Superior even skin tone and anti-ageing benefit of a combination of 4-hexylresorcinol and niacinamide

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Abstract

Objectives: To demonstrate the synergistic effect of 4-hexylresorcinol (4-HR) with niacinamide in boosting anti-melanogenic efficacy in vitro and establish the in vivo efficacy and safety of the combination in a human trial.

Methods: Primary human epidermal melanocytes and 3D pigmented skin equivalents were treated with 4-HR, niacinamide, and their combinations for their effect on pigmentation. This was followed by a randomized, double-blind, split-face clinical study in Chinese subjects, and effects on skin tone, hyperpigmentation, fine lines and wrinkles, hydration, and skin firmness were measured for a 12-week study period.

Results: In vitro tyrosinase enzyme activity studies showed that 4-HR is one of the most potent tyrosinase inhibitors. The combination of 4-HR and niacinamide showed a synergistic reduction in melanin production in cultured melanocytes and lightened the 3D skin equivalent model. In vitro as well as in the human trial, the combination of 4-HR and niacinamide showed significantly improved efficacy over niacinamide alone on hyperpigmentation spots as measured by L*, the visual appearance of fine lines and wrinkles in crow’s feet and perioral area and skin firmness, with no product-related adverse events.

Conclusions: A formulation containing a combination of 4-HR and niacinamide delivered superior skin tone and anti-ageing benefits significantly better than niacinamide alone with no adverse events. This study demonstrates that a product designed to affect multiple pathways of melanogenesis, inflammation, and ageing may provide an additional treatment option, beyond hydroquinone and retinoids, for hyperpigmentation and ageing.

KEYWORDS

cell culture, human volunteer trial, niacinamide, resorcinols, skin physiology/structure, spectroscopy
INTRODUCTION

Skin colour results from the action of multiple biological pathways that primarily control melanin synthesis and its distribution. Additionally, skin colour management is commonly achieved by using ingredients that target these biological processes. Niacinamide is a well-recognised bioactive for even skin tone and an effective treatment for skin hyperpigmentation disorders and for various other dermatological conditions [1–3]. Resorcinols substituted with alkyl groups and aryl groups have been explored in the past as tyrosinase enzyme inhibitors. Within the resorcinol class, 4-HR (4-substituted alkyl resorcinol) is an effective tyrosinase inhibitor that is currently being used in the food and medical industries [4, 5]. 4-HR has been demonstrated through in vitro and human trials to improve hyperpigmentation through its tyrosinase inhibition activity and its anti-inflammatory potential [6, 7]. Kaur et al. [8] further established 4-HR as an NF-κB inhibitor in fibroblasts which could regulate collagen and elastin synthesis and improve photodamaged skin and clinical signs of ageing. In a recent review, Chaudhuri [9] has reported multiple benefits of 4-HR, including evidence for its anti-microbial, antioxidant, anti-glycation effects, along with its effect on extracellular matrix proteins. Successful human trials for skin tone improvement, wrinkle, and aging support the significance of 4-HR for skincare and suggest further exploration of the molecule for various skincare benefits [8, 9].

Improvement of skin appearance is a highly desirable consumer need worldwide. However, in the absence of a potent pigmentation modulator such as a high dose of hydroquinone, consumer perceivable spot lightening is challenging to achieve with cosmetic ingredients. The alternative is to combine actives with complementary or synergistic pathways of pigmentation control to enhance the efficacy of the formulation. While many cosmetic topical products are already available in the market containing either niacinamide or resorcinols, their complementary mechanisms of action against skin colour, photodamage, and clinical signs of ageing had not been explored in
combination earlier. Therefore, in the present studies, we explored the possibility of using a combinatorial approach to enhance the efficacy of these two cosmetic ingredients.

Furthermore, to the best of our knowledge, no prior studies have investigated the synergistic effects of 4-HR and niacinamide formulation in boosting clinical efficacy on skin pigmentation and ageing. Therefore, through in vitro methods, we explored the possible mechanism of action of 4-HR and its combination with niacinamide on skin pigmentation. This was followed by the demonstration of superior efficacy and safety of the cosmetic actives for both hyperpigmentation and ageing in a randomized, double-blind, split-face human trial.

