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ORIGINAL ARTICLE

Modulation of autophagy by an innovative phytocosmetic preparation (*Myrothamnus flabelifolia* and *Coffea arabica*) in human fibroblasts and its effects in a clinical randomized placebo-controlled trial

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Abstract

Background: Autophagy is a natural and evolutionary mechanism that reduces cell toxic components and reutilizes metabolites to provide energy and renew cell function, which is linked to a wide range of age-related diseases, including those that affect the skin. Positive modulation of autophagy is useful to treat skin disorders and new active herbal products are potential candidates as autophagy modulators.

Aims: The present study aimed to evaluate the effects of a phytocosmetic formulation containing *Myrothamnus flabellifolia* leaf and *Coffea arabica* seed plant extracts (MflCas) on the ubiquitin-proteasome and autophagy markers in human dermal fibroblasts, and investigate its topical skin effects in a randomized, simple-blind, and placebo-controlled trial.

Methods: Human dermal fibroblasts were used to determine proteasome activity, protein carbonylation, LC3B protein, and lipofuscin production by luminescence and immune-enzymatic assays, and to determinate gene expression of autophagy biomarkers (Atg5, Atg7, EI24, EIF2A, Park2, foxo1, and mTOR) by RT-PCR. A clinical trial was conducted to evaluate the effects of MflCas on the hand, face, and forearms skin features after treatment by 56 days.

Results: Topical treatment with MflCas improved several skin features of volunteers, mainly skin aging and pigmentation signals. On the hand skin, MflCas 2% after 56 days of treatment, reduced the spots length (30.8%), skin contrast (42.2%), and increased skin homogeneity (63.2%) and skin lightening effect (1.4%). On the face skin, topical treatment after 56 days reduced the spots length (21.5%), wrinkles area (8.1%), and wrinkles volume (5.6%) with an increment in face skin homogeneity (59.5%). These effects were related to the ability of MflCas to reduce proteasome activity protein carbonylation, and lipofuscin level, increase LC3B production, downregulate Atg7 and mTOR genes, and upregulate Park2 gene expressions.

Conclusions: The phytocosmetic preparation containing *Myrothamnus flabellifolia* leaf and *Coffea arabica* seed modulated ubiquitin-proteasome and autophagy process,

representing an innovative and safe herbal preparation to improve skin features, mainly acting as skin anti-aging and lightening agent.

KEYWORDS
autophagy, coffee, resurrection plant, skin aging, skin pigmentation, ubiquitin-proteasome system

1 | INTRODUCTION

Two cell protein degradation systems, the ubiquitin-proteasome system (UPS) and autophagy, are essential to promote cellular homeostasis, degrading proteins into small polypeptides and maintaining amino acid recycling, energy balance, and control protein quality.[1] While UPS is responsible for degrading 80%–90% of proteins, mainly short-lived, abnormal, denatured, and damaged proteins, autophagy is responsible for the degradation of long-lived and aggregated proteins and impaired cellular organelles such as mitochondria, peroxisome, ribosomes, and pathogens.[1] Defects in the cell's ability to remove damaged proteins and organelles via ubiquitin-proteasome and autophagy process is the basis for the development of a lot of age-related diseases, such as neurodegenerative disorders, diabetes, obesity, and skin aging manifestations promoted by intrinsic and extrinsic factors.[1]

Autophagy, a physiological and conserved catabolic pathway that delivers excess, aggregated, or damaged proteins and organelles, unwanted macromolecules, and invading pathogens to lysosomes for degradation, plays important roles in maintaining cellular and tissue homeostasis.[2,3] Defects in autophagy machinery are closely linked to a wide range of human diseases, representing an innovative target for the action of the new pharmacological and non-pharmacological approaches to control several autophagy-related disorders, such as skin aging.[4] Although there is a small number of studies focusing on the impact of autophagy on the pathogenesis of skin diseases, the autophagy machinery in keratinocytes, melanocytes, dermal fibroblasts, skin macrophages, Langerhans, and dendritic cells provides new insights for the development of new products and therapeutic approaches to treat skin diseases.[5] Among these cell types, autophagy has been demonstrated to be preferably impaired in melanocytes compromising melanogenesis and in aged dermal fibroblasts, resulting in deterioration of dermal integrity, skin fragility, and markedly changes in the levels of skin aging-related autophagy markers.[5] Considering skin as the first-line defense of the body, autophagy can be an important process to protect the skin from external damage and environmental stimuli. Recently, autophagy modulation in skin cells has emerged in several studies to provide new insights and the basis for the development of innovative products to treat skin diseases, mainly intrinsic and extrinsic skin aging.[3,5]

Herbal products and natural compounds of plant origin are a rich source for the development of the new therapeutic approaches since plant species contain multiple-target compounds acting by different signaling pathways with potential application to prevent and treat several diseases related to human skin function. Based on this, our research group has been interested in studying different herbal preparations with therapeutic action on several skin conditions to improve cell skin function and skin health focusing on topical agents able to modulate autophagy processes. For this purpose, we studied a topical standardized phytocosmetic preparation containing leaves of *Myrothamnus flabellifolia* Welw. and seeds of *Coffea arabica* L., two plant species containing chemical compounds, mainly trehalose and caffeine, respectively, which modulate autophagy.[6,7]

The present study aimed to evaluate the effects of a phytocosmetic formulation containing *Myrothamnus flabellifolia* leaf and *Coffea arabica* seed plant extracts on the ubiquitin-proteasome and autophagy markers in human dermal fibroblasts, and investigate its topical skin protective effects in human volunteers using a randomized, simple-blind, and placebo-controlled clinical trial.

2 | MATERIAL AND METHODS

2.1 | Phytocosmetic preparation

The phytocosmetic standardized preparation (MflCas), manufactured and provided by Chemyunion Ltd., is a hydroglycolic plant extract containing leaves of *Myrothamnus flabellifolia* Welw. (Myrothamnaceae botanical family) and seeds of *Coffea arabica* L. (Rubiaceae botanical family). The INCI name of this product is Propanediol (and) *Coffea arabica* (Coffee) seed extract (and) *Myrothamnus flabelifolia* leaf extract (and) water.

2.2 | In vitro studies for evaluation of autophagy biomarkers in human fibroblasts

2.2.1 | Proteasome activity

Normal human dermal fibroblasts (NHDF) cells (passage 16) were cultured overnight at a 10.000 cells/well density in a white 96-wells plate with NHDF culture medium (Sigma-Aldrich/Merck KGaA), which was replaced with fresh medium including betulinic acid at 20 μg/ml (positive control) and MflCas at 75 and 37.5 μg/ml (test herbal drug). An untreated cell group was cultured in a regular growth medium and plated in the same experimental conditions (control group). Cell viability was previously checked by staining with Trypan Blue solution (Bio-Rad Lab., Inc.). After 24 h of incubation with each product, chymotrypsin-like

proteasome activity was kinetically measured in all samples for 60 min. Measurements were taken each for 2.5 min. Luminescence generated from proteasome cleavage of a peptide substrate added to the samples was quantified using Proteasome-Glo™ chymotrypsin-like cell-based assay kit (Promega Corp.).

