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Article *in* International Journal of Cosmetic Science · January 2014

DOI: 10.1111/ics.12117 · Source: PubMed

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Bakuchiol: a retinol-like functional compound revealed by gene expression profiling and clinically proven to have anti-aging effects

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Received 04 November 2013, Accepted 25 January 2014

Keywords: anti-ageing clinical, bakuchiol, DNA microarray, histology, retinol

Synopsis

OBJECTIVE: The study was undertaken to compare the skin care related activities of retinol and bakuchiol, a potential alternative to retinoids. Retinol is a pivotal regulator of differentiation and growth of developing as well as adult skin. Retinoic acid is the major physiologically active metabolite of retinol regulating gene expression through retinoic acid receptor – dependant and independent pathways.

METHODS: Comparative gene expression profiling of both substances in the EpiDerm FT full thickness skin substitute model was undertaken. Furthermore, type I, III and IV collagen, as well as aquaporin 3 expression was analyzed by ELISA and/or histochemistry in human dermal fibroblasts and/or Epiderm FT skin substitutes.

RESULTS: Bakuchiol is a meroterpene phenol abundant in seeds and leaves of the plant *Psoralea corylifolia.* We present evidence that bakuchiol, having no structural resemblance to retinoids, can function as a functional analogue of retinol. Volcano plots showed great overall similarity of retinol and bakuchiol effects on the gene expression profile. This similarity was confirmed by the side-by-side comparison of the modulation of individual genes, as well as on the protein level by ELISA and histochemistry. Retinol-like functionality was further confirmed for the upregulation of types I and IV collagen in DNA microarray study and also show stimulation of type III collagen in the mature fibroblast model. Bakuchiol was also formulated into a finished skin care product and was tested in clinical case study by twice-a-day facial application. The results showed that, after 12 weeks treatment, significant improvement in lines and wrinkles, pigmentation, elasticity, firmness and overall reduction in photo-damage was observed, without usual retinol therapy-associated undesirable effects.

CONCLUSION: Based on these data, we propose that bakuchiol can function as an anti-ageing compound through retinol-like regulation of gene expression.

Résumé

OBJECTIF: L'étude a été menée pour comparer les activités liées aux soins de la peau du rétinol et du bakuchiol, une alternative potentielle aux rétinoïdes. Le rétinol est un régulateur essentiel de la différenciation et de la croissance de la peau en développement ainsi que la peau des adultes. L'acide rétinoïque est le principal métabolite physiologiquement actif du rétinol qui régule l'expression des gènes par des voies dépendantes et indépendantes du récepteur de l'acide rétinoïque.

MÉTHODES: Un profilage comparatif d'expression génétique de ces deux substances dans le modèle substitut de la peau EpiDerm FT a été entrepris. La synthèse des collagènes de type I, III et IV et de l'aquaporine 3 dans des fibroblastes dermiques humains normaux ont été analysés par ELISA et/ou en histochimie dans le modèle de peau EpiDermTM FT.

RÉSULTATS: Bakuchiol est un phénol meroterpène abondant dans les graines et les feuilles de la *Psoralea corylifolia*. Nous présentons des preuves que bakuchiol, n'ayant aucune ressemblance structurelle avec les rétinoïdes, peut fonctionner comme un analogue fonctionnel de rétinol. Les diagrammes de type Volcano montrent la grande similitude de l'effet du rétinol et du bakuchiol sur l'expression des gènes. Cette ressemblance a été aussi démontrée par la comparaison de la modulation de l'expression de gènes particulaires, appartenant a de différentes groupes fonctionnelles. La fonctionnalité rétinol -like a été confirmée par la régulation à la hausse du collagène de type I et IV et aquaporine 3 au niveau de proteines par ELISA et histochimie. Le bakuchiol a également été formulé dans un produit de soin de la peau et a été testé dans une étude clinique avec deux applications par jour au visage. Les résultats ont montré que, après le traitement de douze semaines, une amélioration significative a été observée dans les rides et ridules, la pigmentation, l'élasticité, la fermeté et la réduction globale des dommages du photo-vieillissement, sans les effets indésirables habituels associés à la thérapie au rétinol.

CONCLUSION: Sur la base de ces données, nous proposons que le bakuchiol peut fonctionner comme un composé anti- vieillissement grâce à la réglementation de l'expression des gènes similaire au rétinol.

Introduction

Retinoids have been first defined as a family of naturally occurring compounds comprised of vitamin A (retinol) and its derivatives, such as vitamin A aldehyde (retinal) or vitamin A acid (retinoic acid). Retinoic acid is considered to be the active form of vitamin A and is involved in gene regulation, leading to effects ranging from hyperplasia to differentiation and apoptosis of normal and cancer cells [1, 2]. The conversion of retinol to retinal by the retinol dehydrogenases is considered to be the rate-limiting step for the biosynthesis of retinoic acid [3]. In addition to retinol dehydrogenases, P450s 1A1, 1A2, 1B1 and 3A4 have been shown to be involved

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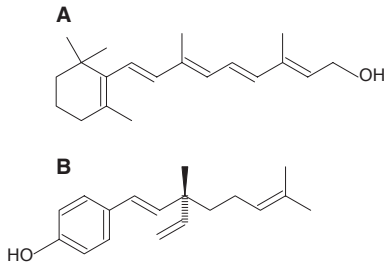

Figure 1 (A) Retinol. (B) Bakuchiol.

in the metabolism of retinoic acid [4]. Human dermal fibroblasts treated with retinol metabolize it to retinoic acid, demonstrating the bioactivity and bioavailability of retinol in the dermis [5].

Retinol is found in a variety of foods such as beef, calf, chicken liver, eggs and fish. It is also present in vegetables such as carrots, squash, sweet potatoes, pumpkin and cantaloupe. Subsequent research has resulted in a much larger class of natural and synthetic compounds that are termed retinoids due to their functional – although not always structural – similarity to vitamin A [6].

The epidermis is one of the major targets for the retinoic acid signalling in adult organism. Classic retinoid activities are mainly achieved through the transcriptional regulation of specific genes via two classes of nuclear hormone receptors, the retinoic acid receptors (RAR) and retinoid X receptors (RXR), each with three isotypes (α, β, γ) and multiple isoforms [7–9]. All-*trans* retinoic acid preferentially binds RARs and its 9-*cis* isomer, 9-*cis* retinoic acid, binds both RARs and RXRs. The predominant retinoid receptors found in skin are RARγ and RXRα [10]. Retinoic acid receptors exert their effects in epidermis through direct binding to epidermal genes [11, 12] or interference with signalling of other transcription factors [13].