**MATERIALS AND METHODS**

**Chemicals & reagents**

4-HR (Kumar Organics, Bangalore, India), 4-butylresorcinol (4-BR, TCI, Chennai, India), 4-phenylethylresorcinol (4-PER, Spechem, Chennai, India), hydrogen phosphate and potassium dihydrogen phosphate (S.D Fine-Chemical Ltd, Mumbai, India), hydroquinone and potassium chloride (Merck, Darmstadt, Germany), sodium chloride (Fischer Scientific, Waltham, USA), ethanol absolute 99.9% (Commercial alcohols, Ontario, Canada) and tissue solubiliser -Solvable (Perkin Elmer, Boston, USA). Kojic acid, arbutin, niacinamide, disodium triton X 100, RIPA buffer, 3-methyl benzothiazolinone-2(3H)-hydrazone (MBTH), 3,4-dihydroxyphenylalanine (DOPA), tyrosine, phenylmethanesulphonyl fluoride (PMSF), glacial acetic acid, calcein-AM, and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich, St. Louis, USA, and neutral red dye from HiMedia, Kennett Square, USA. S9 insect cell line, High Five (H5) insect cell line, and Cellfectin II were purchased from Invitrogen, Waltham, USA; BacPAK baculovirus rapid titer kit (Clontech, Mountain View, USA); procollagen Type I C-peptide (PIP) EIA kit (Takara, San Jose, USA); micro-BCA protein assay kit (Thermo Scientific, Waltham, USA).

**Cell culture**

Neonatal foreskin primary human epidermal melanocytes, adult primary human dermal fibroblasts, and their respective culture reagents were sourced from Cascade Biologics, Portland, USA. Melanocytes were maintained in Medium 254-CF supplemented with human melanocyte growth supplement, and fibroblasts were maintained in Medium 106 supplemented with low serum growth supplement. In addition, both media were supplemented with 10 U/ml penicillin G and 0.1 mg/ml streptomycin sulphate. The cells were maintained at 37°C in a humidified incubator with a 5% CO₂ atmosphere. Maintenance and sub-culturing of cells were carried out as per the manufacturer’s instructions. Cells between passages 4–6 were used for experimentation.

**Tyrosinase enzyme activity assay**

**Generation of synthetic recombinant human tyrosinase protein**

**Preparation of synthetic human tyrosinase gene, sequence optimized for insect cell expression**

Full-Length rHuTyrase gene [isoform 1 of P14679 (TYRO_HUMAN) reviewed, UniProtKB/Swiss-Prot], with EcoRI and HindIII ends, was synthesised and provided by Geneart, LA, USA. The soluble version, that is, without the trans membrane region, was designed as follows: transmembrane region (TMR) in huTyrase protein sequence was predicted using online tools DAS (Dense Alignment Surface), HMMTOP (Hidden Markov Model Topology), SOSUI, TMHMM (Trans Membrane Helices; Hidden Markov Model), and MOBYLE. As a consensus, the amino acid residue 476 was identified as the first residue in the TMR. Therefore, a TMR-less (TMRL) construct was terminated at the 472nd amino acid residue serine (LEQAS- leucine, glutamic acid, glutamine, alanine, and serine), ahead of the start of the TMR. Thus, the TMRL construct lacks both the TMR as well as the cytoplasmic tail of the full-length enzyme. A soluble version was then prepared by polymerase chain reaction (PCR), using the synthetic FL rHuTyrase gene as the template. The TMRL gene was amplified by PCR, gel-purified, and ends were trimmed with EcoRI and HindIII.

**For cloning**

The gene was synthesised and subcloned into the pFASTBAC1 vector into EcoRI/HindIII sites. The screening was carried out by PCR, and the plasmid was isolated from PCR-positive clones, restriction digested for analysis, and sequenced. Transformation of the sequence was carried out using a pFASTBAC1 clone into E. coli DH10Bac to generate bacmids; bacmid was isolated by the alkali lysis method, and the sequence was confirmed again by PCR. This was followed by transfection into Sf9 insect cells.

**Preparation of bacmids & infection of insect cells**

Sf9 cells were maintained in SF900III SFM medium to obtain logarithmic phase culture at a cell density of 1.5–2 million cells/ml. High Five (H5) cells were maintained in Express Five SFM medium to obtain logarithmic phase culture at a 1.5–2 million cells/ml cell density.
Transfection of log-phase Sf9 cells was carried out using Cellfectin II with Truncated Tyr bacmid constructs.