2.2.2 | Protein carbonylation evaluation

The protein carbonylation induced by hydrogen peroxide was also evaluated in normal human dermal fibroblasts using a bicinchoninic acid assay kit (BCA, Fisher Scientific) for total protein determination and protein carbonyl content assay kit (Abcam, Cambridge, United Kingdom) for carbonylated protein evaluation in a Synergy h1 hybrid multi-mode microplate reader (Promega Corp.).

2.2.3 | LC3B (microtubule-associated protein 1A/1B light chain 3B) protein determination

The LC3B determination was evaluated in Human dermal fibroblasts, neonatal (HDFn C-004-5C; Cascade Biologics) by fluorescence assay using specific antibodies (primary antibody LC3B rabbit polyclonal from Invitrogen; LI0382; Thermo Fischer Scientific) and optical fluorescence microscope Leica—DM 6000B coupled with a camera of 2.8 MP Leica DFC7000T (Leica Microsystems GmB). Imagens were captured using the software LAS (Leica Application Suite v.4.12) and evaluated by fluorescence intensity emitted by labeling specific antibodies. The semi-quantification of pixels generated by the fluorescence emitted by LC3B protein in the acquired images was evaluated using the software ImageJ®.

2.2.4 | Lipofuscin determination

Cellular senescence by lipofuscin immunofluorescence was also evaluated in human dermal fibroblasts, neonatal, using the antibody-enhanced detection of senescent cell reagent (SenTraGor™, Arriani Pharmaceuticals, Attica, Greece) and an optical fluorescence microscope Leica—DM 6000B coupled with a camera of 2.8 MP Leica DFC7000T. Imagens were captured using the software LAS (Leica Application Suite v.4.12) and qualitatively evaluated by fluorescence intensity emitted by labeling specific antibodies. The semi-quantification of pixels generated by the fluorescence emitted by lipofuscin in the acquired images was evaluated using the software ImageJ®.

2.3 | Gene expression of autophagy biomarkers in human fibroblasts

HDFn were prepared as previously reported, and cells cultures were separately incubated with three non-cytotoxic concentrations of MflCas (75, 150, and 300 μg/ml) for 24 h. After that, cell lysates were collected for further RNA extraction and gene expression evaluation. From the cell lysates, the total RNA was extracted using a PureLink™ (Life Technologies) and quantification be spectrometry using a NanoDrop Lite (Thermo Fischer Scientific). The RT-PCR assay was performed on StepOnePlus equipment (Applied Biosystems). For gene expression analysis, a commercially available test system was used (TaqMan® Assays Gene Expression) for follow genes here describes. ATG5 (autophagy-related 5 protein): Hs00169468_m1; ATG7 (autophagy-related protein 7): Hs00893766_m1; EI24 (etoposide-induced protein 2.4): Hs00903035_g1; EIF2A (eukaryotic translation initiation factor 2A): Hs00230684_m1; PARK2 (Parkinson protein 2): Hs01038322_m1; FOXO1 (forkhead box O1 protein): Hs00231106_m1; mTOR (mechanistic target of rapamycin kinase): Hs00234508_m1; UBC (ubiquitin): Hs00824723_m1, Applied Biosystems) with the appropriate primers and probes. The experimental conditions were as follows: 48°C for 30 min for reverse transcription, 95°C for 10 min for activation of AmpliTaq Gold® DNA polymerase, followed by 50 cycles of 94°C for 15 s and 60°C for 1 min for denaturation and rewarming, respectively. The relative amount of mRNA was calculated by a mathematical model for relative quantification and gene expression analysis in real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method.

2.4 | Clinical and experimental assessment

A clinical randomized, single-blind placebo-controlled trial was conducted with two groups of female volunteers (total = 68 volunteers), receiving a topical formulation containing placebo base (34 volunteers), which consists of self-emulsion system Uniox C (Cetearyl Alcohol [and] Polysorbate 60) 12%, Hebeatol CG (Xylityl sesquicaprylate [and] Caprylyl Glycol) 1% as preservative, and water as the solvent, or a base formulation containing MflCas 2% (34 volunteers). The volunteers applied on the facial skin, hands, and forearm sufficient amounts of topical formulation (placebo or MflCas 2%), gently spreading, twice a day (morning and evening). Every morning, all volunteers of both experimental groups also applied a sunscreen formulation. Specific inclusion and non-inclusion criteria are described in Appendix S1. All criteria were evaluated by a dermatologist.

The volunteers were subjected to 56 days topical treatment with Placebo or MflCas 2% formulations in the facial, hands, and forearm areas. The obligations imposed to volunteers were the following: (a) do not apply other similar treatments on the experimental area; (d) do not apply aesthetic treatments such as exfoliating, Botox, and peeling; (c) to respect use conditions of tested treatments; (d) conservation of hygiene and/or makeup habits; and (e) do not use makeup in the experimental area during control days in the research center.

In this randomized single-blind placebo-controlled clinical trial, we determine the clinical effects of topical application of placebo formulation and MflCas 2% formulation after 28 and 56 days of treatments, using the quantification of several clinical, skin

macroscopic, and biomechanical parameters. The evaluation of these parameters in volunteers of both experimental groups was made by a technician and a dermatologist before any treatment (Day 0), and 28 (Day 28) and 56 (Day 56) days after treatments. For all measurements at Day 0, 28, and 56, volunteers were subject to a previous rest by 30 min in a room with controlled humidity and temperature.

2.4.1 | Analysis of the hand skin of volunteers

Hand images were acquired by a Digital Canon Camera EOS 60 D (Canon) using the following attributes: shutter exposure time 1/60, aperture F20, sensor sensitivity (ISO) of 500, the flash intensity of 1/4, and automatic flash AWB. Spots lightening and improvement of skin homogeneity were performed using a Chroma Meter CR 400 (Konica Minolta Sensing Americas, Inc.). The measurements were performed on two areas of each volunteer hand, the first area with spots and a second area without spots. The luminance parameter was used to evaluate skin lightening with a range between 0 (darker) to 100 (lighter), whereas the skin homogeneity was determined using the color contrast between spots and adjacent areas through of difference between luminance value of spot area and the luminance value of the adjacent area.

2.4.2 | Analysis of forearm skin of volunteers

Images of the anterior region of the forearm were acquired using Visioscan® equipment (Courage & Khazaka electronic GmbH) to determine skin roughness and anisotropy.

