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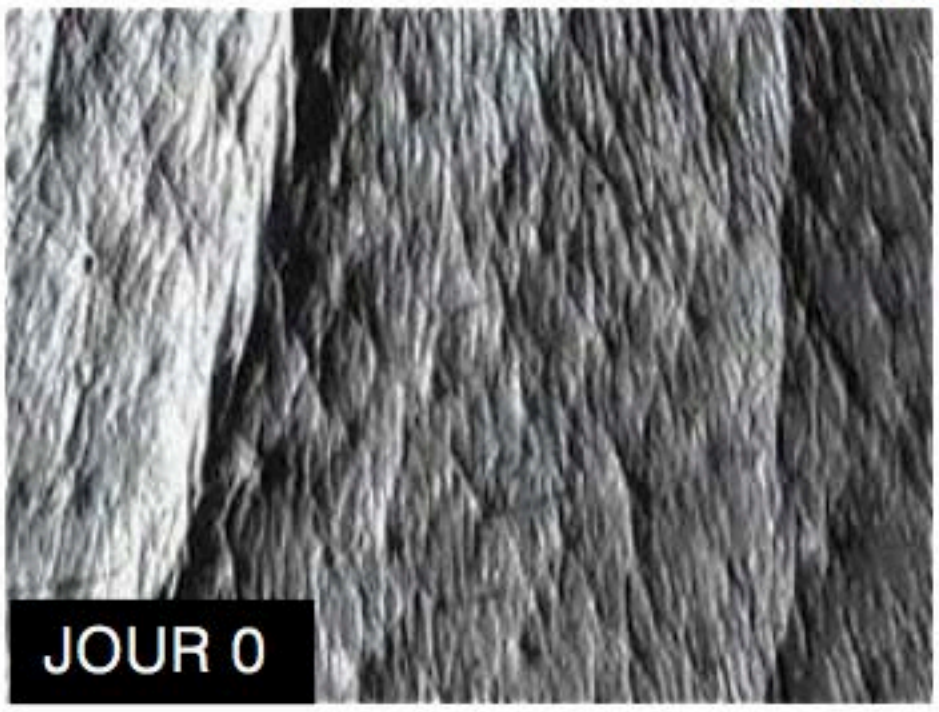

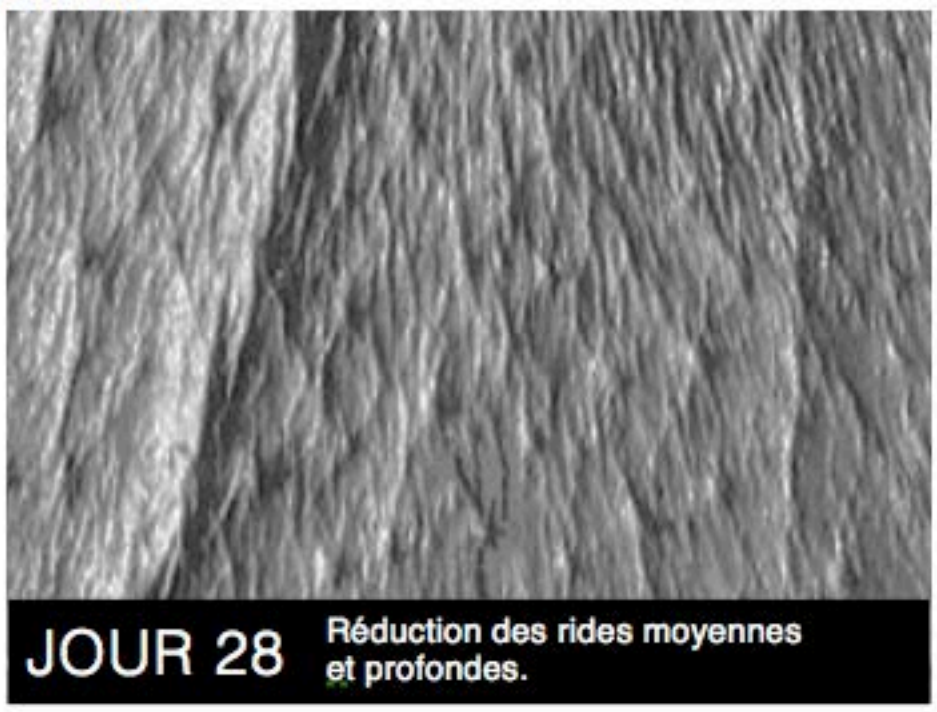

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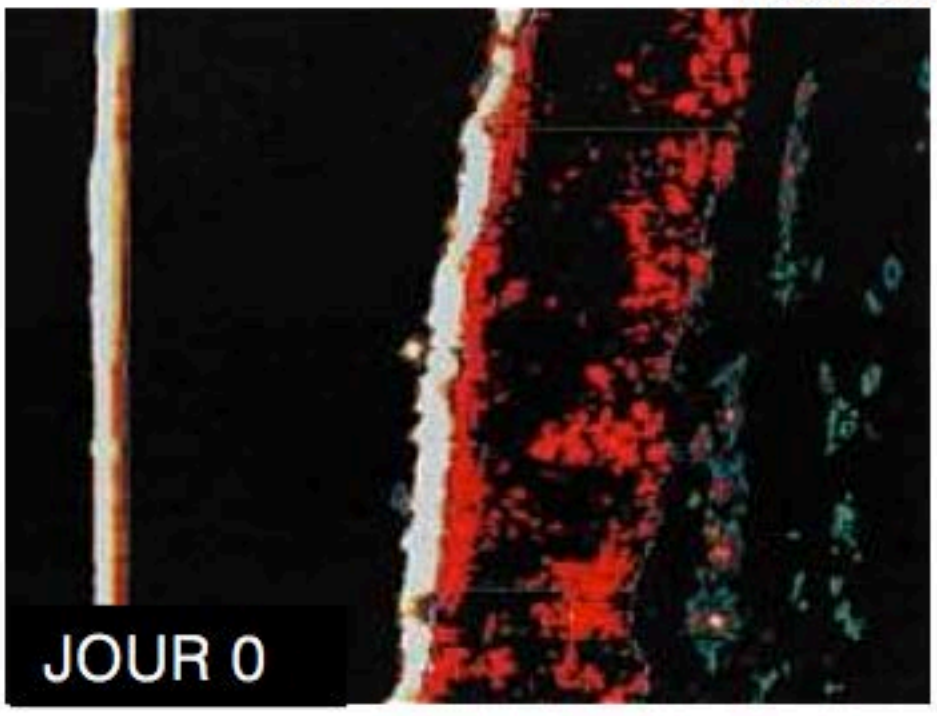

