cosmetics

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Review

Harmonizing In Vitro Techniques for Anti-Aging Cosmetic Ingredient Assessment: A Comprehensive Review

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Abstract: Skin grows old due to intrinsic factors, such as age and associated hormonal changes, and external factors, like solar radiation and chemical substances to which we are exposed. With age, skin shows thinning, laxity, pallor, increased dryness, and the appearance of wrinkles, attributed to a decrease in collagen and elastin produced by fibroblasts. Several theories explain skin aging, including the free radical formation, neuroendocrine, and mitochondrial decline theories. Based on a scientific understanding of skin behavior, different in vitro methods are used to evaluate the effects of new ingredients in cosmetics. Commonly used methods include anti-collagenase, anti-elastase, and anti-hyaluronidase activity alongside techniques utilizing skin cells or 3D models. Although these methods are recognized and widely used, they lack standardization. In this review, a literature search has been conducted to examine the characteristics and variations of these methods across the laboratories. A key issue identified in this review is that many papers provide insufficient detail regarding their protocols. Moreover, the number of studies using cells is less significant than the ones determining enzyme inhibition. Our findings revealed that, in many cases, there is limited information available, underscoring the urgent need to initiate a comprehensive standardization process for the methodologies used to demonstrate anti-aging activity.

Keywords: anti-aging; in vitro techniques; anti-collagenase; anti-elastase; anti-hyaluronidase; standardization

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1. Introduction

Aging is a physiological process resulting from the passage of time, with well-known triggering mechanisms and observable changes. The skin exhibits a series of characteristic alterations, such as loss of collagen, atrophy of the dermis, degeneration of the elastic network, and loss of hydration, inducing wrinkle formation [1]. Different studies have demonstrated the effect of decreased mitochondrial function on skin aging, promoting wrinkle formation and changes in pigmentation [2].

The appearance of the skin differs from young to old, as is shown in the following figure (Figure 1), where we can observe that changes in fibroblasts, keratinocytes and melanocytes as well as in the extracellular matrix (ECM) result in a dysfunctional epidermal barrier, thinner dermis and induction of chronic inflammation [3,4].

The changes observed in the skin result from the combination of different intrinsic and extrinsic factors. Intrinsic factors are the consequence of the general aging process, and extrinsic factors include air pollution, ultraviolet (UV) exposure, diet, and smoking, among others [4,5].

Individuals often attempt to conceal the effects of aging on their skin, and consequently, numerous surgical procedures are available to reverse these effects and transform the appearance of aged skin into a more youthful look. However, not only can a better skin appearance be achieved through surgical means, but cosmetics also play a significant role. The cosmetic industry has developed numerous formulations aimed at producing

anti-aging effects, all of which are based on the pursuit of the best ingredients and extensive research. There exists a recommendation about the characteristics that ingredients of anti-aging cosmetics should achieve, including antioxidant activity and should also prevent the degradation of skin components, e.g., collagen, elastin or hyaluronic acid, as well as protection against increased melanin synthesis [6]. Various methods exist to demonstrate the anti-aging effects of cosmetics, from tests on volunteers conducted with finished products [7–9] to in vitro methods employed for the discovery of new ingredients [10]. These methods are based on the current understanding of the cutaneous aging process. Many of these methods have been described in the recent excellent review by Cruz et al. in 2023 [11]. In that review, examples of studies utilizing different methods are presented, though not in depth. In contrast, the objective of this manuscript was to explore whether standardized protocols exist by analyzing the most widely used methods with a focus on material and method descriptions. We go beyond surface-level comparisons by critically highlighting the key discrepancies between these methods and offering new insights that have not been addressed in previous reviews.