2.4.3 | Analysis of facial skin of volunteers

Three facial images (front, left, and right sides) of volunteers were acquired using Visia CT equipment (Canfield Scientific, Inc.) and standardized using a digital Canon Camera EOS 60D for further analysis of spots lightening, wrinkles, expression lines, and face remodeling using the FrameScan® software (Teradyne). For determining skin (facial and hands) evenness and spots lightening were used the total surface of spots and Haralick homogeneity index, whereas facial wrinkles and expression lines were also evaluated using the FrameScan® software considering the following parameters: visibility coefficient (number of visible wrinkles within a selected area) and occupancy rate (area occupied by the wrinkle within the assessed area). The face oval remodeling was analyzed using the images from Visia CT equipment and the software Image J® as previously described.[8]

Pictures from periorbital areas were used to determine skin relief, wrinkles, and expression lines in an Optical 3D Skin Measuring Device PRIMOS® Compact 5.075 (GFMesstechnik, GmbH). For this analysis, the following parameters were used: volume expressed in mm^3, the SA parameter (media between skin depth and elevation) in the assessed area, and Smax parameter (direct measurement of wrinkles depth).

2.4.4 | Clinical assessment

The clinical dermatological evaluation was performed by a dermatologist on Day 0, Day 28, and Day 56 as described in Appendix S1.

3 | RESULTS

3.1 | Effects of MflCas on the autophagy markers in human fibroblasts

Luminescence signal data relative to proteasome activity were obtained at each time point for 60 minutes for each experimental group (Figure 1A). The results demonstrated both betulinic acid and MflCas at 37.5 and 75 μg/ml significantly increased chymotrypsin-like proteasome activity when compared with the control group. Considering 10 minutes are necessary to stabilize the reaction, this time was used to compare relative proteasome activity among the experimental groups (Figure 1B). The results demonstrated treatment with MflCas at 37.5 and 75 μg/ml significantly stimulated proteasome activity by 45.1 and 43.9%, respectively, whereas the treatment with betulinic acid at 20 μg/ml was able to significantly stimulate proteasome activity by 140.4%.

The detoxifying and protective effects of MflCas on NHDF after protein carbonyl accumulation induced by H_2O_2 were assessed by protein total levels, protein carbonylation, and protein carbonylation relative to total protein levels. The results demonstrated H_2O_2 significantly decreased in 76.5% of the total protein levels when compared with the control group (Figure 1C). The cytotoxic effects of H_2O_2 were reduced by vitamin C and all concentrations of MflCas, with a concentration-dependent response (Figure 1C). The damage induced by H_2O_2 was characterized by a significant increment in protein carbonylation levels (22.7%) when compared with the control group (Figure 1D), whereas MflCas at concentrations of 150 and 300 μg/ml significantly reduced the protein carbonylation in 13 and 24%, respectively, when compared with H_2O_2 group (Figure 1D). Treatments with MflCas at concentrations of 150 and 300 μg/ml protected against protein carbonylation in similar levels to those induced by vitamin C, which did not significantly differ from the control group. We also evaluated the ratio of protein carbonylation with total protein levels as a marker of carbonyl groups per cell. The results demonstrated H_2O_2 significantly increased protein carbonylation by 6.75-fold when compared with the control group (Figure 1E). Treatment of NHDF with MflCas at 75, 150, and 300 μg/ml significantly reduced protein carbonylation levels relative to protein content by 79.1, 83.9, and 89.4%, respectively, when compared with H_2O_2, similar those effects of vitamin C decreasing protein carbonylation by 89.9% (Figure 1E).

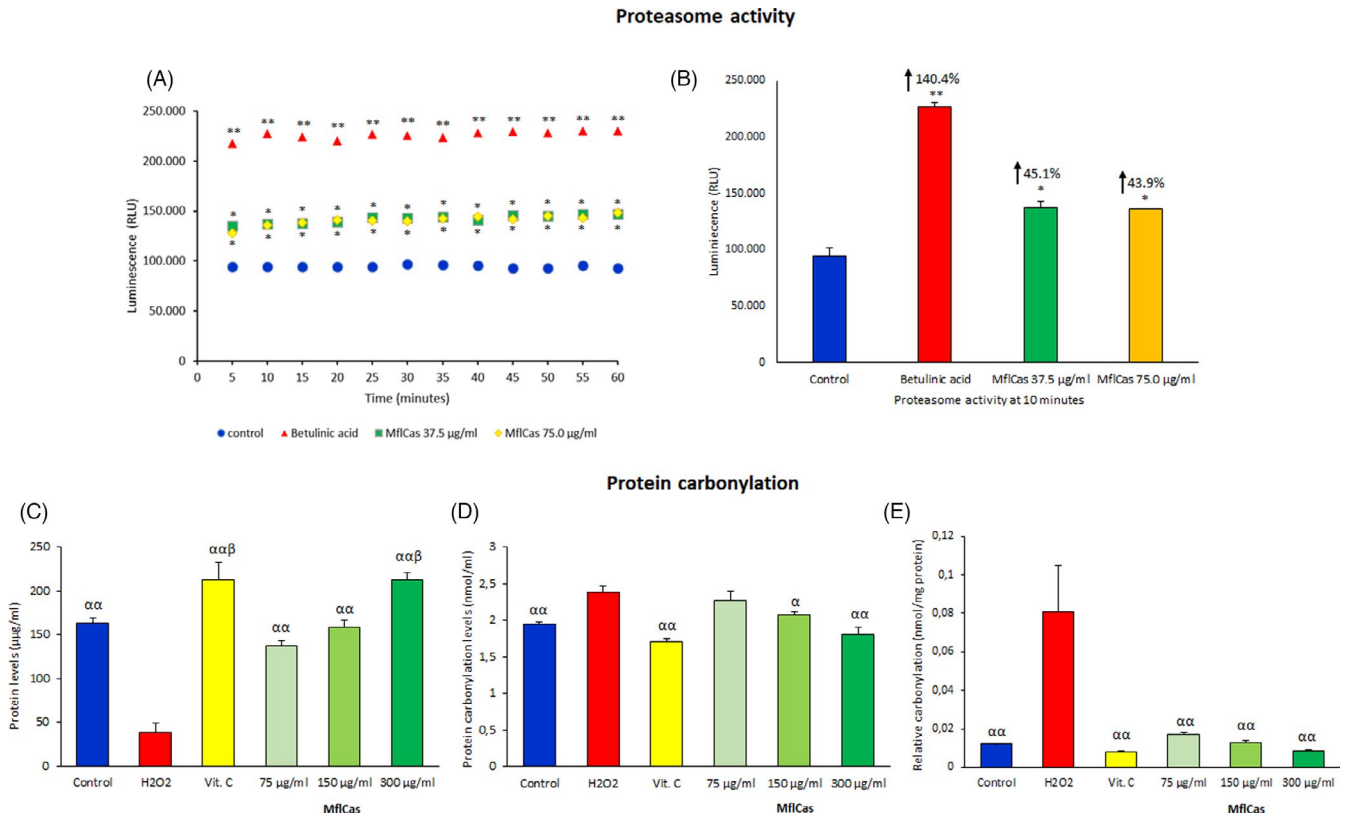

FIGURE 1 Detoxifying effects of MflCas phytocosmetic preparation on proteasome activity and protein carbonylation in Normal human dermal fibroblasts. (A) Time evolution of proteasome activity; (B) Proteasome activity at 10 min; (C) Protein levels; (D) Protein carbonylation levels; (E) Protein carbonylation relative to protein content. Abbreviation: RLU, relative luminescence units; $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{\alpha}p < 0.05$ vs. hydrogen peroxide (H_2O_2); $^{\alpha\alpha}p < 0.01$ vs. hydrogen peroxide (H_2O_2); $^{\beta}p < 0.05$ vs. control

