Review Article

A Review on Evaluation of Efficacy of Anti-Aging Cosmetic Formulation

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ABSTRACT

Skin aging is a multifactorial process that causes visible changes like wrinkles, loss of elasticity, and pigmentation of the skin. Aging is influenced by both internal and external factors. To guarantee both customer satisfaction and scientific credibility, the increasing demand for anti-aging cosmetics calls for a thorough assessment of their effectiveness. The purpose of this review is to give a summary of the methods used to evaluate anti-aging cosmetics, emphasising their applicability, benefits, and drawbacks. Cellular and molecular processes like collagen synthesis, elastase inhibition, and oxidative stress reduction can be studied using in vitro models. A more physiologically relevant platform for evaluating penetration, repair, and biomarker modulation is provided by ex vivo studies that use human skin explants. Clinical trials and other *in vivo* methods continue to be the gold standard for evaluating safety and effectiveness in real time. Clinical trials and other in vivo methods, which are frequently backed by non-invasive imaging, continue to be the gold standard for evaluating safety and effectiveness in real time. Complementarily, in silico techniques, use computational modelling to optimise formulations, predict bioactivity, and speed up compound screening. Combining these techniques shows that no single method is adequate; instead, a combination yields the most accurate evaluation of cosmetic efficacy. To sum up, the methodical use of complementary approaches guarantees anti-aging cosmetics' scientific legitimacy as well as consumer trust. Future developments in AI-powered simulations, bioengineered skin models, and next-generation imaging technologies should improve efficacy evaluation even more and spur industry innovation.

Keywords: Wrinkle reduction, Molecular docking, Anti-senescence effects, MTT assay, mRNA sequencing.

INTRODUCTION

The largest and most intricate organ in the human body, the skin serves as a barrier of defence between the body and the outside world and carries out numerous essential tasks.[1]. All living things experience skin ageing, which is a complicated, multifactorial biological process influenced by both exogenous (extrinsic) and endogenous (intrinsic) factors.[2] Exogenous or extrinsic factors (such as pollution, ionising radiation, chemicals, and toxins) and endogenous or intrinsic factors (such as genetics, cellular metabolism, and hormonal or metabolic processes) combine to influence the complex biological process of skin ageing. All these elements work together to cause cumulative structural and physiological changes that gradually alter each layer of the skin and change the appearance of the skin, especially in areas that are exposed to the sun [3]. However, distinct features like dryness, wrinkles, increased susceptibility to bruises, loss of elasticity, and mottled dyspigmentation [4]. It can be used to identify the characteristics of ageing skin, whether they are extrinsic or intrinsic. Since anti-aging cosmetics are frequently used to stop the skin's surface from showing signs of ageing, it's critical to assess their effectiveness using a variety of techniques, such as in vitro, in vivo, ex vivo, in silico, and instrumental approaches. The various evaluation techniques for determining the effectiveness of anti-aging products are displayed in Table 1 below.

Table 1: The various evaluation techniques for determining the effectiveness of anti-aging products

***In silico* methods**	
Molecular Docking Study	To predict the binding affinity of compounds with protein receptors
In Silico Prediction of Skin Absorption and Toxicity Assessment	Prediction of skin absorption and toxicity assessment by used the pkCSM webserver
***In vivo* methods**	
In vivo human testing by comparing treated and untreated skin areas	Study carried using healthy individuals by comparing treated and untreated skin areas.
In vivo Clinical and Instrumental Skin Evaluation	non-invasive imaging tool for evaluating skin parameters
***In vitro* methods**	
I. Cell Proliferation & Senescence	a. Cell Proliferation Assay b. Anti-Senescence Assay
II. ECM Protein Evaluation	a. Collagen Synthesis Assay b. Elastin Synthesis Assay
III. Cell Proliferation & Senescence	a. Anti-Collagenase Assay

	b. Anti-Elastase Assay c. Enzyme Inhibition Assay
IV. Pigmentation & Skin Barrier	a. Melanin, Procollagen, Filaggrin Quantification
V. Antioxidant Assays	a. DPPH Radical Scavenging Assay b. ORAC Assay c. Total Phenolic/Flavonoid d. Content (TPC/TFC)
Instrumental methods	
I. HPTLC	Identification of bioactive compounds in plant extracts; phenolic profiling
II. HFUS (High-Frequency Ultrasound Imaging)	Non-invasive evaluation of skin aging and effectiveness of cosmetic products
III. PRIMOS Lite 3D Scanner	Wrinkle analysis (surface roughness, Ra value)
IV. DermaLab USB Elasticity Probe	Measurement of skin elasticity (Young's modulus)
V. V. DUB® SkinScanner	Dermal density measurement
VI. CR-2600D Spectrophotometer	Skin tone measurement (brightness, redness, yellowness)
VII. Visioline (Shadow Analysis)	Wrinkle length and depth; skin topography (crow's feet scale)
VIII. CUPRAC Assay	Antioxidant capacity at physiological pH
IX. FRAP Assay	Antioxidant reducing power
***Ex vivo* methods**	
I. Structural Studies	a. Histological Analysis b. Barrier Protein Analysis
II. Functional Studies	a. Viability Assays b. Permeation and Retention Studies c. Hydration Assessment
III. Imaging and Topography	a. 3D Skin Imaging
IV. Inflammation & Protection	a. Cytokine and Inflammatory Marker Assessment b. Antioxidant/UV Protection Studies