Retinoic acid and its derivatives have been used as therapeutic agents for numerous skin conditions from psoriasis to acne and were also found to be clinically effective against wrinkles [14]. Retinol (Fig. 1A) application is believed to be a more efficient method to deliver retinoic acid to the skin cells than direct treatment with retinoic acid [15]. However, retinoid therapy using even the newer analogues is still restricted by many undesirable side effects, such as irritation, dryness, peeling, erythema and a sensation of burning on the skin [16, 17]. These side effects often result in non-compliance and discontinuation of therapy. Therefore, there is a definite need to develop improved retinoid compounds. Such compounds should have similar but not identical gene expression pattern as compared with retinol, ideally, resulting in retinol-like beneficial effects, without having retinol-like undesirable side effects. Here, we build on our preliminary findings [18] and report that bakuchiol (Fig. 1B) – a meroterpene phenol from seeds of the plant *Psoralea corylifolia* [19, 20] – exhibits such retinol-like functionality.

Materials and methods

Test materials

Retinol (Fig. 1A), trade named Retinol 50 C (INCI name Retinol and Polysorb 20), was purchased from BASF (Florham Park, NJ, U.S.A). This product is a yellow oil containing 50% vitamin A in polysorbate 20 and stabilizer system consisting of 3.5% BHT and 1% BHA. Bakuchiol (Phenol, 4-[1E,3S)-3-ethenyl-3,7-dimethyl-1,6-octadinenyl; Fig. 1B)l is a phenolic compound with a monoterpene side chain [19]. Bakuchiol belongs to a rare group of terpenoids in which the aromatic ring system is derived from phenylpropane unit. The material used in this study is obtained from edible seeds of *Psoralea corylifolia,* which is psoralene-depleted Bakuchiol (trade named Sytenol® A; INCI name Bakuchiol) with a purity of about 95%.

DNA microarrays

EpiDerm FT tissues were obtained from Mattek (Ashland, MA, U.S.A.; cat. no. EFT 212) and cultured according to the manufacturer's instructions. The test materials – Retinol (50%) and Bakuchiol (100%) were dissolved in DMSO at 10 mg mL^{-1} (Retinol) and 5 mg mL^{-1} (Bakuchiol), and further dilutions were made in type I sterile water. Test materials were assayed at 10 μg mL^{-1} (Retinol) and 5 μg mL^{-1} (Bakuchiol) against 0.1% DMSO as a control. The incubation time with skin tissues was 2 days. After incubation, skin tissues were harvested, frozen in liquid nitrogen and subjected to total RNA extraction with Qiagen kit (Frederick, MD, U.S.A). The quality of extracted RNA was validated twice by electrophoresis (after extraction and before microarray analysis).

Samples were hybridized and data were analysed using human OneArray platform from Phalanx Biotech (Palo Alto, CA, U.S.A.). The Excel file yielding information on over 30 000 probes was then further processed in house to retain only differences with low *P* values (*P* value cut off was 0.05) and high fold-change (the cut off value for fold-change was 2.0).

Collagen ELISA

Retinol and bakuchiol were assayed at 10 μg mL^{-1} on normal human fibroblasts grown in DMEM with 5% calf serum (Hyclone, Salt Lake City, UT, U.S.A.). For type I and IV collagen quantification, neonatal human dermal fibroblasts (low passage; American Type Culture Collection, Manassas, VA, U.S.A. cat. no. PCS-201-010, lot no. 58243223) were used. For type III collagen quantification, human epidermal fibroblasts from a 68-year-old female donor (p. 5, Zen-bio, cat. no. KR-F) was used. Cells were exposed to test materials for 3 days (type I collagen quantification) or 7 days (type III and IV collagen quantification). Afterward, cell-culture conditioned media were harvested and assayed for type I, type III or type IV collagen by sandwich ELISA using affinity-purified antibodies, followed by streptavidin-avidin-HRP conjugate and ABTS, according to a standard ELISA protocol [21, 22]. The colorimetric signal proportional to collagen content was quantified with the Bio-Rad microplate spectrophotometer 3550-UV at 405 nm with background subtraction at 660 nm and analysed with Microplate Manager v.2 software for Macintosh (BioRad, Hercules, CA, U.S.A.).

Histochemistry

Fort type IV collagen and aquaporin three visualization retinol and bakuchiol were dissolved at 50 mg mL^{-1} in DMSO. Test samples were then further diluted in type I sterile water and tested at final concentrations 10 μg mL^{-1} (Retinol) and 5 μg mL^{-1} (Bakuchiol) using EpiDermFT tissues from Mattek. Tissues were equilibrated for 24 h and incubated with test materials or water (negative control) for 96 h, afterward they were rinsed and fixed in 10% buffered formalin. Paraffin sections of these tissues were stained with a biotinylated antibody against type IV collagen (cat.

no. 1340-08 Southern Bio, Birmingham, AL, U.S.A.) or AQP3 (cat. no. sc-9885, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and developed with the ABC peroxidase staining components (Santa Cruz Biotechnology). Slides were mounted in Permmount and observed on Nikon (Tokyo, Japan) Eclipse TS100 inverted microscope at 100 magnifications. Microphotographs were taken with Canon Rebel digital camera mounted on the Nikon TS100 microscope.

Clinical study

General

Seventeen healthy but photo-aged subjects were recruited to this blind study (all females; age range 40–65 years). All subjects read, understood and signed an informed consent. All subjects had abstained from the use of moisturizing products and used only simple soap, for at least 1 week prior to treatment conditions. All test products were supplied in identical containers. Subjects were instructed on the use of the cream – twice daily morning and evening applications to the entire face for 3 months. Clinical assessments of the skin of the face were performed for all participants at baseline and following 4, 8 and 12 weeks of product use. The following parameters were assessed at each visit by an expert grader: Fine Lines/Wrinkles, Roughness and Dryness, Skin Tone, Skin Elasticity and Firmness, Radiance, Brightening and Overall Eye Area Appearance. Assessment for each parameter was performed at baseline using the following five-point ordinal severity scale: 0 = None; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Severe.

Silicone analysis profilometry

At each visit, a single silicone replica was made of the target area and a photographic record was kept of this target for subsequent relocation. Comparative analysis of skin profilometry was conducted, using surface roughness and wrinkle depth analysis. The heights of the replicated wrinkles were measured using Miyomoto Surftest profilometer. Ry (depth) and Ra (mean roughness) were recorded at each time of measuring operation. The area scanned from each sample was clearly mapped so as to determine the same area in respective weeks 4, 8 and 12 samples.

Photo booth

At each time point, a series of high resolution digital photographs was collected using a photo booth equipped with Canon G7 Digital Camera 10 MP, 6× zoom. Subject positioning was reproduced upon return visit. A light booth was used so as to provide controlled reproducible light conditions. The booth consists of an array of 8 equally spaced fluorescent tubes in a semicircular configuration. The software-driven system allows the position and expression of the test subjects to be aligned to a high degree.

Method of assessment & product application

Baseline

The expert grader performed assessment of the panelist's face and eye area for all the parameters as described before. Photographs were conducted using a photo booth with a three-point head restraint with photographs taken with frontal view, 45 degrees to the right and 45 degrees to the left at each time point (Day 0, Weeks 4, 8 and 12). A replica ring was used to delineate the wrinkle site in the crow's feet area. Silflo was applied on the site, allowed to dry for approximately 5 min, and the replica was removed gently from the site.