Immunofluorescence images in HDFn cells demonstrated treatment with different concentrations of MflCas increased LC3B protein amounts when compared with basal control (Figure 2). A semi-quantification of fluorescence expression showed MflCas promoted a significative concentration-dependent response on the LC3B synthesis of the 35.3, 55.2, and 94.6% at concentrations of 75, 150, and 300 µg/ml, respectively, when compared with the control group after 16 h of incubation (Figure 2). The group supplemented with FBS 1%, the positive control, demonstrated an increase of 51% on the LC3B protein synthesis at the same experimental conditions. The effects of MflCas at 300 µg/ml were also significantly increased when compared with the FBS group (Figure 2).

Data of immunofluorescence analysis demonstrated UVA irradiation strongly induces senescence in cells exposed to radiation, as noted by intense lipofuscin expression in the control group (Figure 2). The treatment of cells with 37.5, 75, and 150 µg/ml of MflCas was able to reduce the lipofuscin synthesis in HDFn when compared with UV irradiated group (Figure 2). The treatment with MflCas significantly reduced lipofuscin in 17, 20, and 17% at concentrations of 37.5, 75, and 150 µg/ml, respectively, when compared with the UV control group (Figure 2).

The effects of the MflCas on the gene expression of several markers of autophagy in the culture of human fibroblasts were selective for some genes as presented in Figure 3. MgflCas phytocosmetic preparation significantly reduced ATG7 gene expression on the highest test concentration (300 µg/ml), with no effects in lower concentration (Figure 3). In the same concentration, MflCas significantly reduced in 12% the mTOR gene expression (Figure 3). On the other hand, the Parkin gene was upregulated after cell culture treatment with MflCas at a concentration of 300 µg/ml, increasing its expression by 64% when compared with the control group (Figure 3). MflCas phytocosmetic preparation produced no effects on the ATG5, EI24, EIF2A, FOXO1, and ubiquitin gene expression (data not shown).

3.2 | Effects of MflCas 2% on the skin of volunteers

The effects of MFlCas phytocosmetic preparation at 2% in the clinical randomized, single-blind placebo-controlled trial demonstrated an improvement in several hand and face skin features of volunteers after treatment by 28 and 56 days (Figure 4). The results demonstrated MflCas 2% induced a significant reduction of 25.9% and 30.8% on the hand spots length of volunteers after treatment for 28 and 56 days, respectively, whereas placebo formulation produced no effects (Figure 4A). These data were accompanied by a significant

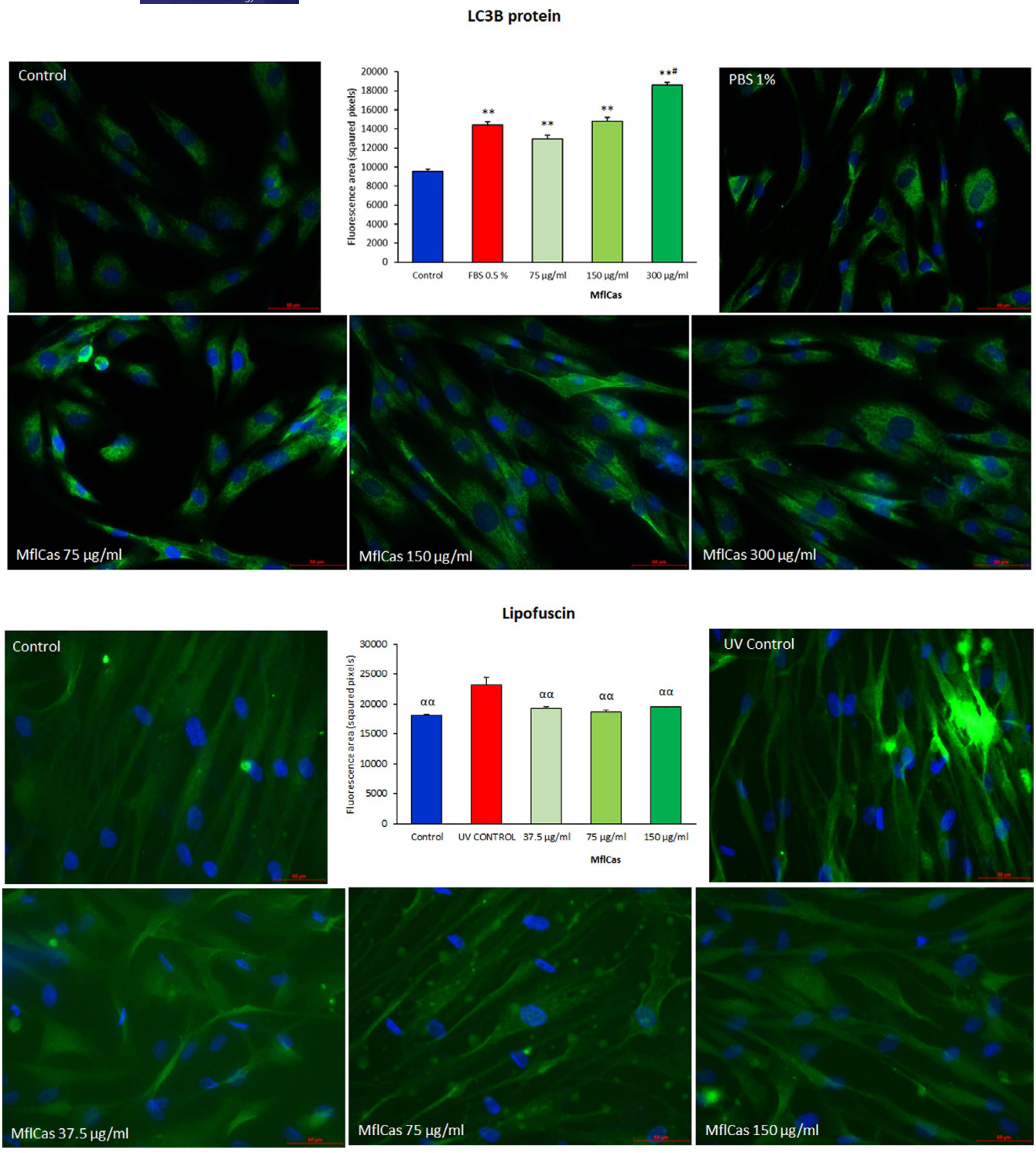

FIGURE 2 Effects of MflCas phytocosmetic preparation on LC3B protein and lipofuscin in human dermal fibroblasts, neonatal. $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. PBS 1%; $^{\alpha\alpha}P < 0.01$ vs. control group