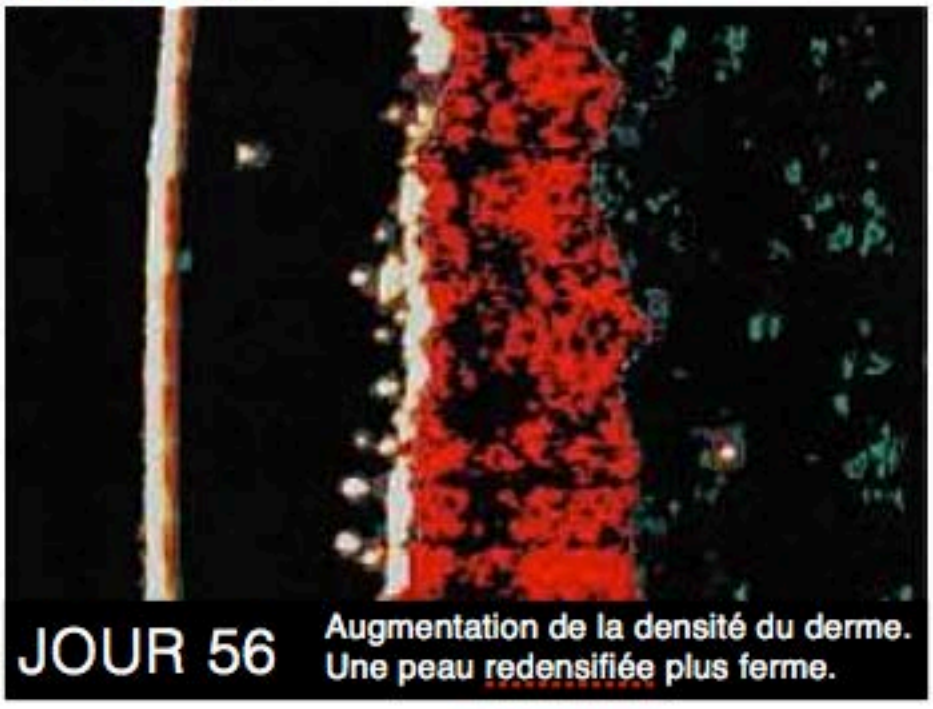

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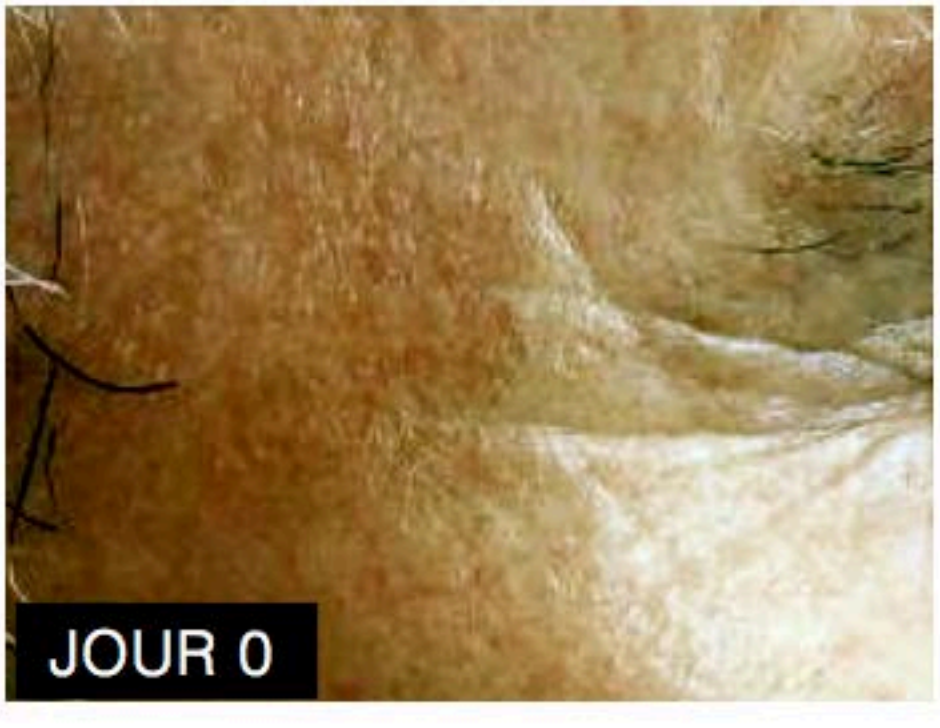

Modulating testosterone pathway: an efficient strategy to tackle male skin aging?

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Abstract: In men, the level of testosterone decreases with age. At the skin level, the result is seen by a decrease in density and in a lower elasticity. Identifying compounds able to increase the level of testosterone appear an attractive strategy to develop new anti-aging bioactive ingredients for men. Reverse pharmacognosy was successfully applied to identify new natural compounds able to modulate testosterone level. Among several *in silico* hits, Phyto-androzyme was retained as a candidate having the greatest potential to become an active ingredient. This result was then validated *in vitro* on **aromatase and 5-alpha-reductase type I and II,** two types of enzymes implicated in the degradation of free testosterone. Indeed, Phyto-androzyme was identified as an inhibitor of aromatase, with an IC50 of about 50 µM. In addition, Phyto-androzyme was shown to be an inhibitor of 5-alpha-reductase type I, with an IC50 of about 75 µM. Taken together, these data indicate that Phyto-androzyme modulates testosterone levels and its structure has the potential to serve as a lead for future designs of highly selective inhibitors of 5-alpha-reductase type I.

Résumé : Chez l'homme, le taux de testostérone diminue avec l'âge. La conséquence au niveau de la peau est une perte d'élasticité, une fragilité accrue et une perte de densité. Trouver des molécules capables de restaurer ce taux de testostérone apparait comme une stratégie attrayante pour lutter contre le vieillissement cutané chez l'homme. La pharmacognosie inverse a été appliquée avec succès pour l'identification de nouveaux composés naturels capables de moduler le taux de testostérone. Nous avons sélectionné le Phyto-androzyme parmi les touches identifiées *in silico* comme le meilleur candidat

potentiel pour devenir un ingrédient actif. Ce résultat a été, ensuite, validé *in vitro* sur l'aromatase et la 5-alpha-réductase, deux enzymes impliquées dans la dégradation de la testostérone libre. Le Phyto-androzyme s'est effectivement avéré être un inhibiteur de l'aromatase avec une IC50 de l'ordre de 50 µM. De plus, nous avons montré que le Phyto-androzyme inhibait également la 5-alpha-réductase de type I, avec une IC50 d'environs 75 µM. L'ensemble de ces données indique que le Phyto-androzyme module le niveau de testostérone et constitue une nouvelle touche pour la conception d'inhibiteurs actifs et hautement sélectifs de la 5-alpha-réductase de type I.

INTRODUCTION

Testosterone plays a key role in the development of male reproductive tissues such as the testes and prostate as well as promoting secondary sexual characteristics such as increased muscle and bone mass and hair growth. In addition, testosterone is essential for health and well being as well as preventing osteoporosis. A progressive decrease in androgen production such as testosterone is common in aging men and well documented [1, 2, 3]. The clinical picture of andropause syndrome is characterised by a diminished sexual desire and erectile capability, fatigue, depression, skin alterations, a decrease in intellectual activity, in lean body mass, in body hair and in bone mineral density that result in osteoporosis, in an increase of the visceral fat and obesity [4, 5].