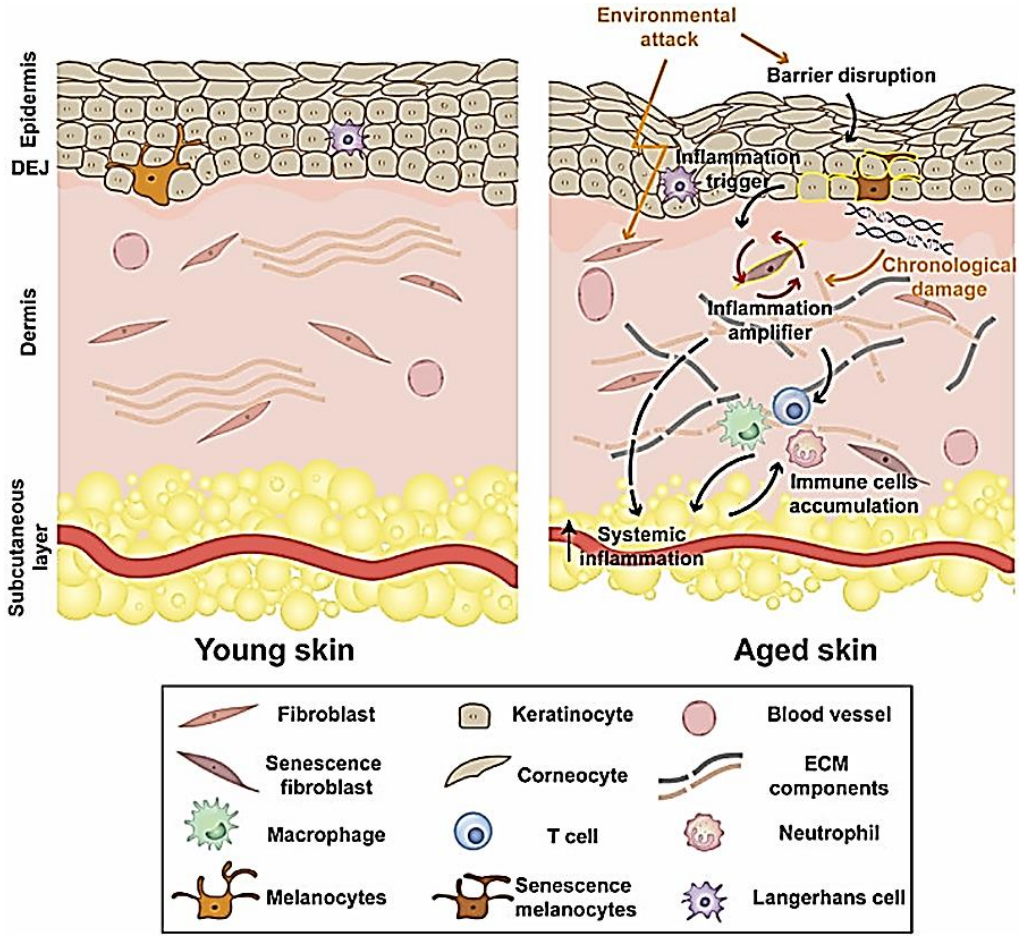

Figure 1. Schematic representation of skin aging and the involved cells. Reprinted from Ref. [4].

In cosmetic research, various methods have been used to explain the same biological activity. However, significant discrepancies arise due to differences in protocols—such as incubation time, temperature, solvents, wavelength readings, signal types, and the use of standards or controls. These methodological variations can lead to inconsistent or even erroneous conclusions despite using the same materials. In this review, we aim to thoroughly analyze these discrepancies, providing a detailed comparison of the most employed techniques and highlighting the risks of misinterpretation stemming from protocol inconsistencies.

2. Materials and Methods

A search was conducted across PubMed following the recommendations of the PRISMA statement [12]. The search was performed by combining representative keywords, namely anti-aging cosmetics, and was limited to the title, abstract, or index words. This first search was refined by including only the documents published between 2003 and 2023. Then, a second search among the documents retrieved was performed, including keywords such as in vitro, anti-aging, and cosmetic, and it was limited to the last 10 years.

The found documents have been classified in an Excel (Microsoft Office 2016) sheet where the following fields have been recorded: authors, title of the work, journal, year of publication, type of document (article, review, book chapter, conference abstract, etc.), and language. Then, those records not written in English, as well as those that were not original articles (reviews, conference abstracts, clinical studies, letters, book chapters, etc.), were discarded. The documents were assessed for eligibility by screening the title and abstract.

Finally, we have analyzed the different methodologies and the differences among the different papers using the same methodology. For each methodology, a minimum of ten articles have been reviewed, and the information recorded.

3. Results and Discussion

In the first PubMed search, we looked for papers containing the keywords "antiaging" and "cosmetics" to explore research related to anti-aging activity in the cosmetic industry in the last twenty years. The number of studies obtained was 1118, and the evolution from 2003 to 2013 is presented in Figure 2. The studies in 2024 have not been included because they are not representative of the total number of studies for the entire year since we are only halfway through the year. We can see a progressive increase in the number of studies from 2003, but a more significant increase is observed in the last 7 years, including 2020; nevertheless, it was the year of the pandemic.

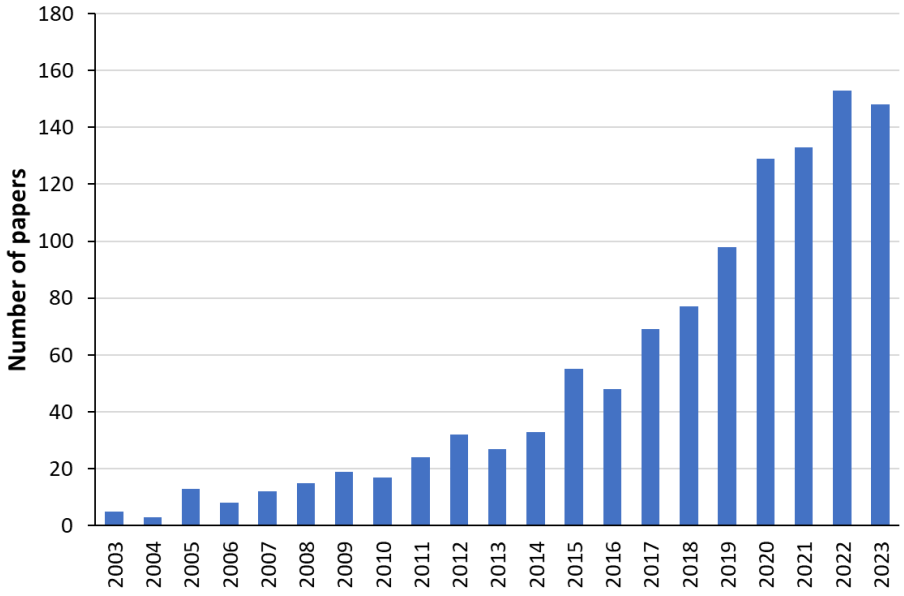

Figure 2. Evolution of the number of papers related to anti-aging cosmetics from 2003 to 2023.

Similarly, we searched for papers related to the in vitro methods to study the anti-aging effect of cosmetics in the last ten years (2013–2023). The results are presented in Figure 3, where a progressive increase can be observed in the number of studies, with a total of 258 studies.

Finally, we conducted an analysis of the various studies found in the last ten years focusing on the different methods proposed for evaluating anti-aging activity. In our analysis, we considered the methodological differences as well as cited references to methods by

the different authors. In the following subsections, we explain the main findings for each of the methods analyzed.

