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Current Methods for the Discovery of New Active Ingredients from Natural Products for Cosmeceutical Applications

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ABSTRACT

Cosmeceuticals are designed to serve a dual purpose: to provide desired esthetical effects and to treat dermatological conditions. Natural products derived from plants and marine organisms are a novel source of potential cosmeceutical active ingredients for incorporation into new formulations due to consumer demands. Contrary to common perceptions, most regulatory agencies do not view cosmeceuticals as being a separate category from cosmetics; thus, these products are not regulated accordingly, thereby forcing the consumer to rely on the self-regulatory policies of the cosmetics industry. Cosmeceuticals are advertised as having capabilities that include anti-aging, anti-acne, solar-protective, wound healing, and skin whitening. Such traits normally comprise several biological activities. In order to ensure the safety and efficacy of these products, active ingredients employed in the formulations must undergo a series of tests. In this review, *in vitro* (enzymatic and cellular) and *in vivo* tests employed to evaluate the potential of new cosmeceutical active ingredients are discussed, and new trends that are being explored by the cosmeceutical industry are described.

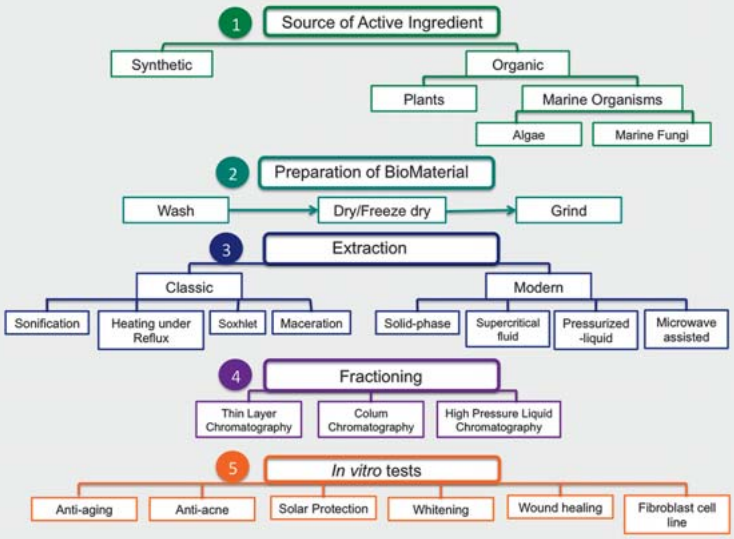
ABBREVIATIONS

CFU	colony forming units
DMAB	para-Dimethylaminobenzaldehyde
ECM	extracellular matrix
EGCG	epigallocatechin gallate
FDA	Food and Drug Administration
FALGPA	N-[3-(2- furyl)acryloyl]-Leu-Gly-Pro-Ala
HA	hyaluronic acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-sulfonic acid
ISO	International Organization for Standardization
L-DOPA	L-3,4-dihydroxyphenylalanine
MED	minimal erythema dose
SPF	sun protection factor
UVA-PF	UVA protection factor

Introduction

The use of topical aides to improve physical appearance dates back worldwide to 6000 years in human history [1, 2]. At present, the cosmetics industry accounts for $445 billion dollars in sales annually, making it a highly desirable and competitive industry [3]. The flowers, seeds, roots, leaves, and fruits of plants have been used for their cosmetic properties, a practice that continues to this day [2].

The term cosmeceutical, referring to a hybrid substance between a cosmetic and a pharmaceutical, was first described by Raymond E. Reed in the 1960s as a substance that complies with the following points: (1) external application; (2) produces useful and desired results; (3) has desired esthetical properties; and (4) meets chemical, physical, and medical standards [2, 4–6]. Thus, cosmeceuticals combine the esthetical properties of a cosmetic with the efficacy of a dermatological drug. Modern cosmeceuticals must achieve the accomplishment of 2 specific benefits:



▶ **Fig. 1** Basic steps needed for the discovery of new cosmeceutical active ingredients from natural products.

an immediate response (like a cosmetic) and a prolonged effect (like a pharmaceutical) [7]. Nevertheless, in recent years, the term has expanded to include any cosmetic that has or claims to have purported medicinal properties [5].

Most governmental offices worldwide, like the FDA in the United States, do not regularly recognize the term cosmeceutical, while Korea and Japan legally recognize 3 categories of products: cosmetics, functional cosmetics, and drugs [8]. This lack of recognition of this term has resulted in the grouping of these products as cosmetics or drugs, which complicates regulation [9]. In fact, most products are only self-regulated by the cosmetics industry [7].

One of the most widely spread misconceptions about cosmeceuticals is that the claims present in labeling and advertising are substantiated and approved before the products appear on the market. However, such claims are not always true [5]. Cosmeceutical products often use marketing to construct a "story" that links a certain active ingredient to the purported claims and benefits of the formulation [8]. Since no cosmetic company would consciously damage its own reputation with a problematic product that contains untested ingredients, the industry is in need of assays to test the cosmeceutical ability of new potentially active ingredients, especially those extracted from natural sources [7, 8].

This review aims to give a condensed overview of the process for new cosmeceutical development and to describe the most common tests employed in the development of new cosmeceutical active ingredients while also presenting new trends being explored by the cosmeceutical industry.

Cosmeceutical Development

The formulations of functional cosmetics, or cosmeceuticals, must include active ingredients that are recognized as safe and that provide some beneficial effect [7, 8]. These active ingredients can be either synthetic (like most vitamins) or derived from natural sources (extracts from plants, microorganisms, and animals). The use of specially designed assays to test the active ingredients can substantiate the claims of a new cosmeceutical [8]. An abbreviated guide to the steps necessary to obtain cosmeceutical active ingredients is presented in ▶ **Fig. 1** and more extensive accounts are explained in the following paragraphs.

As shown in ▶ **Fig. 1**, the first step in the design of new cosmeceuticals is the selection of the source of the active ingredient. Plants have been employed by humans for hundreds of years to satisfy an array of different needs, including those of food, shelter, medicine, and cosmetics [10], as they are among the most bountiful sources of new ingredients present in the form of primary or secondary metabolites [11, 12]. Plants are rich in endogenous antioxidants due to their existence in habitats that have severe UV radiation [13, 14]. Plant metabolites with potential cosmeceutical applications include a varied list of structurally diverse compounds, such as phenolics, polyphenols, flavonoids, terpenoids, steroids, steroidal saponins, sterols, sugars, polysaccharides, lignans, carotenoids, organic acids, anthocyanins, and coumarins [2, 15]. In addition, plant extracts are considered safe and have the requisite characteristics of cosmetic ingredients mandated by the FDA [7]. The cosmetics industry currently uses an array of plants, mostly belonging to the Asteraceae, Lamiaceae, Fabaceae, Poaceae, Malvaceae, and Rosaceae families [2]. Furthermore, studies are being conducted among native Canadian, Sri Lankan, and African plant species to find new ingredients [15–17].