I. In Silico

The purpose of this study was to assess the anti-aging activity *in silico*. An online server that offered visual datasets depicting the molecular structures and diagrams showing the connections between the molecules was used to identify the active site or receptor binding sites.[1]

a) Molecular Docking Study

Molecular docking is frequently used to forecast a compound's affinity for binding to protein receptors[5]. The computer-generated three-dimensional structure of tiny ligands can be positioned into a receptor using molecular docking in a few different orientations, conformations, and locations. This allows us to forecast the molecule's affinity for the specific receptor site[6].

AutoDock Vina 1.2.5 was used for docking in this investigation, treating ligands as flexible and proteins as rigid. For molecular dynamics (MD) simulations, the binding pose with the lowest docking score was chosen. UCSF Chimaera and ChimeraX were used to visualise protein–ligand interactions in three dimensions, and Discovery Studio Visualiser was used to create two-dimensional interaction diagrams[7].

In a study titled "Anti-aging Activity, In Silico Modelling and Molecular Docking from Sonneratia Caseolaris," Eka Siswanto Syamsul et al. used AutoDock Vina to perform molecular docking (MD) on three target proteins: hyaluronidase (1FCV), ROS (3ZBF), and collagenase (966C). Van der Waals (VDW) bonds of proteins and ligands were set to 1.0 with a net atomic charge cutoff of 0.25 to evaluate non-polar interactions; other bonds were left unaltered. The Glide cluster-based method was used for docking, and the ligand with the lowest docking score was thought to have the strongest binding affinity 1.

In their study "Antioxidant and Anti-Skin Ageing Potential of Selected Thai Plants: In Vitro Evaluation and In Silico Target Prediction," Kamonwan Chaikhong et al. used AutoDockTools 1.5.6's default protocol for molecular docking studies.

With grid box dimensions of 40 × 40 × 40 points for elastase and 60 × 60 × 60 points for tyrosinase, the grid spacing was set at 0.375 Å. For elastase, the grid box centres were (x = 12.58, y = 9.36, z = 2.251), and for tyrosinase, they were (x = −10.044, y = −28.706, z = −43.443). The Lamarckian Genetic Algorithm (GA) was used for docking simulations with default settings. AutoDockTools 1.5.6 and Discovery Studio Visualiser were used to analyse and display the docking results [1].

b) *In Silico* Prediction of Skin Absorption and Toxicity Assessment

In their study "Anti-Aging Potential of the Two Major Flavonoids Occurring in Asian Water Lily Using In Vitro and In Silico Molecular Modelling Assessments," Bodee Nutho et al. used the pkCSM webserver to predict toxicity and logKp (skin permeation).

The ProTox 3.0 webserver, which was accessed on March 29, 2024, was used to predict the compounds' oral toxicity (acute rodent toxicity) and toxicity classes [7].

Skin permeability, which represents the possibility of transdermal drug delivery, is one of the crucial elements in the application of topical formulations. Thus, they used the pkCSM webserver to further perform in silico prediction of skin permeability, expressed as logKp (cm/h). Effective drug penetration is indicated by high skin permeability, which is defined as a logKp less than −2.5 cm/h. Que-3-Rha and Kae-3-Gal's logKp values in the study were −2.735 (Table 3), indicating that both compounds had favourable skin permeability [7].

The ProTox 3.0 webserver was also used to predict acute oral toxicity.

II. *In vivo* Anti-Aging Effect Study

Healthy volunteers chosen based on their sex, age, skin type, and possible skin conditions participate in in vivo studies. Participants are split up into two groups. One group is given the cosmetic product that contains the active ingredient being studied, while the other group is given a placebo that doesn't contain the active ingredient.[8]

a) *In vivo* human testing by comparing treated and untreated skin areas: -

The study "In vitro and in vivo anti-aging effects of compounds isolated from Artemisia iwayomogi" by Kyung Young Kim et al. is based on the idea of comparing treated and untreated skin areas to assess the anti-aging (anti-wrinkle) effect of a topical formulation containing Artemisia iwayomogi extract straight from *in vivo* human testing. Clinical data demonstrating its potential as a natural anti-aging cosmetic ingredient will be presented in this study [9].

