Vitro–Culture: Disadvantages
Safety narrative and perception	Highest perceived naturalness; Continuity with diet (ingested vesicles).	Conventional residues can undermine safety perception if not rigorously screened.	Transparency on absence of agrochemicals; Traceable, controlled manufacturing.	Consumers may view as less 'natural'; Unfamiliar vesicles from dedifferentiated cells.
Regulatory alignment	Organic sourcing and robust QC facilitate safety dossiers; Strong alignment with natural-origin claims.	Residue management and variability increase regulatory scrutiny; Need extensive documentation.	Process control, SOPs, acceptance criteria suit cosmetic QMS; Clear CoA framework.	Must document removal of media residuals; Justify biological relevance vs. native tissues.
QC and release testing	Multi-omics fingerprinting (Raman/lipidomics/miRNA), NTA/TRPS, pesticide panels (LC/GC-MS), microbiology; batch blending.	Higher analytical burden to ensure comparability across seasons/lots.	Defined residuals panels (PGRs/antibiotics/solvents), orthogonal purity assays, potency assays tailored to skin.	Complex residuals control mandatory; Orthogonal purification (diafiltration/SEC/SPE).
Best-fit use cases	Premium 'natural/organic' lines; claims built on stress-enriched bioactivity and provenance storytelling.	Products requiring extreme uniformity without blending may struggle.	Large-scale lines needing high consistency; Platforms requiring precise tech dossiers and audits.	Applications tolerant to engineered elicitation; When transparency on in vitro origin is acceptable.

2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; CapEx: capital expenditure; CoA: certificate of analysis; DoE: design of experiments; GC: gas chromatography; JA: jasmonic acid; LC: liquid chromatography; MS: mass spectrometry; NTA: nanoparticle tracking analysis; OpEx: operational expenditure; PGRs: plant growth regulators; QC: quality control; QMS: quality management system; ROM: reactive oxygen metabolite; SA: salicylic acid; SEC: size-exclusion chromatography; SOPs: standard operating procedures; SPE: solid-phase extraction; TEER: transepithelial electrical resistance; TRPS: tunable resistive pulse sensing.

5. PDEV Extraction Methods

Once the source (raw materials or in vitro cultures) is identified, isolation proceeds as a modular, orthogonal technology designed to enrich bona fide PDEVs while preserving membrane integrity and cargo bioactivity. Rather than relying on a single operation, the workflow combines complementary separation logics—size and hydrodynamics to condition complex botanical matrices, buoyant density to discriminate vesicles from colloidal look-alikes, and surface affinity to refine biologically relevant subpopulations [55]. In premium cosmetic contexts, this architecture converges on a gentle, release-grade cascade in which clarified feeds are concentrated and washed under low shear, then resolved by selective partitioning to deplete soluble proteins, polysaccharides, pigments, and other co-extracts. Around this backbone, optional analytical and enrichment modules can be interleaved to tune purity versus yield without compromising scalability or introducing harsh process conditions.

What follows is a concise, non-proprietary catalogue of the principal isolation methodologies used within this framework, presented as a high-level guide suitable for methods sections and technical dossiers (Table 5).

The recovery of apoplastic fluid using the vacuum infiltration–centrifugation (VIC) method [56] provides a gentle means of accessing the native extracellular space without causing cellular rupture. This approach yields PDEVs with minimal intracellular contamination and serves as an excellent starting point. After clarification and microfiltration, the fluid is processed through TFF capture and SEC polishing, with optional density flotation or asymmetric flow field-flow fractionation (AF4) for cosmetic-grade refinement [57]. Cold-press or homogenate extraction, involving mechanical expression, generates abundant feedstreams including those from peels or pomace. Due to the higher content of pectins, proteins, and pigments in these matrices, a standardized finish is beneficial. Following clarification and microfiltration, the feed is captured by TFF and polished using SEC, optionally coupled with density flotation or AF4, to eliminate co-extracted substances while preserving bioactivity.

Differential centrifugation (DC) and sedimentation provide a staged clarification process that prepares complex fluids for more refined separations without applying harsh stress [58]. This step commonly precedes the canonical finish, which consists of TFF capture followed by SEC polishing, with optional density flotation or AF4, to ensure clean inputs for downstream processing. Sequential microfiltration using progressively finer pore sizes stabilizes the feed by removing microbes and coarse debris. This prepares the sample for subsequent steps, where it is captured by TFF and polished by SEC with optional density flotation or AF4, maintaining low shear and protecting delicate structures. Ultrafiltration (UF) and TFF serve as the primary capture and buffer-exchange module in the standard purification cascade [59,60]. Clarified and microfiltered liquids are typically captured using TFF and then polished by SEC [61], with optional density flotation or AF4 [62] to ensure scalability and gentle handling of the material. UC, either through pelleting or cushion-based methods, is a useful option when chromatographic resources are limited. In practice, it is often integrated into the membrane chromatography workflow, where samples are later processed via TFF and SEC [63], with optional flotation or AF4 [64], to reduce co-pelleting and improve purity. Buoyant-density UC using iodixanol or sucrose gradients introduces an orthogonal density-based separation [65]. This technique distinguishes extracellular vesicles from lipoprotein-like particles and aggregates. It is commonly positioned after TFF capture and either before or after SEC polishing as the “optional density flotation” step within the canonical sequence [66].

SEC functions as the gentle release step, separating components based on hydrodynamic size [67]. Within the core pipeline, feeds that have been clarified and captured by

TFF are refined by SEC, again with optional density flotation or AF4. This step effectively depletes soluble proteins and polysaccharides while preserving membrane-bound structures, whereas AF4 offers high-resolution, stationary-phase-free fractionation of PDEV subpopulations [68]. Typically used after TFF capture, it represents the "optional AF4" branch in the standard cascade, providing analytical or semi-preparative refinement of the product.

Immunoaffinity capture, targeting specific EV surface epitopes, enables highly selective enrichment for mechanism-focused studies or high-value fractions. It is most effective when applied after TFF and before or after SEC, allowing selective enrichment while preserving the integrity of the overall cascade [69]. Lectin-based affinity capture, which recognizes surface glycans, is similarly positioned post-TFF and adjacent to SEC or AF4. This method is particularly valuable when isolating glyco-defined PDEV subsets within the standard workflow [70]. Affinity-based separation using heparin or glycosaminoglycans (GAGs) provides a strong orthogonal selector that can significantly improve purity. Typically, it is inserted between the TFF capture and SEC polishing stages, maintaining the efficiency and continuity of the overall purification cascade.

Microfluidic separation techniques—including deterministic lateral displacement (DLD), inertial, and acoustofluidic methods—offer gentle, label-free sorting. These are often integrated as optional refinements following TFF and either preceding or following SEC or AF4. They enable the development of compact, continuous pipelines suitable for scalable applications [71]. Finally, polymer precipitation methods, such as polyethylene glycol (PEG) precipitation, offer high apparent recovery but relatively low specificity [72]. When employed, these methods are usually followed by TFF capture and SEC polishing (with optional density flotation or AF4) to regain selectivity. As such, they are more appropriate for preliminary screening rather than production of high-purity or premium products.

Selecting and executing a plant EV isolation strategy for cosmetic use requires balancing purity, integrity, and scalability under transparent, reproducible conditions. Regardless of whether the source is raw biomass or in vitro cultures, three safeguards are non-negotiable: (i) gentle handling (low shear, cold chain) to preserve membrane function and native cargo; (ii) orthogonal purification (at minimum, membrane capture and a selective polish) to deplete proteins, polysaccharides, pigments, and colloidal look-alikes; and (iii) matrix-specific risk control, i.e., agrochemical residue panels and microbiology for raw feeds, and residual phytohormones/antibiotics/solvents for culture media. Method choice should be fit-for-matrix (e.g., VIC for leaf/peel apoplast; homogenate/TFF-SEC for pulps and roots) and fit-for-purpose (e.g., analytical AF4 or affinity modules when subpopulation definition matters; density gradients when research-grade resolution is required). For premium releases, polymer precipitation is best avoided; TFF followed by SEC (with or without density flotation or AF4) remains the most defensible backbone.

Table 5. Isolation techniques for plant-derived extracellular vesicles: advantages and disadvantages.

Technique	Advantages	Disadvantages
Apoplastic fluid recovery	Low intracellular carryover; High extracellular specificity; Good purity–integrity balance for soft tissues.	Moderate throughput; Requires dedicated setup; Less suitable for hard tissues.
Cold-press/juice or homogenate extraction	High feed throughput; Compatible with agro by-products (peels/pomace); Versatile across matrices.	Complex co-extracts (pectins, pigments, proteins) demand robust downstream polishing.

Table 5. *Cont.*

Technique	Advantages	Disadvantages
DC or sedimentation	Universal, gentle front-end; Simplifies complex fluids prior to fine separations.	Poor selectivity among nanoscale species; must be paired with selective steps.
Sequential microfiltration	Reduces bioburden and coarse debris; Stabilizes feed for membranes or chromatography.	Risk of fouling and retention of large EV aggregates; Pressure control needed.
UF and TFF	Scalable capture and buffer exchange; Low shear; Efficient removal of small solutes.	Potential non-specific adsorption; Performance sensitive to shear or transmembrane pressure control.
UC (pellet or cushion)	Widely available; Rapid enrichment when chromatography is limited.	Co-pellets proteins/aggregates; Possible mechanical stress on EVs.
Buoyant-density UC	High resolving power; Separates EVs from lipoprotein-like particles and aggregates.	Time- and equipment-intensive; Added complexity and lower throughput.
SEC	Gentle, low-shear polishing; Efficiently depletes proteins and polysaccharides; Preserves bioactivity.	Fraction dilution requires reconcentration; Performance depends on column and load.
AF4	High-resolution, stationary-phase-free fractionation; Profiles EV subpopulations.	Specialized instrumentation; Limited preparative throughput.
Immunoaffinity capture	High selectivity for targeted subsets; Powerful for mechanism-driven fractions.	Limited capacity; Higher cost; Potential conflicts with clean-label claims.
Lectin affinity capture	Enriches glyco-defined subsets; Informative for plant-specific membrane biology.	Possible co-capture of non-vesicular glycoproteins; Requires gentle elution.
Heparin or GAG affinity capture	Robust, easy-to-implement orthogonal selector; Can sharpen overall purity.	Lower specificity than antibodies; Strong ionic-strength dependence.
Microfluidic separations	Very gentle, label-free sorting; Integrable into continuous compact pipelines.	Current throughput modest; Requires bespoke devices and know-how.
Polymer precipitation	High apparent recovery; Minimal setup and cost.	Low specificity; Co-precipitates proteins and polysaccharides; Generally unsuitable for premium products.

AF4: asymmetric flow field-flow fractionation; DC: differential centrifugation; GAG: glycosaminoglycan; TFF: tangential flow filtration; SEC: size-exclusion chromatography; UC: ultracentrifugation; UF: ultrafiltration.