Product application

Test Materials were distributed to the subjects. Subjects were asked to gently massage a small amount of the test material to the crow's feet and eye area and then smooth over the whole face. They were asked to apply twice a day for 12 weeks. A study diary was given to the panelists to list the time of application, the dates and any subjective comments that they might have in regard to the test product.

Weeks 4, 8 and 12

Panellists returned to the study site after week 4, 8 and 12 of product use. At each study visit, panellists were clinically evaluated in the same manner as at the baseline visit.

Statistical analysis

For each of the parameters, percent improvement from baseline was calculated to express the efficacy of the product at each time point (4, 8 and 12 weeks). Using the *t*-test, the statistical significance of the net change from baseline (pre-application) to each subsequent time point was assessed. Statistical significance was defined at the $P = 0.05$ or less level (corresponding to a 95% or greater confidence level).

Formulated product

Formulation details are given in Table V.

Results and discussion

Bakuchiol is a meroterpene phenol abundant in seeds and leaves of the plant *Psoralea corylifolia* [19, 20] and has also been isolated from other plants, such as, *P. grandulosa* [23, 24], *P. drupaceae* [25], *Ulmus davidiana* [26], *Otholobium pubescens* [27] and *Piper longum* [28]. It is widely used in Indian as well as Chinese medicine to treat a variety of diseases. Bakuchiol has been reported to possess anti-inflammatory [24, 29–31], antioxidant [32–34], anti-tumor [35, 36], anti-bacterial [37], cytotoxic [38], heptaprotective [39] and caspase-3 depended apoptosis [40] effects. The cytotoxicity of bakuchiol is mainly due to its DNA polymerase 1-inhibiting activity [41]. Recently, anti-acne activity of bakuchiol has been reported [42]. Here, we demonstrate the anti-ageing and retinol-like functionalities of bakuchiol using DNA microarray, ELISA, histochemistry and clinical case studies.

As human skin naturally ages, it becomes thin, lax and finely wrinkled. Of these changes, fine lines & wrinkles and uneven pigmentation are most easily appreciated clinically with severity correlating strongly with age. We have demonstrated through a pilot clinical study that topical 0.5% bakuchiol treatment improves clinical appearance (% improvement vs. baseline) of naturally aged/photo-aged human skin.

DNA microarrays

As retinol affects the expression of a vast array of genes, comparative gene expression profiling with retinol is a suitable method to identify retinol-like compounds [43]. Here, we applied this method

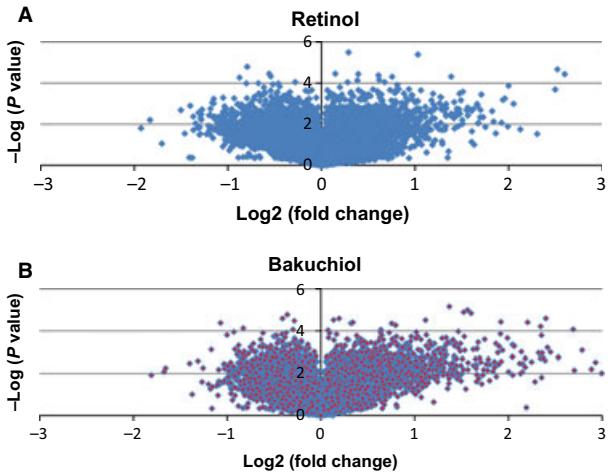

Figure 2 (A) Volcanic plot of DNA microarray data – Retinol. (B) Volcanic plot of DNA microarray data – Bakuchiol.

to reveal retinol-like properties of bakuchiol. These proprieties were first evidenced on the whole genome scale by comparing the shapes of volcano plots of retinol- and bakuchiol-treated skin substitutes (Fig. 2A,B). Volcano plot is a scatter graph used to identify meaningful changes in large datasets, such as data from DNA microarray analysis, by plotting significance versus. fold-change on the y- and x-axes, respectively. Data points with low *P*-values (highly significant) appear towards the top of the plot, and with *P* value of 0.05 being set as the threshold for statistical significance, all points situated above the value of 1.3 on the y axis are statistically significant. The finding that the overall shapes of retinol and bakuchiol volcano plots are similar was the first indication of the functional analogy between the two compounds. This was further confirmed by the analysis of retinoid-binding and metabolizing genes; the expression was often – but not always – similarly modulated (Table I). Interestingly, both RARB and RARG are up-regulated, as expected, by retinol but not by bakuchiol, indicating a possible advantage of the latter in terms of side effects. Also retinol had no effects on CRBP II and CRBP IV, whereas bakuchiol showed significantly higher up-regulation. Furthermore, retinol causes down regulation of CRABP1 gene whereas bakuchiol causes up-regulation, and whereas bakuchiol showed a dramatic up-regulation (82-fold vs. placebo) of LRAT retinol showed 12.3-fold (still a very significant) increase. These results indicate that besides being a retinol functional analogue, bakuchiol may enhance the availability of endogenous retinol.

Retinol and bakuchiol also showed similar modulation of genes implicated in ECM and DEJ (Tables II and III, respectively). ECM is the material that forms the bulk of the dermis, excluding water and cells. Proteins and complex sugars form most of the dermal ECM, and they are arranged in an orderly network fibres and ground substances organized by physical entanglements, opposing ionic charges, chemical covalent bonding and cross-linking into a biomechanically active polymer. This scaffolding structure with regional tensile strength provided by collagens, elasticity by elastins,

Table I Description, fold-change in the DNA microarray experiment and role of modulated retinoid binding and metabolizing genes (R: retinol; B: bakuchiol)