Alternative sources of potentially active ingredients include marine organisms, which produce molecules that are chemically distinct from those of terrestrial sources. Polyphenols, for instance, are composed of phlorotannins rather than the usual structures, which possess biological activities that are attractive for the cosmeceutical industry [18–20]. Other metabolites of interest from marine organisms include polysaccharides, carotenoid pigments, collagen, chitooligosaccharide derivatives, enzymes, peptides, and other natural products [21–23]. Of special

▶ **Table 1** Plant secondary metabolite extraction methods. Table constructed with information modified from [24].

Method	Equipment	Sample loading	Extraction	Solvent volume	Notes
Percolation	Yes	Plant material is wetted with the solvent and loosely packed in the container; more solvent is added to maintain the plant material covered	After soaking the percolator valve is open slightly in order to collect solvent saturated with the solutes	Large Is replaced constantly	
Soxhlet	Yes Commercial options	Wetted plant material is loosely packed in the container; sufficient solvent is added to collecting flask (no overflow)	The solvent is refluxed through the sample for a determined number or cycles	Small Solvent is continuously recycled	The heat needed to drive the extraction may destroy heat liable compounds
Maceration	No Erlenmeyer flasks can be used	The plant material is wetted with the solvent and loosely packed in the container; more solvent is added to cover the sample	The sample with solvent is allowed to stand and extract; typically saturated solvent is decanted and filtered; new solvent is added, stirred, and allowed to macerate overnight	Dependent of the number of solvent changes (commonly no more than 3)	To accelerate the process, sonication or constant stirring on a shaking table can be employed; for small sample sizes, extractions can be carried in closed tubes using a shaking mixer

interest are macro- and microalgae, which can be grown in a rapid and cost-effective manner *in vitro*. Some current examples include the use by a Greek company of sea fennel in sun care products and the use by an Italian company of Guam seaweed for anti-cellulite formulations [23]. Additionally, marine fungi have a great potential for the production of secondary metabolites, although few of these compounds are suitable for use as cosmetic ingredients [22].

Once the source of a possible active ingredient has been selected, the biological material is prepared. Generally, plants are first washed, then dried or freeze-dried, and finally they are ground to homogenize the sample and to provide the largest surface contact with the solvent system. Several extraction methods with the use of water or organic solvents, including sonification, heating under reflux, Soxhlet extraction, maceration, and percolation, can be employed (▶ **Table 1**). The choice of a solvent system is dependent on the type of metabolite being extracted. For extraction of hydrophilic compounds, polar solvents, like methanol or ethanol, are used, while the extraction of hydrophobic compounds requires nonpolar solvents like dichloromethane or hexane [25]. Modern extraction techniques include solid-phase, supercritical-fluid, pressurized-liquid, microwave-assisted, and surfactant-mediated techniques. These avant-garde technologies are more environmentally friendly than their predecessors and carry a set of technological advantages [26–28]. For example, ultrasound-assisted extraction methods use the power of ultrasound to break cell walls, causing the release of inner cell components. The main advantages of the technique include reduction of the amount of power, solvent, and the time need for extraction [28–31]. Additionally, it can be used for thermolabile compounds [28, 30]; however, a comprehensive evaluation of the characteristics of the plant such as moisture content and particle size of the bioactive compound needs to be done to guarantee a high recovery [28, 31]. Another interesting new method is supercritical fluid extraction, which uses a liquid in a state that has features of both a liquid and a gas. The supercritical fluid can enhance extraction yields as it can diffuse simply through solid materials because of its better transport properties [28, 32]. It is a good process for the extraction of compounds that are thermally stable or have high boiling points; nevertheless, the high maintenance needed by the equipment and other factors such as supercritical fluid, raw materials, and co-solvents can prove disadvantageous [28, 29, 33]. Finally, microwave-assisted extraction is one of the most advanced extraction techniques. Its efficiency is owed to its ability to heat the matrix internally and externally without a thermal gradient, resulting in a higher efficiency of extraction with less time, energy, and solvent volume needed [28, 29, 33]. However, the method does present some drawbacks, including the need for processes like filtration or centrifugation to remove solid residues. Moreover, the method is restrictive when it comes to nonpolar compounds extractions and can alter the chemical structure of target compounds [28, 29, 33, 34].

Additionally, in order to make the extraction process more "green" or "environmentally friendly," several changes can be made, including the use of alternative solvents, such as water and agro- or bio-solvents (renewable resource produced from biomasses, such as bio-ethanol) to reduce the consumption of energy and renewable biological materials [25, 35].

Plant and marine organisms produce a great number of metabolites, and extracts are formed by a combination of compounds. In order to be used in cosmeceutical formulations, some extracts must be fractioned to isolate compounds with biological activities. Usually, the extracts are first fractioned in several parts that contain molecules of similar polarity or molecular size. The fractions are then tested *in vitro,* using some of the techniques listed below in the Bioactivity analysis of cosmeceutical properties section for cosmeceutical activities. Then the active fractions are further separated to isolate the active metabolite using techniques like thin-layer chromatography, column chromatography, flash

▶ **Table 2** *In vivo* and *In vitro* tests employed for the safety assessment of cosmetics. With information from [38, 39].

Assessment	*In vivo* model	*In vitro* model
Acute toxicity	▪ Administration of the ingredient (by oral, dermal, or respiratory route) to rabbits, rats, or mice for the calculation of the dose causing 50 % of animal deaths (LD_{50})	▪ Software packages based on quantitative structure activity relationships (QSAR) models ▪ Predict toxicity based on chemical structures
Skin corrosion or irritation	▪ Use of rabbits or other species such as guinea pig and mouse ▪ Human volunteers	▪ EpiSkin: Dermal substrate generated on type I bovine collagen matrix, with a film of type IV human collagen, upon which is laid all epidermal layers of native skin. ▪ EpiDerm: Human epidermal keratinocytes as fully-differentiated epidermal tissue. ▪ TER: Skin from young rats. OECD 430 [40] ▪ SkinEthic: Normal human keratinocytes cultured on inert polycarbonated filters ▪ epiCS: Normal human epidermal keratinocytes from a single neonatal donor supplied in 24-well formats. ▪ RHE: Reconstructed human epidermis OECD 431 [41] ▪ *In vitro* membrane barrier test OECD 435 [42]
Eye irritation	▪ Draize conventional test (use of albino rabbits) ▪ Human volunteers	▪ EpiOcular: 3D model, Non-keratinized epithelium prepared from normal human keratinocytes. ▪ HCE: 3D model, immortalized human corneal epithelial cells
Skin sensitization	▪ Animal Tests: – Mouse local lymph Node assay (LLNA), – Nonradioactive modifications: LLNA-DA and LLNA-BrdU ELISA – Guinea pig maximization test by Magnusson and Kligman (GPMT) – Buehler occluded patch test in the guinea pig ▪ Human volunteers: patch test	▪ Direct peptide: Haptenation process is reproduced in vitro ▪ Reactivity assay KeratinoSens: Immortalized adherent human keratinocyte cell line (HaCaT cell line), transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE) 1
Genotoxicity	▪ Comet test in animals (rats)	▪ Micronucleous test: Formation of "micronucleus" (part of a chromosome not incorporated into one of the daughter nuclei during cell division)
Carcinogenicity	▪ Animal tests: induction of tumors or cancer in rats, mice, or rabbits via oral or dermal administration of the test subject.	▪ BALB/c 3T3: Based on the malignant transformation of immortalized embryonic mouse fibroblasts ▪ SHE: Syrian hamster embryo cell
UV-induced toxic effects/ phototoxicity	▪ Animal tests ▪ Human volunteers	▪ 3T3 NRU: Uses normal BALB/c 3T3 mouse fibroblasts to measure the concentration dependent reduction in neutral red uptake by the cells after exposure to a test material either in the presence or absence of UVA light.

chromatography, and high-performance liquid chromatography, among others [25, 36].