6. Minimal Characterization for PDEVs

Once the source (raw material or in vitro culture) and the isolation workflow have been defined, a rigorous identity characterization is required to demonstrate that the preparation consists of bona fide PDEVs rather than generic lipid vesicles. At a minimum—and in this order—the baseline physical evidence should document: (i) size and size distribution (with particle number) to place the entities within the expected vesicular domain and exclude non-vesicular colloids; (ii) cargo composition (the endogenous molecular fingerprint typical of EVs, as opposed to empty or arbitrarily loaded lipid particles); and (iii) the surface electrokinetic zeta potential (ζ-potential) as a readout of membrane charge

state and colloidal stability consistent with vesicular membranes. These three pillars—size, content, and potential—constitute the minimal, orthogonal proof of vesicular nature on which deeper biochemical and functional analyses can be built.

6.1. Physical and Chemical Characterization of Plant-Derived Extracellular Vesicles

For single-particle and bulk light-scattering or translocation methods should be applied in combination, as each interrogates different observables and has characteristic biases. NTA yields number-based size histograms and absolute counts (particles·mL^{-1}) by tracking Brownian motion of individual particles [73]; dynamic light scattering (DLS) reports a hydrodynamic intensity-weighted mean diameter and polydispersity index (PDI) at high speed and low sample consumption [74]; TRPS provides calibrated single-particle sizes and counts by measuring transient resistive events through size-defined nanopores [75]. Concordance across these readouts places the preparation within the expected vesicular domain for PDEVs—typically mode/mean diameters ~50–300 nm with DLS PDI $\lesssim$ 0.2–0.3 in well-resolved lots—and anchors working stock levels (after concentration by TFF or equivalent) in the 10^9–10^{11} particles·mL^{-1} range [76]. Because species, tissue of origin, isolation sequence (e.g., differential centrifugation followed by TFF capture, SEC polish with or without density gradient or AF4), buffer composition, temperature, and storage history can shift apparent sizes and broaden distributions, acquisition parameters and sample handling must be reported transparently (dilution factors to avoid NTA track overlap, viscosity and temperature settings for all instruments, DLS attenuator choice, TRPS pore, pressure and calibration bead lots).

The morphological validation is best addressed by transmission electron microscopy (TEM) and, when available, cryo-TEM, which directly visualize bilayer-bounded spherical/ovoid vesicles and disclose non-vesicular contaminants (lipoprotein-like particles, amorphous protein/pectin aggregates) and processing artifacts (cup-shaped deformation in negative stain) [77,78]. Cryo-TEM preserves near-native ultrastructure (membrane thickness on the order of a few nanometers) at the cost of lower contrast and higher methodological overhead. TEM offers speed and throughput but demands cautious interpretation of dehydration effects. Optional atomic force microscopy (AFM) can complement these views with topography and deformation profiles on adsorbed vesicles, though tip–sample interactions and substrate choice influence apparent dimensions [79,80].

The ζ-potential measured by electrophoretic light scattering/laser Doppler velocimetry (ELS/LDV) (or derived from TRPS mobility) provides a compact descriptor of surface charge and colloidal stability highly sensitive to pH and ionic strength; in low-salt, near-neutral buffers, PDEVs commonly exhibit negative ζ-potentials around -10 to -30 mV, with predictable shifts under acidified or high-ionic conditions that must be specified alongside results [81,82]. To position these identity metrics against purity and yield, total protein (e.g., bicinchoninic acid assay: BCA or micro-BCA) should be quantified and paired with particle counts to derive a particle-to-protein ratio—a pragmatic, albeit indirect, index of co-purified solubles (higher ratios generally indicating cleaner preparations) [83,84]. SDS-PAGE banding and LC–MS/MS proteomics can further fingerprint vesicular cargo versus matrix proteins (e.g., wall enzymes, abundant soluble proteins) and are particularly informative when comparing lots purified by different trains (pelleting vs. TFF or SEC) [67,85–87]. Across all measurements, controls and validation are essential instrument calibration with traceable beads (for NTA, TRPS and DLS), buffer blanks to identify scattering backgrounds, replicate dilutions to check for coincidence or multiple scattering, and stability pulls to document drift (size growth, PDI inflation, ζ-potential collapse) under intended storage (cold chain, cryoprotectants, freeze–thaw). Finally, for topical or mucosal deployment, formulation-facing physicochemical checks—pH, osmolality, viscos-

ity/rheology pre- and post-spiking with vesicles—should be recorded to ensure vehicle compatibility (e.g., skin-compatible pH ~4.5–6.5) and to guard against conditions that erode membrane integrity or promote aggregation. Taken together, the integrated constellation of size/distribution/concentration, direct morphology, ζ-potential, and protein context constitutes the minimal, orthogonal physical evidence that a preparation is vesicular, stable, and internally consistent; it also establishes batch comparability and provides the quantitative foundation upon which biochemical marker panels and functional assays can credibly build. Table 6 summarizes the minimal, orthogonal physical measurements for PDEVs, including techniques, advantages and disadvantages, typical values, and reporting guidance.

6.2. Cargo Characterization of Plant-Derived Extracellular Vesicles

After determining the source (raw botanical tissue versus in vitro culture) and finalizing the isolation method, the next critical step is characterizing the cargo. This involves rigorously analyzing the vesicle contents to determine whether their molecular profile supports classification as PDEVs, while excluding the possibility that they are merely generic lipid nanoparticles or matrix-derived colloidal particles.

Unlike mammalian EVs, where broadly conserved markers (CD9, CD63, CD81, TSG101, ALIX) enable relatively uniform workflows, plants lack a universally accepted, cross-species marker set [7]. Homologues may be absent, structurally divergent, tissue-restricted, or variably recovered across species and processes, which precludes a simple "copy-paste" of mammalian panels. Accordingly, identity and quality have to rest on a plant-adapted, multi-analyte framework that integrates lipidomics, proteomics, and RNA profiling with positive and negative marker logic, supported by method transparency (i.e., source, extraction, purification) and orthogonal physical evidence (i.e., size, morphology, ζ-potential).

On the lipid axis, plants exhibit membrane chemistries that are both distinctive and diagnostically useful [88,89]. PDEVs frequently show enrichment of phytosterols (β-sitosterol, campesterol, stigmasterol), which modulate bilayer order and packing, glycosylceramides and glycosyl inositol phosphorylceramides (GIPCs), hallmarks of the plant plasma membrane, and very-long-chain sphingolipids (e.g., long-chain base t18:1 with C24–C26 acyls), which are far less prominent in animal counterparts [90–92]. Depending on tissue and process, galactolipids including monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) derived from plastid membranes may be detected [93], alongside vesicular phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and frequently phosphatidic acid (PA) often reported as relatively abundant in plant EV-like fractions and implicated in trafficking [24]. A shotgun or LC-MS/MS lipidomic fingerprint that documents these classes—in tandem with the absence or depletion of animal-type features (e.g., sphingomyelin)—constitutes a persuasive biochemical lineage signal. Beyond class annotation, molecular-species resolution (double-bond geometry, chain length) adds discriminatory power for batch comparability and for tracing process effects (e.g., oxidative shifts, solvent exposure, storage).

Table 6. Physical characterization of plant-derived extracellular vesicles: advantages and disadvantages.

Parameter	Why	Techniques	Advantages	Disadvantages	Typical Value Target	Reporting Note
Particle size, size distribution and concentration	Core identity of vesicular domain; affects uptake, tissue diffusion, biodistribution, and formulation behavior.	NTA (single-particle size histograms and particles/mL); DLS (hydrodynamic diameter, PDI); TRPS (single-particle size and count via nanopore).	NTA: resolves polydispersity and gives absolute counts. DLS: fast, low volume. TRPS: calibrated, high precision.	NTA: operator-sensitive; fluorescence often needed. DLS: biased by large aggregates; PDI conflates multimodality. TRPS: narrow pore ranges; per-sample optimization.	Mode/mean size: ~50–300 nm. DLS PDI: $\leq$0.2–0.3 (well-resolved). Working stocks: 10^{9}–10^{11} particles/mL (method-dependent).	Pair size with concentration; specify buffer/temperature; Disclose isolation (e.g., clarify with TFF capture followed by SEC polish with or without gradient or AF4).
Morphology and membrane integrity	Confirms vesicular architecture; Excludes non-vesicular colloids and aggregates.	TEM (negative stain); Cryo-TEM (vitrified, near-native).	TEM: high contrast, throughput. Cryo-TEM: preserves native morphology.	TEM: dehydration artifacts (cup shape). Cryo-TEM: lower contrast, higher expertise/ instrument burden.	TEM: spherical/ovoid vesicles with intact bilayer, minimal debris; Cryo-TEM: Absence of crystalline/precipitate artifacts.	Use imaging to corroborate sizing data; Include representative micrographs and prep conditions.
Zeta (ζ) potential and surface electrokinetics	Proxy for surface charge and colloidal stability; Influences aggregation, interfacial interactions, excipient compatibility.	ELS/LDV; TRPS-derived zeta (mobility-based).	ELS/LDV: standardized, fast. TRPS-zeta: single-particle resolution.	ELS/LDV: strongly buffer-dependent (pH/ionic strength), sensitive to contaminants. TRPS: narrow dynamic window, pore calibration needed.	Typically -10 to -30 mV in low-salt, near-neutral buffers.	Always report pH, ionic strength, temperature, interpret shifts with formulation changes.
Protein content and purity indices	Contextualizes yield and co-purified solubles; Supports batch comparability.	BCA/micro-BCA (total protein); Particle-to-protein ratio (NTA/TRPS $\div$ protein); SDS-PAGE; LC-MS/MS proteomics.	BCA: robust, simple. Particle-to-protein: quick purity proxy. SDS-PAGE and LC-MS: fingerprints cargo vs. contaminants.	BCA: totals vesicular and non-vesicular proteins. Particle-to-protein: no universal cutoff. Omics: higher analytical overhead.	Higher particle-to-protein ratios indicate cleaner preps (no single gold value—benchmark vs. your TFF or SEC lots).	Report assay, standards, linearity; Compare ratios across lots; Include representative gel/omics profiles.