time-dependent increase of 44.7% and 63.2% in the hand skin homogeneity, at Day 38 and Day 56, respectively, while placebo increased the skin homogeneity only after 28 days of treatment, without effects at Day 56 (Figure 4B). We also evaluated the ability of MFlCas 2% to induce skin lightening effect on the hands of volunteers. The data demonstrated MflCas 2% significantly increased 2.0 and 1.4% the skin lightening (L parameter) after treatment by 28 and 56 days, respectively, whereas placebo formulation was ineffective to promote skin lightening effect (Figure 4C). Finally, the contrast (based on L parameter) between the skin and skin spots was also evaluated and the results demonstrated MflCas 2% significantly reduced 37.5% and 42.2% this parameter when compared with Day 0, whereas placebo was ineffective (Figure 4D). The hand pictures demonstrated the combinatory effects of MflCas 2% on the general aspects of skin, including

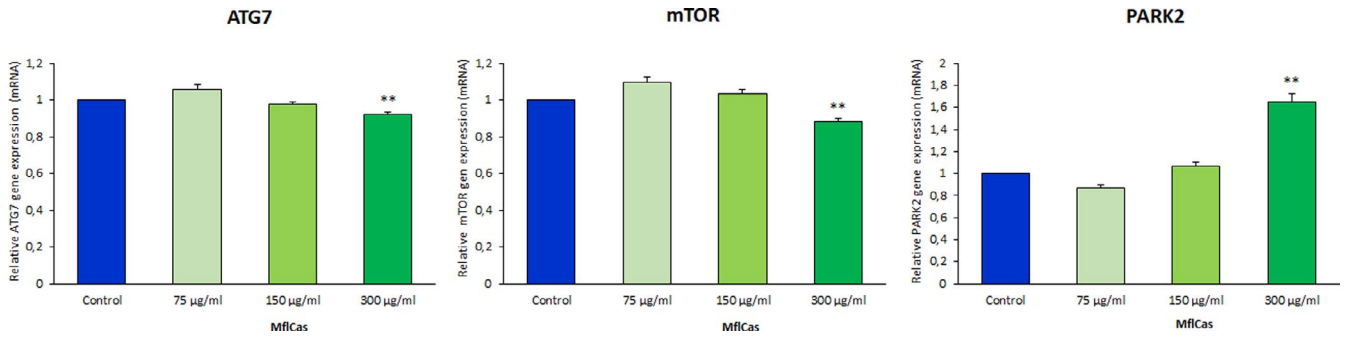

FIGURE 3 Effects of MflCas phytocosmetic preparation on gene expression of autophagy markers in human dermal fibroblasts, neonatal. $^{**}p < 0.01$ vs. control

reduction of spots length and skin contrast, as well as increasing skin hand homogeneity with lightening effect (Figure 4E).

In the second set of evaluations, we evaluated the effects of MflCas 2% topical application in several parameters on the facial skin of volunteers. The length of facial spots was evaluated and the results demonstrated both MflCas 2% and placebo formulations significantly reduced the spot length at Day 28 and Day 56 when compared with respective Day 0 (Figure 5A). MflCas 2% formulation was also able to significantly increase facial skin homogeneity, improving in 44 and 21.5% the skin homogeneity at Day 28 and Day 56, respectively, whereas placebo improves skin homogeneity by 14.3% after 28 days of treatment with no effects at Day 56 (Figure 5B). These results were accompanied by a simultaneous reduction in facial wrinkles area, with a similar protective effect at Day 28 and Day 56 after treatment with MflCas 2%, whereas placebo produced similar reduction only at Day 28, with no effects after treatment by 56 days (Figure 5C). In addition, MflCas 2% also reduced 5.6% of facial wrinkle volume after 28 and 56 days of treatment (Figure 5D). Although placebo reduced the wrinkle volume by 8.2% at Day 28, this effect was not maintained at Day 56, when wrinkle volume was similar to Day 0 (Figure 5D). Face pictures of volunteers also demonstrated the general improvement of skin features when compared with the placebo group (Figure 5).

Finally, the anisotropy index was also evaluated on the forearm of volunteers after treatment with placebo or MflCas 2% by 56 days. The measurements were performed at Days 28 and 56 after treatments and compared with measurements of Day 0 before treatment. The results demonstrated MflCas 2% significantly reduced the skin anisotropy by 8.3% after 56 days of treatment, whereas placebo significantly increased in 9.7% the skin anisotropy after 28 days of treatment (Figure 4F).

3.3 | Product acceptability

The volunteers manifested no skin reaction, including itching, redness, irritation, hypersensibility symptoms, discomfort after use of placebo, and MflCas 2% for 56 days. Thus, the majority of volunteers referred (data no shown) product is easily applicable with a pleasant texture, nice appearance, smother, and firmer as well as they were satisfied and would use the product again.

4 | DISCUSSION

Oxidative stress is a major factor involved in aging and age-related diseases, producing several effects in all cells of the live organisms and associated with several chronic and degenerative diseases. On the skin, intrinsic and extrinsic aging factors produce a lot of effects, particularly facial wrinkles, changes in facial expression lines, spots in hand and faces, roughness, and loss of skin elasticity. The main effect of oxidative stress during the aging process is protein oxidation, which induces protein aggregation with consequent loss of cell viability and dysfunction.[9,10] The deleterious effects of protein aggregation are endogenously counteracted by proteolytic processes, including ubiquitin-proteasome system and lysosome-based autophagy pathways. However, these processes are completely compromised during the aging natural process or in age-related diseases. Products with the ability to positively modulate these degradative processes on the skin can be potentially useful to reduce the speed of the cell aging process and improve skin features.

Topical application of MflCas 2% formulation produced several skin health improvement effects on the skin hand and facial features of volunteers in a clinical trial study. The beneficial effects of the MflCas phytocosmetic preparation on the hand skin were evidenced by a significant reduction of the spots and an increase of skin homogeneity and lightening. On the face skin, MflCas 2% gel also significantly reduced skin spots with consequent better skin homogeneity and lightening, and these effects were accompanied by a significant reduction of the face wrinkles and expression lines. In addition, MflCas reduced the skin anisotropy, which indicated the preferential alignment of collagen fibers in the dermis and it is increased in aging and age-related diseases.[11] The observed clinical effects were closely related to the ability of the phytocosmetic preparation to modulate two main cell protein degradation pathways, the ubiquitin-proteasome system, and the autophagy process. Here, we demonstrated that MflCas acted both increasing proteasome activity and modulating several markers of the cell autophagy machinery, mainly LC3B synthesis, and regulation of ATG7, mTOR, and Parkin gene expressions. In addition, MflCas also acted reducing lipofuscin production induced by ultraviolet radiation and the protein carbonylation induced by hydrogen peroxide in NHDF cells, acting as a detoxifying and protective agent.