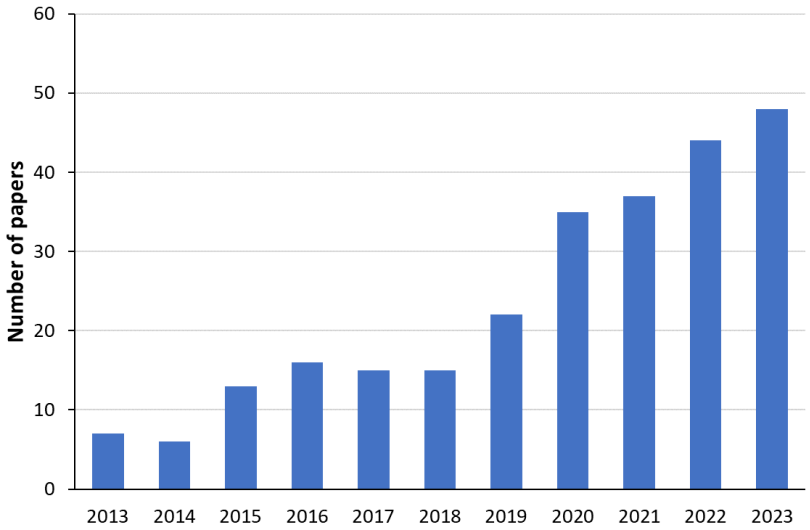

Figure 3. Evolution of the number of papers from 2013 to 2023 related to the in vitro methods to study anti-aging effects of cosmetics.

3.1. Methods Based on Antioxidant Capacity

As aging induces oxidative stress on the skin, it is essential to use straightforward assays to evaluate the potential anti-aging effects of cosmetics and their ingredients. These assays mainly focus on demonstrating the antioxidant activity. Some studies combined the evaluation of antioxidant ability with the effects on different enzymes, whereas other studies exclusively utilized the antioxidant effect to demonstrate anti-aging activity. Figure 4 shows the number of papers using the different assays used to evaluate the antioxidant capacity. The most commonly used method is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay used either as a standalone method [13–15] or in combination with 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [16–19] or with ABTS and ferric reducing antioxidant power (FRAP) [20–22]. Other assays for studying antioxidant capacity, which have been used only in a few studies, include the measurement of antioxidant power (AP). This parameter quantifies both the antioxidant capacity and reactivity, determined by electron spin resonance, and has been employed in a single study with vitamin C as a positive control [23]. Additionally, the total antioxidant capacity (TOAC) [24] and the cupric reducing antioxidant capacity (CUPRAC) spectrophotometric methods were used when polyphenols and flavonoids were present [24,25]. Moreover, the inhibition of lipid peroxidation [14,15], the metal chelation ability [14,15,17], the oxygen radical absorbance capacity assay (ORAC) [25] or the Folin–Ciocalteu assay [19,20] were also reported.

One important aspect of these assays is the use of a well-known antioxidant chemical as a positive control or reference control to better identify and classify the antioxidant capacity of the compounds or extracts assayed. We found that the chemicals most used in the studies analyzed were ascorbic acid or vitamin C and Trolox, independent of the assays performed. In the case of the DPPH assay, ascorbic acid is reported in seven studies [13–15,18,21–23], while other two reported the use of Trolox [19,25], and finally, only one study [24] used butylated hydroxytoluene [BHT] as a reference control. For ABTS, ascorbic acid is found in three studies [18,21,22] and Trolox in another three [17,20,25]. An analysis of FRAP indicates that BHT was used as a reference control [24], but a calibration curve with $FeSO_4$ [21,22,24] or Trolox is also described [20,25]. Finally, for the other assays (CUPRAC, OAC, TOAC, lipid peroxidation, chelation, and Folin–Ciocalteu), besides Trolox [25], the use of gallic acid [19,20], EDTA [14,15,17] and vitamin E [14,15] are reported.

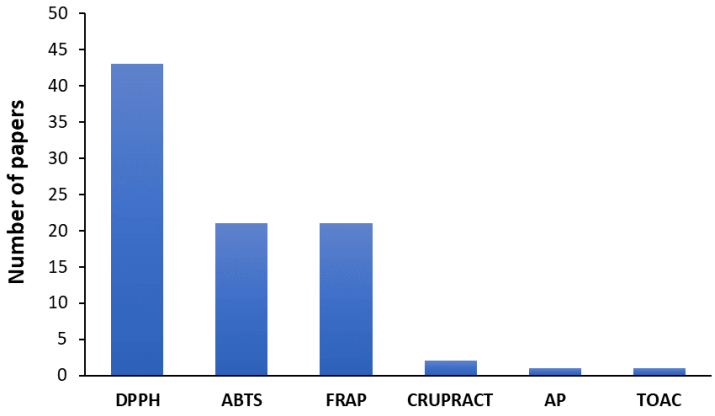

Figure 4. Number of studies using different antioxidant assays to evaluate the anti-aging activity of cosmetic ingredients in the last ten years.

3.2. Methods Based on Enzyme Inhibition

Oxidative stress is primarily responsible for triggering the activation of matrix metalloproteinases (MMPs), such as collagenases, elastases, and hyaluronidases. These enzymes contribute to the degradation of collagen, elastin, and hyaluronic acid (HA), which are key components of the extracellular matrix (ECM) essential for maintaining skin elasticity, firmness, and regeneration. Hyaluronic acid, a non-sulfated glycosaminoglycan, plays a crucial in the dermal and epidermal compartments of the skin by retaining water, promoting skin rejuvenation, and increasing viscosity. Skin hydration is dependent on the hyaluronic acid bound to water in the dermis and the vital area of the epidermis [26]. During the aging process, hyaluronic acid levels decrease due to the synthesis of hyaluronidase, an enzyme that degrades hyaluronic acids, leading to a loss of skin strength, flexibility and moisture. Consequently, one anti-wrinkle strategy involves prolonging skin moisture by preserving HA contents in the skin. Therefore, a method to study anti-aging activity is to determine the inhibitory effect of cosmetics on the various enzymes responsible for the degradation of the extracellular matrix components.

Tyrosinase is another enzyme associated with aging, playing a crucial role in the synthesis of melanin, a pigment produced by melanocytes through melanogenesis. Melanin provides a protective function for the skin against environmental factors, particularly ultraviolet (UV) radiation. However, excessive melanin production can lead to skin disorders such as melasma and age spots, contributing to premature aging. Consequently, the inhibition of tyrosinase activity is a strategy employed in anti-aging cosmetics [27]. Figure 5 illustrates the various assays used in the analyzed studies to inhibit different enzymatic activities as a strategy to demonstrate anti-aging effects.