Table 6. *Cont.*

Parameter	Why	Techniques	Advantages	Disadvantages	Typical Value Target	Reporting Note
Minimal "EV vs. lipid vesicle" differentiation (physical layer)	Support bona fide EV claim vs. empty/synthetic lipid particles.	Concordant EV size domain with TEM/Cryo-TEM bilayer; Physical/biochemical cargo evidence (endogenous macromolecules); ζ-potential consistent with membranes.	Orthogonal, non-invasive, foundational for identity.	Not sufficient alone: must be integrated with biochemical markers and functional assays.	EV-like size; Intact bilayer; ζ within stable window; Endogenous macromolecules present.	State that physical identity is the minimum layer; follow with marker panels and functional tests.
Formulation-relevant physicochemical parameters	Ensure safety/compatibility and preserve vesicle structure within the vehicle.	pH (meter); Osmolality (osmometer); Viscosity/rheology (rotational); Optionally surface tension.	Straightforward QC; Informs excipient optimization.	Excipients can drift values over time; Stability monitoring required.	Skin-compatible pH ~4.5–6.5; Osmolality/viscosity tuned to vehicle (e.g., gels, serums).	Report pre- and post-spike values (after EV addition), assess under ICH-like conditions.
Stability and storage (physical QA)	Verify that size/ζ/morphology remain within acceptance criteria across shelf life.	Time-course NTA/DLS; ζ-tracking; repeat TEM/Cryo-TEM; stress tests (freeze–thaw, agitation, thermal).	Anticipates failure modes; supports label claims.	Added testing burden; container/extractables can confound.	No universal cutoffs—define lot-specific specs (e.g., Δ size, Δ ζ thresholds).	Prefer cold chain; Consider trehalose for frozen stocks; Avoid repeated freeze–thaw; Specify container/ closure system.
Documentation and transparency	Enable reproducibility, comparability, and regulatory review.	Full source and process disclosure; unified analytics across lots; controlled blending if used.	Builds trust; facilitates audits and tech transfer.	Under-reporting undermines claims.	—	Disclose species/tissue: organic vs. conventional vs. in vitro; Isolation train (e.g., clarify with TFF capture followed by SEC polish with or without gradient or AF4); Buffer/pH/ionic strength; Storage; Define acceptance criteria for size/PDI, ζ, particle-to-protein, morphology.

DLS: dynamic light scattering; ELS/LDV: electrophoretic light scattering/laser Doppler velocimetry; LC: liquid chromatography; MS: mass spectrometry; NTA: nanoparticle tracking analysis; PDI: polydispersity index; QC: quality control; SEC: size-exclusion chromatography; TEM: transmission electron microscopy; TFF: tangential flow filtration; TRPS: tunable resistive pulse sensing.

Proteins provide complementary evidence but must be interpreted with plant biology in mind. While no single protein is universal, a pragmatic positive panel can be assembled. In selected species and experimental systems, tetraspanin-like proteins (e.g., TET8 in Arabidopsis) and secretory/fusion machinery (e.g., PEN1/SYP121, EXO70 isoforms, components of the exocyst) have been linked to EV release and are frequently enriched [94,95]. More broadly, annexins (membrane-binding and Ca^{2+}-responsive proteins), aquaporins (e.g., PIP1/PIP2, integral to water transport and reported on plant vesicular membranes), and heat-shock proteins (HSP70/HSP90, chaperones upregulated under stress and repeatedly detected in plant EV preparations) recur across species [96–98]. These putative positives should be counterbalanced by negatives markers of compartments that ought to be absent or strongly depleted in clean vesicle isolates: RuBisCO (RbcL/RbcS) as a chloroplast sentinel, calnexin/BiP for endoplasmic reticulum, histone H3 for nucleus, VDAC and cytochrome c for mitochondria, catalase for peroxisomes, actin/tubulin for cytoskeleton, and, critically in plant matrices, oleosins to rule out oil bodies (oleosomes) that can co-isolate with lipidic nanoparticles [85,99,100]. Detection can be staged from SDS-PAGE/Western blot and ELISA to label-free or targeted LC-MS/MS proteomics, which quantifies enrichment-depletion patterns and enables construction of species-resolved protein atlases [101]. Where feasible, protease-protection assays (with or without detergent) help distinguish luminal from surface-exposed proteins, strengthening the vesicular interpretation.

RNA cargo is central whenever cross-kingdom signaling or gene-regulatory claims are made. Plant vesicles commonly harbor small RNAs, including conserved miRNA families (miR156, miR159, miR164, miR166, miR168), siRNAs, and tRNA-derived fragments; fragmented mRNAs are often detectable. Because adsorption of extracellular RNA to lipid surfaces can confound assays, RNase-protection experiments (with or without detergents) and density-resolved sampling are recommended to verify encapsulation [102–104]. Next Generation Sequencing (NGS)-based small-RNA profiling with spike-in standards, library QC, and bioinformatic depletion of host-matrix sequences can define reproducible signatures, while RT-qPCR or digital PCR is appropriate for lot-release quantification of sentinel miRNAs. Functional plausibility increases when RNA findings co-vary with lipid and protein contexts expected to support vesicle biogenesis and stability. A comprehensive cargo view should also encompass low-molecular-weight bioactives that are frequently invoked in cosmetic narratives. PDEVs from fruits, leaves, and seeds often co-carry polyphenols (e.g., quercetin/kaempferol glycosides, catechins, chlorogenic, ferulic and caffeic acids), terpenoids (e.g., ursolic/oleanolic acids), carotenoids (β-carotene, lutein), and tocopherols [8,105–107]. These can be membrane-associated or lumenal, and their retention is sensitive to oxidation, pH, and excipients. LC-MS/GC-MS metabolomics and targeted assays (e.g., for total phenolics with MS confirmation) document presence and stability across TFF and SEC purification, storage, and formulation, connecting molecular content to anti-oxidant, anti-inflammatory and barrier-support claims. For microbiome-facing applications, sugar conjugates and lipid mediators may be particularly informative.

Cargo characterization must be contextualized by source and process. Raw-tissue PDEVs often reflect in vivo stress biology, showing stress-responsive proteins, PA-rich lipid profiles, and antioxidant metabolites; however, they are more susceptible to co-extraction of soluble proteins and wall components (pectins, extensins) that can mask or dilute vesicular signatures. In vitro-derived PDEVs can be compositionally narrower but more reproducible; elicitation regimes (e.g., jasmonate/salicylate pulses, controlled UVB/blue light, nutrient or oxygen shifts) can partially restore stress-enriched cargo, ideally without compromising batch control. Across both sources, negative-marker discipline and orthogonal analytics are

the main safeguards against false positives driven by lipoprotein-like particles, oil bodies, or protein–polyphenol aggregates.

Methodologically, the minimum credible cargo package combines (i) lipidomics that captures phytosterols, GIPCs/glycosylceramides, and vesicular phospho-/sphingolipid species; (ii) proteomics that documents enrichment of EV-compatible proteins (e.g., tetraspanin-like where applicable, PIP1/PIP2 aquaporins, annexins, HSP70/90) alongside depletion of organelle and cytoskeletal markers (RuBisCO, calnexin/BiP, VDAC/cytochrome c, histone H3, actin/tubulin, oleosin); and (iii) RNA profiling (small-RNA NGS with RNase-protection controls and targeted qPCR for sentinel families). These layers should be acquired on density-resolved or SEC-enriched fractions (not crude homogenates) and reported with full method metadata (buffers, detergents, enzyme treatments, library prep kits) to enable inter-study comparability. Where claims hinge on specific bioactives (e.g., antioxidant or soothing effects), quantitative retention of the relevant molecules through processing and shelf life should be demonstrated, with stress-testing to capture degradation pathways (e.g., polyphenol oxidation, lipid peroxidation). Finally, data integration matters: a robust dossier defines acceptance criteria (e.g., presence and absence rules for negatives; quantitative thresholds or enrichment factors for positives; stability windows for key metabolites), codifies lot-release tests, and ties molecular readouts to functional assays in skin-relevant systems (anti-ROS/ROM, cytokine modulation, barrier metrics). Because plants are inherently diverse, absolute universals are unlikely; instead, the goal is a species- and process-aware fingerprint that is reproducible within a given platform and transparent enough for regulatory review and technology transfer. In this way, cargo characterization moves beyond a checklist to become the central evidentiary bridge between vesicle identity, mechanistic plausibility, and product claims for PDEV-based formulations. In Table 7, positive and negative markers for PDEVs were suggested along with assays, rationale, and acceptance logic.

In Table 8, a species-aware positive set (lipid and protein and RNA) is built and paired with mandatory negative controls (RuBisCO, calnexin/BiP, VDAC/cytochrome-c, histone H3, oleosin). Acquire data on SEC-enriched or density-resolved fractions (not crude), report buffers and conditions, and set lot-release criteria (e.g., presence of phytosterols and GIPCs; enrichment of PIP1/PIP2 or annexins; absence of RuBisCO/oleosin; detection of sentinel miRNAs with RNase protection). This yields a reproducible, plant-aware fingerprint suitable for QC, comparability, and regulatory dossiers. Table 8 includes suggested positive and negative markers for PDEVs, assays, rationale, species/tissue examples, and acceptance thresholds (lot release) as practical guidance. Thresholds are illustrative and should be locked per platform using reference lots (median $\pm$ 2 SD).

Table 7. Cargo markers of plant-derived extracellular vesicles: suggested panels for characterization.

Class	Markers to Target	Assay/Readout	Rationale	Notes/Acceptance Logic
Lipids: membrane hallmarks	Phytosterols: β-sitosterol, campesterol, stigmasterol; Sphingolipids: glycosylceramides, GIPCs; Phospholipids: PC, PE, PA, PI; Galactolipids: MGDG, DGDG (context-dependent)	LC-MS/MS (shotgun or targeted) for class/species quantification; ratios (PA/PC/PE); presence/absence of sphingomyelin.	Plant-specific membrane chemistry supports vesicular lineage; Phytosterols/GIPCs and PA-rich profiles align with plant EV biology.	Expect phytosterol/GIPC signatures and low or absent sphingomyelin; track oxidation markers and chain-length distributions for batch comparability.
Enriched proteins (positive markers)	Tetraspanin-like (e.g., TET8/TET13, species-dependent); Secretory/traffic (PEN1/SYP121, EXO70 isoforms); Aquaporins (PIP1/PIP2); Annexins; Heat-shock proteins (HSP70/HSP90).	SDS-PAGE/Western blot; ELISA; label-free or targeted LC-MS/MS; protease protection (with or without detergent) to map luminal vs. surface.	Recurrently detected in plant EV studies; Indicate membrane origin, secretion machinery, and stress adaptability.	Use species-aware panels; Require enrichment vs. source matrix and co-depletion of negatives.
Depleted proteins (negative controls)	Chloroplast: RuBisCO (RbcL/RbcS); ER: Calnexin, BiP; Mitochondria: VDAC, Cytochrome c; Nucleus: Histone H3; Cytoskeleton: Actin, Tubulin; Oil bodies: Oleosin; Peroxisome: Catalase.	Western blot; ELISA; LC-MS/MS; set depletion thresholds (absent or less compared to source).	Rules out organelle carryover and non-vesicular contaminants (e.g., oil bodies, cytoskeletal debris).	Clean PDEVs show absence or strong depletion; Persistent signals trigger process optimization (clarify by TFF, SEC, or gradient).
RNA cargo: identity and plausibility	Small RNAs: miR156, miR159, miR160, miR166, miR167, miR168, miR172; siRNAs; tRNA-derived fragments; mRNA fragments (contextual).	Small-RNA NGS with spike-ins; RNase protection (with or without detergent) to prove encapsulation; RT-qPCR or dPCR for sentinel miRNAs.	RNA cargo is a defining EV feature and underpins cross-kingdom signaling narratives.	Require encapsulation evidence; Report library QC and mapping; Use endogenous references or spike-ins for lot release.