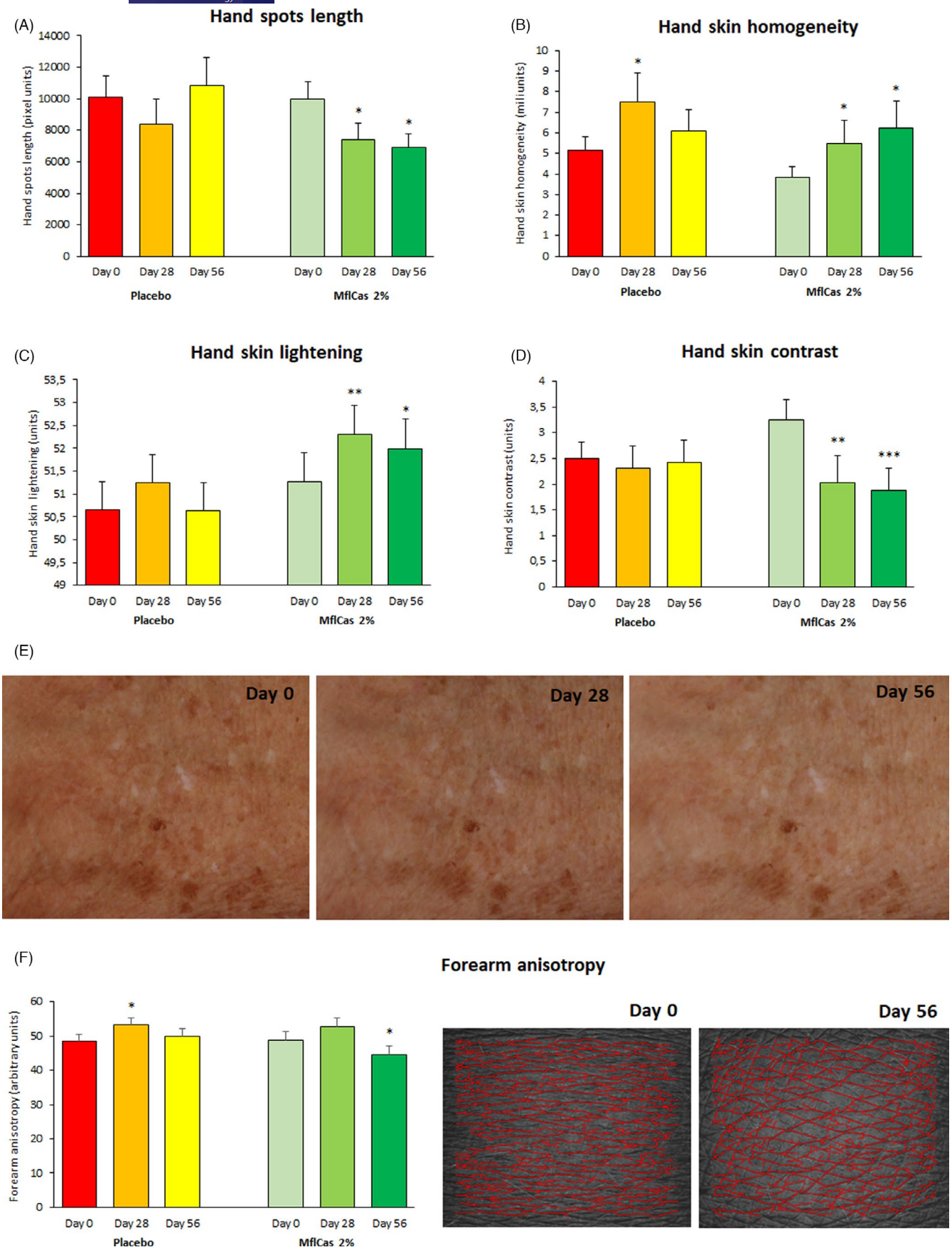

FIGURE 4 Effects of placebo and MflCas 2% formulations on hand and forearm skin features in a clinical trial. (A) hand spots length; (B) hand skin homogeneity; (C) hand skin lightening; (D) the contrast between hand spots and skin; (E) pictures of the hand of selected volunteers before treatment (Day 0) and after treatment by 28 (Day 28), and 56 days (Day 56); (F) forearm anisotropy index. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$ vs. Day 0 of same experimental group

The beneficial effects of MflCas on the ubiquitin-proteasome system were evidenced by increased proteasome activity on the human fibroblasts. Although the mechanism of the ubiquitin-proteasome system is not clear, there is a decrease in proteasome activity under oxidative stress conditions as a result of the intrinsic and extrinsic aging process in age-related diseases.[1,9] The interplay between the proteasome and oxidative stress has been corroborated by data demonstrating that cells treated with proteasome inhibitors induce the production of high levels of reactive oxygen species, which is closely related to the modulation of several markers of redox regulation such as nicotinamide adenine dinucleotide (NADH), kealch-like ECH-associated protein 1 (keap-1), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2).[1,2,11] This imbricated process induces several post-translational changes in several proteasomal subunits, including glutathionylation, protein oxidation leading to proteasomal carbonylation, proteasomal glycoxidation with lipid peroxidation products.[12] Among these processes, carbonylation has been highlighted due to its potentiality to escape degradative processes and irreversibility to produce high-molecular-weight protein aggregates that accumulate with age, promoting serious cytotoxic effects.[13] Oxidative stress conditions induced by hydrogen peroxide can lead to depolymerization of protein filaments and proteasome suppression, contributing to the accumulation of carbonylated proteins and cell dysfunction.[12–14] In our study, cell oxidative stress induced by hydrogen peroxide increased, as expected, the total and relative protein carbonylation, and at the same time, dramatically reduced protein levels in human fibroblasts, whereas MflCas treatment counteracted the oxidative stress, reducing the total and relative production of carbonylated proteins and increasing the availability of protein. These effects were directly related to increased proteasome activity, which protected cells of cytotoxic effects induced by oxidative stress and partially explains the improvement of skin features and anti-aging effects observed in volunteers who received MflCas topical application.