After confirmation that the isolated compound has cosmeceutical activity, it is tested *in vitro* using fibroblasts, a cell type that represents the main dermic population and is considered a good safety model due to its responsiveness (see Section 4a). The goal of the test is to maintain homeostasis of the ECM and elevate the production of some important dermal components, such as collagen and elastin. The isolated active ingredient is then placed in suitable vehicles and tested *in vivo* in human clinical studies [7, 37]. The best formulations can then be released to the marketplace.

It is important to point out that during this process the extracts are constantly tested for activity and toxicity so as to ensure safety. The guidelines used for cosmetics can be employed to test the safety of cosmeceuticals. Cosmetics are required to be safe for consumer use in accordance with the directions in the labeling or in a customary or expected way [9]. Typically, in order to assess the safety of a particular cosmetic, the manufacturer must test the safety of each ingredient present in the formulation. In this respect, a "natural" or "organic" (words often used to describe plant extracts or natural products) ingredient is not guaranteed to be safe and thus must be tested like any other cosmetic ingredient [9]. Standard safety tests are listed in ▶ **Table 2**. Changes in legislation, specifically in the European Union, have encouraged the creation of *in vitro* tests so as to evaluate the safety of cosmetic ingredients, since the use of animal tests was prohibited in Europe in 2004 for cosmetics and in 2009 for cosmetic ingredients [38]. A recent review by Almeida et al. [38] has provided insights into the *in vitro* models for safety and toxicity assessments of cosmetic ingredients and some of the *in vitro* tests currently accepted by the Organization for Economic Co-operation and Development. Traditional *in vivo* tests are listed in ▶ **Table 2**.

Bioactivity Analysis of Cosmeceutical Properties

Cosmeceutical products can be classified according to the type of activity provided by the active ingredients. Marketing teams usually advertise the benefits of cosmetics with the use of undefined phrases like "anti-aging" and "skin toning" that can imply a range of biological activities [8]. The following section lists those cosmeceutical properties that are most important and recurrent and the most common tests that are used to demonstrate the biological activities of ingredients.

Anti-aging

The skin is an organ that easily presents signs of aging due to the weakening of the elasticity and strength of the skin, which results in wrinkle formation [43–44]. Two types of aging affect the skin: intrinsic or age-dependent and extrinsic or photoaging [16, 45–46]. Intrinsic aging is affected by various factors, such as telomere shortening, the imbalance between free radicals and antioxidants, and hormonal changes, while photoaging is promoted by exposure to UV solar radiation [43–45, 47–49].

The skin consists of 3 layers: the epidermis, dermis, and hypodermis [16, 50]. The outer most part of the skin, epidermis, is followed by the dermis, which is mainly formed by the EMC, fibroblast, and protein fibers composed of collagen and elastin [16, 51]. Collagen accounts for about 80% of dry skin weight and is responsible for the tensile strength and maintaining flexibility of the skin. Elastin fibers, on the other hand, provide elasticity to the skin. HA helps to retain moisture of the skin as well as its structure and elasticity. It is also involved in rapid tissue proliferation, regeneration, and repair [16, 52, 53]. Aging causes decreases in the levels of collagen, elastin, and HA via several pathomechanisms (a mechanism by which a pathological condition occurs), including enzymatic degradation of the elastin fibers and collagen networks and the attack of fibroblast components by free radicals, which are responsible for the formation of collagen, elastin, and HA [44, 54, 55].

Active ingredients that possess inhibitory actions against elastase, collagenase, and hyaluronidase (enzymes that degrade elastin, collagen, and HA, respectively), as well as antioxidant activities, could help to decelerate skin aging [44]. The following paragraphs detail the methods employed for the testing of anti-aging enzymatic activities. Antioxidant activity assays are omitted since they have been extensively discussed in other reviews [56].

Anti-collagenase test

The most commonly used anti-collagenase test, developed by Van Wart and Steinbrink in 1981 [57], is based on the enzyme-substrate interaction between collagenase, typically derived from *Clostridium histolyticum*, and FALGPA, a synthetic proteolytic compound that results in a reduction in absorbance between 324 and 345 nm. Pre-incubation of the enzyme with an inhibitor, such as a bioactive compound or plant extract, results in the limited breakdown of FLAGPA and conservation of the absorbance [44]. Most plant extracts have low yields, and in order to economize reagents, it is more efficient to employ micro-scale methods, such as that described in the study by Chattuwatthana and Okello [44], where 20 µL of 50 mM 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid buffer with 0.36 mM calcium chloride (pH 7.4 at 37 °C) is placed in the wells of a 96-well microtiter plate together with 20 µL of collagenase derived from *C. histolyticum*. Then the test substance (plant extracts, fractions) is added and the mixture is allowed to react in the dark at 37 °C for 20 min. To separate wells, 20 µL of EGCG was added as a positive control and 20 µL of 18.2 M Ω water was added as a negative control. After the first incubation period, 40 µL of FALGPA working solution is added to each well to a final reaction volume of 100 µL. The microplate is then incubated in the dark at 37 °C for 30 min. As the final concentration of the reaction mixture, 0.8 mM FALGPA, 0.16 U/mL of collagenase, and 0.023 mg/mL of EGCG were used as a positive control. Finally, the absorbance of the solution is determined using a microplate reader at 335 nm. A blank is used to correct the background absorbance of the plant samples and controls. The percentage of collagenase inhibitory activity is then calculated using Eq. 1.

$$\text{collagenase inhibition } (\%) = \left(1 - \frac{S}{C}\right) \times 100 \quad (1)$$

Where S is the corrected absorbance of the samples containing the collagenase inhibitor (enzyme activity in the presence of the sample) and C is the corrected absorbance of the negative control (enzyme activity without the sample) [44].