The catabolism of testosterone

At the age of thirty, men's testes produce 95% of androgen hormones, especially testosterone (about 10 mg a day). About 98% of all circulating testosterone is inactivated through binding to plasmatic proteins whereas only the remaining two percents (called free testosterone) are responsible for all physiological activities of the hormone. Half of the blood plasma protein bound share is strongly associated to sex hormone binding globulin (SHBG) while the other half is only weakly bound to albumin. This albumin-coupled testosterone becomes part of the free testosterone **[6].**

In aging men, estrogen level raises, resulting in an increase of SHBG and subsequently a global reduction of free testosterone. After the age of 40 years, the capability for SHBG

to bind testosterone augments about 40% which causes a decrease of libido associated with all the symptoms of aging and andropause **[7].**

The key control switches for androgen regulation lie in two converting enzymes: (i) either 5α reductase I and II isoenzymes convert testosterone to dihydrotestosterone (DHT), or (ii) testosterone is converted to a lesser extend to 17β estradiol by CYP-19 aromatase, a metabolic pathway which occurs in both sexes **[8].** The activity inhibition of aforementioned protein could therefore adjust the level of free testosterone. A further metabolic pathway modulating the free testosterone level in blood plasma is its biotransformation by 5-alpha reductase enzymes that convert testosterone into dihydrotestosterone (DHT). Two subtypes of this reductase (type I and II) have been characterised. According to many authors, DHT is responsible for prostate hypertrophy **[9]** and also for hair loss (also known as androgenic alopecia) **[10, 11].**

Cosmetic skin problems arise with sebogenesis or *Acne* which *is* caused by a combination of endocrinic and bacterial agents leading to skin inflammation. Its androgenic components are male hormones (testosterone and *dihydrotestosterone*), besides specific *acne* bacteria **[12, 13]**. The activity inhibition of aforementioned enzymes should allow not only the increase of free testosterone levels but also the reduction of side effects due to its metabolite DHT. That part of testosterone which was not converted into DHT is now transformed into estradiol, an active estrogenic metabolite in presence of CYP-19 aromatase (also called estrogen synthase) **[8].** It belongs to the P450 cytochrome family, which controls the level of estrogens, the transformation of androgen, androstenedione and testosterone into estrogens. In men, an elevated level of estrogens may result in serious health problems mostly due to the feminizing effects (gynecomastia). Inhibiting CYP-19 aromatase could reduce the bioconversion of testosterone into estradiol and consequently, the risks associated with high level of estrogens, especially during andropause **[14].**

Testosterone and skin

With ageing, a decrease in hormone level at the skin level modulates the epidermal skin moisture, elasticity and skin thickness. It has been demonstrated that hormone replacement therapy is able to improve these parameters of skin aging in male [15].

In pharmacy, several delivery systems have been developed to supply exogenous testosterone. This strategy is not easily applicable for cosmetic applications and presents unacceptable adverse effects, such as irritation, contact dermatitis [16]. Moreover, an increase in testosterone concentrations should also increase the DHT levels, the role of which is implicated in hair loss or androgenic alopecia. For these reasons, the use of testosterone for topical applications, especially in cosmetics, seems quite limited. An alternative would be the discovery of a natural product able to increase the testosterone level in elder males.

Phyto-androzyme

Phyto-androzyme (Fig. 1), 2-(4-hydroxy-3-prop-2-enyl-phenyl)- 4-prop-2-enyl-phenol, is a molecular constituent of *Magnolia officinalis* Rehder & Wilson *or Magnolia grandiflora* L, a Chinese medicinal plant. Its known pharmacological effects comprise anti-inflammatory, antithrombotic, anti-arrhythmic, anti-oxidative, central depressant, muscle relaxant and anxiolytic effects [17, 18, 19]. In the past decades, a plethora of research work suggested that Phyto-androzyme possesses anticancer properties with potential implications in cancer treatment [20, 21, 22, 23, 24]. Phyto-androzyme skin application

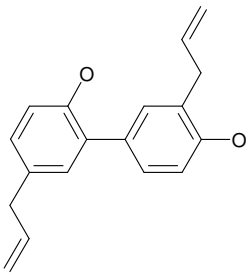


Figure 1 : Structure of Phyto-androzyme, a hydroxybiphenyl compound.

Reverse pharmacognosy

Pharmacognosy is the study of the pharmacochemistry of natural raw materials, mainly, but not exclusively extracted from plants, for pharmaceutical, dietary and cosmetic purposes [25]. It leads to bioactive molecules after extraction, purification, characterization and bioassays.

In some previous publications, we have already introduced and explored the usefulness of a new concept called “reverse pharmacognosy” (RPn) [26]. It is similar to “reverse pharmacology” [27] as small molecules are used as probes to evaluate their effects on a biological system, but differs from reverse pharmacology by its final goal. RPn aims at finding applications for substances of biological origin and their sources (mostly plants). In addition, RPn identifies new biomolecular targets or new biological pathways. It allies chemoinformatic tools and traditional knowledge in search of plants with new applications. The first step is a screening procedure either through *in silico* or *in vitro* methods or even a combination of both to identify botanical, pharmaceutical or cosmetic properties of biomolecule*s*. During the second step, a query in the plant - molecule

relational database finds registered plants containing the searched compound. Since RPn is complementary to ordinary pharmacognosy, it requires two or three specific devices: a virtual screening tool such as Selnergy **[28]** and/or an *in vitro* screening platform as well as a database with cross links between plants and molecules.

In the present study RPn is applied to find a new modulator of testosterone levels to develop an anti-aging formula for man cosmetics.

MATERIALS AND METHODS

The *Greenpharma* database (GPDB)

GPDB was developed to exploit the steadily growing botanical data, natural chemical structures and the knowledge obtained from biological tests with vegetal extracts or isolated molecules or from scientific literature in general. In addition, it covers phytotaxonomic issues like family, genus and species of organisms, their common names and synonyms, and the target organs – in traditional medicines – as well as ethnic groups from which the data was collected. GPDB has demonstrated its usefulness upon accelerating the discovery of anti-inflammatory compounds [29]. Currently, GPDB contains 150,000 molecule and 161,000 organism entries.

In the present work, we focus on this set of natural molecules as input for Selnergy screening on our targets of interest.

***In silico* screening with Selnergy™**

Protein 3D structures were either retrieved directly from published crystal structures at the Protein Data Bank (PDB, http://www.rcsb.org/pdb) [30] or built by homology modelling. The structures of SHBG and aromatase correspond to the PDB entries: 1D2S and 3EQM, respectively. In the case of 5α-reductase 1 no crystal structure was available, and its 3D model was generated by homology modelling. The accepted assumption for this approach is that two proteins with almost identical and highly phylogenetically related (=homologous) amino acid sequences will share similar 3D structures. Hence, 5α-reductase 1 model was safely constructed based on the crystal structure of 5β-reductase 1 (PDB: 3CAS), with a 40% of sequence homology, in view of a commonly accepted threshold of 30 to 25% of minimal homology for a length of 80 to 100 residues. Homology modelling was performed with Biopolymer and Composer modules within the Sybyl 8.0 package [31]. Virtual screening was conducted with Selnergy™ [28]. A candidate was considered as "accepted" for virtual screening if it fulfilled the following conditions. From a test set of molecules consisting of ligands with known target activities and others randomly selected, Selnergy ranks that candidate among the best scoring structures.