Table 7. *Cont.*

Class	Markers to Target	Assay/Readout	Rationale	Notes/Acceptance Logic
Small-molecule bioactives: cosmetic relevance	Polyphenols: quercetin and kaempferol glycosides, catechins, chlorogenic, ferulic, and caffeic acids; Isoprenoids: carotenoids (β-carotene, lutein), tocopherols; Triterpenoids: ursolic and oleanolic acids.	LC-MS/GC-MS metabolomics (targeted/untargeted); Stability tracking across processing and storage.	Support claimed activities (antioxidant, soothing, barrier support); Often co-packaged with PDEVs.	Demonstrate retention through isolation and shelf-life; Link dose to in vitro potency (anti-ROS/ROM, cytokines, TEER).
Glycan features: supportive	GIPC headgroups; lectin-reactive glycan motifs (e.g., ConA, WGA, PNA species-dependent).	LC-MS for glycosphingolipids; Western blot, ELISA or bead capture for lectin.	Plants exhibit distinctive glycosylation; Supports plant membrane identity.	Lectin data are supportive, not definitive; Consider cross-reactivity and gentle elution.
Process/quality indices (contextual)	Particle-to-protein ratio; Lipid-per-particle; Enrichment factors for positives; Depletion factors for negatives.	Combine NTA or TRPS with BCA assay; Lipid quantification with particle counts; Define acceptance criteria in SOPs.	Quantifies purity and comparability beyond single markers.	No universal cutoffs: benchmark against your TFF and SEC (with or without gradient or AF4) platform and specific in lot-release criteria.

DGDG: digalactosyldiacylglycerol; dPCR: digital Polymerase Chain Reaction; GC: gas chromatography; GIPCs: glycosyl inositol phosphorylceramides; LC: liquid chromatography; MGDG: monogalactosyldiacylglycerol; MS: mass spectrometry; NTA: nanoparticle tracking analysis; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; QC: quality control; ROS: reactive oxygen species; ROM: reactive oxygen metabolites; RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction; SOPs: standard operating procedures; TEER: transepithelial electrical resistance; TRPS: tunable resistive pulse sensing.

Table 8. Cargo markers for plant-derived extracellular vesicles: panels with species/tissue examples and lot-release thresholds.

Class	Markers to Target	Assay/Readout	Rationale	Species/Tissues Examples	Acceptance Thresholds (Lot Release)	Notes/Acceptance Logic
Lipids: membrane hallmarks	Phytosterols (β-sitosterol, campesterol, stigmasterol); Plant sphingolipids (glycosylceramides, GIPCs); Phospholipids (PC, PE, PI, PA); Galactolipids (MGDG, DGDG, context-dependent).	LC-MS/MS (shotgun/targeted) with class/species quant; PA/PC/PE ratios; presence/absence of sphingomyelin.	Plant-specific membrane chemistry; Phytosterols/GIPCs and PA-rich profiles support plant vesicular lineage and trafficking roles.	Ginger rhizome vesicles: PA-enriched profiles; Grapefruit/grape vesicles: phytosterols and glycosylceramides recurrent; Spinach/leaf vesicles: GIPCs abundant; Tomato/fruit vesicles: galactolipids detectable depending on process.	Phytosterols and GIPCs present ($\geq$ref lot median $\pm$ 2 SD); sphingomyelin $\leq$ LOD; PA/PC ratio within platform window (e.g., ref median $\pm$20%); lipid hydroperoxides $\leq$ 5% of total unsaturated lipids.	Track oxidation (hydroperoxides) and chain-length distributions; compare to SEC or TFF-polished reference lots.
Enriched proteins (positive markers)	Tetraspanin-like (TET8/TET13, species-dependent); Secretory/traffic (PEN1/SYP121, EXO70 isoforms); Aquaporins (PIP1/PIP2); Annexins; HSP70/HSP90.	SDS-PAGE/Western blot; ELISA; LC-MS/MS (label-free/targeted); Protease protection (with or without detergent).	Recurrently detected in plant EV studies; Indicate membrane origin, secretion machinery, and stress adaptability.	Arabidopsis apoplast: TET8-like and PEN1/SYP121; Citrus peel vesicles: annexins and HSP70/90; Grape skin vesicles: PIP2 aquaporins by proteomics.	Positives enriched $\geq$3–5$\times$ vs. source matrix (or $\geq$ref median $\pm$ 2 SD); Protease protection indicates partial luminal localization for a subset.	Use species-aware panels; Evidence of enrichment should co-occur with depletion of organelle negatives.
Depleted proteins (negative controls)	Chloroplast: RuBisCO (RbcL/RbcS); ER: Calnexin, BiP; Mitochondria: VDAC, Cytochrome c; Nucleus: Histone H3; Cytoskeleton: Actin, Tubulin; Oil bodies: Oleosin; Peroxisome: Catalase.	Western blot; ELISA; LC-MS/MS; define depletion thresholds (absent or less compared to source).	Rules out organelle carryover and non-vesicular contaminants (oil bodies, cytoskeletal debris).	Leaf-derived: RuBisCO is common. It must be strongly depleted; Seed/fruit oils: oleosin must be absent to exclude oleosomes; Roots: VDAC/cytochrome-c flags mitochondrial leakage.	Negatives: absent or $\leq$10% of source-normalized signal (or $\leq$ref lot median $\pm$ 2 SD); oleosin not detected by Western blot or MS.	Persistent negatives trigger process optimization (clarify, TFF, SEC, or gradient); Document LOD/LOQ for each assay.
RNA cargo: identity and plausibility	Small RNAs: miR156, miR159, miR160, miR166, miR167, miR168, miR172; siRNAs; tRNA-derived fragments; mRNA fragments (contextual).	Small-RNA NGS with spike-ins; RNase protection (with or without detergent) to prove encapsulation; RT-qPCR or dPCR for sentinel miRNAs.	RNA cargo is a defining EV feature and underpins cross-kingdom signaling narratives.	Fruit vesicles (grape, grapefruit, orange): miR156/159/168 families; Ginger vesicles: abundant small RNAs with RNase protection; Rice/wheat apoplast: siRNAs in stress contexts.	RNase protection index $\geq$ 70% retention (with or without detergent control); sentinel miRNAs detected with Ct $\leq$ 30 (qPCR) or copies above LLOQ (dPCR); library QC within platform metrics (e.g., % adapter-dimers $\leq$ spec).	Report encapsulation controls; Map reads with host-matrix depletion; Set lot-release Cp/Ct windows around reference lots.

Table 8. *Cont.*

Class	Markers to Target	Assay/Readout	Rationale	Species/Tissues Examples	Acceptance Thresholds (Lot Release)	Notes/Acceptance Logic
Small-molecule bioactives: cosmetic relevance	Polyphenols (quercetin or kaempferol glycosides; catechins; chlorogenic, ferulic, or caffeic acids); Isoprenoids (carotenoids, as β-carotene and lutein; tocopherols); Triterpenoids (ursolic or oleanolic acids).	LC-MS/GC-MS metabolomics (targeted/untargeted); Stability tracking across processing and storage.	Support claimed activities (antioxidant, soothing, barrier support); Often co-packaged with PDEVs.	Apple peel: chlorogenic acid, phloridzin, quercetin glycosides; Grapes: resveratrol and catechins; Citrus: hesperidin/naringin; Tomato: lycopene; Green tea: EGCG-family catechins.	Marker retention $\geq$ 70% of post-SEC baseline over intended shelf-life; Oxidation products $\leq$ platform threshold (e.g., $\leq$10% of parent area).	Tie marker levels to in vitro potency (anti-ROS/ROM, cytokines, TEER) and monitor under ICH-like stability.
Glycan features: supportive	GIPC headgroups; lectin-reactive motifs (ConA, WGA, PNA—species-dependent).	LC-MS for glycosphingolipids; Western blot, ELISA or bead capture for lectin.	Plants exhibit distinctive glycosylation; Supports plant membrane identity.	Arabidopsis: GIPC-rich PM mirrored in EVs; Wheat/rice EVs: WGA-reactive GlcNAc motifs; Citrus peel vesicles: lectin profiles consistent with glycosphingolipid enrichment.	Lectin enrichment factor $\geq$ 2$\times$ vs. buffer and non-EV fractions; Cross-reactivity assessed with negative controls.	Lectin data are supportive, not definitive; Ensure gentle elution to preserve membranes.
Process/quality indices (contextual)	Particle-to-protein ratio; lipid-per-particle; enrichment/depletion factors for positives/negatives.	Combine NTA or TRPS with BCA assay; lipid quantification with particle counts; define acceptance criteria in SOPs.	Quantifies purity and comparability beyond single markers.	Grape/citrus lots (TFF and SEC) show higher particle-to-protein than pelleting-only; Ginger vesicles maintain lipid-per-particle with controlled oxidation.	Particle-to-protein $\geq$ platform cutoff (e.g., $\geq$ref median $\pm$ 2 SD); Lipid-per-particle within $\pm$ 20% of reference; negatives at/below LOD.	No universal cutoffs: benchmark vs. your platform; Lock lot-release specs and document calculation methods.

DGDG: digalactosyldiacylglycerol; dPCR: digital Polymerase Chain Reaction; GC: gas chromatography; GIPCs: glycosyl inositol phosphorylceramides; LC: liquid chromatography; LLOQ: lower limit of quantification; LOD: limit of detection; LOQ: limit of quantification; MGDG: monogalactosyldiacylglycerol; MS: mass spectrome-try; NTA: nanoparticle tracking analysis; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethano-lamine; PI: phosphatidylinositol; QC: quality control; ROS: reactive oxygen species; ROM: reactive oxygen metabolites; RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction; SOPs: standard operating procedures; TEER: transepithelial electrical resistance; TRPS: tunable resistive pulse sensing.

7. Functional Testing and Biological Relevance of PDEVs in Cosmetics

Functional validation is non-negotiable for any product containing PDEVs. Physicochemical and molecular fingerprints establish identity and purity, but only biological activity under relevant conditions can substantiate intended use and support claims in cosmetics. A defensible workflow integrates cellular uptake, proximal activation readouts (including intracellular Ca^{2+} dynamics), and mechanistic efficacy assays aligned with the end-use (e.g., soothing, anti-inflammatory, antioxidant, photoprotection, barrier support, or regeneration) executed across diverse skin cell types, donors, and models.