**Preparation of P1 viral stock**

Spent medium was harvested, centrifuged to remove the cell debris, and stored at 4°C. This stock was denoted as P1 baculovirus, which was further amplified to generate P2 & P3 viral stock. Log-phase Sf9 cells in a shake flask at ~10^6 cells/ml (50 ml) were infected with 5 ml of P1 baculovirus stock (assuming P1 titre of 10^8 pfu/ml). Baculovirus was harvested at 60% cell viability (~4–5 days post-infection). P3 viral stock was generated using P2 viral stock by the same method. A BacPAK baculovirus rapid titre kH5kit was used to determine P3 virus titre and was in the range of ~35–50 million pfu/ml.

**Infection of Sf9 and H5 cells with baculovirus carrying TMRL tyrosinase.** Log-phase cultures of H5 and Sf9 cells (~10^6 cells/ml) were infected with P3 baculovirus at MOIs of 1, 3, and 5. Cells were harvested and pelleted 1, 2, and 3 days post infection. Both Sf9 and H5 cells appeared completely infected, and viability values were much reduced compared to the respective controls.

**Enrichment of tyrosinase**

The cell’s supernatant was centrifuged using concentrating filters of 10 kD.

**Preparation of human melanocyte lysate**

Human melanocytic lysate (HML) was prepared as described earlier by Uchida et al. [10], starting from darkly pigmented cultures of primary human melanocytes. The minor alteration introduced was the reduction of PMSF concentration to a final concentration of 0.1 mM in the lysis buffer. The protein concentration in resultant lysates was estimated using the standard Bradford assay, and the prepared samples were stored as 0.2ml aliquots at ~70°C. Once thawed, the aliquots were diluted (e.g., to final 0.05 µg/µl concentration) into appropriate volumes of stock using 83 mM phosphate buffer (pH 6.8) before use.

**DOPA substrate method**

This was carried out using 96-well plates with 100 µl reaction volume. Briefly, 10 µl of various (10X) concentrations of tyrosinase inhibitors were mixed with 10 µl stock of 20 µg/ml of recombinant tyrosinase protein in 0.5 M phosphate buffer (pH 6.8). In addition, 20 µl of fresh 5mM DOPA stock was added. Volume was adjusted to 100 µl in all conditions using double distilled water. Appropriate no inhibitor controls (reflecting 100% activity level), inhibitor solvent controls, and no enzyme controls (0% activity – maximal possible inhibition theoretically achievable, as well as a measure of uncatalysed background DOPA) were also set up in parallel. The reaction mixtures were incubated in the dark at 37°C for 30 mins. Optical density was measured in a Tecan plate reader using a 470nm filter.

**Cellular in-situ tyrosinase enzymatic activity assay**

A 2 × 10^4 cells were seeded in each well of two 96-well plates, and cultures were left undisturbed for 24 h in a 5% CO2 incubator at 37°C. At 24 h post seeding, cultures in both plates were treated identically with 4-HR, niacinamide, and 4-HR+niacinamide with the vehicle (ethanol) and left undisturbed for 72 h. After 72 h of incubation, one plate was used to determine viable cell counts using the Neutral Red Dye (NR) exclusion assay. The second plate was progressed for the determination of in situ tyrosinase activity. For the latter, cultures were rinsed twice with phosphate-buffered saline (PBS, 1X) and permeabilised with 40 µl of 0.5% Triton X 100 for 1 h on an ice bed. In situ tyrosinase activity was visualised by adding 60 µl of 50-mM sodium phosphate buffer (pH 6.8) containing 2 mM DOPA and 4 mM MBTH for 1 h at 37°C. The reaction was stopped by adding 100 µl of ice-cold 10% trichloroacetic acid (TCA) and then centrifuged at ~300 g for 10 mins at 4°C. The soluble supernatant was separated from the pellet, and the OD was read in a TECAN M1000 plate reader at a 540-nm filter. Tyrosinase activity was expressed after correction for cell numbers (activity/NR) and represented as a percentage of control.