Gene	Full name	Function & comments
CRBP I; CRBP II; CRBP IV	Cellular retinol binding protein I, II & IV	CRBP I:R = 2.6; B = 4.2 CRBP II: R = NS; B = 4.1 CRBP IV: R = NS; B = 3.1 CRBP I mediates the cellular uptake of retinol, solubilizes and detoxifies it for further transport within the cytoplasm and presents it to the appropriate enzymes to biosynthesize retinoic acid
N6AMT2	N-6 adenine-specific DNA methyltransferase 2	R = NS; B = −2.1 Retinoic acid resistance might be overcome by the use of epigenetic modifying agents such as DNA methyl transferase inhibitors. Down-regulation provided by bakuchiol may reduce retinoic acid-induced toxicity
TIG1	Tazarotene-inducible gene 1	R = 13.2; B = 12.9 Retinoid acid (RA) receptor-responsive gene. The expression of this gene is found to be down-regulated in a variety of human cancers as well as in acne, rosacea and psoriasis. Up-regulation by bakuchiol may provide a solution to problem skin. Anti-acne clinical study results of bakuchiol has recently been reported [40]
DHRS9	Dehydrogenase/reductase SDR family member 9 precursor	R = 5.5; B = 11.6 DHRS9 is involved in converting retinol to retinal and then to retinoic acid, the rate-limiting step for the biosynthesis of retinoic acid
RETSAT	All-trans- 13,14-dihydroretinol saturase	R = −2.9; B = −2.8 RETSAT expression is involved in adipocyte differentiation
LRAT	Lecithin-retinol acyltransferase	R = 12.3; B = 82.2 Retinol esterification with long-chain fatty acid by LRAT is the key step in both absorption & storage of retinol.
CYP1A1; CYP1A2	Cytochrome P450	CYP1A1: R = 4.0; B = 4.9 CYP1A2: R = 3.6; B = 6.7 In addition to retinol dehydrogenase, P450s 1A1 and 1A2 genes are the major human P450s
RARB; RARG	Retinoic acid receptor beta -1; Retinoic acid receptor gamma -1	RARB: R = 5.6; B = NS RARG: R = 1.8; B = NS The actions of retinoids are generally mediated by the retinoic acid receptors (RARs alpha, beta, and gamma) and the retinoid X receptors (RXRs alpha, beta, and gamma). Both RARB and RARG are up-regulated, as expected by retinol but not with bakuchiol

Table II Description, fold-change in the DNA microarray experiment and roles of modulated genes coding for ECM components [(R: retinol; B: bakuchiol]

Gene	Full name	Function & comments
COL1A2; COL4A6; COL9A2; COL9A3;	Collagen 1A2; Collagen 4A6; Collagen 9A2; Collagen 9A3	COL1A2: R = 3.3; B = 1.9 COL4A6: R = 6.4; B = 11.2 COL9A2: R = 5.6; B = 6.7 COL9A3: R = 4.1; B = 5.8 Collagens provide scaffolding structure with regional tensile strength and elasticity; Degradation leads to fine lines and wrinkles. Comparable fold-change observed with four collagen genes with the exception of COL3A1. This may be due to the use of neonatal tissue in the skin substitutes. ELISA study with mature fibroblasts showed stimulation of type III collagen.
EMILIN3 EMILIN1	Elastin microfibril interface-located protein 3; Elastin microfifibril interface-located protein 1	EMILIN3:R = 2.2 (NS); B = 9.1 EMILIN1: R = −3.4; B = −2.4 The EMILINs are a family of glycoproteins of the extracellular matrix; Widely distributed in several tissues associated with elastin and localized at the interface between amorphous elastin and microfibrils
PI3	Peptidase inhibitor 3/Elastase-specific inhibitor	R = 2.5; B = 2.7 Bakuchiol and retinol are expected to maintain the desired level of elastin required for maintaining the connective tissue structure due to their up-regulation of elastase-specific inhibitor gene PI3
FLRT2; FLRT3	Fibronectin-like domain-containing leucine-rich transmembrane protein 2	FLRT2: R = NS; B = 13.9 FLRT3: R = 2.8 (NS); B = 6.1 Fibronectins maintain the shape of cells and matrix stability
HAS3	Hyaluronan synthase 3	R = 10.8; B = 19.2 Hyaluronans are important for the maintenance of a highly hydrated extracellular matrix in tissues and is also involved in cell adhesion and supports cell migration. It is synthesized by hyaluronan synthases, such as HAS-3
AQP3	Aquaporin 3	R = 3.5; B = 4.3 Aquaporin 3 is the water/glycerol transporting channel protein expressed in the epidermis which helps maintain the right level of skin hydration, elasticity and barrier recovery. Both retinol and bakuchiol up-regulated AQP3

Table III Description, fold-change in the DNA microarray experiment and roles of modulated DEJ genes (R: retinol; B: bakuchiol)

Gene	Full name	Fold-change, function & comments
COL4A6 COL17A1	Collage alpha-6 (IV) Collagen alpha-1(XVII)	COL4A6: R = 6.4; B = 11.2 COL17A1: R = 3.6; B = 8.7 Lamina densa mostly consists of type IV collagen, perlecan and nidogen. Through a complex inter- and intramolecular interactions type IV collagen forms supra molecular networks that influence cell adhesion, migration, and differentiation. Type XVII collagen is the protein component of anchoring fibrils that fortifies the attachment of the epidermis to the dermis.
PLEC1 or HD1	Plectin I (Hemidesmosomal protein 1)	R = 2.7; B = 6.8 Plectin, a 500-KDa protein and a constituent of the intracellular component of hemidesmosomes, attaches intermediate filaments to both hemidesmosomes and plasma membranes of basal keratocytes
ITGB4; ITGB6; ITGB8; ITGA6	Integrin beta-4; Integrin beta-6; Integrin beta-8; Integrin alpha-6	ITGB4: R = 3.5; B = 8.0; ITGB6: R = 7.5; B = 7.7 ITGB8: R = 0; B = 3.9; ITGA6: R = 0; B = 3.6 Integrins are transmembrane glycoproteins and a major component of hemidesmosomes. They mediate the transfer of information between the extracellular matrix and the interior of the cell, thereby aiding in modulating the organization of the cytoskeleton, proliferation and differentiation. Main functions: (a) attachment of the cell to the ECM and (b) signal transduction from ECM to the cell
LAMA3; LAMC2	Laminin subunit alpha-3 precursor; Laminin subunit gamma-2 precursor	LAMA3: R = 4.7; B = 11.0; LAMC2: R = 2.7; B = 7.8 Laminins are the major non-collagenous proteins in lamina densa and are integral part of the structural scaffolding in almost every tissue; Involved in cell differentiation, migration, adhesion as well as phenotype and survival.
CDH1	E-Cadherin	R = 9.4; B = 21.6 One of the most important and ubiquitous types of adhesive interactions required for the maintenance of solid tissues is that mediated by E-cadherin. E-cadherin has important functions in pluripotency and maintenance of the differentiated state of cells

adhesiveness by structural glycoproteins, compressibility by proteoglycans – hyaluronans and communicability by transmembrane receptors, such as integrins, which exchange information between the cytoskeleton-bound cellular elements and between cells and the dermal extracellular matrix, provides a unique homeostatic tensegrity function to the skin [44, 45]. DEJ, in turn, provides cohesion between dermis and epidermis. With age, both, ECM and DEJ gradually deteriorate resulting in skin thinning and morphological

Table IV Comparative collagen stimulatory effects of bakuchiol and retinol as determined by ELISA in human dermal fibroblasts. The stimulatory effect is expressed as the % of water control (100%)

Test material (10 μg mL^{-1})	Collagen I	Collagen III	Collagen IV
Bakuchiol	147	150	119
Retinol	119	148	100

flattening [46]. Retinol is a well known inhibitor of those processes, and the finding that bakuchiol has retinol-like functionality at the ECM and DEJ levels makes it an interesting novel candidate for anti-ageing applications.