Enough healthy women between the ages of 30 and 50 were used in the procedure. The study does not accept volunteers who have a history of skin conditions. To determine the skin irritations, a patchtest was conducted. Following safety confirmation, a cream containing 1% water fraction of Artemisia iwayomogi was applied to the right corner of the right eye, and a control patch was applied to the left eye's corner. After washing your face, apply a cream twice a day. Over the course of the eight-week test, the anti-wrinkle properties were compared to the area that received a control patch (the area that was not treated) [9].

Safety measures: Participants abstained from UV exposure and did not use any additional anti-wrinkle cosmetics.

b) *In vivo* Clinical and Instrumental Skin Evaluation: -

Similar to the study mentioned above, Massimo Milani et al. conducted an in vivo study of the topical preparation in human subjects in their article "Anti-Aging Efficacy of Melatonin-Based Cream: Clinical and Instrumental Skin Evaluation." They also used an ANTERA 3D computer-assisted skin analysis evaluation to measure the melanin content and the coarse and fine wrinkles of the periorbital area at baseline and two months after treatment [10]. ANTERA 3D is a non-invasive, objective imaging method for assessing skin parameters.

This study aims to assess the safety and anti-aging effectiveness of a MelatosphereTM-based cream on women's facial skin ageing by measuring changes in wrinkles, pigmentation, and general skin condition using objective 3D imaging (ANTERA 3D) and clinical evaluations. ANTERA 3D reconstructs the surface in two and three dimensions by using a computer to analyse the differences between these images. Skin colour, wrinkles, texture, haemoglobin, melanin, pores, depressions, and elevation can all be assessed using ANTERA 3D images.[10].

A suitable number of women aged ≥45 with mild to moderate facial skin ageing were chosen for this study. The study does not include participants who are allergic to any of the ingredients in the cream. For two months in a row, a cream based on MelatosphereTM was applied to the face twice a day with two fingers. After two months, the melanin content and the coarse and fine wrinkles of the periorbital area were assessed using the ANTERA 3D device. Counting the returned tubes was another way to verify patient compliance[10].

III. *In Vitro* Models for Anti-Aging Efficacy Assessment

The most common in vitro models for anti-aging activity assessment are the ones evaluating antioxidant, anti-collagenase, anti-elastase, anti-hyaluronidase, anti-tyrosinase, anti-inflammatory, antiglycation, or moisturizing activity, and the induction of skin cellproliferation/anti-senescence effects or the inhibition of matrix metalloproteinase production. Each with their specific characteristics, these assays require the use of skin cell cultures, absorbance or fluorescence reading, polymerase chain reaction (PCR), and/or Western blotting.

a) Induction of Skin Cell Proliferation and Anti-Senescence Effects

This type of study has been extensively performed in plant-derived compounds. An example is the study performed by Gu et al.

The study investigated the potential anti-senescence effect of bamboo leaf flavonoids on HaCaT cells, which are an immortalized human keratinocyte line. To assess this, researchers chemically induced senescence in HaCaT cells using 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH). The cells were then treated with different concentrations of bamboo leaf flavonoids for 48 hours. The materials required included HaCaT cells, AAPH, and the standard 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reagent, which was used to evaluate cell growth. The results showed that flavonoids at concentrations between 5 and 80 µg/mL effectively countered the negative impact of AAPH on cell growth. However, a higher concentration of 160 µg/mL led to greater inhibition, indicating a dose-dependent response. Further analysis with mRNA sequencing and Western blotting revealed that the flavonoids reduced the

expression of p53, p21, and p16, which are genes linked to cell senescence. At the same time, the flavonoids increased Lamin B1 expression, suggesting that they help reduce senescence-associated cell cycle arrest. Fluorescent staining confirmed these findings.

Overall, the results suggest that bamboo leaf flavonoids may be a promising treatment for delaying cellular senescence and addressing age-related diseases[11].

b) Anti-Collagenase Activity

The study looked at the anti-aging potential of Maclura pomifera methanolic extract (MPM). It focused on how well MPM can inhibit collagenase activity, using Clostridium histolyticum as the enzyme source and N-[3-(2-furyl) acryloyl]-Leu Gly-Pro-Ala (FALGPA) as the substrate. MPM, collagenase enzyme, FALGPA, and epigallocatechin gallate (EGCG) were used as a positive control in the experiment.

To conduct the assay, the extract was incubated with the collagenase enzyme for 15 minutes. After that, the substrate was added, and absorbance was measured right away to find out how much enzyme activity was inhibited. The results showed that MPM strongly inhibited collagenase, achieving 84.55 ± 1.99% inhibition after 20 minutes and 94.68 ± 2.42% inhibition after 40 minutes at a concentration of 1 mg/mL. This indicates that the inhibitory effects increased over time. The positive control, EGCG, showed similar inhibitory results. These findings suggest that MPM has strong anti-collagenase properties and could be a promising candidate for anti-aging uses[11].

c) Enzyme Inhibition Assay for Anti-Aging Flavonoids:

The enzyme inhibition assay investigated the anti-aging potential of two major flavonoids, Quercetin-3 O-rhamnoside (Que-3-Rha) and Kaempferol-3-O-galactoside (Kae-3-Gal), which are prominent compounds found in Asian water lily extracts. I believe moreover, the goal was to evaluate their inhibitory effects on key skin-aging enzymes: collagenase, elastase, and tyrosinase. The materials required for this assay included the flavonoid standards (Que-3-Rha and Kae-3-Gal), enzyme sources (collagenase, elastase, tyrosinase), specific substrates for these enzymes, buffer solutions, and instruments like a spectrophotometer or microplate reader to measure absorbance.