**Neutral Red Dye exclusion assay**

As mentioned above, for one plate post incubation, the culture medium was replaced with 200 µl of neutral red reagent and incubated for 4 h. The NR solution was removed by aspiration, and the insoluble cell-bound dye was dissolved with 200 µl of desorption solution (25 ml distilled water + 25 ml 100% ethanol + 0.5 ml glacial acetic acid). Spectrophotometric absorbance was measured at 520/660 nm in a TECAN plate reader.

**Cell viability and melanin content assay**

Melanocyte cultures were set up as mentioned earlier. After 24 h, cultures were treated with test materials and left undisturbed for a further period of 72 h. Comparative vehicle controls of 0.1%(v/v) DMSO or media controls (for aqueous actives) were also set up simultaneously. At the end of the incubation period, cell viability was determined using the Calcein-AM method.
Briefly, cell culture spent media were removed, and cells were washed with 0.2 ml of PBS in Ca–Mg free solution. Next, 1 µM Calcein-AM was added (0.2 ml/well), including the control wells without cells. Plates were covered with aluminium foil and incubated for 30 mins at 37°C in the regular CO2 incubator. Calcein fluorescence was then measured (excitation at 490 nm and emission at 520 nm) in the TECAN plate reader. Calcein is light-sensitive; all operations using Calcein were carried out with minimal exposure to light.

After the Calcein fluorescence readings were obtained, the media were drained, and 0.15 ml of 1N NaOH (in 10% DMSO) was added per well. Cells were lysed by resuspension and incubation (60°C for 1 h). Then, 0.12 ml of this lysate was transferred to a fresh 384-well plate, and absorbance was measured at 405 nm in a TECAN plate reader.

**Melanin content measurement**

The tissue melanin was extracted in 150 µl of Solvable on a dry bath at 60°C for 12–16 h with gentle shaking. 100 µl of the supernatant was transferred to a 384-well plate, and absorbance was measured at 490 nm. Values are expressed as the mean percentage (%) change in melanin over control.

**Procollagen production**

A 1 x 10^4 fibroblasts were seeded in each well of 96-well plates and cultures left undisturbed for 24 h in a 5% CO2 incubator at 37°C. After 24 h, cultures were treated with test materials and left undisturbed for a further period of 48 h. Comparative vehicle controls of 0.1% (v/v) DMSO or media controls (for aqueous actives) were also set up simultaneously. At the end of the incubation period, the cells were lysed with RIPA buffer, and the lysate was used to measure procollagen levels by ELISA using the

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**FIGURE 1** Inhibition of recombinant human tyrosinase enzyme activity by 4-HR. Dose-dependent inhibition of tyrosinase enzyme activity by 4-HR was performed using synthetic recombinant human tyrosinase enzyme protein and DOPA as the substrate. The data are representative of at least three independent sets of experiments, and the values are mean ± standard deviation (SD) [Colour figure can be viewed at wileyonlinelibrary.com]
procollagen Type I C-peptide (PIP) EIA Kit as per the manufacturer’s instructions. In addition, total protein was also measured from the same lysate using the micro-BCA protein assay kit as per the manufacturer’s instructions.

**Clinical study design**

This was a randomised, double-blind, split-face product application, 12-week study with 44 subjects completing all study phases. The study was conducted according to the protocol and in accordance with ethical guidelines. The study was conducted between November and February on Chinese females in Shanghai after receiving approval from the Shanghai Clinical Research Centre Ethical Committee (SCRC-IEC-2014-23-01).

The subjects were of Chinese origin between 35 and 60 years of age and in generally good health. Subjects with at least three hyper-pigmented spots (the diameter or the length of the spot was >2 mm, spot type limited

**Figure 2** Effect of 4-HR on melanin content in melanocytes after 72 h of treatment. The values are mean ± SD of three independent sets of experiments. *p < 0.05; statistically significant versus kojic acid, arbutin, and hydroquinone; \( ^{\text{5}}p < 0.05; \) statistically significant versus arbutin and hydroquinone

**Figure 3** Effect of different ratios of 4-HR and niacinamide combination on melanin content in melanocytes after 72 h of treatment. The values are mean ± SD of three different sets of experiments. *p < 0.05; statistically significant versus control; \( ^{\text{5}}p < 0.01; \) statistically significant versus all treatments; \( ^{\text{6}}p < 0.005; \) statistically significant versus 4-HR (1 µM) and 4-HR (10 µM); \( ^{\text{6}0}p < 0.005 \) statistically significant versus 4-HR (1 µM)