Validation of DNA microarray results by ELISA and histochemistry

Collagens secreted by dermal fibroblasts are major components of the skin extracellular matrix (type I and type III collagens) and basement membrane (type IV collagen). In aged and photodamaged skin, the new collagen pool is decreased due to the inferior amount and quality of dermal fibroblasts. Therefore, we chose to measure select collagens by ELISA and histochemistry methods to validate the data obtained by DNA microarrays. The results, summarized in the Table IV, confirm the upregulation of types I and IV collagen in DNA microarray study and also show stimulation of type III collagen in this mature fibroblast model.

To determine whether the stimulation of type IV collagens in cell culture translates into a more robust collagen expression in 3D skin substitute tissue, EpiDermFT tissues were incubated with bakuchiol or retinol at 10 μg mL^{-1}, and histological sections were stained with anti-type IV collagen antibodies (Fig. 3). The stain revealed a stronger type IV collagen signal at the dermal–epidermal junction, as compared with the water control, further corroborating the DNA microarray and ELISA results. This collagen stimulatory effect observed in cell culture seems to be due to the selective metabolic activation of collagen synthesis in fibroblasts, because at 10 μg/mL^{-1}, bakuchiol or retinol did not enhance cell proliferation (results not shown).

Water homeostasis of the epidermis is essential for the normal function of the skin and stratum corneum (SC) hydration. It is a determinant of skin appearance, mechanical properties, barrier function and metabolism. In addition, it is indispensable in maintaining proper water balance of the body. Dehydration of SC is a typical feature of skin ageing, especially in photo-aged skin and of

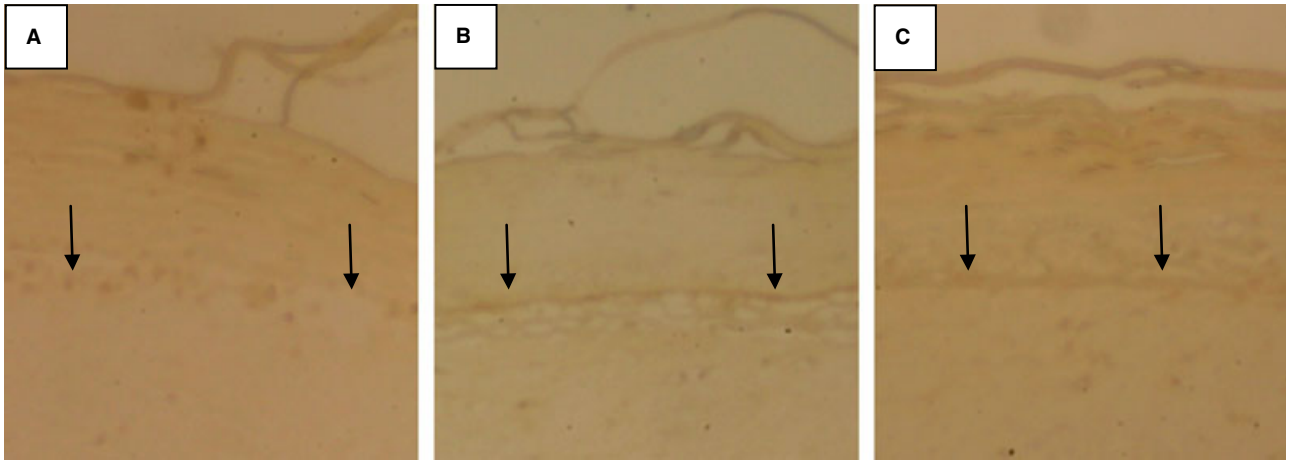

Figure 3 Effect of Retinol (B) and Bakuchiol (C) on type IV collagen expression compared with non-treated control (A) in human EpidermFT (full thickness) tissue substitutes. Arrows indicate dermo-epidermal juncture (basement membrane), where collagen IV is localized. Note darker band in (B) and (C) as compared to (A) at this level, indicating greater type IV collagen expression.

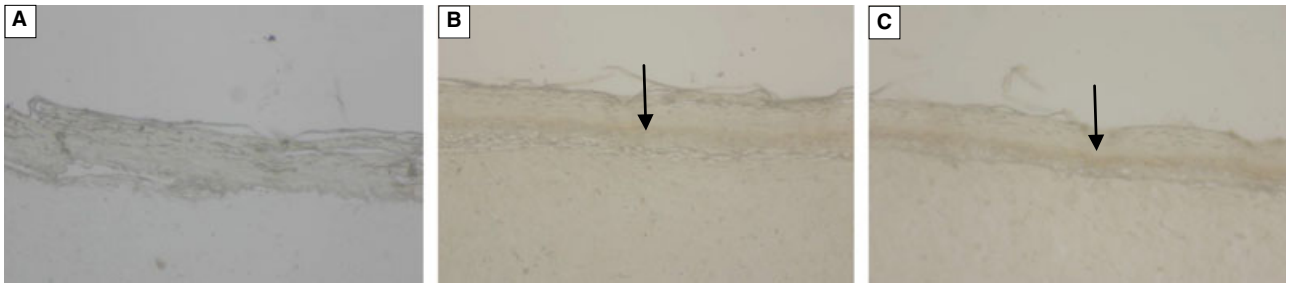

Figure 4 Effect of Retinol (B) and Bakuchiol (C) on aquaporin-3 expression, compared with non-treated control (A) in human EpidermFT (full thickness) tissue substitutes. Arrows indicate aquaporin-3 staining in the basal layer, where this protein is principally localized.

Table V Formulation details of bakuchiol

Ingredient	Trade name/supplier	% w/w
Phase A		
Glyceryl stearate and PEG-100	Arlacel 165/Uniqema	1.50
Arachidyl alcohol, Behenyl alcohol, Arachidyl glucoside	Montanov 202/Seppic	4.00
Dimethyl isosorbide	Arlasolve DMI/Uniqema	3.00
Isohexadecane	Permethyl 101A/Presperse	8.00
Dimethicone	Dow Corning 200, 100 cst/Dow Corning	2.00
Bakuchiol	Sytenol A/Sytheon	0.50
Phase B		
Water		69.75
Propylene Glycol	Propylene glycol/Lyondell	2.00
Pentylene Glycol	Hydrolite-5/Symrise	3.00
Xanthan Gum	Vanzan NF/Vanderbilt	0.25
Phase C		
Dicaprylylether	Cetiol OE/Cognis	4.00
Hydroxyethylacrylate (and) sodium acryloyldimethyl taurate copolymer	Sepinove EMT 10/Seppic	1.00
Phenoxyethanol (and) Caprylyl glycol	Optiphen or Microcare PHG	1.00
Total		100.00

Table VI Subjective evaluation by expert – % improvement vs. baseline

Parameters	4 Weeks	8 Weeks	12 Weeks
Roughness & dryness	65	81	90
Fine lines/wrinkles	5	16	29
Skin tone	14	29	39
Skin elasticity/firmness	13	20	39
Radiance	24	40	52
Skin brightening	13	35	46
Eye area appearance	8	24	46

Table VII Subjective evaluation by panelists – % improvement vs. baseline

Parameters	4 Weeks	8 Weeks	12 Weeks
Roughness & dryness	35	40	48
Fine lines/wrinkles	19	25	23
Skin tone	12	19	29
Skin elasticity/firmness	14	23	25
Radiance	11	17	30
Skin brightening	10	14	29
Eye area appearance	22	24	35

several diseases, for example, eczema, atopic dermatitis, psoriasis and hereditary ichthyosis (retinol 3.5 fold and bakuchiol 4.3 fold, Table II) [47–50]. To determine whether the increase of the !!! expression of water channel AQP3 gene observed in DNA microarrays translates into orthotropic increase at the protein level.