Proteasome activity reduction has been closely related to increased production of lipofuscin in fibroblasts due to decreased activity of lysosomal proteases and lipases.[10] Moreover, lipofuscin is reported as the long-term result of a decreased degradation of oxidized proteins by binding to proteasome with an important role in autophagy, mainly in the macroautophagy process.[15] Lipofuscin, an intracellular fluorescent pigment usually accumulated in aged cells, are granules containing 20%–50% of protein components, 30%–70% of lipids mainly triglycerides, free fatty acids, cholesterol, and phospholipids, 4%–7% of carbohydrates, and traces of vitamins and transition metals, which accumulates in cells due to impaired mitochondrial/lysosomal and proteasomal activities upon oxidative stress or cellular damage lipases.[10] Based on this, lipofuscin may be recognized as both a marker of autophagy and proteolytic function, mainly macroautophagy and proteasome activity, useful to identify the effects of new products with skin anti-aging and depigmentation properties. In the present study, MflCas phytocosmetic preparation reduced lipofuscin production and its effects can be related to increased proteasome activity, suggesting that beneficial anti-aging and skin lightening effects with reduced face and hand spots observed in the volunteers were also related to MflCas ability to modulate proteolytic activity reducing protein accumulation as evidenced by reduced lipofuscin deposition.

Besides its effects on the proteasome system, MflCas also modulated the autophagy machinery to produce skin lightening and anti-aging effects, beneficially affecting different markers of the autophagy process, including LC3B protein as well as the ATG7, mTOR, and PARK2 gene expression.

LC3B (microtubule-associated protein 1A/1B light chain 3B) is a structural microtubule-associated group of proteins of autophagosomal membranes with three members, LC3A, LC3B, and LC3C, which are widely reported as key markers of autophagy and member of ATG8 (autophagy-related protein 8) family used to quantify autophagosome formation.[16,17] Besides LC3 proteins, several autophagy-related genes, including Atg3, Atg5, Atg7, Atg10, and Atg12 are involved in two ubiquitylation-like modifications, Atg12-conjugation, and LC3-modification, which are essential for autophagosome formation.[16,17] While Atg12-conjugation is a key pathway for the formation of pre-autophagosomes, LC3-modification is essential for the formation of autophagosomes.[16,17] In this imbricated signaling pathway, Atg7 activated both Atg12 and LC3-I to form a membrane-bound LC3-II, which promotes the fusion of autophagosomes with lysosomes.[16] These data indicated LC3 functions as an autophagosomal membrane marker, which has been used as a pharmacological target to evaluate candidate autophagic modulators via quantification of autophagosome formation.[17] Although we observed a reduction of Atg7 gene expression, MflCas phytocosmetic preparation was able to increase the production of LC3B protein using a green fluorescent protein, the most appropriate assay to measure autophagic activity in living mammalian cells.[17] The observed data demonstrated MflCas positively modulated LC3B protein independent on Atg7 activation.

The mechanistic target of rapamycin is a serine/threonine kinase that regulates cell growth, proliferation, and survival as well as protein synthesis and autophagy. mTOR forms the complex mTORC1 and mTORC2, the first widely recognized as the mTOR complex that stimulates the synthesis of proteins, lipids, and nucleotides and blocks the autophagy process.[8] Since amino acids are key regulators of mTORC1 activation and amino acid deprivation is a potent autophagy inducer, there is an inverse coupling between autophagy and mTORC1 activation,[8,18] indicating that mTOR is a pharmacological target to modulate the autophagy process and control several autophagy-related diseases. Rapamycin, a natural compound with immunosuppressant properties, was the first compound reported as an inductor of autophagy by inhibiting the mTOR signaling pathway, originating the name of this important mediator of metabolic processes.[8] mTOR has been implicated in the regulation of lifespan, aging, and age-related diseases and processes, including cellular senescence, immune responses, cell stem regulation, mitochondrial function, protein homeostasis, and autophagy.[19] Consequently, products that inhibit the mTOR signaling pathway are potentially useful to control or treat aging

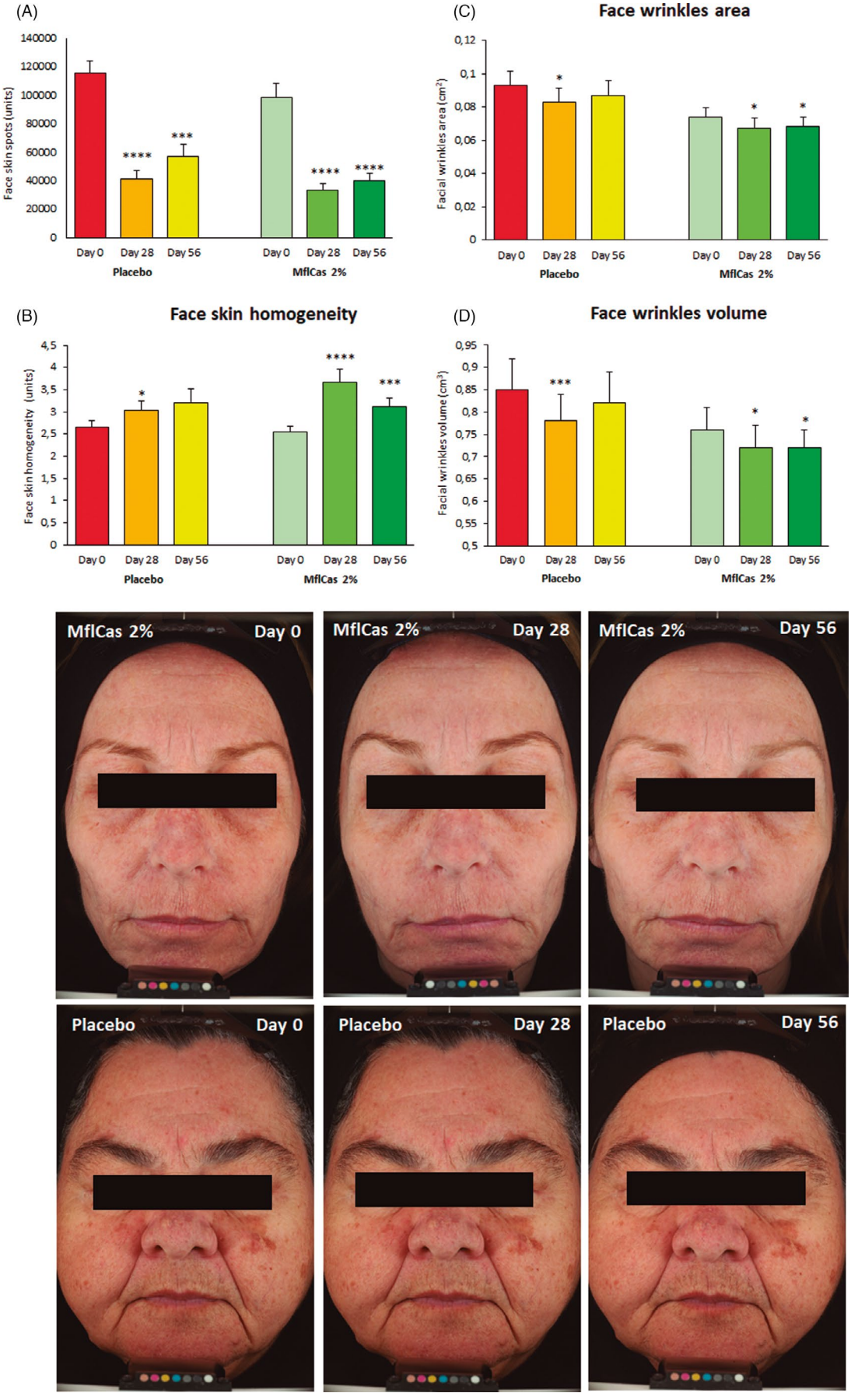

FIGURE 5 Effects of placebo and MflCas 2% formulations on face skin features. (A) face spots length; (B) face skin homogeneity; (C) face wrinkles area; (D) face wrinkles volume; face pictures of placebo and treated volunteers before treatment (Day 0) and after treatment by 28 (Day 28), and 56 days (Day 56). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$ vs. Day 0 of same experimental group

and age-related diseases, as observed in the present study that demonstrated MflCas phytocosmetic preparation inhibited mTOR gene expression in human fibroblasts, contributing with beneficial effects on the skin features in volunteers received a topical application of MflCas 2% formulation.