Functional testing (Figure 2) is not a mere supplementary step but a core pillar of scientific validation for non-animal EVs in cosmetic and regenerative applications. Only through systematic, biologically relevant, and mechanistically informed testing can the promise of these natural nanocarriers be responsibly translated into effective, safe, and scientifically credible products.

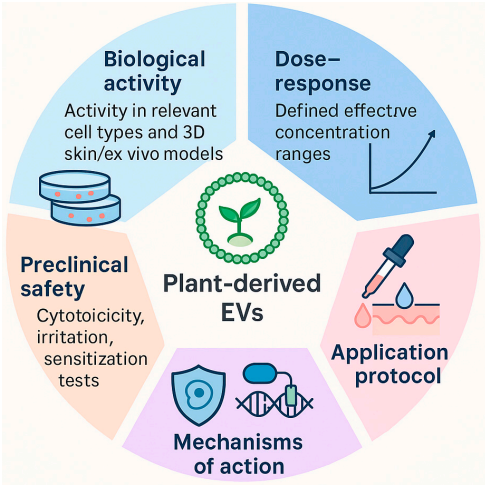

Figure 2. Key validation steps for plant-derived extracellular vesicles (PDEVs) in cosmetics: assessment of biological activity, definition of effective doses, delivery protocol, safety evaluation, and mechanistic evidence.

7.1. Cellular Uptake and Competency to Deliver Cargo

PDEV internalization should be demonstrated in human skin-relevant cells, including primary dermal fibroblasts, epidermal keratinocytes, melanocytes, sebocytes, microvascular endothelial cells, and immune sentinels (e.g., monocyte-derived macrophages, Langerhans-like dendritic cells) [108–111]. PDEVs should be labelled by membrane dyes (e.g., PKH26/PKH67, DiD/DiO) or more specific strategies (e.g., clickable lipids, protein tags, or fluorescent RNA cargo) to quantify uptake by confocal microscopy (by z-stacks, colocalization with endo-lysosomal markers), high-content imaging, or flow cytometry. Stringent controls should be included to exclude dye micelles and adsorption: dye-only sham controls, trypan blue quenching (to suppress extracellular fluorescence), 4 °C uptake (energy-dependence), and pathway inhibition (e.g., dynasore, chlorpromazine, filipin) to map endocytosis routes. Report positive cell percentage and median fluorescence per cell (or voxelized intensity), with dose–response and time courses.

7.2. Intracellular Calcium Dynamics (Proximal Activation and Signaling Competence)

Intracellular Ca^{2+} flux is a sensitive, early readout of EV-cell engagement that couples to migration, differentiation, secretion, and barrier homeostasis [40,112–116]. Measure Ca^{2+} should be performed in fibroblasts, keratinocytes, endothelial cells, and sensory neuron–keratinocyte co-cultures. Fluo-4 AM ($\Delta F/F_0$ peak, area under the curve, rise/decay kinetics), Fura-2 ratiometry (340/380 nm), or genetically encoded sensors (GCaMP6s) in

engineered models should be used. Specificity should be established with extracellular Ca^{2+} chelation (EGTA), intracellular buffering (BAPTA-AM), store-depletion (thapsigargin) to probe SOCE (Orai1/STIM1), and pathway blockers (e.g., suramin for purinergic receptors, U73122 for PLC, La^{3+}/Gd^{3+} for mechanosensitive channels). ATP or ionomycin should be included as positive controls. Peak amplitude ($\Delta F/F_0$), AUC, EC_{50} from concentration–response curves, and fraction of responding cells should be reported. As acceptance guidance, PDEVs at intended use levels should elicit consistent, non-cytotoxic Ca^{2+} transients (e.g., $\geq$30% median $\Delta F/F_0$ over vehicle in $\geq$2 cell types) without desensitizing responses.

7.3. Antioxidant and Photoprotective Activity

Target cells should be challenged with H_2O_2, UVA/UVB, visible/blue light, or PM-like oxidative mixtures [30,34,117–119]. Then, cellular ROS (by DCFDA), mitochondrial superoxide (by MitoSOX Red), lipid peroxidation (by BODIPY-C11), and DNA damage (by γH2AX/CPD immunostaining) should be quantified. In keratinocytes and fibroblasts from young vs. aged donors, or across Fitzpatrick phototypes I–VI, PDEVs with antioxidant cargo should reduce ROS by $\geq$20–30% vs. vehicle, preserve mitochondrial potential (TMRE), and limit γH2AX foci after UVB.

7.4. Anti-Inflammatory and Soothing Effects

An in vitro inflammation model should be established using TNF-α, IL-1β, LPS, or SLS (for irritancy) [36,41,120–122]. Cytokine levels, including IL-6, IL-8, TNF-α, CXCL1, as well as anti-inflammatory markers IL-10 and TGF-βshould be quantified via ELISA or multiplex bead assays. This should be complemented by analysis of the NF-κB p65 nuclear translocation (immunofluorescence) or luciferase reporter assays, along with assessment of the COX-2/PGE_2 signaling pathway. Experiments should be conducted in keratinocytes (including donor lines mimicking atopic or sensitive skin), macrophages, and Langerhans-like dendritic cells.

Functionally relevant PDEVs should down-tune pro-inflammatory outputs (e.g., $\geq$25% reduction in IL-6/IL-8) without immunosuppression, evidenced by no loss of basal cell viability or barrier protein expression.

7.5. Barrier Integrity and Homeostasis

In keratinocyte monolayers or reconstructed epidermis, transepithelial electrical resistance (TEER) and paracellular permeability using FITC-dextran should be evaluated, along with the expression of tight junction (TJ) proteins (ZO-1, claudin-1, occludin) and terminal differentiation markers (filaggrin, loricrin, involucrin) via immunofluorescence, qPCR, or Western blot [123–126].

In keratinocytes from dry-skin or aged donors, PDEVs that improve barrier function should induce a $\geq$10–20% increase in TEER, decrease permeability, and upregulate filaggrin and claudin-1 following barrier stress (e.g., SDS or calcium-switch challenge).

7.6. Regeneration and Remodeling of Extracellular-Matrix

In dermal fibroblasts derived from adult versus aged donors, as well as from diabetic or keloid-prone skin (representing models of impaired or dysregulated wound healing), scratch wound closure, transwell migration, and extracellular matrix (ECM) production should be evaluated. Key readouts should include procollagen-I C-peptide (via ELISA), hydroxyproline, tropoelastin, and fibrillin-1, alongside MMP-1, MMP-3, and TIMP-1 levels to monitor matrix turnover and exclude a catabolic shift. Additionally, activation of the TGF-β/SMAD2/3 pathway and modulation of MAPK signaling should be assessed [8,127,128].

PDEVs with pro-regenerative activity are expected to enhance COL1A1 and ELN expression by approximately 1.5–2-fold, without inducing a disproportionate increase in MMP-1.

7.7. Angiogenesis and Vascular Support (Wound-Healing Relevance)

In microvascular endothelial cells derived from dermal and diabetic donors, angiogenic potential should be assessed through Matrigel tube formation assays, cell migration, and the expression of key angiogenic markers, including VEGF-A, eNOS, and angiopoietins [128–131].

PDEVs with pro-regenerative effects should promote increased tube length and branching compared to vehicle controls, without inducing pro-inflammatory activation.

7.8. Pigmentation and Photo-Evenness (Optional, Claim-Dependent)

In melanocyte monocultures and melanocyte-keratinocyte co-cultures, melanin content, tyrosinase activity, and MITF/TYR/TRP1 expression should be evaluated. For post-inflammatory hyperpigmentation models, modulation of PAR-2crosstalk and melanocyte dendricity should be examined [132–135].

7.9. Microbiome-Aware Endpoints (Skin Ecology)

In keratinocyte–commensal co-culture models with Staphylococcus epidermidis and Cutibacterium acnes, the expression of antimicrobial peptides (e.g., β-defensin-2, LL-37), cytokine secretion, and biofilm dynamics should be evaluated. In ex vivo human skin, microbial community composition via 16S rRNA sequencing, where applicable and depending on the intended claim, should be monitored [136].

PDEVs with skin-compatible activity should preserve microbial homeostasis, exhibiting no signs of dysbiosis while enhancing epithelial innate immune defenses.

7.10. Advanced Models for Translational Relevance

Reconstructed human epidermis (RHE) and full-thickness 3D skin equivalents comprising stratified keratinocytes over a fibroblast-populated dermal matrix should be used to evaluate topical PDEV penetration, retention, and bioactivity. Visualization techniques such as fluorescent labeling, confocal imaging, and Raman microscopy can be employed to track distribution. In ex vivo human skin explants (including facial, scalp, and palmoplantar regions; from young and aged donors across Fitzpatrick phototypes I–VI), assess endpoints such as epidermal thickness, cell proliferation (Ki-67), collagen and elastin content (by Masson's trichrome or Picrosirius red staining), cytokine expression, and wound closure kinetics. Organ-on-chip skin platforms incorporating perfusion and immune components are recommended for mechanistic or claim-critical studies.

Some illustrative examples across diverse skin contexts include:

Aged facial skin (phototype II–III, ex vivo): Topical application of PDEVs reduces UVB-induced ROS and IL-6 in the epidermis, increases filaggrin and claudin-1 expression, and moderately increases the thickness of viable epidermal layers.

Atopic-like keratinocytes (primary donor-derived): PDEVs attenuate TNF-α/IL-4-induced NF-κB activation, enhance TEER, and restore filaggrin expression.

Diabetic dermal fibroblasts: PDEVs promote scratch wound closure, upregulate COL1A1, and elicit pro-regenerative calcium transients (including thapsigargin-sensitive SOCE), without excessive MMP-1 induction.

Scalp sebocytes and keratinocytes: PDEVs mitigate blue-light-induced ROS, normalize IL-8 secretion, and maintain lipid homeostasis; cellular uptake is confirmed via confocal microscopy.

Palmoplantar keratinocytes (barrier-thick skin): PDEVs enhance TEER and reduce FITC-dextran permeability following SLS challenge, accompanied by robust calcium signaling indicative of barrier pathway engagement.

Fitzpatrick phototypes IV–VI in melanocyte co-cultures: PDEVs modulate tyrosinase activity and melanocyte dendricity under UV-mimetic stress, without triggering pro-inflammatory responses.

Table 9 summarizes the experimental controls and comparators, study design requirements, functional acceptance threshold, and statistics/reporting guidance for PDEVs.

Table 9. Functional controls, comparators, and acceptance threshold in plant-derived extracellular vesicles for functional testing and biological relevance in cosmetics.