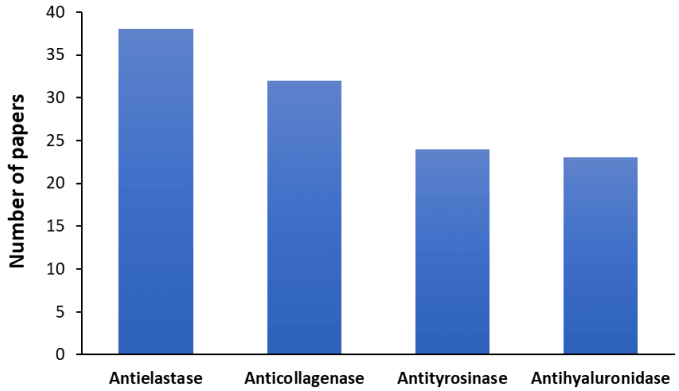

Figure 5. Number of papers using the effect of different enzymes as an assay to evaluate the anti-aging activity of cosmetic ingredients in the last ten years.

Elastase, a member of the MMPs family, breaks down elastin fibers, leading to the formation of wrinkles, loss of skin elasticity, and, consequently, skin aging. Reducing elastase levels is a strategy for achieving anti-aging effects. The anti-elastase assay involves the inhibition of the elastase enzyme, determined by a spectrophotometric method using porcine pancreatic elastase and N-succinyl-Ala–Ala–Ala–p-nitroanilide (AAAPVN) as a substrate. Table 1 presents the different protocols used in the evaluated papers, highlighting differences in wavelength, incubation time with the enzyme and the use of positive controls. The information about the origin of the elastase and the units are not always described in the material and methods.

Table 1. Characteristics of the anti-elastase assay to predict the anti-aging activity of cosmetics as described in the studies analyzed.

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Acero et al., **2024** [28]	NI, 0.03 U/mL	Yes	S, 410 nm	RT, 10 min	[29]
Altyar et al., **2020** [30]	NI, 3.33 mg/mL	Yes	S, 381–402 nm	NI, 15 min	[31]
Andrade et al., **2021** [32]	NI, 1 U/mL	Yes	S, 405 nm	25 °C, 10 min	[33]
Bakrim et al., **2022** [34]	PPE, 3.33 mg/mL	Yes	S, 400 nm	NI, 15 min	[35]
Barak et al., **2022** [24]	PPE, 3.33 mg/mL	Yes	F, Ex 365 nm–Em 410 nm	37 °C, 15 min	[36,37]
Buarque et al., **2023** [38]	PPE, ≥4.0 U/mg protein	No	S, 410 nm	NI	[33]
Castejón et al., **2021** [39]	PPE, 1 U/mL	Yes	S, 420 nm	30 °C, 15 min	[40]
Chaiyana et al., **2021** [41]	NI	Yes	NI	NI	[42]
Dymek et al., **2023** [43]	PPE, 10 µg/mL	No	S, 405 nm	25 °C, 15 min	NI
El-Nashar et al., **2022** [44]	Assay Kit	Yes	F, Ex 400 nm–Em 505 nm	37 °C, 5 min	[45]
Jiratchayamaethasakul et al., **2020** [46]	PPE, 7.5 U/mL	No	S, 410 nm	25 °C, 10 min	[29]
Jugreet et al., **2022** [47]	PPE, 4.9 U	Yes	S, 405 nm	37 °C, 5 min	[48]
Lee, et al., **2020** [49]	PPE, WS: 1 U/mL	No	S, 410 nm	25 °C, 20 min	[50]
Lim et al., **2022** [51]	NI, 0.1 U/m	No	S, 410 nm	37 °C, NI	[52]
Madan et al., **2018** [53]	Assay kit	No	S, 410 nm	25 °C, 15 min	[54]
Michalak et al., **2023** [55]	Assay Kit (NE)	Yes	F, Ex 400 nm–Em 505 nm	37 °C, 5 min	[56]
Nutho, et al., **2024** [57]	PPE, NI	Yes	S, 410 nm	NI	[50]
Pagels et al., **2022** [58]	NI, 1 U/mL	No	S, 405 nm	37 °C, 10 min	[59]
Tawfeek et al., **2023** [60]	PPE, 3.33 mg/mL	Yes	S, 400 nm	NI, 15 min	[35]
Vaithanomsat et al., **2022** [61]	NI, 7.5 U/mL	Yes	S, 410 nm	25 °C, 20 min	[42]
Wang, et al., **2024** [62]	NI, 600 mU/mL	Yes	S, 410 nm	25 °C, 15 min	[63]
Wichayapreechar et al., **2024** [64]	PPE, 1 mM	Yes	S, 410 nm	RT, 5 min	[52]
Widowati et al., **2016** [65]	PPE, 0.5 mU/mL	No	S, 410 nm	25 °C, 15 min	[52]
Xu et al., **2022** [66]	NI, 0.5 U/µL	No	S, 405 nm	37 °C, 15 min	[67]
Younis, et al., **2022** [68]	PPE, 3.33 mg/mL	Yes	S, 400 nm	RT, 15 min	[31]

* Origin and concentration of the enzyme used; F—fluorimetry; NE—neutrophil elastase; NI—not indicated; PPE—porcine pancreatic elastase; RT—room temperature; S—spectrophotometry.

As observed, the majority of studies evaluated (64%) used positive controls epigallocatechin gallate (EGCG) the most used control [24,41,61,62,64,68], kojic acid [34,60], oleanolic

acid [28,57], and ursolic acid [32,47] were other substances used as control, and in three studies the positive control was not provided [30,39,44]. Spectrophotometry is the preferred technique to evaluate the inhibition of the elastase enzyme because only three studies used fluorimetry. Also, two studies reported the use of an elastase enzyme assay kit. Regarding the steps and conditions of the reaction, 17 studies incubated the samples with the enzyme prior to adding the substrate, whereas 3 reported a preincubation with the substrate before adding the elastase, the other three incubated both enzyme and substrate, and in two articles is not reported. These differences, together with those in time and temperature, can be attributed to the different protocols followed; however, Thring et al. [52] are reported by three studies [51,64,65] with differences in incubation time and temperature and preincubation with the elastase enzyme [64,65] or with the substrate [51].