The general procedure involved incubating each flavonoid with the target enzyme and its substrate under controlled conditions. After incubation, enzyme activity was quantified by measuring the release of reaction products that absorb light at specific wavelengths using the spectrophotometer. The percentage inhibition was calculated by comparing the enzyme activity in the presence of flavonoids to control samples without flavonoids. Results demonstrated significant anti-aging activity for both compounds. Que-3-Rha showed notable inhibition of collagenase (60.24 ± 7.59%) and elastase (50.28 ± 7.24%) but had moderate tyrosinase inhibition (46.54 ± 6.17%). Kae-3-Gal exhibited strong inhibition across all three enzymes: collagenase (59.84 ± 8.13%), elastase (55.56 ± 7.56%), and tyrosinase (51.14 ± 6.89%). These findings align with previous studies endorsing flavonoids as effective anti-aging phytochemicals, supporting their potential use in cosmetic, cosmeceutical, or phytopharmaceutical applications for skin aging prevention[12].

d) Evaluation of Elastin Synthesis in NHDF Cells

The determination of total elastin content in human fibroblasts was achieved using a colorimetric assay kit (Fastin TM ElastIN Assay Kit, Biocolor Life Science Assesses, UK), which employs chemo-binding to identify different types of -elastic materials, such as both soluble and insoluble varieties of the molecule, by measuring various dye-bound & insolent forms of each elasticity. To perform the analysis, fibroblast cells were divided into 12 wells with an equal number of cells at each concentration (1.8105 cells per well) and kept in a humidified atmosphere for 37 °C, along with 5% CO2. Following a 48-hour period, cells were subjected to the same collagen synthesis test and controls were present after being treated with control doses. Cells were obtained after 24 hours of treatment, with the elastin content being measured in accordance with kit manufacturer's instructions. A linear regression standard curve was used to measure the average absorbance at specific wavelengths, and these values were then applied to a series of experiments in triplicate for each sample of elastin, as well as duplicated tests. The measurement of elastin changes caused by treatment in fibroblast cultures was reliable using this method[13].

e) Collagen and elastin evaluation

Skin explants were rinsed with PBS after treatment. The samples were fixed in 4% formaldehyde for 1 hour at room temperature. The fixed tissues were transferred into 15 mL tubes containing 70% ethanol and stored at 2–8 °C. Samples were dehydrated gradually using increasing concentrations of ethanol. Dehydrated tissues were embedded in paraffin. Paraffin sections were cut and mounted onto glass slides. Sections were stained with Masson's trichrome, and we followed the instructions that came with the kit (Sigma-Aldrich). Then prepared slides and then took photos of them using a Zeiss Axio Observer 7 inverted microscope with a Moticam 5+ camera[14].

f) Evaluation of melanin, type I procollagen, and filaggrin production in human skin explants

This study was an **in vitro human skin explant model** using abdominal skin from a 44-year-old female donor. Skin fragments were cultured at the air–liquid interface, treated with AHHF-010, and exposed either to UV radiation (for melanin and type I procollagen analysis) or to SLS barrier disruption (for filaggrin evaluation). Melanin levels were assessed by Fontana-Masson staining, while procollagen and filaggrin expression were measured by immunofluorescence, with ImageJ used for quantitative analysis.

Human abdominal skin was obtained from a healthy female donor during abdominoplasty in this study. Following

soaking skin fragments in 4°C saline solution for a maximum of 24 hours, they were then dermatomized to 600 m thickness and cut into 1.5 mm sections. For 1 hour, samples were incubated at 4°C in culture medium containing gentamicin and amphotericin B to provide aseptic treatment. The samples were then placed on permeable inserts and cultured in an air-liquid interface culture system, with the latter being left at 37°C for 2 hours at room temperature before being subjected to AHHF-010 (25–30 mg/cm2) after stabilization. The melanin content and production of type I procollagen were measured after 7 days with UV exposure for 10 sessions. All treatments were successful. J/cm²). AHHF-010 was used to test for filaggrin levels, which were then measured after incubating for 48 hours and disrupting the skin barrier with 5% sodium lauryl sulfate (SLS).