Table VIII Silicone replica analysis using profilometry: % reduction vs. baseline

Parameters	4 Weeks	8 Weeks	12 Weeks
Wrinkle depth	−7%	−13%	−20%
Skin roughness	−2%	−10%	−21%

EpiDermFT skin substitutes were incubated with retinol, bakuchiol and compared with the negative (water) control using immunohistochemistry. It was found that both retinol and bakuchiol increased AQP3 expression (Fig. 4) as visualized by more intense brown coloration in retinol and bakuchiol – treated tissues located, as expected, at stratum spinosum and corneum level. Recently, Bellemere *et al.* [51] have shown the effects of all-trans retinoic acid (ATRA) on AQP3 expression and function both *in vitro* and ex vivo. ATRA treatment increased a rapid accumulation of AQP3 transcripts in cultured normal human epidermal keratinocytes. Also in our model of EpiDermFT skin substitute, both retinol and bakuchiol increased AQP3 expression.

Clinical study

Taken together, these results prompted us to test bakuchiol clinically. The formulated product containing 0.5% bakuchiol (no moisturizer or any other active ingredients or sunscreen included in this formulated product; Table V) was applied twice daily to the whole face. Sixteen subjects of seventeen enrolled completed the study. One subject discontinued due to protocol violation. Each of the parameters was graded on a semi-quantitative scale from 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe). With regard to subjective evaluation by experts and panelists, the obtained results for the entire subjects are summarized in Tables VI and VII, respectively. Evaluations were performed at baseline and then at 4, 8 and 12 weeks and the results compared versus. the baseline.

Retinol is used widely in cosmetic products for reducing the appearance of the signs of ageing and photo-damage [52]. Retinol is a precursor of retinoic acid. Once it penetrates skin, it is sequentially oxidized to retinoic acid, causing retinoic acid-like effects, but is notably less irritating. Currently, most of these products contain 0.1% or lower of retinol. However, even at this low concentration, some irritation can occur [53]. It seems that bakuchiol has acceptable skin tolerability.

Analysing the data (Table VI vs. Table VII), it is evident that % improvement score for roughness & dryness and radiance given by expert is significantly higher versus. the panelists' self-evaluation. It is interesting to note that % improvement score given by the subjects, on the other hand, for fine lines and wrinkles and eye area appearance is significantly higher than the score given by the expert. Most of the parameters were improved significantly more after 8 weeks compared to 4 weeks of application of bakuchiol, showing a certain degree of cumulative beneficial effect over time.

Results obtained from silicone replica analysis using profilometry are summarized in Table VIII. Comparison of results of day 0 (base line) vs. 4, 8 and 12 weeks treated skin provided −7%, −13% and −20% reduction in wrinkle depth (Ry), respectively. All three results are statistically significant ($P \leq 0.01$). Comparison of results of day 0 (baseline) versus. 4, 8 and 12 weeks treated skin yielded

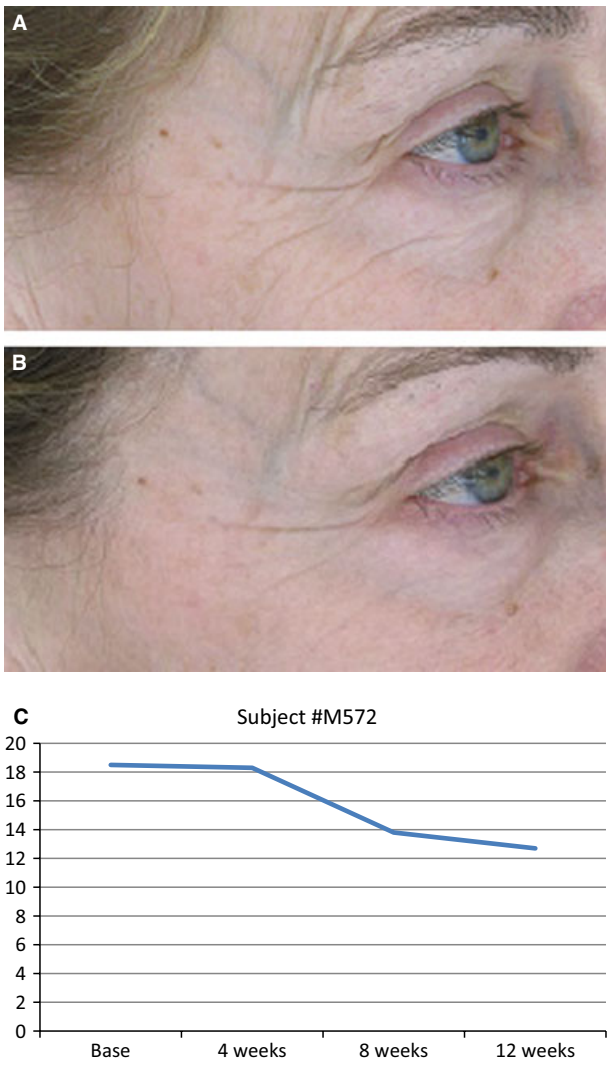

Figure 5 (A) Subject no. M572; Right view; Pre application. (B) Subject no. M572; Right view; 12-week treatment. (C) Change in wrinkle depth in μm.

−2%, −10% and −21% reduction in skin roughness (Ra), respectively. The results at 8 and 12 weeks are statistically significant ($P \leq 0.004$).

The photos (Fig. 5; before and after treatment) shown here are representative of the results that have been obtained with bakuchiol treatment for 12 weeks.

Clinical grading and comparative analysis of skin profilometric measurements were performed at baseline and after 4, 8 and 12 weeks of application. After 8 weeks of daily application, a significant reduction in the wrinkle depth and roughness was observed with the product vs. baseline. These changes were even greater after 12 weeks of product application. For example, wrinkle depth reduction (Fig. 5C) is significantly more after 8 weeks compared to 4 weeks of application of bakuchiol (subject no. M572) showing a certain degree of cumulative beneficial effect over time.

The significant improvement in fine lines and wrinkles, elasticity, firmness and overall reduction in signs of photodamage including even toning effects observed after 12 weeks of treatment provided the ultimate validation of the *in vitro* results and were in line with the retinoid-type functionality of bakuchiol.