The degradation of collagen is induced by collagenases, a type of metalloproteinases produced by keratinocytes and fibroblasts. Levels of these specific metalloproteinases are elevated in aged skin [69]. Thus, one strategy to reduce wrinkles is the inhibition of these enzymes, which is a method used to evaluate the anti-aging activity of cosmetics. The proposed in vitro method involves the spectrophotometric determination of enzyme activity using collagenase from *Clostridium histolyticum*. While this methodology has been described in various studies, there are notable differences among them. Table 2 presents several studies employing this in vitro method, highlighting variations in incubation time, the wavelength used to determine the enzymes and the references related to the methodology. It is also important to note that some studies lack crucial details such as incubation time and temperature, or when provided, the information is inconsistent.

Table 2. Characteristics of anti-collagenase assay to predict the anti-aging activity of cosmetics as described in the studies analyzed.

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Acero et al., **2024** [28]	CH, 0.8 U/mL	Yes	S, 340 nm	25 °C, 15 min	[52]
Altyar et al., **2020** [30]	CH, 0.8 U/mL	Yes	S, 335 nm	NI, 15 min	[52]
Andrade et al., **2021** [32]	CH, 1 U/mL	Yes	S, 450 nm	37 °C, 10 min	[52,70]
Ashmawy, **2023** [71]	Assay Kit	Yes	F, Ex 490 nm–Em 520 nm	25 °C, 15 min	[70]
Bakrim et al., **2022** [34]	CH, 0.8 U/mL	Yes	S, 490 nm	NI, 15 min	[35]
Barak et al., **2022** [24]	CH, 0.8 U/mL	Yes	NI	NI, 15 min	[72]
Buarque et al., **2023** [38]	CH, 0.8 g/L	No	S, 345 nm	NI	[70]
Castejón et al., **2021** [39]	Assay Kit	No	NI	NI	NI
Chaiyana et al., **2021** [41]	CH, 0.16 U/mL	Yes	S, 340 nm	37 °C, 15 min	[42]
El-Nashar et al., **2022** [44]	Assay Kit	Yes	F, Ex 490 nm–Em 520 nm	RT, 15 min	[67]
Jiratchayamaethasakul et al., **2020** [46]	CH, 200 U/mL	No	S, 550 nm	43 °C, 60 min	[73]
Jugreet et al., **2022** [47]	Assay Kit CH, 0.2 U/mL	Yes	F, Ex 4850 nm–Em 515 nm	37 °C, 15 min	[74]
Madan et al., **2018** [53]	Assay Kit	No	S, 345 nm	37 °C, 20 min	[65]
Michalak et al., **2023** [55]	Assay Kit	Yes	F, Ex 490 nm–Em 520 nm	RT, 15 min	[75]
Nutho et al., **2024** [57]	CH, NI	Yes	S, 335 nm	NI	[50]
Pagels et al., **2022** [58]	NI, 1 U/mL	No	S, 345 nm	37 °C, 15 min	[32,70]

Table 2. *Cont.*

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Tawfeek et al., **2023** [60]	CH, 0.8 U/mL	Yes	S, 490 nm	NI, 15 min	[35]
Vaithanomsat et al., **2022** [61]	NI, 0.5 U/mL	Yes	S, 340 nm	37 °C, 15 min	[52]
Wichayapreechar et al., **2024** [64]	CH, 0.8 U/mL	Yes	S, 335 nm	RT, 10 min	[52]
Widowati et al., **2016** [65]	CH, 0.01 U/mL	No	S, 335 nm	37 °C, 20 min	[52]
Xu et al., **2022** [66]	NI, 2 U/mL	Yes	F, Ex 320 nm–Em 405 nm	37 °C, 15 min	[76]
Younis, et al., **2022** [68]	CH, 0.8 U/mL	Yes	S, 490 nm	RT, 15 min	[52]

* Origin and concentration of the enzyme used; CH—clostridium histolyticum; F—fluorimetry; NI—not indicated; RT—room temperature; S—spectrophotometry.

In a similar way as in the case of anti-elastase activity, the majority of studies used positive controls (73%), and EGCG was the preferred one in 50% of them [24,30,32,41,61,64,66,68]. Only seven studies cite the same reference [52], but one of them uses a very different wavelength (490 nm instead of 340 nm) [68]. Additionally, fluorometric determination kits are available, showing differences in excitation and emission wavelengths and incubation temperature. In contrast to the case of elastase, almost all studies (80%) incubate the collagenase enzyme before adding the substrate to start the reaction.

The inhibition of hyaluronidase is of significant importance due to its critical role in skin aging. Consequently, skincare cosmetic products with high efficacy against hyaluronidase activity have been well developed. The activity of hyaluronidase is determined using UV spectroscopy by measuring the amount of N-acetylglucosaminoglycan formed from the degradation of hyaluronic acid using hyaluronidase from bovine testes [77]. Table 3 outlines the various protocols used, highlighting the different measurement procedures, including spectrophotometry, fluorimetry, turbidimetry, and gel electrophoresis.

Table 3. Characteristics of the anti-anti-hyaluronidase assay to predict the anti-aging activity of cosmetics as described in the studies analyzed.