The quantification of melanin was achieved through Fontana-Masson staining with ImageJ software, while procollagen and filaggrin proteins were detected through immunofluorescence stainning with specific antibodies and by ImageFileJ measuring fluorescence. A clinical trial that involved 33 female participants aged 40 to 55 years with Fitzpatrick skin types I–IV who used cosmetics was approved by the Ethics Committee. The criteria for exclusion included pregnancy, skin diseases, recent retinoic acid treatments, excessive sun exposure, and lifestyle changes. Consistent adherence to contraceptive, dietary, and cosmetic practices was observed by the participants throughout the study. A combination of ex vivo and clinical studies was used to evaluate the impact of AHHF-010 on skin pigmentation, collagen production (where applicable), barrier function, and overall clinical effectiveness[15].

g) Assays:

These tests show how well Hibiscus sabdariffa fights oxidants and could help with skin aging caused by oxidative stress and enzymes.

i. **DPPH Radical Scavenging Assay**

This test checks how well an extract can fight off DPPH, a free radical that's usually a deep purple color. Antioxidants in the extract give hydrogen or electrons to DPPH, turning it into a yellow thing. This makes the solution absorb less light at 517 nm. Basically, you mix the extract with a DPPH solution, let it sit in the dark for half an hour, and then measure how much light it absorbs. You then figure out the percentage of DPPH that the extract wiped out compared to a control. The test gives you an IC50 value, telling you how much extract you need to stop 50% of the DPPH radicals. A lower IC50 means the extract is a better antioxidant.

ii. **Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) Assays**

We tested the extract to see how many phenolics and flavonoids it had. These tests show the amount of antioxidant compounds inside. For phenolics, we used a special reagent that turns blue when it reacts with them, and we can measure this at 765 nm. For flavonoids, we used aluminum chloride, which turns yellow, and we can detect it at 510 nm. The results tell us how much gallic acid (for phenolics) and quercetin (for flavonoids) are in the extract. If there are high levels, it usually means it has good antioxidant properties.

iii. **Enzyme Inhibition Assays (Anti-aging relevance)**

We check if the extract can stop enzymes such as collagenase, elastase, and hyaluronidase. These enzymes break down collagen, elastin, and hyaluronic acid, which are needed for young-looking skin. Usually, we mix the enzyme with the extract and something it can act on. Then, we check how active the enzyme is with a special machine. If the enzyme is less active, it means the extract might help keep skin from aging by protecting it.

iv. **Oxygen Radical Absorbance Capacity (ORAC) Assay**

ORAC measures how well an antioxidant can grab peroxyl radicals using a fluorescent marker. We measure how the fluorescence fades because of radicals. Then, we measure how much protection the extract gives compared to Trolox (a normal antioxidant). Higher ORAC numbers mean the extract is a stronger antioxidant[16].

IV. INSTRUMENTAL METHODS: -

1. **HPTLC**

HPTLC offers several advantages: minimal to no sample preparation, faster analysis times, higher detection sensitivity, and represents a highly cost-effective technique.

HPLC phenolic profile

HPTLC provides significant advantages in discovering anti-aging compounds by allowing the simultaneous analysis of different herbal samples with potent bioactivity. Multi-detection capability and its ability to be integrated with *in vitro* bioassays and chemometrics make HPTLC a powerful tool for efficiently screening and assessing the bioactivity of complex extracts.

Usually, HPTLC studies serve as bio-guiding methods to identify biologically active compounds, facilitating their preparative isolation or direct structural analysis, often through hyphenation with spectroscopic techniques. Although bioautographic assays provide directly linked bioactivity information to specific chromatographic zones, such bioactivity is estimated from processes that are governed on the surface of the sorbent and may differ from similar processes that take place in the solution (liquid media). Additionally, some bioautographic assays are difficult to optimize.

On the other hand, the HPTLC-chemometric approach relies on mathematical methods and can effectively reveal the fingerprint-activity relationship. By treating the chromatogram as a multivariate signal carrying vast amounts of information, chemometric HPTLC fingerprinting eliminates the need to identify and interpret every peak or only significant one. Additionally, regression techniques

enable the identification of specific compounds responsible for bioactivities, providing deeper insights into the relationship between chemical profiles and biological effects The major drawbacks of the chemometric-based approach are related to model-building limitations. To develop general models for the selection of a greater number of bioactives, a more diverse set of plant extracts is required for model training. However, by increasing sample diversity, the ability of the models, especially those based on linear approaches, to appropriately describe data variability is limited, often resulting in statistically poor performances. On the other hand, if the more coherent set of samples is considered (congeneric plants), good statistically performed models are obtained, but a smaller number of bioactives will be identified.

Nevertheless, both approaches (bio-guided assays, and chemometric-based HPTLC methods), can be combined to extract information about bioactives in plant extracts.[17]

2. **HFUS**

High-frequency ultrasound skin imaging analysis (HFUS) is a non-invasive technique that allows a unique approach to the analysis of skin aging, as well as in evaluating the effectiveness of dermatological and cosmetic products, especially for skin rejuvenation.