An unattended post-processing procedure was performed to discard docking solutions falling outside active site: a spatial fit criterion is taken into account to determine whether a ligand was "correctly" docked into the binding cavity. This goal was achieved by comparing the distances between two centroids: one defining the active site of the studied protein and the other defining the docked compound. Each measured distance

centroid to centroid: (d_{C-C}) served as the spatial fit criterion. Any docking position with a d_{C-C} > 4 Å was considered as being "out of the protein active site", and the protein/ligand pair was discarded from further analysis. As Selnergy can propose several poses for the same molecule, the docked pose with the shortest d_{C-C} distance was selected. A macro was written in SPL and implemented to automate this procedure. The SPL macro is distributed by Tripos. Finally, the automated screening and docking processes end, when an expert inspects the outcome on intuitive grounds in order to discard false-positives according to the similarity of their binding mode with reference co-crystallised ligands.

Biological assays: aromatase and 5-alpha reductase activity tests

The aim of the study was to determine the *in vitro* potency of putative inhibitors – found by our *in silico* screening tool, Selnergy – of the aromatase enzyme using human placental microsomes as source of the enzyme and tritiated water [32]. The approved aromatase inhibitor letrozole served as a reference compound.

Furthermore, in the present study the *in vitro* potency of Phyto-androzyme as an inhibitor of the 5α-reductase isoenzymes type I and type II was investigated. Transfected human embryonic kidney cells (HEK293), stably expressing the respective isoenzymes I or II (HEK293-5α1 = HEK I, HEK293-5α2 = HEK II), were used as a whole-cell test system [33]. On the basis of the enzymatic conversion of the ^{14}C-labelled substrate androstenedione to the 5α-reduced product dihydroandrostenedione, the inhibitory

potency was measured by photo-stimulated luminescence and compared to those of finasteride [34, 35], a well-known 5α-reductase inhibitor.

Aromatase assay

Microsomal aromatase enzyme preparation

The microsomal fraction was prepared from freshly delivered human term placenta. The tissue was washed in ice cold 0.15 M KCl and freed of membranes and blood vessels. The tissue was placed in 0.25 M sucrose (1.0 mL/g tissue) and cut into small pieces with surgical scissors. The tissue was homogenised in an Ultraturrax T25 using twenty 10 s bursts at 20,000 rpm with 50 s cooling periods. Portions (50 mL) of the homogenate were subjected to a Kinematica Polytron ultrasonic homogeniser using five 15 s bursts at speed 5 with 15 s cooling periods. The homogenate was centrifuged at 20,000 g for 50 min (Hitachi centrifuge). The clear supernatant was centrifuged at 148,000 g for 65 min (LKB, Ultraspin). The pellets were washed in phosphate buffer (0.05 M; pH 7.4) using a teflon homogeniser. The washed pellets were pooled, dissolved in about 50 mL phosphate buffer and washed twice by centrifugation at 100,000 g for 60 min. The final pellet was resuspended in 45 mL phosphate buffer and 100 µL aliquots were made from a stirring suspension, snap frozen and stored at -70°C. Protein contents were determined by the method of Lowry *et al*. [36], using a SPECTRAmax Plus384 from Molecular Devices and BSA as a standard.

In vitro aromatase inhibition experiment

Incubations were performed at 37°C containing [1β-^{3}H]-androstenedione (250 nM), excess NADPH (0.24 mM), 20 µg of human placental protein and phosphate buffer (0.05 M; pH 7.4). The final volume of the incubation mixture was 1.0 mL. Control incubations were performed without inhibitor and the background was determined in an incubation without any enzyme. The inhibitor was dissolved in MeOH and serially diluted to reach the three test concentrations of 100, 10 and 1 µM. Letrozole was tested at 10 nM.

The reaction was started by the addition of protein and stopped after 20 min by the addition of dichloromethane (10 mL). Following extraction, the tubes were centrifuged at 2,000 g for 5 min in a bench top centrifuge. 0.5 mL of the aqueous phase was removed and placed in a tube to which 1.0 mL of 5% charcoal suspension (Norit A) was added. After shaking the tubes for at least 15 min at room temperature, the tubes were centrifuged at 2,500 g for 10 min. Two 0.5 mL aliquots were removed, added to scintillation vials containing 10 mL scintillation fluid (Quickszint 212, Zinsser Analytic) and counted in a liquid scintillation counter (1209 RACKBETA, LKB Wallac). Counts from the control incubations containing no enzyme were subtracted from each incubation counts.

Data analysis

Results were expressed as percent inhibition values relative to untreated controls. Inhibition rates were calculated out of the mean conversion rates with (n=2) and without inhibitor (n=4).

5α-Reductase assay

Cell culture

HEK I and HEK II cells were cultivated in DMEM (pH 7.4) with 10% FCS, Penicillin/Streptomycin (100 U/mL and 100 µg/mL) and 0.5 mg/mL of Geneticin-418-sulfate. Inhibition assays were performed with HEK I and HEK II cells seeded at a concentration of 0.25 x 106 cells per 1.9 cm^2 and incubated for 20 h for attachment, doubling and differentiation in a humidified 5 % CO_2 atmosphere at 37 °C.

In vitro inhibition assays

Test compounds were dissolved in DMSO and serially diluted in DMEM to reach the three final test concentrations of 100 µM, 10 µM and 1 µM. Incubation mixtures containing 0.24 mM NADPH and 50 nM [4-^{14}C]-androstenedione at a final volume of 500 µL were pre-incubated at 37 °C for 10 min. Finasteride used as an internal control was also dissolved in DMSO and diluted to the test concentrations of 100 nM and 800 nM. Controls containing the solvent only (1 % DMSO) were treated the same way.

The reaction was started by removing the culture medium and adding the pre-warmed incubation mixture to the cell layers. After 30 min (HEK I) or 15 min (HEK II), respectively, the reaction was stopped by removing the supernatant. For the extraction of

product and non-converted substrate, 500 µL ethyl acetate was added to each sample. After 10 min shaking, samples were centrifuged for 5 min for phase separation and the supernatant was transferred into fresh tubes. After evaporation of the solvent, the dried residues were reconstituted in 25 µL of acetone.

The reconstitutes were spotted on a HPTLC plate (20 cm × 10 cm, Silicagel 60F254 with concentrating zone). The HPTLC plates were run twice in a freshly prepared solution of dichloromethane : diethylether (8:2) as solvent. Imaging plates were exposed to the HPTLC plates for 48 h. The imaging plates were scanned using a PhosphoImager and the spots corresponding to [4-^{14}C]-androstenedione (A) and [4-^{14}C]-dihydroandrostenedione (DHA) were integrated using the corresponding software.