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Acero et al., **2024** [28]	HBT, 1.5 U/µL	Yes	S, 600 nm	37 °C, 10 min	[78]
Altyar et al., **2020** [30]	HBT Type I-S, NI	Yes	S, 585 nm	NI	[79]
Ashmawy, **2023** [71]	HBT, 7900 U/mL	Yes	S, 585 nm	37 °C, 20 min	[80]
Bakrim et al., **2022** [34]	NI, ST: 1.5 mg/mL	Yes	S, 600 nm	100 °C, 3 min	[35]
Barak et al., **2022** [24]	NI	No	S, 600 nm	37 °C, 20 min	[81,82]
Castejón et al., **2021** [39]	HBT Type I-S, 2100 U/mL	Yes	S, 585 nm	37 °C, 20 min	[83]
Chaiyana et al., **2019** [42]	HBT, 0.1 g/mL	No	SDS-PAGE	37 °C, 48 h	NI
El-Nashar et al., **2022** [44]	HBT, 7900 U/mL	Yes	S, 585 nm	37 °C, 20 min	[84]
Jiratchayamaethasakul et al., **2020** [46]	NI, 8 mg/ml	No	S, 585 nm	37 °C, 20 min	[85]
Lee, et al., **2020** [49]	HBT, 3000 U/mL	No	S, 570 nm	37 °C, 40 min	[86]
Lim et al., **2022** [51]	NI, 585 U/mL	No	T, 600 nm	37 °C, 10 min	[87]
Madan et al., **2018** [53]	NI	No	T, 540 nm	37 °C, 10 min	[88]
McCook et al., **2015** [89]	HBT type IV-S, NI	Yes	T, 595 nm	NI	[90]

Table 3. *Cont.*

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Pagels et al., **2022** [58]	NI, 900 U/mL	No	S, 560 nm	37 °C, 30 min	[91]
Tawfeek et al., **2023** [60]	NI, 1.5 mg/mL	Yes	S, 600 nm	NI	[35]
Tomou et al., **2021** [92]	HBT Type I-S, 400 U/mL	No	S, 590 nm	37 °C, 20 min	[77]
Vaithanomsat et al., **2022** [61]	NI, 1.5 U/mL	Yes	S, 600 nm	37 °C, 10 min	[52]
Wang, et al., **2024** [62]	NI, 500 mU/mL	Yes	S, 530 nm	37 °C, 20 min	[93]
Widowati et al., **2016** [65]	HBT Type I-S, NI	No	T, 600 nm	37 °C, 10 min	[29]
Younis, et al., **2022** [68]	NI, 1.5 mg/mL	No	F, Ex 545 nm–Em 612 nm	NI	[77,94]

* Origin and concentration of the enzyme used; F—fluorimetry; HBT—hyaluronidase from bovine testes; NI—not indicated; S—spectrophotometry; T—turbidimetry.

Only 50% of the studies analyzed used a positive control to compare the anti-hyaluronidase activity of the extracts or compounds assessed. Among the chemicals used as standards, oleanolic acid [28,61] and tannic acid [39,89] were the only ones used in more than one study.

Finally, Table 4 presents various studies using the anti-tyrosinase assay as a method to evaluate anti-aging activity.

Table 4. Characteristics of the anti-anti-tyrosinase assay to predict the anti-aging activity of cosmetics as described in the studies analyzed.

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Acero et al., **2024** [28]	NI, 200 U/mL	Yes	S, 475 nm	37 °C, 15 min	[95]
Andrade et al., **2021** [32]	Mushroom, 5000 U	Yes	S, 450 nm	37 °C, 5 min	[96]
Bakrim et al., **2022** [34]	Mushroom, 2500 U/mL	Yes	S, 475 nm	NI	[35]
Castejón et al., **2021** [39]	Mushroom, 50 U/mL	Yes	S, 475 nm	37 °C, 5 min	[83]
Chaiyana et al., **2021** [41]	Mushroom, NI	Yes	NI	NI	[97]
Dymek et al., **2023** [43]	Mushroom, 0.2 mg/mL	No	S, 475 nm	NI	[98]
El-Nashar et al., **2022** [44]	Assay Kit	Yes	S, 510 nm	25 °C, 10 min	NI
Herawati et al., **2022** [99]	Mushroom, ≥1000 U/mg of activity	Yes	S, 492 nm	RT, 5 min	[100]
Jiratchayamaethasakul et al., **2020** [46]	Mushroom, 1500 U/mL	No	S, 490 nm	37 °C, 12 min	[101]
Lasota et al., **2024** [16]	Mushroom, 500 U/mL Murine, 20 µg protein lysate	Yes	S, 450 nm	RT, 10 min RT, 4 h	[102]
Lim et al., **2022** [51]	Mushroom, 6 U/mL	No	S, 450 nm	37 °C, 10 min	[103]
Mohamadi et al., **2022** [104]	Mushroom, 50 mM	No	S, 490 nm	20 min	[105]
Nutho et al., **2024** [57]	Mushroom, 0.2 mg/mL	Yes	S, 475 nm	NI	[106]

Table 4. *Cont.*

Study	Enzyme *	Controls	Technique	Incubation	Reference Method
Pagels et al., **2022** [58]	NI, 50 U/mL	Yes	S, 475 nm	25 °C, 5 min	[107]
Patathananone et al., **2023** [108]	NI, 250 U/mL	No	S, 495 nm	37 °C, 10 min	[109]
Tawfeek et al., **2023** [60]	Mushroom, 2500 U/mL	Yes	S, 475 nm	NI	[35]
Vaithanomsat et al., **2022** [61]	NI, 1380 U/mL	Yes	S, 475 nm	NI, 10 min	[110,111]
Younis, et al., **2022** [68]	Mushroom, 2500 U/mL	Yes	S, 475 nm	NI	[112]

* Origin and concentration of the enzyme used; NI—not indicated; RT—room temperature; S—spectrophotometry.

All methods employ spectrophotometry with wavelength ranging from 450 to 495 nm, differing in incubation time and concentrations used except in the case of [99], which only indicates the specific activity provided by the supplier. Some studies do not indicate the origin of tyrosinase, but we assume they used tyrosinase from mushrooms. Moreover, Lasota et al. [16] have also used tyrosinase obtained from murine melanoma cells. The use of kojic acid as positive control is reported in 67% of the studies, and only [39] used quercetin as a well-known anti-tyrosinase chemical.