Module	What to Include	Acceptance Thresholds	Notes/Design Details
Comparators	Formulation vehicle; Size- and lipid-matched liposomes; Heat-inactivated PDEVs; RNase/Protease with or without detergent to probe cargo dependence; EV-depleted supernatant to control soluble factors.	—	Verify liposomes are size-matched (DLS/NTA); Confirm inactivation (loss of activity, preserved size/morphology); Include dye-only and 4 °C controls in uptake studies.
Design	Dose–response ($\geq$4 doses spanning intended use); Time courses; $\geq$3 donors per condition (include sensitive/atopic, aged, diabetic fibroblasts, multiple phototypes); Blinding where feasible.	Minimum set met prior to efficacy claims.	Randomize plate positions; Pre-register endpoints; Define stopping/failure criteria a priori; Replicate across days/batches.
Functional gates (platform-specific)	Uptake; Calcium dynamics; Antioxidant; Anti-inflammatory; Barrier; Regeneration.	See sub-rows below.	Use same lot across assays where possible; Align doses to topical-equivalent exposure.
Uptake	Internalization in $\geq$2 skin-relevant cell types (e.g., keratinocytes, fibroblasts, immune cells) by confocal or flow cytometry.	$\geq$80% positive cells at intended dose; Energy-dependence and dye-control sanity checks passed.	Include trypan blue quench; 4 °C condition; Endocytosis inhibitors to map pathways.
Calcium dynamics	Fluo-4/Fura-2/GCaMP readouts in fibroblasts, keratinocytes, endothelial cells (with or without sensory neuron co-cultures).	Reproducible transients: median $\Delta F/F_0 \geq 30\%$ over vehicle; AUC increase; Attenuated by expected blockers; No cytotoxicity.	Probe SOCE (EGTA/BAPTA, thapsigargin); Test purinergic/PLC blockers (e.g., suramin, U73122).
Antioxidant	Cellular ROS (DCFDA), mitochondrial ROS (MitoSOX), lipid peroxidation (BODIPY-C11) under oxidative challenge (H_2O_2, UV).	$\geq$20–30% ROS reduction vs. vehicle in keratinocytes and fibroblasts.	Include $\Delta\Psi m$ (TMRE) and DNA damage (γH2AX/CPDs) as supportive endpoints.

Table 9. *Cont.*

Module	What to Include	Acceptance Thresholds	Notes/Design Details
Anti-inflammatory	Cytokines (IL-6, IL-8, TNF-α) and anti-inflammatory mediators (IL-10, TGF-β) by ELISA or multiplex; NF-κB reporter by IF.	≥25% decrease in IL-6/IL-8 (or equivalent) in inflamed keratinocytes/macrophages; No viability penalty.	Model with TNF-α/IL-1β/LPS/SLS; Confirm specificity and lack of broad immunosuppression.
Barrier	TEER and FITC-dextran permeability; TJ proteins (ZO-1, claudin-1, occludin); Differentiation markers (filaggrin, loricrin, involucrin).	TEER increase ≥10–20% with reduced permeability and improved TJ staining.	Use calcium-switch or SLS stress; Test in RHE/3D skin equivalents for translational relevance.
Regeneration	Scratch/wound closure; Migration (trans-well); ECM markers (COL1A1, ELN, fibrillin-1), MMP-1/3, TIMP-1.	COL1A1/ELN up-regulation ≥1.5–2.0× without excessive MMP-1 (or with compensatory TIMP-1).	Assess TGF-β/SMAD and MAPK; Include aged/diabetic/keloid-prone donor cells.

DLS: dynamic light scattering; ECM: extracellular matrix; NTA: nanoparticle tracking analysis; RHE: reconstructed human epidermis; ROS: reactive oxygen species; TEER: transepithelial electrical resistance; TJ: tight junction.

Table 10 summarizes examples of biological functions of PDEVs in skin-relevant contexts, the suggested assays or models for their evaluation, and, where applicable, measurements of intracellular calcium levels.

Table 10. Plant-derived extracellular vesicles isolated from different plant sources: examples of functional evaluation and recommended assay.

Plant Source (Species/Tissue)	Reported Biological Functions (Examples)	Evaluation Assays/Models (Recommended Assay)
Grapefruit (*Citrus paradisi*): fruit vesicles	Antioxidant; Anti-inflammatory; Macrophage modulation; Uptake in skin cells.	Confocal/flow uptake (PKH/DiD); ROS (DCFDA) under UV/chemical stress; cytokines (IL-6/TNF-α ELISA) in keratinocytes/macrophages; Ca^{2+} imaging (Fluo-4) in fibroblasts
Grape (*Vitis vinifera*): skin/juice vesicles	Antioxidant/photoprotection (polyphenols); Barrier reinforcement; Soothing.	ROS (DCFDA, MitoSOX) after UVB/UVA; TEER and FITC-dextran in keratinocyte monolayers/RHE; TJ IF (ZO-1, claudin-1); Ex vivo skin explants histology.
Ginger (*Zingiber officinale*): rhizome vesicles	Anti-inflammatory; Pro-regenerative (migration/closure); Antioxidant; Immunomodulatory.	Cytokines (IL-6/IL-1β/TNF-α) ELISA; NF-κB reporter; Fibroblast scratch and trans-well; Ca^{2+} transients (Fluo-4/Fura-2; SOCE probes); Uptake by confocal.
Green tea (*Camellia sinensis*): leaf vesicles	Antioxidant (catechins); Blue-light protection; Anti-inflammatory; Barrier support.	ROS assays post blue light/UVA; IL-8/COX-2 readouts; TEER and TJ markers; Mitochondrial potential (TMRE) and γH2AX DNA damage limits.

Table 10. *Cont.*

Plant Source (Species/Tissue)	Reported Biological Functions (Examples)	Evaluation Assays/Models (Recommended Assay)
Apple (*Malus domestica*): peel/pulp vesicles	Antioxidant; Barrier homeostasis; Soothing; Regenerative cues in fibroblasts.	ROS (DCFDA) with or without UV; TEER and permeability; Ca^{2+} signalling in keratinocytes/fibroblasts; collagen-related transcripts (COL1A1) by RT-qPCR.
Tomato (*Solanum lycopersicum*): fruit vesicles	Photoprotection; Antioxidant; Anti-inflammatory under UV challenge.	UVB models with ROS (DCFDA), γH2AX/CPDs immunostaining; IL-6/IL-8 ELISA; Uptake by confocal.
Broccoli (*Brassica oleracea* var. *italica*): leaf/floret vesicles	Antioxidant/cytoprotective; Anti-inflammatory; Potential Nrf2 pathway engagement.	ROS and lipid peroxidation (BODIPY-C11); Nrf2/ARE reporter assays; Cytokine profiling; Ca^{2+} dynamics in keratinocytes under irritant stress.
Aloe vera: leaf gel vesicles	Regenerative (wound closure); Anti-inflammatory; Barrier hydration/support.	Scratch closure kinetics; Procollagen-I ELISA/hydroxyproline; TEER and TJ proteins; cytokines (TNF-α/IL-1β challenge).
Rice (*Oryza sativa*): apoplastic/callus vesicles	Anti-inflammatory; Microbiome-compatible signaling; Barrier support.	Cytokines in keratinocytes/macrophages; Co-culture with skin commensals (biofilm/AMPs); TEER in RHE; Ca^{2+} imaging for epithelial signalling.
Wheat (*Triticum aestivum*): leaf/apoplast vesicles	Barrier maintenance; Soothing/anti-irritant; Antioxidant under chemical stress.	TEER and permeability after SLS/calcium-switch; ROS (DCFDA); IL-6/IL-8 modulation; Uptake and Ca^{2+} readouts in keratinocytes.
Strawberry (*Fragaria* × *ananassa*): fruit vesicles	Antioxidant (anthocyanins); Soothing; Photoprotection; Pigmentation-evening support.	ROS (DCFDA) with or without UV; γH2AX/CPDs; Melanin/tyrosinase assays in melanocyte–keratinocyte co-cultures; Ca^{2+} imaging in keratinocytes; TEER.
Rosemary (*Rosmarinus officinalis*): leaf vesicles	Anti-inflammatory; Antioxidant (carnosic acid/carnosol context); Barrier reinforcement.	IL-6/COX-2/NF-κB assays; ROS (DCFDA); TEER and TJ IF; Fluo-4/Fura-2 Ca^{2+} transients in fibroblasts/keratinocytes.
Sage (*Salvia officinalis*): leaf vesicles	Anti-inflammatory; microbiome-balance support; Antioxidant.	Cytokines (IL-6/IL-8); keratinocyte–microbiome co-culture (*S. epidermidis*/*C. acnes*) with AMPs; ROS assays; Ca^{2+} readouts.
Blueberry (*Vaccinium corymbosum*): fruit vesicles	Antioxidant/anti-photoaging (anthocyanins); ECM preservation.	ROS (DCFDA, MitoSOX) after UV; MMP-1 suppression; COL1A1/ELN qPCR; Ca^{2+} dynamics in fibroblasts.
Pomegranate (*Punica granatum*): pericarp/seed vesicles	Antioxidant; Anti-inflammatory; Elastase/MMP modulation.	ROS; IL-6/TNF-α ELISA; Elastase/MMP-1 activity; Collagen markers; Ca^{2+} in dermal fibroblasts.

Table 10. *Cont.*

Plant Source (Species/Tissue)	Reported Biological Functions (Examples)	Evaluation Assays/Models (Recommended Assay)
Olive (*Olea europaea*): leaf/fruit vesicles	Antioxidant (oleuropein context); Barrier support; Soothing.	ROS; TEER and permeability; TJ IF (claudin-1/ZO-1); Ca^{2+} in keratinocytes; Ex vivo skin retention imaging.
Cucumber (*Cucumis sativus*): peel/pulp vesicles	Soothing; Hydration/barrier homeostasis; Anti-irritant.	TEER; transepithelial permeability; IL-8 reduction after SLS; Ca^{2+} signaling in keratinocytes under irritant challenge.
Carrot (*Daucus carota*): root vesicles	Antioxidant (carotenoids); Pro-regenerative effects; Photoprotection.	ROS with or without UV; Scratch closure; COL1A1/ELN expression; Ca^{2+} imaging in fibroblasts.
Turmeric (*Curcuma longa*): rhizome vesicles	Anti-inflammatory (NF-κB/MAPK modulation); Antioxidant; Soothing.	NF-κB luciferase/IF; cytokines (IL-6/IL-8); ROS; Ca^{2+} readouts; Uptake by confocal.
Chamomile (*Matricaria chamomilla*): flower vesicles	Soothing/anti-irritant; Barrier repair support.	IL-8 reduction after SLS; TEER restoration; TJ markers; Ca^{2+} signals in keratinocytes.
Licorice (*Glycyrrhiza glabra*): root vesicles	Anti-inflammatory; Pigmentation modulation; Antioxidant.	Cytokines; Tyrosinase activity/melanin content; ROS; Ca^{2+} imaging in melanocyte–keratinocyte models.
Calendula (*Calendula officinalis*): flower vesicles	Regenerative/wound healing; Anti-inflammatory; Angiogenic support.	Scratch/trans-well migration; Procollagen assays; tube formation (endothelial cells); cytokines; Ca^{2+} in fibroblasts/endothelium.
Basil (*Ocimum basilicum*): leaf vesicles	Antioxidant; antimicrobial balance; Soothing.	ROS; keratinocyte–microbiome co-culture; IL-6; Ca^{2+} readouts; TEER.