Anti-elastase test

The basic principles of the anti-elastase test, as first described in the study by Kraunsoe et al. [58], are that elastase directly breaks down the elastin substrate, causing a change in the absorbance that can be measured with a spectrophotometer [44]. Ndlovu et al. [16] in their study described a modified micro-method, where the wells of a 96-well plate are filled with 25 µL each of 0.1 M HEPES buffer (pH 7.5), the test sample (1.4 mg/mL), and elastase (1 µg/mL). Blank wells are filled with 75 µL of HEPES buffer and negative controls with 25 µL elastase and 50 µL HEPES buffer, while positive controls received 25 µL each of elastase, HEPES buffer, and elafin/N-methoxysuccinyl-Ala-Ala-Pro-Chloro (10 µg/mL). Solvent controls consist of 25 µL each of elastase, HEPES buffer, and a 10% solution of the solvent to dissolve the sample. Running color controls of each of the samples tested using 150 µL of HEPES buffer and 25 µL of the sample is recommended. Then the plate is incubated at room temperature (25 °C) for 20 min. Afterward, 100 µL of the substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (1 mM) is added and the plate is further incubated at 25 °C for 40 min. Absorbance is then read at 405 nm using a microplate reader, and the percentage of inhibition is calculated with Eq. 2:

$$\text{Enzyme Inhibition } (\%) = \left[\left(\frac{A_{\text{control}} - A_{\text{test sample}}}{A_{\text{control}}}\right)\right] \times 100 \quad (2)$$

Where A_{control} is the absorbance of the buffer, elastase, and solvent, and $A_{\text{test sample}}$ is the absorbance of the buffer, elastase, and extract or elafin/N-methoxysuccinyl-Ala-Ala-Pro-Chloro.

Hyaluronidase activity

There are different methods to evaluate hyaluronidase activity [59], which include the classical turbidimetric [60, 61], viscometric [62], and colorimetric [63] methods and the more modern spectrophotometric [64–66], fluorogenic [67], radiometric [68], and agarose plate-based [69–70] methods in addition to an ELISA-like method [71–73], high-performance liquid chromatography [74], and zymography [75], among others. The fluorimetric method, which is based on the Morgan-Elson reaction [76] and was first modified by Reissig et al. [77], then by Takahashi et al. [59], is among the most practical and commonly used assays. The procedure is as follows: 25 µL of calcium chloride (12.5 mM), 12.5 µL of the test sample (2.8 mg/mL), and hyaluronidase (1.5 mg/mL) are placed into a 2-mL test tube. The blank control contains 25 µL of distilled water, the negative control 12.5 µL of distilled water, the positive control 12.5 µL of aurothiomalate (2.8 mg/mL), and the solvent control 12.5 µL of the solvent used for dissolution of the extracts. To all tubes, except the blank control, 12.5 µL of the enzyme was added. The tubes were then incubated in a water bath at 37 °C for 20 min. Afterward, 100 µL of the substrate HA (1 mg/mL in 0.1 M acetate buffer; pH 3.5) was added and the tube was incubated for an additional 40 min. Then 25 µL of KBO_2 (0.8 M) was added to all tubes, which were placed in a water bath at 100 °C for 3 min. After cooling to room temperature (25 °C), 800 µL of DMAB (4 g of DMAB in 40 mL of acetic acid and 5 mL of 10 N HCl) was added and the tubes were incubated for 20 min. Afterward, the contents of the tubes were transferred to the wells of a 48-well plate. Fluorescence was detected using a spectrophotometer at an excitation wavelength of 545 nm and an emission wavelength of 612 nm. The percentage of inhibition was calculated using Eq. 2, where $A_{control}$ was the absorbance of the buffer, hyaluronidase, and the solvent and A_{sample} was the absorbance of the buffer, hyaluronidase, and extract or sodium aurothiomalate [16, 59].

Anti-acne

Acne vulgaris is a chronic inflammatory skin disease affecting approximately 85% of the population at some point in life, typically starting at the ages of 12–14 y [78–80]. It is characterized by the presence of comedones, papules, cysts, and nodules [78–81]. While the condition is not life threatening, it causes significant psychological morbidity due to scaring and disfigurement if left untreated [79]. Typically, acne affects the skin of the face, trunk, and upper arms [82]. The localized placement of the condition allows for the use of topical treatments that reduce or eliminate the need for oral drugs [82]. Acne is caused by multiple factors, including androgen-mediated stimulation of sebaceous gland activity, follicular hyperkeratinization, hormonal imbalance, and inflammation caused by stimulation of the innate immune system via several pathways, including external bacterial infection. *Propionibacterium acnes* and *Staphylococcus epidermidis* are the major bacterial causes of acne [80, 83–85]. According to the study by Han et al. [86], the crucial steps in the control of acne are avoidance of bacterial colonization and inflammation of the pilosebaceous units. Therefore, tests employed for the discovery of ingredients with potential anti-acne activities rely on the antibacterial and anti-inflammatory properties of the test substance.

Agar disk-diffusion method

The agar disk-diffusion method, which was developed in 1940, is currently the most commonly used test to determine antimicrobial activity [87, 88] and is the official method for routine susceptibility testing of cultures of either patient isolates or of samples acquired from the ATCC. In this procedure, agar plates are inoculated with a standardized inoculum of the test microorganism, such as *P. acnes* (ATCC 6919), *S. epidermidis* (ATCC 12228), or *Staphylococcus aureus* (ATCC 25913) in the case of anti-acne testing. Briefly, sterilized filter paper disks, approximately 6 mm in diameter, are loaded with 20 µL of the test substance at an established concentration and subsequently are placed on an inoculated plate at an appropriate distance. Positive controls are confirmed antimicrobials against the test microorganisms, such as triclosan at 5 mg/mL, which has antimicrobial activity against acne-causing bacteria [89, 90]. As a negative control, 20 µL of the solvent used for the preparation of the extract are used. The Petri dish is then incubated under appropriate conditions. The test substance diffuses into the agar and active extracts inhibit bacterial germination and growth. To assess the antibacterial capacity of the samples, the diameters of the bacterial growth inhibition zones surrounding each paper disk are measured [88, 91].

Minimum inhibitory concentration

Once the antimicrobial activity of the plant extracts had been confirmed, the lowest concentration of the extract to inhibit bacterial growth is measured as the minimum inhibitory concentration with the use of the following micro-scale method. The well of each column (1–12) of a 96-well plate is filled with 50 µL of sterilized nutrient broth (except for the first well of each column). Then 50 µL of plant extract at the appropriate concentration (similar to that employed in the agar diffusion test) is added to the first well of columns 4–12 (each extract in triplicate), which is serially diluted in the following 7 consecutive wells of the columns (50 µL), while discarding 50 µL of the last wells of each column. Next, 50 µL of sterilized nutrient broth and 50 µL of the bacterial inoculum (10^9 CFU/mL) are added to each well to a final volume of 150 µL. The first 3 wells served as controls. The first well is the positive control that contained an anti-acne drug, such as isotretinoin (1 mg/mL), which is serially diluted as with the plant extracts. The wells of the second column, as a negative control, contained 50 µL of the solvent, 50 µL of the bacteria inoculum, and 50 µL of nutrient broth. Finally, the third column serves as a sterility control, where the wells contained only the nutrient broth (150 µL). The plates are then incubated at 37 °C for 24 h. After incubation, the indicator dye 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium is added to each well, which indicated bacterial growth by a red-pink color, while growth inhibition is indicated by the lack of color change [85, 92].