For the evaluation and clinical diagnosis of aging, we highlight the technique of skin image analysis utilizing high-frequency ultra sound (HFUS), which, in addition to non-invasive, has shown great potential for application and characterization of skin changes in depth resulting from aging, and which can contribute greatly to the advancement of dermatological and cosmetic science.8 The HFUS imaging device is based on the principle of transmitting short pulses of ultrasound energy to a certain tissue, and the receipt of these reflected waves (called echoes), resulting from ultrasound- tissue interaction.9 A propagation ultrasound wave can interact with the tis sue, and the results are reflection, refraction, dispersion, absorption, and attenuation, but it is the echoes reflected by the tissue and picked up by the transducer that represents the ultrasound-tissue interac tions responsible for forming the sonographic image.9 For a complete evaluation of the skin, the use of HFUS is essential, because the high frequency allows detailed real- time visualization of the epidermis and dermis layers and subcutaneous tissue, allowing an in- depth characterization of various skin parameters.10- 13 In this context, the purpose of this study was to describe the main information found in the literature regarding the evaluation of skin aging, as well as the strategies carried out to minimize these effects, through the HFUS technique.

it can be said that, along with other non- invasive skin-evaluation techniques, HFUS imaging analysis is an integral tool for the accurate, efficient characterization that provides a new perspec tive for the clinical diagnosis of aging, generating quantitative data that in favor of the accuracy of the diagnosis of the skin condition and allows a higher level of evaluation of the efficacy of dermatolog ical procedures and cosmetic products.[18]

3. **PRIMOS Lite 3D**

For evaluation of wrinkle improvement, a PRIMOS Lite 3D Face and Skin Scanner Analyzing System (GFMesstechnik GmbH, Berlin, Germany) was utilized. The outer corner of the right eye was measured three consecutive times with the PRIMOS Lite after placing subjects' face onto a special PRIMOS face-held-equipment (GFMesstechnik GmbH) and focusing the outer corner of eye on a same pattern of the PRIMOS Lite to prevent the test area from moving. The images adjusted to the same position each time by applying 3D matching and were analyzed with the PRIMOS Lite software (version 5.6E; GFMesstechnik GmbH). The measurement variable Ra (average of all heights and depths to the reference plane) was used for wrinkle analysis as the most common surface roughness index worldwide, which represents the maximal mathematical average of the profile within the entire measurement range. The Ra value decreases with a lower depth of wrinkles, indicating that skin wrinkles were improved.[19]

4. **DermaLab USB elasticity**

For evaluation of skin elasticity improvement, the DermaLab USB elasticity probe (Cortex Technology ApS, Hadsund, Denmark) was applied. After attaching the probe to the skin with tape, the left cheek under the eye was measured only once for prevention of skin fatigue caused by repeated measurement. The DermaLab USB elasticity probe quantifies skin changes and restoring forces in accordance with inhalation of skin and the duration of the inhalation, and the results were analyzed using DermaLab USB analysis software, version 1.09 (Cortex Technology ApS). Young's modulus (E) was used for elasticity analysis, which is the value representing the difference in forces to raise surface skin as much as 1.5 mm, the distance between two infrared sensing wires within the probe. Its unit of measure is the mega pascal (MPa). Young's modulus (E) increases with a higher elasticity, indicating that skin elasticity was improved.[19]

5. **DUB® SkinScanner**

For evaluation of dermal density improvement, an ultrasonographic DUB® SkinScanner (Tpm Taberna Pro Medicum GmbH, Lüneberg, Germany) was applied. After applying ultrasonography gel, 3 cm from the outer corner of left eye was measured by using the probe at a right angle with skin and pressing skin with same pressure. The range of analysis was set in limits from epidermis to upper subcutaneous fat layer. The value increases with a higher density of dermis, indicating that dermal density was improved.[19,20]

6. **CR-2600D spectrophotometer**

For evaluation of skin tone improvement, a CR-2600D spectrophotometer (Konica Minolta, Inc., Tokyo, Japan) was used. The right cheek was measured three consecutive times, and the average value was determined. The L* and

a* of three measurement values were determined as a measure of skin tone. L* indicates brightness, a* indicates red and b* indicates yellow.[19]

7. **C+K Visioline**

Instrumental Test for Wrinkle Length and Depth.[21]

This research investigated the ability of shadow analysis (via the Courage + Khazaka Visioline and Image Pro Premiere 9.0 software) to accurately assess the differences in skin topography associated with photo aging.

When used properly, shadow analysis is effective at accurately measuring skin surface impressions for differences in skin topography. Shadow analysis is shown to accurately assess the differences across a range of crow's feet severity correlating to a 0-8 grader scale. The Visioline VL 650 is a good tool for this measurement, with room for improvement in analysis which can be achieved through third party image analysis software.[22]

8. **CUPRAC**

The CUPRAC (CUPric Reducing Antioxidant Capacity) method of antioxidant measurement, introduced by our research group, is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRAC reagent, Cu(II)-Nc, where absorbance is recorded at the maximal light-absorption wavelength of 450 nm.