Data analysis

Results were displayed as PSL units (Photo-Stimulated Luminescence) and corrected for the background. Conversion rates were calculated according to the following formula:

$$\% \text{ conversion} = \frac{PSL(DHA)}{PSL(DHA + A)} \times 100$$

Inhibition rates, expressed as percent inhibition values relative to untreated controls, were calculated out of the mean conversion rates with (n=2) and without inhibitor (n=2).

Quality control : Acceptability of the assays

As quality controls, positive control inhibitors were included in the assay set-up. The *in vitro* assays are considered as acceptable if it meets the following criteria:

- 50% inhibition of aromatase by letrozole at a concentration of 10 nM (tested concentration corresponding to the IC_{50} of letrozole).
- 50% inhibition of 5α-reductase type I by finasteride at a concentration of 800 nM (tested concentration corresponding to the IC_{50} of finasteride).
- 50% inhibition of 5α-reductase type II by finasteride at a concentration of 100 nM (tested concentration corresponding to the IC_{50} of finasteride) .

Clinical evaluation

Patch test study

Previous to the patch test study, Phyto-androzyme 1% and 5% was evaluated for its irritating potential on the chorio-allantoïdian membrane of egg hens (HET-CAM) and for its mutagenicity potential on the AMES test according to OCDE471. This test relates to the evaluation of the acute cutaneous tolerance of the raw material and was designed and evaluated by Dermscan, France. A basic cream formula was used including 1% and 5% of Phyto-androzyme. The study was realised during 48 hours with an occlusive method on 20 volunteers with normal skin.

Clinical study : evaluation of the product

The randomized, double-blind comparative study was carried out in two parallel groups; one group testing the placebo preparation (cream without active ingredient) and the other group testing the verum product at 1% (that is placebo + 1% Phyto-androzyme). The study protocol was carried out by Dermscan (France). The study was conducted

from October to December. The objectives were to evaluate the anti-aging and redensifying effects of the tested products.

Volunteers

The study involved 40 Caucasian healthy men from 55 to 63 years old (mean: 60±1 years.). The main inclusion criteria were wrinkles on crow's foot and loose skin on the face.

Study protocol

All volunteers were divided exactly into two groups. The verum group received a cream preparation containing 1% of Phyto-androzyme whereas the placebo group received the only vehic*le*, i.e. apparently the same formulation without the active ingredient (Phyto-androzyme). Facial applications (crow's foot) were made twice a day for 2 months. The kinetic data were assessed at three sampling points: Day 0, Day 28 and Day 56. Our assessment criteria were as follows:

- Skin prints analysis with the Skin Image Analyser
- Dermis density by Dermascan.
- Macrophotographs of a crow's foot (for 10 volunteers of each group).

Statistical study

For statistical studies, EXCEL 9.0 version 2000 was used. The statistical method was the Student's paired t-test.

RESULTS

Molecular modelling: virtual screening results from Selnergy

Testosterone level is controlled by numerous enzymes, receptors and carrier proteins. After consulting the underlying metabolism pathways for testosterone in the Kegg database [37], we inspected three enzymes for their relevant biological properties. The rationale to select the target structures in terms of molecular modelling is the following:

- the protein must occupy a key position in the regulation system of maintaining testosterone levels;
- the inhibition of the protein reflects the desired biological properties because it is usually easier to find inhibitors for enzymes or receptors than exogenous activators;
- sufficient structural data exists to conduct virtual screening studies.

We found that aromatase or cytochrome P450 19A1 [38], 5α-reductase type I [39] and sex hormone binding globulin (SHBG) [40] were amenable to further scrutiny.

3D protein models were generated for their posterior use as target structures in *in silico* calculations with Selnergy. Our software allows simultaneous virtual screening (VS) on all three targets in order to detect putative selectivity and/or synergy. Approximately, 150,000 natural compounds of the GPDB database were filtered. Each of the 150,000 molecules was ranked by Selnergy according to its computed interaction energies with

each of the three targets. The top 1% of all docked poses in terms of estimated binding energy was post-processed with the aforementioned procedure to retain only those molecules from successful docking. Table I lists the hand-selected compounds after final inspection. Three compounds were judged as most promising. Then toxicity concerns, procurement and intellectual property issues were documented and evaluated for all three molecules. Two molecules were henceforth discarded, leaving only one molecule as the best candidate: Phyto-androzyme. It is the sole molecule to have a putative activity on all selected targets, thus ranked as the best molecule: it seems to act on two pathways, which inhibition leads to the raise of testosterone rate. The second hit is phloretin but it was abandoned due to its limitation of exploitation in cosmetics: it would be very difficult to get intellectual properties. The third molecule was also excluded due to possible hormonal issues.

Selnergy results are illustrated in Fig. 2, where Phyto-androzyme is docked into aromatase active site. We can observe the complementarity in shape and in electrostatic properties (hydrophathy). Note that one of the ethylene groups is closed to the ferric central ion of the haem group and may interact with the cation.

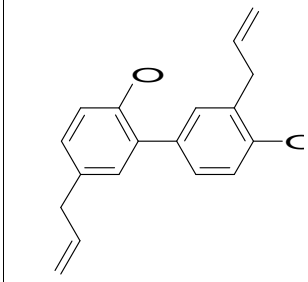
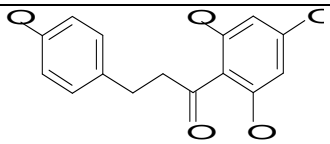
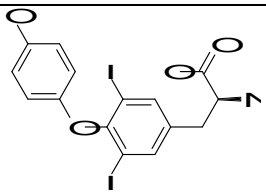
Molecules	Structures	CAS	Targets	Sources
Phyto-androzyme		35354-74-6	Aromatase SHBG 5α-reductase	*Magnolia spp.* (L.)
Phloretin		60-82-2	Aromatase SHBG	*Helichrysum splendidum* ((Thunb.) Less.)
(S)-3,5-Diiodothyronine		1041-01-6	Aromatase	*Undaria pinnatifida* (Harvey) Suringar

Table I: Final selection of putative molecules targeting testosterone pathways.

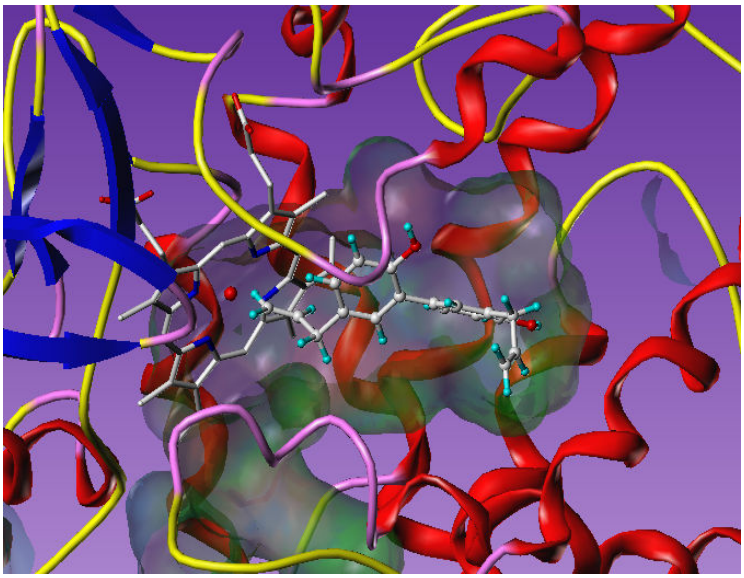


Figure 2: Phyto-androzyme (displayed in balls and sticks style) docked into the aromatase active site. The backbone of the enzyme is represented as ribbons with red colours for helices, blue arrows for beta-sheets. Rose and yellow segments represent turns and loops, respectively. The haem with the ferric central atom (red sphere) is displays as capped sticks. The active site is modelled with a transparent surface: hydrophobic areas appear in brown, while hydrophilic and intermediate zones show blue and green colours, respectively. Carbon atoms are coloured in white, nitrogen in blue, oxygen in red and hydrogen in cyan.