**Figure 4** Effect of 4-HR+niacinamide on melanin content and *in situ* tyrosinase activity in melanocytes after 72 h of treatment. The values are mean ± SD of three different sets of experiments. *\( ^{\text{5}}p < 0.05; \) statistically significant versus 4-HR (1 µM) for melanin and *in situ* tyrosinase activity.
to solar lentigines/freckles/post-inflammatory hyperpigmentation; as determined by study dermatologist) on each side of the face, moderate pigmentation level \( \geq 4 \) (scale 0–9), and fine lines & wrinkles on crow’s feet \( \geq 3 \) (scale 0–9) were included in the study. The subjects agreed to minimise sun exposure and not to use any other creams during the study. Subjects were provided in total two test products, one product for each half face, and instructed to apply \(-0.3g\) on half side of the face, twice per day, according to a randomisation scheme assigned to each test subject for 12 weeks. Written informed consent was obtained from all subjects before their participation.

Consent was also obtained to use the photos without exposure to their identity. In total, 50 subjects enrolled for the study, and six subjects dropped out for non-product-related reasons.

The two test formulations were identical, containing 3% niacinamide, except for the presence of 0.4% 4-HR in one but not the other. Other formulation ingredients (\( \geq 1\% \)) include cyclomethicone (and) dimethicone crosspolymer, cyclopentasiloxane, glycerin, cyclohexasiloxane, glycolic acid, potassium hydroxide solution, distilled water, octylmethoxycinnamate, and conjugated linoleic acid.
Clinical measurements

Clinical evaluations were performed using visual and instrumental measurements. Visual assessment was performed by a well-trained visual assessor using scales on the following parameters: (1) Crow’s feet fine lines and wrinkles, (2) Perioral fine lines and wrinkles, (3) Overall hypo-pigmentation, and (4) Facial skin colour. In addition, well-established instrumental measures were carried out as follows:

1. Skin lightness parameter L* on the forehead and selected hyperpigmentation spots were measured using a skin reflectance Spectrophotometer® CM2600d (Minolta, Japan)
2. Skin elasticity by a suction chamber device, Cutometer® MPA 580 (Courage + Khazaka Electronic GmbH, Germany)
3. Skin hydration of the cheek was measured using a Corneometer® CM 825 (Courage + Khazaka Electronic GmbH, Germany)
4. Skin barrier function was evaluated as Transepidermal Water Loss (TEWL) measured with Aquaflux AF200® (Biox systems, England)

All instrument measures were repeated measures, and the average data were used for data analysis. In addition, digital images by VISIA®-CR (Canfield Scientific, Inc. USA) were obtained to document the results. The camera setup, lighting conditions, and subject’s position were standardised during the study.

All the measurements were performed by the same assessor and same devices for 12 weeks during the winter season in Shanghai from November to February. The examination room was temperature- and humidity-controlled, with the room’s temperature being 22 ± 2°C and its relative humidity being 50 ± 5%.

All subjects were made to sit for at least 20 mins in the examination room before any measurement was taken. All parameters were assessed at baseline, first week, and then for every 4 weeks up to 12 weeks.

**FIGURE 7** Effect of 4-HR+niacinamide skin formulation on background skin colour. (a) Visual assessment on facial skin lightness. *p < 0.05, a significant improvement over baseline. (b) Spectrophotometry measurement of lightness in L* units on forehead skin. *p < 0.05, a significant improvement over baseline. *p < 0.05 a significant improvement over 3% niacinamide treatment. Effect of 4-HR+niacinamide skin formulation on spot colour. (c) Visual assessment of overall hyperpigmentation spots. *p < 0.05, a significant improvement over baseline. (d) Spectrophotometry measurement of lightness in L* units on selected three hyperpigmentation spots. *p < 0.05, a significant improvement over baseline, and *p < 0.005 a significant improvement over 3% niacinamide treatment
**Statistical analysis**

Response for all analysis was represented as a change from baseline, which is calculated as follows:

$$\text{CFB} = \text{Week}(i) - \text{Baseline}$$

where \(i\) = week 1, week 4, week 8, week 12.