The engulfment of mitochondria, also known as mitophagy, is a macroautophagy process by which impaired mitochondria are selectively sequestered and delivered for lysosomal degradation.[20] In this process, PARK2 is selectively recruited from the cytosol to damage mitochondria to trigger autophagy.[21] PARK2 also called Parkin is an E3 ubiquitin-protein ligase that was originally reported to be closely involved in Parkinson's disease and reported to promote ubiquitylation of proteins targeted for the proteasome-dependent protein degradation pathway.[21] However, the key role of PARK2 in the autophagy process to degrade impaired mitochondria indicated PARK2 is a key marker of mitophagy and a pharmacological target for the action of drugs. MflCas phytocosmetic preparation promoted an upregulation of Park2 gene expression in human fibroblasts, suggesting this herbal preparation also acted on the mitophagy process, contributing to impaired mitochondrial degradation.

On the contrary, MflCas produced no effects on the gene expression of several biomarkers of the autophagy process, including EI24 (etoposide-induced 2.4 kb transcript), EIF2A (eukaryotic initiation factor 2 alpha), FOXO1 (Forkhead box protein O1), and Atg5 (autophagy-related protein 5), suggesting that autophagy-mediated effects induced by MflCas in human fibroblasts were no related to these signaling pathways of autophagy.

The leaf of *Myrothamnus flabellifolia* is one of the herbal actives used to produce MflCas phytocosmetic preparation. *Myrothamnus flabellifolia* is one among several medicinal plants collectively called "resurrection plants," a group of poikilohydric plants able to survive in extreme dehydration conditions for a long time. The chemical composition of *M. flabellifolia* includes flavonoids and other polyphenols, alkaloids, terpenoids and triterpenes, anthocyanins, cardiac glycosides, saponins, tannins and phlorotannins, steroids, and sugars.[22] Trehalose is an osmoprotectant disaccharide of *M. flabellifolia* responsible for its dehydration tolerance and a homoiochlorophyllous compound that promotes chlorophylls retention for the photosynthetic process.[22,23] In addition, *M. flabellifolia* is a rich source of sugars, mainly sucrose and trehalose, the last is a disaccharide representing 38.1% of total sugar in the dehydration state, which has been reported as a modulator of autophagy machinery in plants and animals.[6,23–25] Trehalose inhibited the solute carrier 2A (SLC2A), a glucose transporter, and reduces the accumulation of lipids droplets in hepatocytes, triggering beneficial cell autophagy as well as reversed cell dysfunction in human fibroblasts induced by proteasome inhibition, also counteracting the increase of reactive oxygen species, ubiquitination of proteins, increasing LC3 levels and acting as an mTOR-independent autophagy activator.[6,23–27]

Seed of *Coffea arabica*, the second herbal component of MflCas, is the source for preparing the coffee, a functional beverage widely used in the world, which have a great historic, social, economic, agricultural, and scientific importance, representing a source of several active components, mainly caffeine. Coffee consumption has been associated to lower risk of several diseases such as chronic liver disease, diabetes mellitus, obesity, cardiovascular diseases, some types of neoplasia, and chronic inflammation as well as acting as antiviral, antifungal, antibacterial, analgesic, diuretic, central nervous system stimulant, effects closely related to high amounts of antioxidant polyphenol and xanthine compounds with the ability to activate an adaptative cellular response via antioxidant, detoxifying and repair enzymes properties.[28,29] In addition, several studies reported the topical use of different herbal preparations containing coffee as safe after skin application and useful for cosmetic and pharmaceutical industries acting as an antimicrobial, antioxidant, antiallergic, ultraviolet radiation protector, anticellulite, skin wound healing, skin anti-aging, and skin hydrating by increased modulation of aquaporin-3 levels in human fibroblasts.[29–31] The chemical composition of *C. arabica* includes polyphenolic compounds and derivatives, diterpene alcohols, carotenoids, carbohydrates, lipids, heterocyclic compounds, and alkaloids, including caffeine.[29,30] Caffeine, the main active component of *C. arabica*, is reported as a potent activator of autophagy that acts by different mechanisms, increasing LC3 autophagosomal marker expression in neuronal, liver, muscle, and heart cells, inhibiting mTOR complex 1 in the liver, muscle, and heart cells, inducing lipophagy in skeletal myotubes and hepatic cells, and protecting skin from oxidative stress and cell senescence induced by ultraviolet radiation via activation of adenosine monophosphate-activated protein kinase (AMPK)-mediated autophagy in human keratinocytes.[7,32–34]

5 | CONCLUSION

We demonstrated that MflCas phytocosmetic preparation containing extracts of leaves of *Myrothamnus flabellifolia* and seeds of *Coffea arabica* modulated ubiquitin-proteasome and autophagy processes in human fibroblasts, inducing proteins degradation by different mechanisms, including the stimulation of autophagosome formation via positive modulation of LC3B production, downregulation of Atg7 and mTOR, the master regulator of lifespan, aging, and cellular senescence, increase of the impaired mitochondrial degradation by upregulation of PARK2. Thus, MflCas acted to increase proteasome activity and reduce protein carbonylation in human fibroblasts, producing detoxifying effects. As a consequence of these differential mechanisms, MflCas phytocosmetic preparation improved skin features in volunteers, reducing facial wrinkles area and volume, hand and facial spots length, anisotropy, and color contrast as well as increasing hand and facial skin lightening. Since phytocosmetic preparation after topical application produced no skin reactions or discomfort, it is possible to state its use is safe, representing an innovative phytocosmetic preparation, useful to improve skin features, mainly as a skin anti-aging and lightening agent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This study was conducted according to regulation from the National Health Surveillance Agency (ANVISA) of the Brazilian Health Ministry, Helsinki Declaration, and Document of America on the good clinical practices. All volunteers did not have any direct benefit from this study.

DATA AVAILABILITY STATEMENT

Data availability under request to authors.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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