If these predictions are confirmed by experiments, Phyto-androzyme will be a good candidate for an active ingredient. Furthermore, it can be found in *Magnolia spp.* which

is already used in cosmetic for other applications. This is another reason why we selected Phyto-androzyme for further experimental investigations.

Biological activities: Aromatase and 5-alpha reductase assays

Aromatase assay

Phyto-androzyme's potential aromatase inhibition was tested during *in vitro* experiments at three concentration levels of 100 µM, 10 µM and 1 µM (n=2). Their results are displayed in Tab. II, expressed as percentage of inhibition. Letrozole, a 1,2,4-triazole derivative, served as a positive control and was tested at a concentration corresponding to its IC_{50} value (10 nM) in the same test system.

Phyto-androzyme displayed a concentration-dependent inhibition of the aromatase enzyme reaction, indicating a competitive binding of the test item towards the active site of the enzyme. The IC_{50} value of the test item is assumed to be in the range between 40 to 60 µM. In stark contrast, the IC_{50} value of letrozole which was determined within a range between 7 to 10 nM, is more than 3 orders of magnitude lower than the estimated IC_{50} of the test item. Nevertheless, we should bear in mind that Phyto-androzyme is a natural lead compound without any chemical optimisation.

Compound	Concentration	% Inhibition aromatase
Phyto-androzyme	1 µM	0
	10 µM	13
	100 µM	88
Letrozole	10 nM	61

Table II: Inhibition of aromatase *in vitro* by Phyto-androzyme and letrozole.

5α-Reductase assay

Test item Phyto-androzyme was tested *in vitro* for the inhibitory potential towards 5α-reductase type I and type II in a cell-based assay using stably transfected HEK293 cells at 100 µM, 10 µM and 1 µM.

The dual 5α-reductase inhibitor finasteride – an azaandrostene derivative – was tested as a positive control at concentrations of 800 nM and 100 nM, corresponding to the IC_{50} values of finasteride for 5α-reductase type I and 5α-reductase type II, respectively. The results of this study are presented in Tab. III.

Phyto-androzyme inhibited 5α-reductase isoform type I in a concentration-dependent manner. A competitive binding of the test item towards the active site of the enzyme is assumed. The IC_{50} value of the test item for isoform I is estimated to be in the range of 60-90 µM. In contrast, Phyto-androzyme did not display any affinity towards 5α-reductase isoform type II.

Compound	Concentration	% inhibition 5α-reductase type I	% inhibition 5α-reductase type II
Phyto-	1 µM	1	n.i.
	10 µM	11	n.i.
	100 µM	64	n.i.
Finasteride	100 nM	11	55
	800 nM	48	89

Table III: Inhibition of 5α-reductase *in vitro* by Phyto-androzyme and finasteride. n.i.: no inhibition

In comparison, the IC_{50} values of finasteride for 5α-reductase inhibition were found in the range of 800 nM for isoform type I and 100 nM for isoform type II. In this system, finasteride is a 100 fold more potent inhibitor of 5α-reductase type I than Phyto-androzyme which is a natural compound without any prior chemical optimisation.

Due to the fact that the test item Phyto-androzyme did not affect 5α-reductase type II, this compound may be considered as a new lead for a selective 5α-reductase type I inhibitor since this is the first time that a biphenolic scaffold is reported to be selectively active on 5α-reductase type I.

Clinical results

Patch test study

Prior to patch test studies, we verified that Phyto-androzyme has no mutagenic and no irritation effects under AMES and HET-CAM assays. Under the patch test study conditions – 48-hours with an occlusive method on 20 volunteers with normal skin – the 1% skin preparations remained nonirritating after 30 minutes and 24 hours readings. At a 5% level, three volunteers recorded skin irritations and testing was immediately abandoned. The 1% preparations, however, are well tolerated without safety concerns and therefore constitute the highest levels of Phyto-androzyme in use for all our clinical studies.

Clinical study

Anti-wrinkle effect

The preparation without the active ingredient induced no statistically significant variation of the cutaneous relief parameters after 28 or 56 days of administration. After the 56 day period 71% of all volunteers presented a reduced count of typical wrinkles (-8%). After the first 28 days under 1% Phyto-androzyme treatment, the number of micro-relief furrows (-13%) and the number of average wrinkles (-3%) slightly decreased in 69% of the volunteers. Then, after the 56-day-treatment, the prints analyses showed a fairly reduced number of deep wrinkles (-11%). These results reflect both anti-wrinkle and smoothing effects (Fig. 3a and 3b) with statistical certainty: $p=0.058$ that is nearly significant at a p level of $p<0.05$.