Anti-inflammatory activity

The anti-inflammatory capability of the extracts can be assessed by measuring the production of inflammatory cytokines by THP-1 cells (ATCC TIB-202). First, the cells are seeded in the wells of 24-well plates at a concentration of 1×10^6 cells/well. Then the wells are inoculated with *P. acnes* together with the sample at the appropriate concentrations. Dexamethasone at 1×10^{-5} M is used

as a positive control. After 24 h of culture, levels of tumor necrosis factor alpha, interleukin (IL)-1B, IL-6, and IL-8 are measured by flow cytometry and with the use of the BD Cytometric Bead Array Human Inflammatory Cytokines Kit (BD Biosciences) [91, 93].

Solar protection

Solar UV radiation is composed of 3 different UV bands: UVA (315–400 nm), UVB (280–315 nm), and UVC (< 280 nm) [94–98]. Human health is affected both positively and negatively by UV radiation. On the positive side, the principal effects are UVB-mediated production of vitamin D in the skin, improvement of some dermatological conditions, including psoriasis, vitiligo, and atopic dermatitis, and mood improvement due to the release of endorphins [99–101]. By contrast, the negative effects of UV radiation are responsible for a variety of acute and chronic skin problems. Acute responses include sunburn and erythema, while chronic ailments consist of photoaging and photo-carcinogenesis [102]. In fact, UV light is considered the main etiological agent of a large number of skin cancers, sunburn, and oxidative stress [101]. Topically applied sunscreens can be used to prevent damage to the skin caused by exposure to UV light [102–105]. Active ingredients in sunscreens, referred as UV filters, act in different ways either by absorbing UV radiation (organic-based compounds) or by scattering and reflecting UV radiation (inorganic molecules) [102, 106, 107]. Some studies have demonstrated that the combination of the 2 usually results in better UV protection capabilities of the final product [102, 108, 109]. The efficacy of a sunscreen is measured as the SPF, a term coined by Greiter in 1970 [110], with a higher SPF indicating stronger photoprotective activity [101]. Hence, it is important to note that the value represents an evaluation of protection primarily against UVA-2 and UVB radiation [97, 98, 111–113]. SPF is expressed as the UV energy required to produce a MED on protected skin divided by the UV energy required to produce a MED on unprotected skin. MED is the lowest time interval or dosage of UV light irradiation that is sufficient to produce a minimally perceptible erythema (skin reddening and inflammation) [98].

SPF measurement

The only method to measure the efficacy of a sunscreen expressed as SPF that is recognized by the International Organization for Standardization is ISO 244444:2010, an *in vivo* method that involves 10–20 human volunteers [98, 101]. Nevertheless, it is recommended that the *in vivo* method be preceded by *in vitro* measurements during the first stages of the development of active ingredients. One of the most commonly used *in vitro* assays is the Diffey-Robson method [101, 114]. The FDA recommends a modified version of this method for *in vitro* SPF testing [102]. The test compares SPF values of preparations of plant extracts with emulsions containing the same proportion of free sunscreens with the use of the SPF-290S computer-controlled analyzer. Briefly, a surgical patch of Transpore tape with an area of 50 cm^2 is used as the substrate for the experiment. Normally, 2 mg/cm^2 of each test sample is distributed on the substrate surface with a syringe, and a gloved hand is used to stratify the sample with longitudinal movements back and forth. Then transmittance is measured at 6 different points and the results are expressed as average values. To calculate the SPF as an indicator of the UVA/UVB protective property of the test preparation, a calculation is made from the monochromatic protection factor, the solar irradiance, and the erythemal constants according to the Eq. 3:

$$\mathrm{SPF} = \sum_{290}^{400} E_\lambda\, B_\lambda \Big/ \sum_{290}^{400} E_\lambda\, B_\lambda \,/\, \mathrm{MPF}_\lambda \qquad (3)$$

Where MPF_λ is the mean monochromatic protection factor, E_λ is the spectral irradiance of terrestrial sunlight under controlled conditions, and B_λ is the the erythemal effectiveness. The defined condition for E_λ is the midday midsummer sunlight at 40 °C N with a solar zenith angle of 20° [102]. Some studies have evaluated SPF *in vitro* using the models proposed in the study by Gharavi et al. [115] or that by Mansur et al. [116].

UVA-PF

Although useful, SPF is an imperfect measure of skin damage, since UVA light does not primarily cause skin reddening or pain but rather produces invisible damage and skin aging. The UVA-PF can be determined by *in vivo* or *in vitro* methods. One assay is persistent pigment darkening, a Japanese *in vivo* method that is very similar to the SPF test, except that instead of reddening, tanning of the skin is measured. ISO 24443:2012 is an *in vitro* version of the UVA-PF [101]. Other substitutes for *in vivo* testing have been suggested with the use of cells and regenerated tissues [117, 118] or with the use of animals [101, 119–121]. A recent article in the study by Aparecida-Figueiredo et al. [98] assessed the protective efficacy of sunscreen products using a neonatal cell culture of human dermal fibroblasts by evaluating calcineurin depletion induced by UVA radiation. The method consists of determining calcineurin enzyme activity after irradiation in the absence and presence of sunscreen products. Lysates of neonatal human dermal fibroblasts are exposed to 24 J/cm^2 of UVA radiations in the presence or absence of the sunscreen preparations. The sunscreen products are applied on quartz slides to prevent interaction with the cell lysates so that it is possible to evaluate the UV absorption potential of the sunscreen [113, 122–125]. The photoprotective formulations are spread on the quartz slides at a concentration of 2 mg/cm^2 above each well. After irradiation, the lysates are diluted and processed, and then calcineurin activity is determined as described by Aparecida-Figueiredo et al. [98].

Skin whitening

Melanin, the principal molecular determinant of skin color, provides skin protection by limiting the absorption of UV radiation by approximately 50–75 % and scavenging reactive oxygen species [17, 126, 127]. In humans, the biosynthesis of melanin is performed inside melanocytes, a type of cell that contains the enzyme tyrosinase [128]. Tyrosinase catalyzes 2 different reactions: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity), which are transformed into melanin by a series of nonenzymatic reactions [128, 129]. Melanogenesis, the synthesis of melanin pigments, is stimulated by exogenous UV light [128, 130, 131]. However, overproduction of melanin may cause hyperpigmentation of the skin. Some conditions caused by

localized hyperpigmentation in humans include lentigo, nevus, ephelis, a post-inflammatory state, and melanoma in pregnancy [17, 128, 132]. Tyrosinase inhibitors are often employed in cosmeceutical preparations as whitening agents for the treatment of pigmentary disorders [17, 128].