CUPric Reducing Antioxidant Capacity (CUPRAC) method. In this method, developed in the analytical chemistry laboratories of Istanbul University, the copper(II)-neocuproine (2,9-dimethyl-1,10-phenanthroline) reagent can oxidize antioxidant compounds that are soluble in water or oil, itself being reduced to the colored copper(I)-neocuproine chelate complex. As an ET-based method, similar to the FRAP method, the CUPRAC method can react with a leading thiol-group antioxidant, GSH, as opposed to the non-responsive FRAP method [13]. In a further differentiation from the FRAP method employed at pH 3.6 and the Folin method [20] at pH 10, the CUPRAC method measures the antioxidant capacity at nearly physiological pH (i.e. pH 7), so it better simulates the physiological action of these antioxidants. The univalent-charged CUPRAC chromophore ($Cu(Nc)_2^+$) is soluble in both aqueous and organic solvents, enabling the simultaneous determination of hydrophilic and lipophilic antioxidants.[23,24]

9. **FRAP assay**

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as a novel method for assessing “antioxidant power.” Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants.

The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.[25,26,27]

V. Ex vivo evaluation

In the Article “**in vitro, ex vivo and clinical evaluation of anti- aging gel containing EPA and CBD**” by Guy Cohen and his team

Cell and skin organ cultures

All materials came from biological industries (Beit-HaEmek) unless otherwise noted. HaCaT cells were cultured and kept in a humidified incubator at 37°C and 5% CO2 in Dulbecco's modified eagle medium (DMEM; high glucose) supplemented with 10% foetal bovine serum and 1% Penicillin–Streptomycin–Amphotericin B Solution.[28]

With the exception of the positive control, which was applied 30 minutes before irradiation, all treatments were administered 24 hours after UVB stimuli. Echo Pharmaceuticals created the topical formulation, which is utilised in both clinical and ex vivo organ culture evaluation. In addition to the standard base formulation (placebo), preliminary screening revealed that the agents were highly compatible with the (0.1%) CBD, (0.1%) fish oil eicosapentaenoic acid (EPA), and (0.1%) Salvia miltiorrhiza root extract (Labiatae; 0.1%; Draco Natural Products) (data not shown). Salvia miltiorrhiza also contains antioxidant and anti-inflammatory components; EPA (an omega-3) is known to have anti-inflammatory properties and may support membrane integrity; and CBD provides antioxidant, anti-inflammatory, and potentially DNA damage mitigation.[28]

a. AEVA assessment for wrinkles

Stereo cameras and a fringe projection system (AEVA-HE device; Eotech, France) were used to create three-dimensional images of the topography of the skin. The optical system's dual cameras picked up a fringe standard that was projected onto the skin. The deflection in the fringes, which depict a qualitative and quantitative skin profile, was used to compute the 3D effect. The 3D result was created by stereo-combining the camera images. This method was used to evaluate roughness, volume depth, and wrinkle count.[28]

It has been repeatedly demonstrated that the ex vivo human skin culture can be used as a non-animal investigational model in dermo-cosmetic applications and can replicate a few important features of intact tissue. Consequently, the above-mentioned anti-inflammatory effects were confirmed in the ex vivo human skin culture. The viability of epidermal tissues was reduced by about 40% when exposed to UVB rays. While the placebo formulation

had no effect, treatment with the topical formulation of combined CBD and EPA completely restored viability to levels comparable with non-irradiated controls. Topical application dramatically decreased cytokine levels, which is consistent with in vitro results.[28]

"**Acai Oil-Based Organogel Containing Hyaluronic Acid for Topical Cosmetic": *In Vitro* and Ex Vivo Assessment** by Suellen Christtine da Costa Sanches et al conducted a study

b. Ex vivo study of retention in the stratum corneum

To assess the retention times at 2, 4, 6, 8, 24, 48, and 72 hours following the skin permeation study, the skins were kept in the diffusion cells. The donor compartment's excess organogel was taken out, cleaned with distilled water, and then dried with absorbent paper. After that, the tape-stripping process was applied to the skins. In order to extract the stratum corneum from the exposed area, the skin was secured on a Styrofoam support. Twelve adhesive tapes were then pressed and pulled over the skin. The first tape was thrown away to get rid of extra material, and the other tapes were each moved to a tube with two millilitres of methanol and two millilitres of distilled water.