3.3. Methods Based on Cell Culture

3.3.1. Fibroblast Cell Culture

Fibroblasts are the most representative cells found in the dermis, and many studies use primary human dermal fibroblasts [113], human foreskin fibroblast cell lines [28], and murine fibroblast cell lines for in vitro research [104]. Previously, the cytotoxicity of the different ingredients was tested in these cells because ingredients should have poor cytotoxicity for cosmetic applications. In order to demonstrate the anti-aging activity of ingredients, the antioxidant activity of the ingredient on fibroblasts is assessed by measuring intracellular reactive oxygen species (ROS) production on cells in the presence of oxidative stimuli such as tert-Butyl hydroperoxide (TBHP) [113], H_2O_2 [28,104], or UV irradiation at 30 J/cm^2 [114]. Clearly, the protocols of oxidative stress are different using various oxidative stimuli; in the case of H_2O_2, the amount applied varies depending on the cell line used, then 1500 µM H_2O_2 for murine fibroblast, and only 200 µM H_2O_2 for the human foreskin fibroblast cell line.

Fibroblasts are dermal cells responsible for the synthesis of collagen and elastin. It is well-documented that aged fibroblasts exhibit reduced collagen synthesis capacity [115]. Therefore, using in vitro fibroblasts to measure the production of these fibers is a strategy employed to investigate anti-aging effects. Collagen type I (COL1A1) is the predominant structural protein in skin, while collagen type VII (COL7A1) forms anchoring fibrils in dermo-epidermal junctions, contributing to skin mechanical stability. During photoaging, the levels of COL7A1 decrease, weakening the bond between the dermis and epidermis [116].

In the present review, studies focusing on human dermal fibroblasts (HDFs) from multiple donors are highlighted. These studies determined the levels of COL1A1 and COL7A1 proteins after HDFs are exposed to various test products. After demonstrating their antioxidant activity, studies have shown that both Bakuchiol and retinol application significantly increases levels of COL1A1 and COL7A1 [23]. Other investigations using HDFs have evaluated the production of procollagen type I C-peptides assessed by ELISA [51,117,118] and CoL1A1 gene expression by Quantitative Reverse Transcription Polymerase chain reaction (Q-RTPCR) [119]. Additional studies quantified collagen and elastin using respective colorimetric kits [120], Sirius Red-based colorimetric microassay [121], or differentiated collagen types I and III by employing specific antibodies and secondary antibody conjugates [122].

In each instance, the protocols vary in terms of cell seeding density, incubation duration, and the method used for collagen assessment.

Other assays to study the anti-aging activity of ingredients have been based on the determination of the expression of genes encoding tyrosinase, collagenase, elastase, hyaluronidase and hyaluronic acid synthase in human dermal fibroblasts [123] and the production of hyaluronic acid by an enzyme-linked immune sorbent assay (ELISA)-like hyaluronic acid assay [121].

3.3.2. Keratinocyte Cell Culture

The human epidermis is mainly composed of keratinocytes and corneocytes, which are continuously produced through the differentiation of keratinocytes and arranged in multiple cell layers. The evidence suggests that the buildup of senescent cells with age may play a role in age-related skin changes. The senescent cells are characterized by their lack of ability to proliferate, resistance to apoptosis, and secretion of factors that promote inflammation and tissue breakdown. The characteristic thinning of aging skin is partly due to the reduced proliferation and renewal capacity of basal keratinocytes [124].

Some authors have used the ability to enhance keratinocyte proliferation as an indicator of the anti-aging activity of cosmetic ingredients. However, the number of articles related to keratinocyte activity in the evaluated period is significantly lower than those studying the effects on fibroblasts. The proliferation of keratinocytes induced by cosmetics has been studied in HaCaT cells by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay [125].

A model of photoaging can be studied in keratinocytes of the HaCaT cell line exposed to UV radiation, demonstrating the decrease in the production of interleukin 8 (IL8) and prostaglandin E2 after the treatment of the cells with potential antiaging agents [126].

3.3.3. Full Skin Models

A three-dimensional (3D) full-thickness in vitro skin model containing normal human epidermal keratinocytes and normal human dermal fibroblasts has been used to determine the polymerase chain reaction (PCR), the gene expression of genes that play important roles in skin biology as a method to demonstrate the activity of cosmetic ingredients with anti-aging activity. Authors have demonstrated an increase in genes related to keratinocyte differentiation and barrier function [127,128]. When studying gene expression, it is important to select the more significant gene related to the aging process.

The 3D skin model allows for the study of the cosmetic application for seven days and the posterior histology observation. The treatment with anti-aging compounds, such as resveratrol, can demonstrate an increase in collagen expression and stratification of epidermis [129]. The 3D model can be commercial or prepared in a lab [130].

3.3.4. Skin in a Chip

Innovative methodologies can be developed to study the skin. In this context, biochip technology with 3D culture skin-on-a-chip systems employs skin equivalents that mimic the characteristics of the human skin. The pumpless skin-on-a-chip (Figure 6) utilizes a microfluidic chip, creating a biomimetic environment that enables various physiological functions. The system supplies nutrients to cells and removes cell waste via microfluidic channels. Additionally, the pumpless skin-on-a-chip can be engineered to have a physiological residence time that matches the blood flow. Post-treatment analysis of filaggrin, involucrin, keratin 10, integrin, and collagen I genes in human skin on the chip can be used to demonstrate the anti-aging effects [131].