ECM: extracellular matrix; IF: immunofluorescence; RHE: reconstructed human epidermis; ROS: reactive oxygen species; TEER: transepithelial electrical resistance; TJ: tight junction.

8. Regulatory for PDEVs

For PDEVs, physicochemical and molecular profiling provide information about the identity and composition of the ingredient, while the Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TGs) inform regulators about its hazard behavior under standardized, non-animal testing conditions.

In the European Union (EU), and increasingly in other jurisdictions, safety substantiation for cosmetic products must be based on validated in vitro and in chemico methods. Given that PDEVs are biological nanocolloids composed of lipids, proteins, RNAs, pigments, and antioxidants, they may interact with assay components through mechanisms such as light scattering, dye quenching, or redox interference. Therefore, a robust skin safety assessment should integrate appropriate OECD test guidelines with pre-designed

interference controls, utilize both as-formulated products and neat dispersions as test articles, and provide thorough documentation of vesicle stability and dispersion behavior throughout the assay period [137].

Skin corrosion and irritation are addressed with reconstructed human epidermis models: TG 431 (corrosion) and TG 439 (irritation) [138]. These 3D tissue model classify materials by viability thresholds, typically assessed via MTT or Neutral Red (NR) assays, and, in the case of irritation testing, by measuring cytokine release—most commonly interleukin-1α (IL-1α). When testing PDEVs, it is essential to verify that inherent colorants (e.g., polyphenols, carotenoids) or redox activity do not artificially influence viability readouts. This requires performing the color and chemical interference checks outlined in the relevant OECD guideline, incorporating appropriate matrix and vehicle controls, and confirming tissue integrity following exposure. Successful performance in these assays indicates that the test material, when applied topically at intended use concentrations, is neither corrosive nor irritating under the specified test conditions.

Skin sensitization (i.e., allergic contact dermatitis) cannot be predicted solely based on chemical composition; rather, it requires an integrated approach to testing and assessment (IATA) [139]. OECD TG 497 formalizes Defined Approaches (DAs) that combine data from three mechanistically distinct assays: TG 442C (Direct Peptide Reactivity Assay, DPRA) to assess peptide reactivity, TG 442D (KeratinoSens™) to measure Nrf2-ARE pathway activation in keratinocytes, and TG 442E (e.g., h-CLAT, U-SENS™, or IL-8 Luc) to evaluate dendritic cell activation. Collectively, these assays yield a binary hazard classification (sensitizer or non-sensitizer) and, in some cases, provide potency categorization. When applying these methods to PDEVs, particular care must be taken to account for potential interferences such as protein or lipid adsorption to plastic surfaces, instability of dispersions, and background fluorescence or absorbance arising from vesicle cargo. These feasibility assessments should be thoroughly documented, and where necessary, dosing protocols should be adjusted or fluorescence-independent detection methods employed to prevent misclassification.

As many PDEVs contain chromophores such as flavonoids and carotenoids that absorb UVA radiation, phototoxicity represents a plausible safety concern. OECD TG 432, which employs the 3T3 Neutral Red Uptake (NRU) phototoxicity assay, detects UVA-dependent cytotoxicity by comparing cellular viability in irradiated versus non-irradiated conditions [140]. Prior to conducting the assay, it is essential to characterize the test item's UV–visible absorption spectrum to define an appropriate irradiation window and to assess potential interference with the NR dye (e.g., via binding or quenching). If such interference cannot be avoided, any proposed mitigation strategies, such as adjusting the spectral window or employing alternative detection methods, must be scientifically justified and documented in the study plan. A negative result in TG 432 provides strong evidence that the test item poses minimal phototoxic risk under sun-exposed conditions.

Dermal absorption is a critical component of exposure assessment. OECD TG 428, which utilizes Franz diffusion cells with human or porcine skin, quantifies the extent of active ingredient penetration into and through the skin, as well as its retention within specific skin layers [141]. When evaluating PDEVs, it is important to assess both the finished cosmetic formulation and the neat dispersion. Where scientifically appropriate, include size-fractionated controls (e.g., SEC-enriched EVs vs. soluble filtrates) to distinguish vesicle-associated components from unencapsulated (free) cargo. Ensure full mass balance by quantifying analyte distribution across all compartments (donor solution, stratum corneum, viable epidermis/dermis, and receptor fluid). Any observed vesicle instability, aggregation, or other formulation-related changes under the selected test vehicle or dosing conditions should be carefully documented and reported.

Finally, although not specific to skin, a basic genotoxicity battery is typically required for novel active substances. This generally includes OECD TG 471 (the Ames test) to assess bacterial mutagenicity, along with a mammalian chromosomal damage assay such as TG 487 (in vitro micronucleus test), TG 473 (in vitro chromosomal aberration test), or TG 476 (in vitro gene mutation test using mammalian cells) to evaluate clastogenic and mutagenic potential [142,143]. As with other assays, it is important to anticipate and address potential assay interference arising from antioxidant or redox-active components of PDEVs, particularly in colorimetric or fluorescence-based readouts. Any methodological adaptations or controls implemented to mitigate such interference should be clearly justified and documented.

The OECD TGs address regulatory hazard identification and are not intended to replace efficacy evaluations. Concurrently, it is common to perform claim-relevant functional assays such as measurements of barrier integrity via TEER and permeability, assessment of anti-inflammatory cytokine production, quantification of oxidative stress through ROS generation and lipid peroxidation, intracellular calcium flux as an early signaling marker, and expression analysis of regeneration-related markers, including COL1A1 and ELN versus MMP-1 and TIMP-1. While these functional data serve as supportive evidence linking vesicle composition to biological plausibility, the foundation of a robust safety dossier for PDEVs lies in a strategically designed and interference-aware execution of OECD TG 431/439 (corrosion/irritation), TG 497 (sensitization via assays 442C, 442D, and 442E), TG 432 (phototoxicity), TG 428 (dermal absorption), and a genotoxicity screening battery. These assays should be conducted on representative production lots and reported comprehensively, including details on species and tissue source, isolation procedures, buffer composition, particle size and concentration, and vesicle stability.

Nevertheless, the regulation of cosmetic products differs markedly across global jurisdictions, reflecting distinct historical, cultural, and regulatory philosophies regarding consumer protection, industrial innovation, and public health. Among these, the EU, the United States Food and Drug Administration (US FDA), and major Asian markets such as China, Japan, South Korea, and the ASEAN region provide the most influential regulatory models. Although all frameworks share the common goal of ensuring product safety and truthful consumer information, they diverge significantly in scope, enforcement mechanisms, and pre-market control.

In the EU, cosmetics are governed by Regulation (EC) No. 1223/2009, one of the most comprehensive and stringent cosmetic laws worldwide [144]. The EU system is characterized by its strong emphasis on pre-market safety assessment and documentation. Each cosmetic product must be supported by a Product Information File (PIF) containing toxicological data, manufacturing details, and a formal safety assessment performed by a qualified professional. The manufacturer or importer must also designate a Responsible Person who ensures compliance with all regulatory obligations. The EU approach is largely preventive, placing responsibility for safety and conformity on the producer before a product reaches the market. Furthermore, the EU strictly prohibits animal testing for both finished products and cosmetic ingredients, encouraging the adoption of validated alternative methods. Regulatory annexes list prohibited and restricted substances, as well as authorized colorants, preservatives, and UV filters, reflecting a precautionary, science-based philosophy.

By contrast, the US adopts a more flexible and post-market regulatory approach under the Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Fair Packaging and Labeling Act [145]. Historically, cosmetic products in the US have not required pre-market approval, except for color additives. Instead, manufacturers are legally responsible for ensuring product safety, while the FDA intervenes only when products are deemed "adul-

terated" or "misbranded." This system relies heavily on post-market surveillance and industry self-regulation. However, recent reforms introduced by the Modernization of Cosmetics Regulation Act (MoCRA, 2022) are narrowing the regulatory gap with the EU. MoCRA introduces facility registration, mandatory product listing, record-keeping, and recall authority, thus increasing transparency and oversight. Despite these advances, the US framework remains less prescriptive regarding ingredient control, with fewer explicit positive or negative lists compared to the EU. Moreover, animal testing is not legally prohibited, though it is increasingly discouraged by industry practice and state-level legislation.

In Asia, cosmetic regulation is highly heterogeneous, shaped by varying levels of industrial maturity, cultural priorities, and trade integration [146]. China, through the National Medical Products Administration (NMPA), has recently modernized its system under the Cosmetic Supervision and Administration Regulation (CSAR, 2021). Chinese regulation distinguishes between special-use cosmetics (such as sunscreens, whitening, and hair dyes), which require pre-market registration, and general-use cosmetics, which undergo a simplified filing process. For imported products, a domestic Responsible Person is mandatory, ensuring traceability and accountability. Although animal testing was once compulsory for imported cosmetics, recent reforms allow exemptions for general-use products supported by non-animal safety data, aligning China more closely with OECD and EU principles.

Japan's regulatory system, under the Ministry of Health, Labour and Welfare (MHLW), combines pharmaceutical-style rigor with flexibility [147]. Products are divided into "cosmetics," which only require notification, and "quasi-drugs," which make stronger functional claims (e.g., whitening, anti-acne) and require formal pre-market approval. Japan maintains both positive and negative ingredient lists, and its quality and labeling standards are deeply rooted in traditional consumer safety culture. Similarly, South Korea's Ministry of Food and Drug Safety (MFDS) distinguishes between general and "functional" cosmetics, with the latter subject to efficacy verification. The Korean system is recognized for integrating scientific evaluation and innovation-friendly policies, which have supported the global expansion of the K-beauty industry. The ASEAN region (including Singapore, Malaysia, Thailand, and others) operates under the ASEAN Cosmetic Directive (ACD), a harmonized framework modeled on the EU Regulation. The ACD relies on a notification system combined with mandatory maintenance of a PIF and post-market surveillance, promoting regional trade while maintaining safety standards.