To break up the cells, the tubes were vortexed for one minute and then sonicated in an ultrasound bath for fifteen minutes. The spectrophotometer at 530 nm was used to measure the amount of HA present in the supernatant. Three duplicates of the experiment were conducted. These results support the formulation's potential for long-lasting topical action by showing that it allowed for the sustained deposition of HA within the stratum corneum.[29]

c. Ex vivo study of retention in the epidermis and dermis

A scalpel and scissors were used to fix, cut, and mince the skin that was exposed to permeation after the stratum corneum was removed. Evaluations were conducted on retention durations of 2, 4, 6, 8, 24, 48, and 72 hours. To break up the cells, the skins were placed in tubes with two millilitres of methanol and two millilitres of distilled water. They were then sonicated for thirty minutes in an ultrasound bath. A spectrophotometer set to 530 nm was used to measure the amount of HA present in the supernatant that was kept in the dermis and epidermis. Three duplicates of the experiment were conducted. These results collectively show that the formulation supported prolonged hydration by delivering both sustained delivery into deeper layers (epidermis/dermis) and rapid surface deposition (stratum corneum) and potential bioactivity.[29]

Evaluation of Skin Hydration: OG + HA treatment considerably raised the skin's water content in comparison to controls. The formulation's capacity to improve stratum corneum hydration was demonstrated by the progressive improvement in hydration up to 24 hours and its maintenance through 72 hours.[29]

Thermal Examination of the Stratum Corneum DSC, or thermal analysis: A distinctive endothermic transition was seen in control membranes. While OG + HA significantly changed the enthalpy values in a time-dependent manner, OG treatment showed only minor changes. This confirmed that HA interacted with the stratum corneum's lipid-protein structure and enhanced its ability to retain water.[29]

d. Ex Vivo Skin Permeation and Retention Study

The investigation conducted on the OG + HA system at times 1, 2, 4, 6, 8, and 24 hours showed that HA could not penetrate the pig ear's skin membrane, preventing the active ingredient from entering the bloodstream.[29]

Permeation and Retention: Systemic absorption was ruled out because no HA permeation into the receptor compartment was found at any time. Rather, HA gathered in the viable layers and stratum corneum. Over 80% of the HA was localised in the stratum corneum and epidermis/ dermis by 6 hours, indicating sustained topical delivery. Retention rose in proportion to contact time[29].

Vinay Bhardwaj et al conducted study on "In Vitro and Ex Vivo Mechanistic Understanding and Clinical Evidence of a Novel Anti-Wrinkle Technology in Single-Arm, Monocentric, Open-Label Observational Studies"

e. Human Skin Explant Experimentation and Histological Staining

According to the study, the cream-gel formulation encourages the dermal extracellular matrix to remodel (more collagen, elastin, and hyaluronic acid), which is typically desired in anti-aging and anti-wrinkle treatments.

Additionally, it improves epidermal functions, such as boosting cell proliferation (Ki67) and barrier protein levels (filaggrin), which may aid in skin renewal.

Changes were detectable after only three to six days ex vivo, indicating that the formulation has biological effects that happen rather quickly.[30]

f. Histological Changes in Dermal ECM Components

We conducted a histological examination of the primary components of the extracellular matrix (ECM)—collagen, elastin, and hyaluronic acid—to investigate the major structural alterations involved in wrinkle reduction. The dermis of untreated skins had a uniform distribution of total collagen fibres. The papillary dermis close to the dermo–epidermal junction had especially high staining. The papillary area of the dermis had a dense network of longer collagen fibres. The staining analysis showed that the total collagen score increased statistically significantly when compared to the control (+16%) or untreated samples (+25%).

In the test samples, elastin staining showed longer vertical fibres throughout the dermis that had the odd ability to run towards the epidermis more than in control or untreated skins. Additionally, compared to control (+67%) or untreated (+59%) samples, the elastin score in tested skins increased statistically. Additionally, we examined HA in the dermis histologically. From the dermal papillary area to the deep layers of the epidermis, the cream-gel treated skins showed increased staining with gradation. This finding was

demonstrated by a 34% increase in score when compared to the untreated samples.[30]

Systematic Evaluation of Anti-Inflammatory Aging in Dermatological Application Using Multi-Layer Skin Models

3D epidermal skin models were shielded from UVB ray damage by PE. It restored important epidermal barrier proteins and decreased sunburn cells. GPE supported barrier integrity and resilience by increasing filaggrin (+95.6%), loricrin (+146.2%), and transglutaminase 1 (+207.7%) in comparison to UVB-only samples [30].

CONCLUSION

An integrated approach combining in vitro, in vivo, in silico, ex vivo, and instrumental methods is needed to assess the effectiveness of anti-aging cosmetics. Ex vivo models offer physiologically relevant data on penetration and biomarker modulation, in vitro and in silico studies allow for quick screening and mechanistic insights, and in vivo studies—backed by sophisticated instrumental techniques—remain the gold standard for evaluating safety, efficacy, and observable results. The intricacy of skin ageing cannot be adequately captured by any one technique, so their combined use is crucial to maintaining both consumer confidence and scientific validity. Next-generation, evidence-based anti-aging formulations could be developed more quickly thanks to advancements in AI-driven predictive tools, high-resolution imaging, and bioengineered skin models.

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