Tyrosinase inhibition assay

A tyrosinase inhibition assay can be performed to measure the whitening capability of the active ingredients of cosmeceutical preparations. The following is a description of the procedure as reported by Chiari et al. [133] and adapted by Liyanaarachchi et al. [17]. The method uses L-DOPA as the enzyme substrate. Briefly, 20 µL of various concentrations (10–50 µg/mL) of the test substances (plant extracts or other potentially active ingredients), mushroom tyrosinase aqueous solution (10 µL, 50 U/mL), and phosphate buffer (pH 6.8, 80 µL) are mixed and pre-incubated at 37 °C for 5 min. Then L-DOPA (90 µL, 2 mg/mL) is added and the mixture is incubated at 37 °C for 20 min. The amount of dopachrome is measured at a wavelength of 475 nm. Kojic acid dissolved in dimethyl sulfoxide is used as a positive control, while phosphate buffer is used as a blank control. The percent of tyrosinase enzyme is calculated using Eq. 2, where $A_{test\ sample}$ is the absorbance of the sample extract and $A_{control}$ is the absorbance of the assay using the buffer instead of the inhibitor (sample). The half maximal inhibitory concentration can be calculated for test samples with more than 50 % inhibition from the dose-effect curves, at 500 µg/mL.

Wound healing

Wounds are defined as a disruption of the normal anatomical structure and functional integrity of living tissue [134–136]. Wounds can be caused by physical, chemical, or thermal injury [137] and are classified as incisions, lacerations, abrasions, contusions, ulcers, and burns [136]. In response to damage, the tissue undergoes a complicated process of healing, which consists of 3 stages: inflammation, proliferation, and maturation [136]. To facilitate normal wound healing, the use of an appropriate wound therapy and wound care intervention (bandages) is necessary [138, 139]. Wounds that exhibit impaired healing by failure to progress through the normal stages of healing enter a state of pathologic inflammation and are considered chronic in nature. Such wounds are usually associated with ischemia, diabetes mellitus, venous stasis disease, and pressure. Non-healing or chronic wounds result in great health care costs due to the requirement of extensive treatment [136, 140, 141]. Alternative products that employ plant extracts as active ingredients could alleviate such costs. However, it is important to determine the wound-healing capabilities of potential ingredients.

Wound-healing models can be classified as either *in vitro* or *in vivo*. *In vitro* models are generally simple, rapid, involve minimal ethical considerations, and provide insight into the biochemical and physiological processes induced by the test compound [137, 142]. There are several standardized protocols, including proliferation [143], scratch [144], cell invasion [144], cell viability, proteomic, and genomic assays [139]. On the other hand, *in vivo* models are used to determine the degree of re-epithelialization, collagenation, neovascularization, and tensile or breaking strength of wounds. These models include excisions, incisions, superficial wounds, dead space, and burn wounds performed on mammalian models, usually those of rabbits or hamsters [137, 142, 145, 146].

In vitro wound healing models

In vitro cell culture models are classified as monoculture models and co-culture or 3-dimensional cell models. Monoculture models of the epithelium offer a useful platform to study the re-epithelialization process. The cultures consist of keratinocytes (preferably HaCaT cells, an immortalized human skin cell line [147]), primary keratinocytes, or epidermal stem cells [139, 148–151]. The cell line is grown in a monolayer *in vitro* and is subjected to a number of assays relevant to the wound healing process [139]. Many studies have used the presence of cytokines [138, 152–155], growth factors [148, 156–160], or the expression of several markers of the ECM [161, 162] as standards for the measurement of the wound healing processes [139]. Co-culture cell models more closely resemble the *in vivo* epithelial structure and consist of cells that are normally in communication during the wound healing process. An example is the model developed in the study by Sato et al. in 1997 [163], which used keratinocytes and fibroblasts in co-culture. The cell-cell interactions of the Sato model showed enhanced wound healing.

A review by Planz et al. [164] describes a number of modern *in vitro* skin models that could be employed for the assessment of wound healing capabilities of new products. Similarly, a review by Kucharzewski et al. [165] lists novel approaches to *in vitro* wound healing models using skin stem cells.

Different methods are used in order to introduce wounds on cell cultures. For monolayers, a common way to simulate a scratch wound includes the manual disruption of the intact monolayer using pipette tips, indirect physical exclusion by placing a physical barrier into the wells and other especially designed tools [166]

In vivo wound healing models

Even though animal skin does not resemble human skin *in vivo* tests provide important data that can be used to study the complex process involved in wound healing and offer a good platform for the study and testing of new active ingredients [167–168]. Among the most popular animal models used are rodents and rabbits. Rodent skin differs from humans in several ways: first, rodents have a thin epidermis, loose skin adherence, and dense hair which is thought to contribute to accelerate healing [168, 169]; they lack apocrine and eccrine glands [168, 169]; rodents also have a subcutaneous panniculus carnus muscle, which promotes rapid wound contraction; and finally, rodents have stronger immune systems [168, 170]. Nevertheless, their availability, low cost, and small size make them suitable for large-scale studies [168]. Rabbit models, on the other hand, can provide a closer approach to human skin; their cartilage is highly vascularized and can act as a wound bed that forces healing through re-epithelialization and granulation, like humans, rather than contraction [168, 171]. The principal downside of this model is their high breeding costs [168, 172]. Still, animal models are currently superior to *in vitro* models of wound healing and should be considered prior to human testing of potential active ingredients [168].

As mentioned above, several *in vivo* assays can be employed to evaluate the effectiveness or healing potential of possible active ingredients. One of the most commonly used is the excision wound model. This test offers important information on epithelization, the area of wound contraction, and the wound index. Additionally, it can be used to estimate collagen formation (hexosamine) [173, 174]. Briefly, the test animals (normally rats) are anesthetized using ketamine HCl at a dose of 80 mg/kg of body weight via the peritoneal route. Then the hair of the back region of the animals is shaved and an impression is made on the dorsal thoracic region, from which 300 mm^2 of skin is removed, using a scalpel and scissors to create a wound with a depth of not more than 2 mm [175]. To measure the wound area, progressive changes to the damaged tissues can be monitored with a full high-definition camera on predetermined days (e.g., days 2, 4, 8, 12, and 20) until the wound has healed. The photographs can later be used to calculate the area width in mm^2 [176]. The period of epithelization is determined by the time it takes for the scab to drop from the wound, since it is considered the endpoint of complete epithelization [177]. The wound index is measured using the scoring system described in the study by Zaouani et al. [178]. Assessment of hydroxyproline content is used to estimate the amount of collagen, a key marker of wound healing. For hydroxyproline estimation, the protein hydrolysate is prepared as follows: 50 mg of tissue is collected from the wound site and is placed into a glass tube, which has been autoclaved and neutralized to pH 7.0, containing 1.0 mL of 6 N hydrochloric acid. Then 1 mL of the protein hydrolysate is passed to a clean glass tube, while double distilled water is used as a blank control. Afterward, 1 mL of new copper sulfate solution 0.01 M is added to the test tube, followed by 1 mL of 2.5 N sodium hydroxide and 1 mL of 6% (v/v) hydrogen peroxide. The contents are mixed well, and the tube is heated for 5 min in a water bath at 80 °C and is then immediately chilled on ice. Then 4 mL of 3 N sulfuric acid is added to the tube followed by agitation. Later, 2 mL of Ehrlich reagent is added to the test tube, which is then incubated at 70 °C for 15 min in a water bath [179]. Afterward, the optical density is measured at a wavelength of 540 nm using a colorimeter. Finally, the animals are euthanized and the tissue is excised from the wound site for histopathological analysis. The tissue samples are separately stored in conservation solution for microscopic examination [180].