Conclusion

Given the fact that retinol and bakuchiol do not have close structural similarities, yet they exhibit a similar gene expression profile, especially on certain key anti-ageing genes and proteins,which is remarkable. Bakuchiol has several substantial advantages over retinol, including excellent photochemical and hydrolytic stability a good safety profile and ease to formulate due to miscibility with a wide variety of emollients and solubilizers [18]. Bakuchiol can be used during the day due its photostability. Interestingly, bakuchiol is an excellent stabilizer of retinol under photo-oxidative as well as singlet oxygen environments (not discussed here). This property may help reduce oxidative stress caused by retinol when combined with bakuchiol and used at concentrations higher than the physiological limit [54].

This open clinical pilot study needs further confirmation of bakuchiol bioactivity *in vivo*, from vehicle- or benchmark-controlled studies. The similarity of gene expression and protein synthesis stimulation observed in the comparison of bakuchiol with retinol is, however, remarkable and suggests that similar bioactivity *in vivo* is probable. Taken together, this study demonstrates the potential of bakuchiol, a true retinol-like functional compound, to become a key ingredient for dermatological and skin care products.

Acknowledgements

The authors would like to thank Sytheon Ltd for funding this research and to Stephanie Ma for her dedicated assistance in this project.

References

1. Linney, E. Retinoic acid receptors: transcription factors modulating gene regulation, development, and differentiation. *Curr. Top. Dev. Biol.* **27**, 309–350 (1992).
2. Balmer, J.E. and Blomhoff, R. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43**, 1773–1808 (2002).
3. Siegenthaler, G., Saurat, J.H. and Ponec, M. Retinol and retinal metabolism. Relationship to the state of differentiation of cultured human keratinocytes. *Biochem. J.* **268**, 371–378 (1990).
4. Marill, J., Cresteil, T., Lanotte, M. *et al.* Identification of human cytochrome P450s involved in the formation of all-*trans*-retinoic acid principal metabolites. *Mol. Pharmacol.* **58**, 1341–1348 (2000).
5. Bailly, J., Crettaz, M., Schifflers, M.H. *et al.* *In vitro* metabolism by human skin and fibroblasts of retinol, retinal and retinoic acid. *Exp. Dermatol.* **7**, 27–34 (1998).
6. Barnard, J.H., Collings, J.C., Whiting, A. *et al.* Synthetic retinoids: structure-activity relationships. *Chemistry* **15**, 11430–11442 (2009).
7. Mattei, M.G., Petkovich, M., Mattei, J.F. *et al.* Mapping of the human retinoic acid receptor to the q21band of chromosome17. *Hum. Genet.* **80**, 186–188 (1988).