Before analysis of treatment effects, a baseline balance across the treatments was conducted, considering the subject as a random effect. Then, normality assumption was tested, and if data were normal, parametric tests (one sample, t-test/ Paired t-test as appropriate) were performed. Otherwise, as appropriate, corresponding non-parametric methods (Wilcoxon signed rank, Pratt–Lehmann / Mann–Whitney (Wilcoxon rank sum)) were applied. All statistical analyses were carried out at a 5% level of significance. All data were analyzed using SAS (version 9.3 SAS Institute INC, USA) and represented as a change from baseline (CFB) means ± standard error (SE).

**RESULTS AND DISCUSSION**

The amount and distribution of melanin are key determinants of skin colour. Melanin plays a major role in protecting the skin from the sun’s radiation. However, abnormal accumulation of melanin is associated with hyper-pigmentary conditions, including melasma, freckles, and senile lentigines [11]. Tyrosinase enzyme plays a key role in the production of melanin, and many tyrosinase inhibitors have been reported and used by various cosmetic and pharmaceutical industries as skin brightening agents [12]. The commonly used compounds in this class include hydroquinone, arbutin, kojic acid, thiols, and resorcinol. Amongst resorcinols, 4-BR and 4-PER are primary cosmetic ingredients that are under focus for efficacy and safety for skin brightening [13, 14]. Another alkyl resorcinol, 4-hexylresorcinol (4-HR), has also been shown to reduce the appearance of fine lines, wrinkles, and hyper-pigmentation [6, 8].

In our studies with recombinant human tyrosinase protein, using DOPA as the substrate, 4-HR and 4-BR appeared

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**FIGURE 8** (a) Effect on skin ageing parameters through visual assessment of Crow’s feet fine lines and wrinkles. *\(p < 0.05\), a significant improvement over baseline; # \(p < 0.05\), significantly better than 3% niacinamide formulation. (b) Effect on skin ageing parameters through visual assessment of perioral fine lines and wrinkles. *\(p < 0.05\), a significant improvement over baseline; \# \(p < 0.05\) significantly better than 3% niacinamide formulation.
to be the best tyrosinase inhibitors amongst the tested compounds, with the IC_{50} values of 16 and 13 µM, respectively (Figure 1 and Table 1). However, 4-HR appeared to be better in melanin reduction than 4-BR and other compounds when all were tested on primary human melanocytes.

As shown in Figure 2, 4-HR showed the most potent effect on melanin production in melanocytes with an inhibition of 35 ± 1.9% compared to 4-BR (28.6 ± 3%) and 4-PER (23 ± 4%) at similar doses. Therefore, 4-HR was progressed further for the combination studies.

The other class of skin brightening compounds is the one that regulates the transfer and distribution of melanin/melanosomes from melanocyte to keratinocytes and includes soybean extract and niacinamide [15, 16]. Niacinamide is a well-established and commonly used skin-lightening agent and has been reported to have a significant effect on various hyperpigmentary conditions [1, 2, 17]. Therefore, the efficacy of the combination of 4-HR and niacinamide was evaluated for pigmentation and ageing benefit.

For the combination studies, different ratios of 4-HR (1 and 10 µM) with niacinamide (1 and 10 mM) were tested on melanocyte cultures, as shown in Figure 3. The doses of the compounds selected were the maximum non-cytotoxic doses for primary melanocytes (4-HR-10 µM and niacinamide-10 mM) and respective ten times lower doses for combination studies. A significant reduction in melanin could be achieved by combining the two compounds (49.5 ± 2%; p < 0.01) at the maximum non-cytotoxic dose of 10 µM 4-HR and 10 mM niacinamide versus all other treatments (Figure 3; 1 mM niacinamide data not shown). However, a synergistic reduction of 30 ± 0.8% in melanin synthesis was observed only with the combination of 1 µM 4-HR with 10 mM of niacinamide (p < 0.05) versus that of niacinamide alone (7.2 ± 4.3%) or 4-HR alone (7.3 ± 3%).