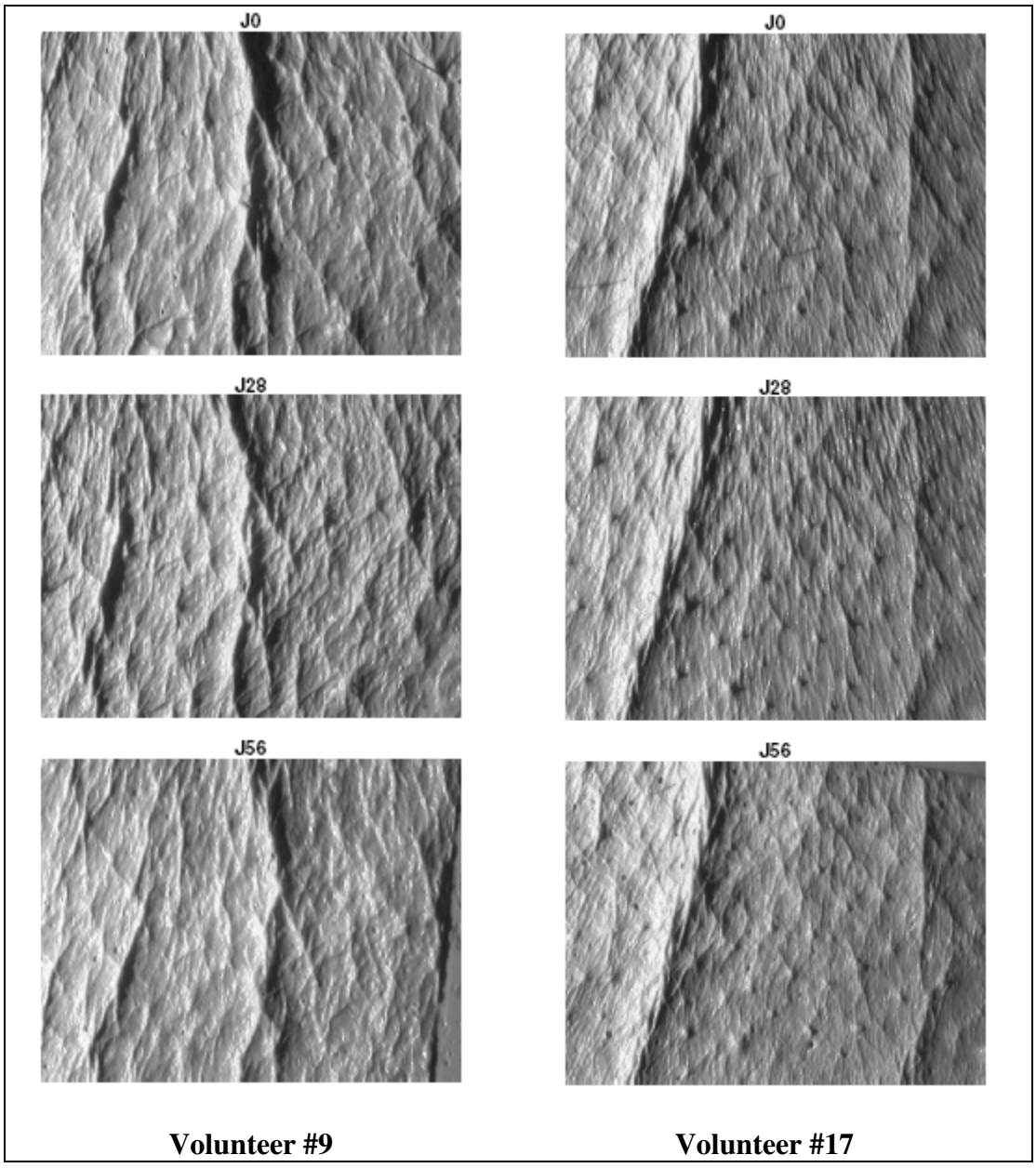


Figure 3a: Anti-wrinkle effect of a cream containing 1% of Phyto-androzyme. Observations taken for two typical volunteers (#9 to the left, and #17 to the right) at start (day 0), halfway (day 28) and study end (day 56).

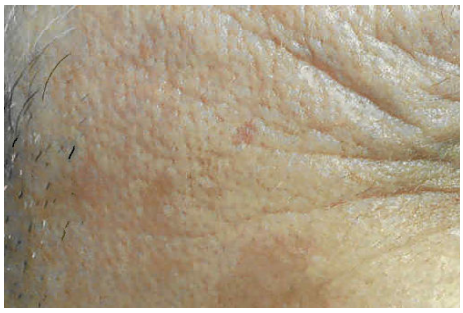
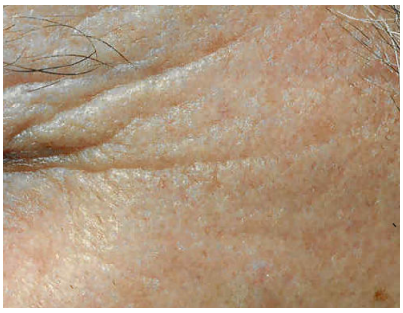
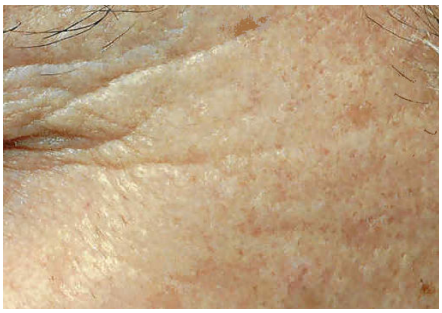
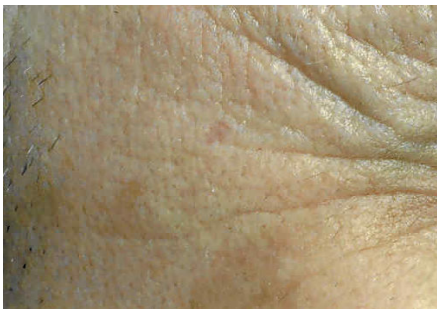
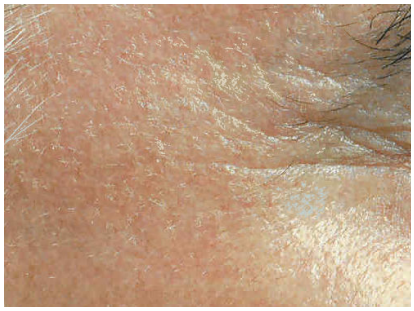
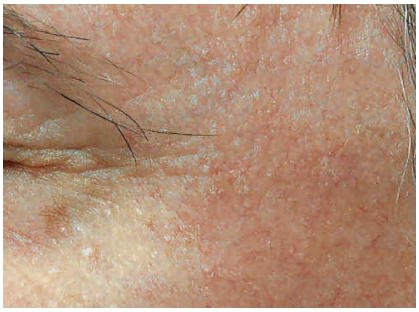
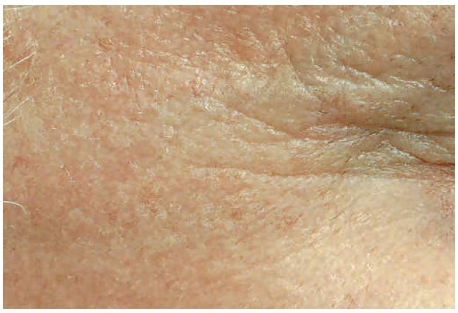
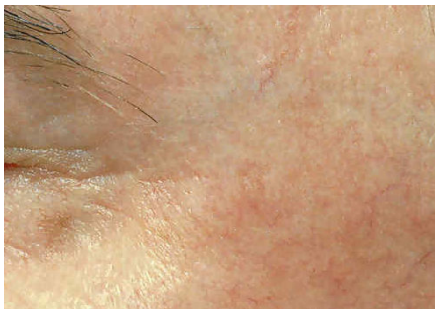
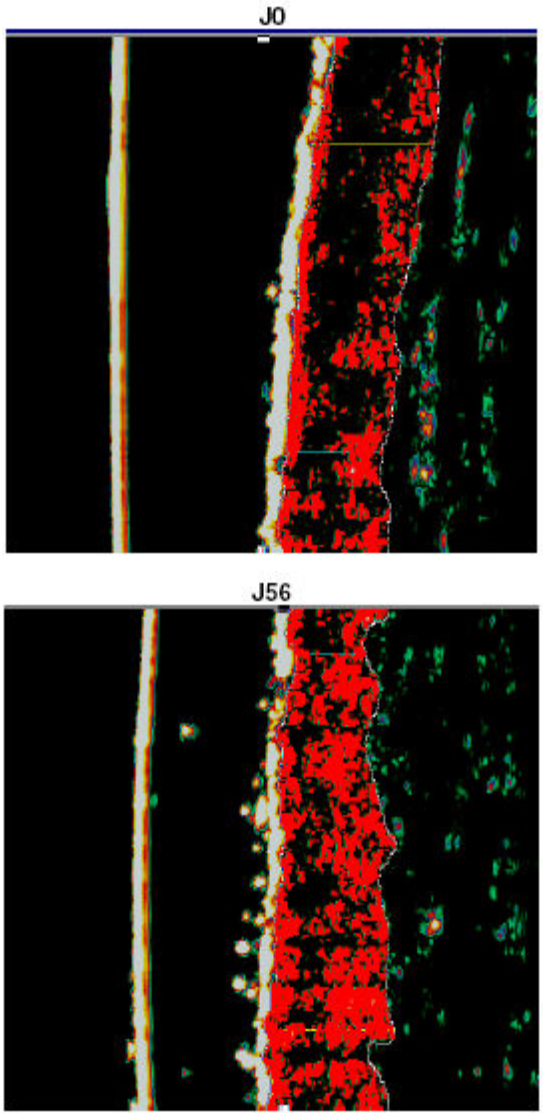
Placebo		**Cream 1% Phyto-androzyme**
Volunteer #22		Volunteer #14
	Day 0	
	Day 56	
Volunteer #16		Volunteer #35
	Day 0	
	Day 56	