8. Chambon, P. The nuclear receptor superfamily: a personal retrospect on the first two decades. *Mol. Endocrinol.* **19**, 1418–1428 (2005).
9. Gann, A.A., Gates, P.B., Stark, D. *et al.* Receptor isofrom specificity in a cellular response to retinoic acid, *Proc. Biol. Sci.* **263**, 729–734 (1996).
10. Fisher, J.G., Talwar, H.S., Xiao, J.H. *et al.* Immunological identification and functional quantitation of retinoic acid and retinoid x receptor proteins in human skin. *J. Biol. Chem.* **269**, 20629–20635 (1994).
11. Jho, S.H., Radoja, N., Im, M.J. *et al.* Negative response elements in keratin genes mediate transcriptional repression and the cross-talk among nuclear receptors. *J. Biol. Chem.* **276**, 45914–45920 (2001).
12. Jho, S.H., Vouthounis, C., Lee, B. *et al.* The book of opposites: the role of the nuclear receptor co-regulators in the suppression of epidermal genes by retinoic acid and thyroid hormone receptors. *J. Invest. Dermatol.* **124**, 1034–1043 (2005).
13. Lee, D.D., Stojadinovic, O., Krzyzanowska, A. *et al.* Retinoid-responsive transcriptional changes in epidermal keratinocytes. *J. Cell. Physiol.* **220**, 427–439 (2009).
14. Kafi, R., Kwak, H.S.R., Schumache, W. *et al.* Improvement of naturally aged skin with vitamin A (retinol). *Arch. Dermatol.* **143**, 606–612 (2007).
15. Fisher, G.J. and Voorhees, J.J. Molecular mechanism of retinoid actions in skin. *FASEB J.* **10**, 1002–1013 (1996).
16. David, M., Hodak, E. and Lowe, N.J. Adverse effects of retinoids. *Med. Toxicol. Adverse Drug Exp.* **3**, 273–288 (1988).
17. Sorg, O., Kuenzli, S. and Saurat, J.H. Side effects and pitfalls in retinoid therapy. In: *Retinoids and Carotenoids in Dermatology*, 1st edn (Vahlquist, A. and Duvic, M. eds), pp. 225–248. Informa Healthcare, New York, London (2007).
18. Chaudhuri, R.K. The miracle of retinol. *Soap Perfum. Cosmet.* 23–24 (2010).
19. Mehta, G., Nayak, U.R. and Dev, S. Meroterpenoids-I: *Psoralea corylifolia* L.-1. Bakuchiol, a novel monoterpene phenol. *Tetrahedron* **29**, 1119–1125 (1973).
20. Prakasarao, A.S.C., Bhalla, V.K., Nayak, U.R. *et al.* Meroterpenoids. II. *Psoralea corylifolia* L. 2. Absolute configuration of (+)-bakuchiol. *Tetrahedron* **29**, 1127–1130 (1973).
21. Dobak, J., Grzybowski, J., Liu, F.T. *et al.* 1,25-Dihydroxyvitamin D3 increases collagen production in dermal fibroblasts. *J. Dermatol. Sci.* **8**, 18–24 (1994).
22. Zhao, H., Alexeev, A., Chang, E. *et al.* Lycium barbarum glycoconjugates: effect on human skin and cultured dermal fibroblasts. *Phytomedicine* **12**, 131–137 (2005).
23. Labbe, C., Faini, F., Coll, J. *et al.* Bakuchiol derivatives from the leaves of *Psoralea glandulosa*. *Phytochemistry* **42**, 1299–1303 (1996).
24. Backhouse, C.N., Delporte, C.L., Negrete, R.E. *et al.* Active constituents isolated from *Psoralea glandulosa* L. with antiinflammatory and antipyretic activities. *J. Ethnopharmacol.* **78**, 27–31 (2001).
25. Lystvan, K., Belokurova, V., Sheludko, Y. *et al.* Production of bakuchiol by *in vitro* systems of *Psoralea drupacea* Bge. *Plant Cell Tiss. Organ. Cult.* **101**, 99–103 (2010).
26. Choi, S.Y., Lee, S. and Choi, W.H. Isolation and anti-inflammatory activity of Bakuchiol from *Ulmus davidiana* var. japonica. *J. Med. Food* **13**, 1019–1023 (2010).
27. Krenisky, J.M., Luo, J., Reed, M.J. *et al.* Isolation and antihyperglycemic activity of bakuchiol from Otholobium pubescens (Fabaceae), a Peruvian medicinal plant used for the treatment of diabetes, *Biol. Pharm. Bull.* **22**, 1137–1140 (1999).
28. Ohno, O., Watabe, T., Nakamara, K. *et al.* Inhibitory effects of Bakuchiol, Bavachin and Isobavachalcone isolated from *Piper longum* on melanin production in B16 mouse melanoma cells. *Biosci. Biotechnol. Biochem.* **74**, 1504–1506 (2010).
29. Ferrandiz, M.L., Gil, B., Sanz, M.J. *et al.* Effect of bakuchiol on leukocyte functions and some inflammatory responses in mice. *J. Pharm. Pharmacol.* **48**, 975–980 (1996).
30. Pae, H.O., Cho, H., Oh, G.S. *et al.* Bakuchiol from *Psoralea corylifolia* inhibits the expression of inducible nitric oxide synthase gene via the inactivation of nuclear transcription factor-κB in RAW 264.7 macrophages. *Int. Immunopharmacol.* **1**, 1849–1855 (2001).
31. Matsuda, H., Kiyohara, S., Sugimoto, S. *et al.* Bioactive constituents from Chinese natural medicines. XXXIII. Inhibitors from the seeds of *Psoralea corylifolia* on production of nitric oxide in lipopolysaccharide-activated macrophages. *Biol. Pharm. Bull.* **32**, 147–149 (2009).
32. Adhikari, S., Joshi, R., Patro, B.S. *et al.* Antioxidant activity of bakuchiol: experimental evidences and theoretical treatments on the possible involvement of the terpenoid chain. *Chem. Res. Toxicol.* **16**, 1062–1069 (2003).
33. Haraguchi, H., Inoue, J., Tamura, Y. *et al.* Antioxidative components of *Psoralea corylifolia* (L.). *Phytother. Res.* **16**, 539–544 (2002).
34. Haraguchi, H., Inoue, J., Tamura, Y. *et al.* Inhibition of mitochondrial lipid peroxidation by bakuchiol, a meroterpene from *Psoralea corylifolia*. *Planta Med.* **66**, 569–571 (2000).
35. Pandey, U., Bapat, K., Samuel, G. and Venkatesh, M. Bioevaluation studies of 125I-Bakuchiol in tumor bearing animals. *BARC News Lett.* **285**, 88–91 (2007).
36. Chen, Z., Jin, K., Gao, L. *et al.* Anti-tumor effects of bakuchiol, an analogue of resveratrol, on human lung adenocarcinoma A549 cell line. *Eur. J. Pharmacol.* **643**, 170–179 (2010).
37. Katsura, H., Tsukiyama, R.I., Suzuki, A. *et al.* In-Vitro antimicrobial activities of Bakuchiol against oral microorganisms. *Antimicrob. Agents Chemother.* **45**, 3009–3013 (2001).
38. Fang, J., Xin-rui, Z., Qi, W. *et al.* Cytotoxic effect and mechanism of bakuchiol and bakuchiol combined with psoralen on HK-2 cell. *Int. J. Autom. Comput.* **24**, 50–58 (2010).
39. Cho, H., Jun, J.Y., Song, E.K. *et al.* Bakuchiol: a hepatoprotective compound of *Psoralea corylifolia* on tacrine-induced cytotoxicity in Hep G2 cells. *Planta Med.* **67**, 750–751 (2001).
40. Park, E., Zhao, Y.Z., Kim, Y.C. *et al.* Bakuchiol-induced caspase-3-dependent apoptosis occurs through c-Jun NH_2-terminal kinase-mediated mitochondrial translocation of Bax in rat liver myofibroblasts. *Eur. J. Pharmacol.* **559**, 115–123 (2007).
41. Sun, N.J., Woo, S.H., Cassady, J.M. *et al.* DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. *J. Nat. Prod.* **61**, 362–366 (1998) Erratum in: *J. Nat. Prod.*, 66, 734 (2003).
42. Chaudhuri, R.K. and Marchio, F. Bakuchiol in the management of acne-affected skin. *Cosmet. Toilet.* **126**, 502–510 (2011).
43. Ishida, S., Shigemoto-Mogami, Y., Kagechika, H. *et al.* Clinically potential subclasses of retinoid synergists revealed by gene expression profiling. *Mol. Cancer Ther.* **2**, 49–58 (2003).
44. Hayden, M.R., Sowers, J.R. and Tyagi, S.C. The central role of vascular extracellular matrix and basement membrane remodeling in metabolic syndrome and type 2 diabetes: the matrix preloaded. *Cardiovasc. Diabetol.* **4**, 9 (2005).
45. Ingber, D.E. The architecture of life. *Sci. Am.* **278**, 48–57 (1998).
46. Timár, F., Soós, G., Szende, G.B. *et al.* Interdigitation index - a parameter for differentiating between young and older skin specimens. *Skin Res. Technol.* **6**, 17–20 (2006).
47. Takenouchi, M., Suzuki, H. and Tagami, H. Hydration characteristics of pathologic stratum corneum–evaluation of bound water. *J. Invest. Dermatol.* **87**, 574–576 (1986).

48. Thune, P. Evaluation of the hydration and the water-holding capacity in atopic skin and so-called dry skin. *Acta Derm. Venerol. Suppl.* **144**, 133–135 (1989).
49. Scheuplein, R.J. and Blank, I.H. Permeability of the skin. *Physiol. Rev.* **51**, 702–747 (1975).
50. Cao, C., Wan, S., Jiang, Q. *et al.* All-trans retinoic acid attenuates ultraviolet radiation-induced down-regulation of aquaporin-3 and water permeability in human keratinocytes. *J. Cell. Physiol.* **215**, 506–516 (2008).
51. Bellemere, G., Von Stetten, O. and Oddos, T. Retinoic acid increases aquaporin 3 expression in normal human skin. *J. Invest. Dermatol.* **128**, 542–548 (2008).
52. Mukherjee, S., Date, A., Patravale, V. *et al.* Retinoids in the treatment of skin aging: an overview of clinical efficacy and safety. *Clin. Interv. Aging* **1**, 327–348 (2006).
53. Oddos, T., Roure, R., Leyden, J. *et al.* A placebo-controlled study demonstrates the long-lasting anti-aging benefits of a cream containing retinol, dihydroxy methyl chromone (DMC) and hyaluronic acid. *J. Cosmet. Dermatol. Sci. Appl.* **2**, 51–59 (2012).
54. Gimeno, A., Zaragozá, R., Vivó-Sesé, I. *et al.* Retinol, at concentrations greater than the physiological limit, induces oxidative stress and apoptosis in human dermal fibroblasts. *Exp. Dermatol.* **13**, 45–54 (2004).