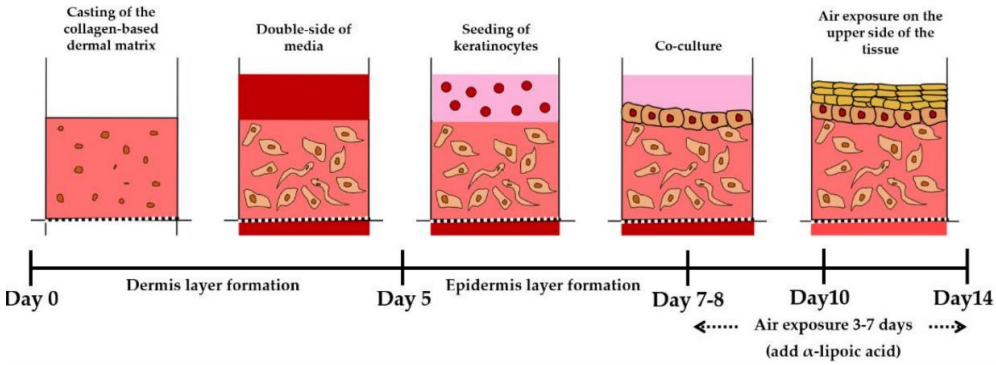

Figure 6. Schematic diagram of the 3D skin-model formation process in pumpless skin-on-a-chip. Reprinted from Ref. [131].

3.3.5. Skin Explants

Another strategy to study anti-aging cosmetics involves utilizing human skin obtained from volunteers irradiated with UV light. Post-treatment, the skin is fixed with 4% formaldehyde and subsequently dehydrated using an ascending concentration of ethanol. The tissue is then embedded in paraffin, sectioned, and mounted on slides. The sections are stained with Masson trichrome following the manufacturer's protocols to assess collagen and elastin production. A qualitative histological analysis revealed a marked increase in these components within the extracellular matrix [126].

Explants have also been used to assess collagen III production by immunostaining skin samples with monoclonal mouse anti-collagen III antibodies and the alkaline phosphatase/RED detection system. The antigen quantity on each slide was quantified by pixel intensity, and the distribution of the red staining within a defined area of the upper part of the dermis was analyzed using ImageJ. This analysis yields a staining score, which was normalized as a percentage relative to the untreated control [122].

In this review, we have analyzed publications from the last five years that studied the anti-aging effects of various cosmetics and cosmetic ingredients, with the aim of identifying the most commonly used methodologies to demonstrate this effect in vitro. It has been observed that most studies focused on demonstrating the antioxidant capacity of the tested products. These constitute the simplest, fastest, and most cost-effective methodologies, as they involve chemical methods that do not require special infrastructure, unlike cell cultures. The DPPH assay has been the most commonly used method to determine the antioxidant effect, as in most cases, this method was used alone [13] and, to a lesser extent, in combination with another assay. However, these in chemico methods are not enough to demonstrate the anti-aging activity; nevertheless, some studies used only these assays.

Knowledge of the skin aging process has enabled the development of simple methods that involve determining the inhibitory effect on enzymes that degrade extracellular matrix fibers, such as elastase and collagenase, leading to loss of firmness and the appearance of age-related wrinkles. Among the studies analyzed, the effects on elastase constitute the most commonly used method, followed by the effects on collagenase.

As previously mentioned, there are fewer studies based on the use of cell culture than those using chemical methods. Fibroblasts, representative cells of the dermis and producers of the extracellular matrix, have been the most commonly used cells. Secondly, the proliferation of keratinocytes has been used as a method to demonstrate a cosmetic's ability to reverse the effects of skin aging. In a few cases, reconstructed human skin models or explants obtained from the surgeries of volunteers have been used.

When the expression of genes is used as a model to demonstrate anti-aging activity, the selection of the more representative genes of this effect is crucial, especially when a large panel is used [127].

4. Conclusions

The number of studies about the applicability of different ingredients, in general from natural origin, for anti-aging cosmetics has increased significantly in the last few years. There are different strategies to demonstrate the anti-aging effect in vitro. Many of these studies focus on the antioxidant activity of these compounds and their ability to reduce the activity of enzymes responsible for age-related changes in the skin. These methods based on the activity of these enzymes present significant differences in protocols because there are no standardized methods, contrary to what happens with the toxicological methods, which are more rigorously defined. The number of studies using cells is less significant than the ones determining enzyme inhibition. Similarly, there are no standardized methods, with differences in the cell number seeded, the incubation time and so on. The lack of standardized methods in both enzyme- and cell-based assays creates challenges for drawing clear, comparable conclusions across studies.

Looking ahead, the establishment of standardized methodologies is essential to ensure the reliability and reproducibility of results. This is especially important as we move toward human trials, where consistency in preclinical findings can help avoid unnecessary or flawed studies involving volunteers. Developing universally accepted protocols for evaluating the anti-aging properties of new ingredients will enhance the credibility of research in this field and facilitate the transition from laboratory studies to real-world applications.

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Abbreviations

3D	three-dimensional.
7-AAD	7-aminoactinomicina D.
AAAPVN	N-succinyl-Ala–Ala–Ala–p-nitroanilide.
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
AP	Antioxidant power.
BHT	Butylated hydroxy toluene.
CH	Clostridium histolyticum.
COL1A1	Collagen type I.
COL7A1	Collagen type VII.
CUPRAC	Cupric reducing antioxidant capacity.
DPPH	2,2-diphenyl-1-picrylhydrazyl.
ECM	Extracellular matrix.
EGCG	Epigallocatechin gallate.
ELISA	Enzyme-Linked ImmunoSorbent Assay.
F	Fluorimetry.
FRAP	Ferric reducing antioxidant power.
HA	Hyaluronic acid.
HBT	hyaluronidase from bovine testes.
HDFs	Human dermal fibroblasts.
IL8	interleukin 8.
MMPs	matrix metalloproteinases.

MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide).
NE	neutrophil elastase.
NI	not indicated.
ORAC	oxygen radical absorbance capacity assay.
PCR	Polymerase chain reaction.
PPE	porcine pancreatic elastase.
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
Q-RTPCR	Quantitative Reverse Transcription Polymerase chain reaction.
ROS	Reactive oxygen species.
RT	room temperature.
S	spectrophotometry.
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis.
T	Turbidimetry.
TBHP	tert-Butyl hydroperoxide.
TOAC	total antioxidant capacity.
UV	Ultraviolet.

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