In Vitro Cell Culture Tests for Advanced Cosmeceutical Applications

As mentioned above, fibroblasts are commonly found in the connective tissue of numerous organs, including the skin, uterus, and gastrointestinal tract, among others. Fibroblasts create ECMs through the secretion of fibrous proteins and ground substances to improve the strength, form, and adhesion capabilities of the structural support of animal organs. The size of fibroblasts vary depending on performance, as active fibroblasts are larger and known to produce modified versions of commonly known proteins, such as collagen, elastin, reticular fibers, and fibronectin [181].

Fibroblasts are employed to test plant-derived cosmeceutical active ingredients *in vitro* [182]. An *in vitro* assay allows for the study of skin models as well as dermal toxicology and the efficacy of products. Some *in vitro* tests using cell cultures are used to assess the anti-aging and detoxification properties of cosmeceuticals by measuring cell proliferation, the oxidative stress response, DNA damage, and collagen formation. In addition, sun protection, skin whitening, and pigmentation are also studied with *in vitro* skin models. Hence, *in vitro* cell culture testing is a crucial tool for the development of cosmeceuticals [181]. The following sections discuss the most important aspects of this technology for the development of cosmeceuticals.

In vitro cell culture of fibroblasts for commercial cosmetics testing

In vitro tests are used to assess the safety of both the ingredients and final cosmeceutical products as well as for justification of efficiency claims. Currently, *in vitro* tests are mostly employed for safety assessment due to the consumer's desire for "cruelty-free" products [6], while *in vivo* tests, which involve volunteers, are used to assess efficacy. However, the use of *in vitro* tests for validation of cosmeceutical claims provides certain advantages, including safety, during the early phases of new product development while offering quicker results and a more cost-effective experimental setting that allows for formulation and screening of ingredient characteristics within ranges that would not be feasible with volunteers [183].

Skin fibroblasts are spindle-shaped cells with long cytoplasmic prolongations derived from multipotent mesenchymal cells, which are closely linked to fibers responsible for dermal architecture and resilience. Additionally, dermal fibroblasts synthesize various growth factors and cytokines that are important in the regeneration processes [183]. Fibroblasts are also suitable for testing the effect of natural compounds on cellular senescence and also the ability of natural compounds to protect from unfavorable environmental conditions [184, 185]. Furthermore, fibroblasts stimulate keratinocyte proliferation and reduce some of the most common signs of skin aging, such as wrinkle formation, elastosis, and loss of skin tone. [186]. The following paragraph describes common techniques to test the characteristics of cultured fibroblasts.

Cultures of human fibroblasts are generally established using a cell line from ATTC. Briefly, the cells are cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic solution (penicillin or streptomycin) at 37 °C with an atmosphere of 5% CO_2/95% air. The cells are subcultured by trypsinization while changing the culture medium every 2–3 d. Cell proliferation is usually assessed using the Mosmann method [187], which is a colorimetric assay based on the reduction of the yellow tetrazolium salt MTT by living cells. In the presence of mitochondrial succinate dehydrogenase found in living cells, the tetrazolium ring of MTT is reduced and forms a violet product, formazan. Thus, the yellow solution becomes purple and the intensity of the coloration is proportional to the number of living cells. Cell proliferation is a desirable effect of plant-derived compounds in cosmeceuticals. For the test, cells at a concentration of 5×10^4 cells/mL in 100 µL of complete culture medium are seeded into the wells of a 96-well

plate, which is then incubated under the same conditions as stated above for 24 h. The medium is then removed, and 100 µL of the samples (potential cosmeceutical ingredient) prepared in DMEM containing 1% (v/v) antibiotic solution or negative control (DMEM containing 1% [v/v] antibiotic solution without sample) is added into the wells. After 48 h, 25 µL of MTT (5% w/v in phosphate-buffered saline) is added to each well and the plates are incubated at 37 °C for 4 h. Afterward, the medium is removed and 200 µL of dimethyl sulfoxide is added to the wells and the plate is incubated for 10 min. Following incubation, absorbance is read at 550 nm using a microplate reader [188]. Cell cytotoxicity can be determined using the sulforhodamine B assay. Briefly, cells (1×10^4 cells/well) are seeded into the wells of a 96-well plate and the plate is incubated for 24 h. Afterward, the cells are treated with different concentrations of the samples for 72 h. The adherent cells are fixed, washed, and dyed, and then the absorbance is measured at 540 nm. The viability of the treated cells is compared with that of the control cells treated with absolute ethanol [189–191]. To determine the antioxidant activity of the proposed active ingredients, the following process can be employed. First, fibroblasts are incubated for 48 h before treatment. Then the culture media is replaced with different concentrations of the test samples and the solvents (absolute ethanol) are dissolved in culture media for 24 h. Then the media is replaced with fresh culture media containing 150 µM H_2O_2 and the plate is incubated for an additional 3 h. The cells are then fixed, washed, dyed with sulforhodamine B, and solubilized with 10 mM $(HOCH_2)_3CNH_2$. Finally, absorbance at 540 nm is measured and cell viability is calculated [189–191]. In order to assess the anti-aging capabilities of the active compounds, collagen production and HA quantification can be performed. For collagen production analysis, dermal fibroblasts at a density of 5×10^4 cells/wells are seeded into the wells of 24-well culture plates in complete culture media. After 24 h, the medium is removed and 2 mL of either the sample, negative, or positive control in DMEM containing 1% (v/v) antibiotic solution is added to each well. The plates are then incubated at 37 °C for 48 h and collagen production is measured using the Sirius red staining procedure. With this method, the medium is removed and the cells are washed twice with phosphate-buffered saline and then fixed for 1 h with 1 mL of Bouin's solution at room temperature. After fixation, the Bouin's solution is removed and the cells are washed twice with distilled water and then stained with 1 mL of Sirius red solution (0.5 g of Sirius red 80 in 500 mL of a saturated aqueous solution of picric acid) for 1 h while shaking at room temperature. Samples are washed successively with distilled water and 0.01 M HCl to remove unbound dye. Finally, the bound dye is solubilized in 500 µL of 0.1 M NaOH for 1 h while shaking and absorbance is read at 550 nm [188]. Lastly, for HA quantification, an ELISA-like HA test plate can be employed following the instructions of the manufacturer (e.g., R&D Systems).