Furthermore, to investigate the reason for synergy in melanin reduction, in situ tyrosinase enzyme activity was measured in melanocytes treated with doses of actives which showed a synergistic reduction in melanin, as

![Figure 9](image-url)
shown in Figure 3, for 72 h. In accordance with melanin reduction, a simultaneous decrease in tyrosinase enzyme activity was also observed (Figure 4), which suggests that mechanisms other than direct inhibition of tyrosinase enzyme activity by 4-HR could be involved, resulting in reduced enzyme production or expression in melanocytes when treated with the combination. It is possible that the synergistic effect on melanogenesis may not just be the tyrosinase enzyme inhibition but also the reported multiple effects of 4-HR and niacinamide on pathways influencing pigmentation [9, 18].

**4-HR plus niacinamide stimulates procollagen synthesis in dermal fibroblasts**

4-HR+niacinamide combination was evaluated for its anti-ageing potential by testing its ability to boost pro-collagen synthesis in dermal fibroblasts at multiple doses (data not shown). Figure 5 shows statistically significant stimulation of procollagen synthesis over the control (33 ± 9%; \( p < 0.05 \)) in fibroblasts only when the cells were treated with higher ratios of 4-HR to niacinamide, unlike in melanin reduction.

Kaur et al. also reported similar findings on the stimulatory effect of 4-HR on collagen and elastin synthesis through inhibition of NF-kappaB, which may be the mechanism for its anti-ageing efficacy [8]. Besides the above mentioned evidence, studies have shown that 4-HR and niacinamide could modulate NF-kB–mediated inflammatory response, which may support their anti-ageing activity [9, 18]. Glycation is another key driver of skin ageing and, together with oxidative stress and inflammation, can lead to loss of collagen structure in the dermis and modify fibroblast functions [19]. Both molecules have also been shown in vitro to prevent or inhibit the glycation of proteins through their antioxidant property and may benefit ageing skin [6, 9, 20].

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**FIGURE 10**  (a) Effect on skin hydration and barrier parameters. Instrumental measurement on skin hydration by using a corneometer. \( *p < 0.05 \), a significant improvement over baseline, \( #p < 0.05 \) a significant improvement over 3% niacinamide treatment. (b) Effect on skin TEWL. Instrumental measurement on TEWL by AquaFlux. \( *p < 0.05 \), a significant improvement over baseline.
4-HR plus niacinamide combination inhibits melanin in 3D skin equivalent models and reduces hyperpigmentation in subjects

The active compounds were formulated at different doses individually and in combination and tested on 3D skin equivalent to decide on the two appropriate treatments to progress to the human trial. As shown in Figure 6a, a dose-dependent reduction in melanin was observed with both individual and combination of actives as well as improvement in L* (Figure 6b), without any cytotoxicity (data not shown).

From this study, the combination of niacinamide (3%) + 4-HR (0.4%) was found to be directionally better (43 ± 5.8%) in melanin reduction and L* (5.23 L*) vs. 35 ± 2.8% with 4.52 L* for 4-HR (0.4%), and 28 ± 3.7% with 4.54 L* for niacinamide (3%), respectively, and was progressed for a 12-week human trial in Chinese subjects. Any other higher doses of ingredients were not considered due to issues related to formulation compatibility and sensory and possible toxicity.

In the human trial, the efficacy of niacinamide (3%) + 4-HR (0.4%) was compared with niacinamide (3%) alone over 12 weeks of product application in subjects; both niacinamide and 4-HR+niacinamide treatment, in general, improved the skin colour and spot lightening against baseline at all assessment time points, as indicated by both visual assessment (Figure 7a,c) (lower value indicates lighter colour) and spectrophotometry CM2600d measurement L* value (Figure 7b,d) (higher value indicates lighter colour), respectively. The improvement of facial skin colour with 4-HR+niacinamide (p = 0.024) was significantly better than niacinamide alone only at week 4 by spectrophotometry measurement L* value (Figure 7b). On the other hand, the improvement on spots with 4-HR+niacinamide was significant to 3% niacinamide alone over all time points (week 2; p = 0.0044, week 4; p = 0.0003, week 8; p = 0.0002, week 12; p = 0.0001) by spectrophotometry measurement L* value (Figure 7d).