Despite their differences, all these systems share several fundamental objectives. They require manufacturers to guarantee product safety, ensure accurate labeling, and prevent misleading claims. Ingredient transparency, traceability, and consumer protection are central across all jurisdictions. However, the degree of regulatory intervention and timing of oversight vary significantly. The EU represents a preventive model, demanding comprehensive evidence of safety before commercialization; the US has traditionally relied on a reactive model, emphasizing post-market enforcement; and Asian systems display a hybrid structure, combining elements of both depending on product category and national priorities. Another notable difference lies in the treatment of innovative or borderline technologies, such as nanomaterials, biotechnology-derived actives, and exosomes. The EU provides explicit definitions and labeling requirements for nanomaterials, while the US lacks formalized nanomaterial-specific rules. Asian regulators are gradually adapting: Japan and Korea have issued guidance documents, and China is actively developing regulatory pathways for novel ingredients under CSAR. Ethical and technological modernization, particularly the gradual transition away from animal testing and toward OECD-aligned in vitro methods, is a unifying trend across all regions. Overall, while the EU, US, and Asian cosmetic regulations are converging toward greater transparency and safety assurance,

their underlying philosophies remain distinct. The EU system prioritizes precaution and scientific rigor; the US favors market freedom balanced by post-market control; and Asian authorities pursue a pragmatic balance between safety, innovation, and trade facilitation.

9. Stability and Storage Considerations for PDEVs

Stability represents a critical, yet frequently underestimated, parameter in the development, formulation, and commercialization of EV-based cosmetic and therapeutic products. Given the biological origin and nano-scale structure of EVs, their integrity and bioactivity are highly susceptible to environmental and formulation-related stressors. Failure to assess and control stability parameters can result in loss of efficacy, inconsistent performance, and even adverse reactions in the final product. PDEVs are especially vulnerable to a variety of destabilizing conditions that may be encountered during production, transport, storage, or incorporation into cosmetic matrices. These include elevated temperature, which may cause vesicle deformation or cargo degradation; repeated freeze–thaw cycles, which can lead to membrane rupture and aggregation; UV exposure, known to oxidize lipid components and degrade nucleic acids; and the presence of preservatives, surfactants, or excipients, which may disrupt membrane integrity or chemically interact with vesicle cargo [148,149]. As such, manufacturers must implement comprehensive stability testing protocols that address both storage stability and formulation compatibility. This includes real-time and accelerated stability studies, in accordance with ICH guidelines or cosmetic product regulatory standards, depending on the intended market. Short-term (1–3 months) and long-term (6–24 months) studies should be conducted under various conditions (e.g., 4 °C, room temperature, 40 °C, with/without light exposure), with periodic sampling to assess critical quality attributes such as vesicle size distribution, PDI, zeta potential, turbidity, and pH [150,151]. Morphological integrity should be confirmed by TEM or Cryo-TEM, while functional stability can be monitored via bioassays relevant to the intended application (e.g., antioxidant activity, cytokine modulation, cell viability support) [24]. Formulation compatibility studies are equally essential, especially when PDEVs are intended for integration into creams, gels, emulsions, or serums. Interactions with emulsifiers, humectants, thickeners, or preservatives can lead to loss of membrane stability or denaturation of bioactive components. Therefore, vesicle behavior in these matrices, both immediately after incorporation and over time, should be closely monitored. Parameters such as pH drift, phase separation, color change, and precipitation must be tracked, and their implications on EV performance must be evaluated [148]. Additionally, studies should confirm whether the biological activity of vesicles is retained within the final formulation, as well as during typical consumer usage conditions (e.g., ambient air exposure, application on skin, exposure to light or body temperature) [152].

For long-term preservation or to improve transportability, lyophilized (freeze-dried) or spray-dried forms of PDEVs may be developed [153,154]. In such cases, the choice of cryoprotectants or stabilizing excipients (e.g., trehalose, mannitol) must be carefully optimized, and the ability to reconstitute vesicles with preserved structural and functional integrity must be demonstrated [155,156]. Redispersibility should be confirmed via NTA or DLS, and retained bioactivity validated through appropriate in vitro assays [157].

Another cornerstone of quality assurance is the demonstration of batch-to-batch reproducibility. Since PDEVs are derived from biologically variable sources, differences in plant cultivar, growth conditions, seasonal variation, and harvesting protocols can significantly alter the vesicle yield, composition, and activity. Therefore, it is imperative to implement traceable and well-documented sourcing protocols, with detailed logging of botanical identity, harvest date and location, plant part used, and processing conditions. Quantitative metrics such as yield per gram of raw material and cargo concentration (e.g., total

protein, RNA content, polyphenol levels) should be consistently reported [158,159]. Every production batch should be accompanied by a CoA that includes all key physicochemical and biological parameters, such as size, concentration, purity markers, residual solvents or contaminants, and assay results for defined biological activities. These CoAs should be made available to downstream users, including formulators, regulatory reviewers, and consumers (in summarized form), as part of a transparent and responsible supply chain. In conclusion, robust stability and reproducibility protocols are not merely a regulatory requirement, but a scientific and ethical imperative to ensure that PDEV-based products are safe, effective, and reliable throughout their intended shelf life and application context.

10. Future Perspectives

As non-animal EVs, and specifically PDEVs, gain traction in cosmetic and regenerative applications, a scientifically robust and regulator-aware foundation becomes essential. The minimal guidelines outlined here are not intended to restrain innovation; rather, they establish a clear baseline for reproducibility, safety, and trust, upon which differentiated technologies and credible claims can be built.

Once PDEVs enter the market, they inevitably intersect with existing regulatory frameworks (e.g., OECD non-animal safety testing, EU cosmetic regulations), which will help address many of the issues discussed in this review and prevent avoidable downstream rework.

For cosmetic application, several obligations follow directly from law and good scientific practice. (i) Preclinical efficacy and safety must rely on cellular and tissue models—not animals—leveraging validated OECD Test Guidelines for hazard identification and robust, skin-relevant functional assays for performance. (ii) Products that pass preclinical gates should transition to human clinical studies (e.g., HRIPT/tolerability; claim-support trials) to substantiate safety and real-world benefit. (iii) Claims must remain cosmetic in scope—benefits such as soothing, barrier support, photo-protection or appearance improvements—without curative/therapeutic positioning that would reclassify the product as a drug. (iv) The production strategy must comply with quality standards suitable for commercial release (e.g., ISO 22716 cosmetic GMP, validated cleaning and cross-contamination controls) and be supported by an adequately sized, qualified plant. (v) Finished products must demonstrate stability—physicochemical, microbiological, and functional—under intended shelf-life and use conditions. (vi) Industrial scalability requires a frank technology choice: routes based on plant cell/tissue cultures can offer compositional control but often face true scale limits and downstream removal challenges for growth factors and antibiotics; routes based on raw biomass scale more readily but demand rigorous controls for residues, batch variability, and agronomic drift. Whichever route is chosen, removal validations, acceptance criteria, and traceable provenance are mandatory.

Formulation strategy is also a critical determinant of product quality. Single-source PDEVs (derived from one plant species) are generally easier to standardize and document, whereas multi-source blends may offer broader bioactivity profiles (e.g., antioxidant, soothing, barrier-supporting effects) at the expense of greater quality control complexity and potential component interactions. Such blends should be justified by data demonstrating additivity or synergy without compromising safety and controlled by lot-specific molecular fingerprints.

The proposed framework aligns with safe-by-design innovation, responsible sourcing, and evidence-based marketing in the natural cosmetics domain. It requires interdisciplinary collaboration among biologists, chemists, dermatologists, engineers, and regulatory experts to ensure that the potential of PDEVs is realized without compromising scientific rigor or public health. From both the formulator's and the end user's perspective, no EV-based

ingredient should be adopted without a comprehensive functional data package that complements OECD hazard testing and enables informed, defensible decisions. At minimum, the dossier should contain: (i) demonstrated biological activity in relevant human cell types or tissue models (e.g., keratinocytes, fibroblasts, RHE/explants), under conditions mimicking intended use. (ii) Dose–response curves and defined effective concentration ranges (with upper no-effect and cytotoxicity limits). (iii) Application protocol and delivery method details (vehicle, contact time, frequency, body sites), including interference controls for nanocolloids. (iv) Preclinical safety data: cytocompatibility, OECD in vitro skin corrosion/irritation, sensitization (defined approaches), phototoxicity (if chromophores present), and dermal absorption for exposure estimation. (v) Mechanistic support for key claims (e.g., barrier markers, cytokine modulation, ROS/lipid peroxidation limits, Ca^{2+} signaling engagement; pathway evidence such as NF-κB/MAPK/TGF-β, where appropriate).

11. Conclusions

A high level of transparency and methodological rigor enables traceability, quality assurance, and consumer confidence while granting innovators the flexibility to differentiate based on biological source, isolation technology, and formulation design. Operating within this framework ensures that PDEVs remain safe, reproducible, and purpose-fit for modern skincare. By adhering to these principles, producers can guarantee the consistency, functionality, and provenance of their vesicle-based ingredients, while consumers and formulators can make informed decisions about efficacy and safety. Only through such shared responsibility can the field progress from a fragmented, largely unregulated landscape toward a transparent, scientifically accountable domain of innovation in natural EV-based therapeutics and cosmeceuticals.

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Abbreviation List

The following abbreviations are used in this manuscript:

2,4-D	2,4-dichlorophenoxyacetic acid
AF4	asymmetric flow field-flow fractionation
AFM	atomic force microscopy
BAP	6-benzylaminopurine

CapEx	capital expenditure
CFD	computational fluid dynamics
CMC	chemistry, manufacturing, and control
CoA	certificate of analysis
DC	differential centrifugation
DGDG	digalactosyldiacyl-glycerol
DLS	dynamic light scattering
DO	dissolved oxygen
DoE	design of experiments
dPCR	digital Polymerase Chain Reaction
ECM	extracellular matrix
ELN	elastin
ELS/LDV	electrophoretic light scattering/laser Doppler velocimetry
EVs	extracellular vesicles
GAGs	glycosaminoglycans
GC	gas chromatography
GIPCs	glycosyl inositol phosphorylceramides
HACCP	hazard analysis and critical control points
IF	immunofluorescence
JA	jasmonic acid
LC	liquid chromatography
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
LOR	limit of reporting
MGDG	monogalactosyldiacylglycerol
MMP-1	matrix metalloproteinase-1
MS	mass spectrometry
NGS	Next Generation Sequencing
NTA	nanoparticle tracking analysis
OECD	Organisation for Economic Co-operation and Development
OpEx	operational expenditure
PA	phosphatidic acid
PAT	process analytical technology
PC	phosphatidylcholine
PDI	polydispersity index
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PGRs	plant growth regulators
PI	phosphatidylinositol
QC	quality control
CQA	critical quality attributes
QMS	quality management system
RHE	reconstructed human epidermis
ROM	reactive oxygen metabolites
ROS	reactive oxygen species
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SA	salicylic acid
SEC	size-exclusion chromatography

SOPs	standard operating procedures
SPE	solid-phase extraction
TAMC	total aerobic microbial count
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
TFF	tangential flow filtration
TG	Test Guideline
TIMP-1	tissue inhibitor of metalloproteinases-1
TJ	tight junction
TRPS	tunable resistive pulse sensing
TYMC	total yeast and mold count
UC	ultracentrifugation
UF	ultrafiltration
VIC	vacuum infiltration–centrifugation

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