In vitro cell culture for skin penetrability

The skin is the primary entrance point for cosmeceuticals, even though one of its principal functions is to act as a barrier to protect the body from the outside environment, effectively preventing large molecules from entering the body. The former presents a challenge for every cosmeceutical formulation, since, in order to target the desired pharmacological and cosmetic responses, the active metabolites must penetrate the skin [182].

The evaluation of percutaneous permeation of molecules is a key step in the testing of dermal and transdermal delivery systems. If the drugs are intended for delivery to humans, the most appropriate setting for assessment is an *in vivo* human model. However, human experimentation may not be possible because of ethical, practical, and economic reasons, particularly in the early phases of development [192].

The most relevant model for the evaluation of *in vitro* skin absorption of drugs is human skin excised from cadavers or obtained from plastic surgeries. However, the availability of human skin is limited; therefore, animal models are often employed. In all cases, it is important to perform *in vitro* screening of drug permeation from different formulations and to identify factors that increase the transdermal flux of drugs to predict the *in vivo* behavior of different drug delivery systems [193].

Full-thickness skin or dermatome tissues have been used for *in vitro* permeation assays. *In vitro* methods enable precise control of experimental variables with use of the simplest protocols. However, *in vitro* assays cannot fully reproduce the complexity of biological systems; thus, *in vivo* evaluations are recommended to validate the results and, if possible, to establish an *in vivo–in vitro* correlation [193].

Most tests are physical experiments that use membranes, which are either mammalian or synthetic in nature, across which the permeation of a chemical is measured experimentally [194]. A timeline of the *in vitro* cell cultures used for skin permeability tests during the past decade is presented in ▶ **Table 3**. The skin parallel artificial membrane permeability assay provides a suitable platform as a fast, cost-efficient, and high throughput technology. This assay also employs a 96-well plate that contains an artificial membrane, mimicking the properties of stratum corneum *in vitro* [205]. This modification of the original method comprises an acceptor plate composed of magnetic stirrers, an artificial membrane, as previously stated, and a patch, all of which are placed over a reservoir (i.e., plate holder) [205].

In vivo tests often present a lack of correlation in the permeation capabilities of molecules across from different application sites in the same animal model due to variations in skin thickness. According to Netzlaff et al. [206], the amount of free fatty acids and triglycerides and the density of hair follicles are important factors causing differences between the skin barriers among species [207]. Rodent skin is typically employed in percutaneous permeation studies, with rat skin presenting more structural similarities to human skin; nevertheless, rodent skin, except rat skin, generally has higher permeation rates than human skin [207–209].

Perspectives in Cosmeceutical Development

Nowadays, the public has become more aware of the importance of skin care and professionals are often consulted to obtain recommendations and make better selections of appropriate products. A cross-sectional survey of dermatology residents and der-

▶ **Table 3** Timeline of the advancements on *in vitro* cell culture tests for skin permeability.

Model	Testing	Results	Reference
Human living skin equivalent models	Disodium isostearyl 2-O-L-ascorbyl phosphate is evaluated as a topical prodrug of amphiphilic vitamin C	Advantageous as a readily available source for skin care applications	[195]
Human skin equivalence *in vitro*	Combinations of additives for development of *in vitro* test	A pronounced effect on the permeability barrier	[196]
Cultured skin cells and snap freezing	To determine the barrier function of skin	A modified barrier dye penetration assay	[197]
Skin membrane	Passive drug permeation	Mechanistic interpretation of permeability beyond their predictive abilities	[198]
Cultured T-cells	DNA vaccination in the skin using microneedles	Induces higher humoral and cellular immune	[199]
In vitro epidermis	Regulations on the production of fatty acids Metabolism and permeability	Site where regulations on the production of fatty acids with more than 26 carbons occur; the barrier capabilities of its permeability are kept by extracellular lipid lamellae.	[200]
A human skin model with cultured cells	Synthesized valiolamine derivatives	N-2(-hydroxycyclohexyl) valioamine shows a stronger inhibitory effect on melanin production voglibose	[201]
Dermal membrane	Microneedles of a novel copper peptide	Ability to deliver other peptides or cofactors across the dermal membrane	[202]
Dermal cells	nanomicelles with high-pressure homogenization to raise skin permeability without irritation	It shows potential as a transdermal delivery system, specifically in cases respecting hyperpigmentation.	[203]
Human kidney cells	molecular mechanisms of the skin permeability barrier	Phospholipase domain-containing protein 1 is directly involved in acylceramide synthesis as a transacylase	[204]

matology faculty members performed in 2015 found that dermatology residents feel that education on these products should be included in residency training [210]. Recognition by the medical community of the variety and functionality of cosmeceutical products available on the market would bring more attention to the industry and, if the claims purported by the products are substantiated by tests like the ones mentioned above, this could lead to the validation of cosmeceuticals as a separate category of products from cosmetics.

Recent studies have shown that antioxidant-rich plant extracts can have dual proposes in a cosmeceutical formulation, apart from the traditional health benefits like anti-aging, anti-inflammatory, and antimicrobial activities, but can also function as preservatives by preventing the oxidation of oils present in cosmeceutical products. Another new trend in cosmeceutical development is the use of delivery systems, such as liposomes, microsomes, transferosomes, lipid nanoparticles, polymeric microparticles, and nanoparticles, to facilitate penetration of the skin barrier by the active ingredients to reach appropriate sites [211].

Awareness of the ecological crisis currently afflicting our planet has resulted in a surge of consumer concerns with the environmental impact of the development, manufacturing practices, and quality control procedures of the cosmetics industry. The industry response has been to create new green cosmetic products and the implementation of new environmentally friendly analytical tools, ingredients, and processes. The most polluting step in cosmeceutical development from natural sources is the extraction of active ingredients. New methods include the use of less polluting solvents or solvent-free processes, like microwave irradiation [211–213].

Finally, as mentioned above, new regulations, specifically in the European Union, have restricted the use of *in vivo* testing to assess the safety of cosmetic ingredients, which has resulted in the creation of new *in vitro* methods [38]. In the coming years, we expect emerging countries in South America, Asia, and Africa to implement more such regulations.

Conclusions

The cosmeceutical industry has continued to grow rapidly, and trends in the marketplace have highlighted the demand for new "natural," "green," and "cruelty-free" products. With this in mind, the discovery of new active ingredients from plants and other organisms has surged. However, the lack of industrial regulations, especially product claims, could lead to consumer dissatisfaction and to subsequent decreased sales. In this regard, the use of tests, especially *in vitro* methods, to validate product claims would be very valuable to recreate physiological conditions on a smaller and simpler scale. Due to the changing regulations for the safety assessment of cosmetics, it is expected that new *in vitro* methods for claim authentication and safety appraisal will be developed and validated in the near future.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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