4-HR and niacinamide synergize to boost the anti-ageing efficacy of the skin formulation

As shown in Figure 8, both 4-HR+niacinamide and niacinamide alone significantly improved crow’s feet and perioral fine lines and wrinkles against baseline at all assessment time points. The superiority of 4-HR+niacinamide versus niacinamide alone was evident on crow’s feet, fine lines, and wrinkles from week 4 onwards (week 4; p = 0.0069, week 6; p = 0.01, week 8; p = 0.014) (Figure 8a), while higher efficacy was seen earlier on at weeks 1 (p = 0.0094).

| Test product | Clinical benefit type | Parameters | Assessment time points |
|--------------|-----------------------|------------|------------------------|
| 4-HR+niacinamide versus niacinamide alone formulation | Anti-ageing/ wrinkles | Visual lines/wrinkles on crow’s feet | x |
| | Visual lines/wrinkles on perioral | x |
| | Skin elasticity and firmness | Cutometer R2 | x |
| | | Cutometer R7 | x |
| | Skin/Spot lightness | Visual overall hyperpigmentation | x |
| | | Visual skin colour | x |
| | | Skin lightness - L* | x |
| | Selected hyperpigmentation spot lightness - L* | x |
| | Hydration | Corneometer reading | x |
| | Skin barrier function | Trans-epidermal water loss | x |

Note: Where x = 4-HR+niacinamide formulation is superior to niacinamide alone formulation. X p < 0.05 significantly better than 3% niacinamide.
and 4 ($p = 0.0076$) for the perioral area, fine lines, and wrinkles; however, this was not sustained over later weeks (Figure 8b).

Firmness and elasticity measurements were performed using a Courage & Khazaka Cutometer at baseline and all assessment time points of the study. The cutometer generates data curves of deformation versus time, from which calculations are performed to give indications relating to the mechanical properties of the skin [21]. The parameter $U_a/U_f$ (R2), which corresponds to the part between the maximal amplitude, indicates the capacity of the skin to deform (biological elasticity) [22]. The $R_7$ ($U_r/U_f$) measurement is commonly referenced as the parameter which reflects skin elasticity. The more the value aims towards 1 (100%) for these parameters, the more elastic the skin is expected. An increase of any one of these parameters translates as improvement of the skin firmness.

In the study (Figure 9a,b), both treatments increased the R2 value and R7 value against baseline at all time points. Furthermore, an improvement over niacinamide alone was observed at all time points for R2 (week 1; $p = 0.0016$, week 4; $p < 0.0001$, week 8; $p = 0.0002$, week 12; $p = 0.0006$) and from week 4 onwards for R7 (week 4; $p = 0.015$, week 8; $p = 0.01$, week 12; $p < 0.056$), with 4-HR+niacinamide for skin firmness.
Skin hydration was measured in the study using a CM825 Corneometer (Courage & Khazaka GmbH). Trans-epidermal water loss was measured as an indicator of the integrity of the skin barrier. Measurements were performed on the side cheek area for all time points for both parameters. An enhanced rate of water loss from the skin indicates that the barrier is damaged and weakened, allowing more water to escape from the skin surface. Healthy skin allows smaller quantities of water to evaporate from the skin’s surface in a more regulated manner.

As shown in Figure 10, both formulations significantly improved TEWL, but no difference was observed between the treatments at all time points (Figure 10b). Both treatments also delivered improvement on skin hydration at all time points. However, a significant difference between the two could be seen only in the 12th week of the study ($p = 0.03$) (Figure 10a).

Thus, as evident from Table 2 and Figures 11 and 12, 4-HR+niaacinamide formulation was significantly better ($p < 0.05$) than niaacinamide alone on many parameters, including improvement of skin colour, spot lightening, and skin firmness.

CONCLUSION

Our study indicates that 4-HR with niaacinamide is a potent combination promoting even skin tone and anti-ageing benefits. We speculate that the effect of the combination is attributable, at least partly, to the anti-tyrosinase enzyme activity of 4-HR and melanosome transfer inhibition by niaacinamide for hyper-pigmented spot reduction. The additional contribution could be due to the multiple mechanisms attributed to both the ingredients like their anti-inflammatory, anti-glycation, antioxidant, and collagen-boosting ability relevant to skin ageing. These studies, thus, demonstrate that a product designed to affect multiple biological processes or pathways may provide a safe and effective treatment option as an alternative to hydroquinone for hyper-pigmentation and retinoids for anti-ageing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

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