Figure 3b: Anti-wrinkle effect of a preparation containing 1% of Phyto-androzyme, on crow's foot of three volunteers. Comparison between study start and end (day 0 and day 56).



Redensifying effect

Figure 4: Dermal redensifying effect of the 1% of Phyto-androzyme preparation at day 0 and day 56. The redensifying effect of the dermis and the reformation of the extracellular matrix came close to statistic significance at $p < 0.05$ ($p = 0.058$)

The negative control product (placebo without active ingredient) induced no significant variation of the dermis density after 56 days of skin treatment. The verum product containing 1% of Phyto-androzyme, however, induced an increase of the dermis density after 56 days in 60% of all volunteers. On average, a increase of the dermis density of +5% was clearly observed (Fig. 4).

DISCUSSION

Our present combined approach with *in silico* screening and clinical testing extends the scientific literature attesting to the field of anti-aging treatment for male skin with respect to the role of Phyto-androzyme's dermopharmaceutical modulation capacity. The latter is based on new evidence gained from our randomized, double-blind comparative study to observe the dermatological effects of Phyto-androzyme. Firstly, the placebo administration did not show any anti-wrinkle or densifying effect. Secondly, the verum preparation containing a modulator of testosterone such as 1% of Phyto-androzyme showed a smoothing and an anti-wrinkle effect as well as a densifying effect. It was demonstrated by other authors that Phyto-androzyme is antioxidant and myo-relaxant [17, 18, 19]. These properties may exert anti-wrinkle effects. However, the

increase of skin density cannot be entirely explained by these properties and may be attributed to Phyto-androzyme property of modulating the testosterone pathway disclosed herein. In view of our preliminary findings, additional clinical studies will be necessary to consolidate this postulate: increasing the testosterone rate in skin may be beneficial for anti-aging and remain to be explored in details.

According to the common literature sources in life science, to date, little original research work has focused on the cosmetically relevant connection between testosterone and skin-aging in male adults. Our study tries to fill in the gap, taking the testosterone – antiaging assumption as the starting point for our working hypothesis. Quite in contrast, the relation between endocrine activity deficiencies and certain age-associated diseases like the andropause (lack of testosterone) has found its way into clinical studies. Intriguingly, attempts to reverse clinical manifestations of senescence by serum hormone level substitution in older individuals are still controversially discussed in the literature [41]. The skin is a peripheral endocrine organ, and aging male skin leads to androgen deficiency which in turn is related to degeneration of skin and internal organs. Age-dependent decrease in tissue androgens in some skin areas was reported even a decade earlier [42]. Hence, topical or even oral hormone substitution treatments were proposed to counteract defects on the skin due to decreasing hormone levels [1, 42, 43]. Older skin shows a lower content of lipids and further experimental work showed diminished surface permeation of certain hormones and drugs [44]. Testosterone insufficiency in aging men includes negative effects on skin and hair. The dermatologic benefits and risks of testosterone replacement therapy have to be studied in short term

and long term trials [45]. In a review about male aging, testosterone substitution was positively evaluated because short-term adverse effects were not found, but in the same paper the authors warn of possible unknown long-term risks of testosterone [46]. More supportive records come from another study where human skin improvements like epidermal thickness, humidity or pigmentation were clinically observed under oral adrenal steroid treatments in both sexes [47]. In contrast, certain risks of hormone replacement therapy were found to be statistically higher in a meta-analysis of published clinical trials and patient monitoring recommended [48]. Over more than 40 years ago, the effects of testosterone injections on the aging male skin was tested and skin improvements became histologically manifest [49]. In 2007, the intracrine or paracrine actions of sexual hormones produced in the skin were reviewed. The paper reports that the androgen receptor densities may have important implications in the development of hyperandrogenism and the associated skin diseases such as acne, seborrhea, hirsutism and androgenic alopecia [3]. Androgen delivery to the aging skin was registered as an invention in 2006 [50]. On a biochemical molecular level, testosterone was found to modulate the phospholipase A2 system in epidermal tissue of rats [51]. In addition, molecular aspects of hair and skin physiology were reviewed and androgenic steroids, their converting enzymes as well as certain proteins further downstream that bind their active metabolites [52].

CONCLUSION

Our combined theoretical and experimental study extends the hitherto known literature about dermopharmaceutical anti-aging treatment for male skin. Its design and technical dimensions show preliminary character but yields sufficient evidence to direct our ongoing research and development with the following implications. Phyto-androzyme inhibits aromatase and 5α-reductase type I in a dose-dependent manner, with an IC_{50} of about 50 µM and 75 µM respectively. These results validate the predictions made by Selnergy. Moreover, a preliminary clinical study demonstrated that the *in vivo* efficacy of a testosterone-like compound, such as Phyto-androzyme, is able to improve the skin aging parameters in men. The topic administration prevents muscle-relaxing effects of Phyto-androzyme that are associated to oral administration as systemic effect. The ascribed anti-oxidative properties may play a stabilizing role known in dermopharmaceutical formulations.

Future development and perspectives:

Additional studies are needed to test the link between testosterone pathway modulation and anti-aging. Phyto-androzyme seems to be a good starting point. Moreover, interestingly, Phyto-androzyme is a selective inhibitor of 5α-reductase type I. To our best knowledge, the number of selective 5α-reductase type I (*vs* type II) inhibitors is sparse. Therefore, Phyto-androzyme is an interesting lead for further iterative cycles of drug developing: the design, synthesis and bioassays to find and test more potent and selective derivatives (hemisynthesis) in order to obtain nM range inhibitors of 5α-reductase type I and devoid of activities in 5α-reductase type II and aromatase.

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