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Plant Small RNAs: A New Technology for Skin Care

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Synopsis

This study was performed on an aqueous extract of baobab seedcake enriched in small ribonucleic acids (RNAs) for cosmetic use. The seedcake is a by-product, obtained from Baobab seeds belonging to the *Adansonia digitata* species. A particular patented extraction process, named plant small RNA (PSR) technology, has been developed to retain some specific nutrient compounds, including small RNAs. Small RNAs, such as microRNAs (miRNAs), play an essential role in gene regulation. The biological potential of this new patented PSR extract was studied in skin fibroblasts and in ex vivo skin. To demonstrate the effect relative to the presence of small RNAs, the same extract in which small RNAs were removed was also tested. After observing the efficacy of PSR extract on collagen expression in ex vivo skin, different markers of senescence were investigated on fibroblasts aged by replicative senescence. The study of the expression of Drosha, an enzyme responsible for miRNA maturation, the expression of miRNA-19b, a biomarker of cellular aging, and the activity of senescence-associated β-galactosidase showed more efficient activity of PSR extract, compared with small RNAs–free extract. Taken together, these studies demonstrate the potential of PSR extract for use in cosmetic end use applications.

INTRODUCTION

Baobab is a tree belonging to the Malvaceae family. *Adansonia digitata* is a species of baobab that grows in Africa's sub-Saharan semi-arid and sub-humid regions. The largest specimen is 30-m tall and has a diameter of up to 11 m.

Baobab, also known as the "tree of life," is an integral part of the lives of indigenous people of African landscape, who use all parts of the baobab for food and in medicinal applications or for cosmetics (1). In particular, the baobab's outstanding fruit is turned into a great variety of products. Pulp of the fruit is used in food. Its seeds are used to produce oil recognized for its beneficial effect on skin. Several studies revealed that baobab's Integral Antioxidant Capacity (IAC) was 10 times higher than orange pulp. The antioxidant power is due partly to the high content of its organic compounds such as ascorbic, citric, malic, and succinic acids. Furthermore, it has been described that all parts of the baobab (including the pulp, leaves, and seeds) not only have higher IACs than orange

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pulp but also higher IACs than strawberry, bilberry, and kiwi pulp (2). The baobab fruit is a powerful anti-inflammatory and antioxidant (3). Of 14 species of "wild edible fruits" evaluated, those from *Adansonia digitata* ranked second for highest phenolic and flavonoid content, which are well-known for their antioxidant properties (4).

The seeds are extracted from the baobab fruit by cracking the hard, outer capsule. The extracted seeds are then washed to remove the powder coating and then air-dried in the hot sun. These seeds can be stored for many months before pressing. Baobab oil is made by a cold pressing process. Baobab oil is an excellent skin moisturizer, which is quickly absorbed by the skin without clogging pores (5). It leaves the skin feeling soft and moisturized. Indeed, thanks to its composition, its richness in omega 6–9 acids, vitamins, sterols, and minerals, organic baobab oil is ideal for use in cosmetics.

Ashland has developed an extract obtained by valorizing oil by-product, the seedcake, which keeps the richness of cold pressing seeds. In particular, we developed an extraction process that allowed us to retain the healthy nutrients and compounds of interest such as phenolic compounds, sugars, proteins, and amino acids of the seeds, while also enriching our extract with small RNAs, named plant small RNA technology (PSR technology) (Figure 1).

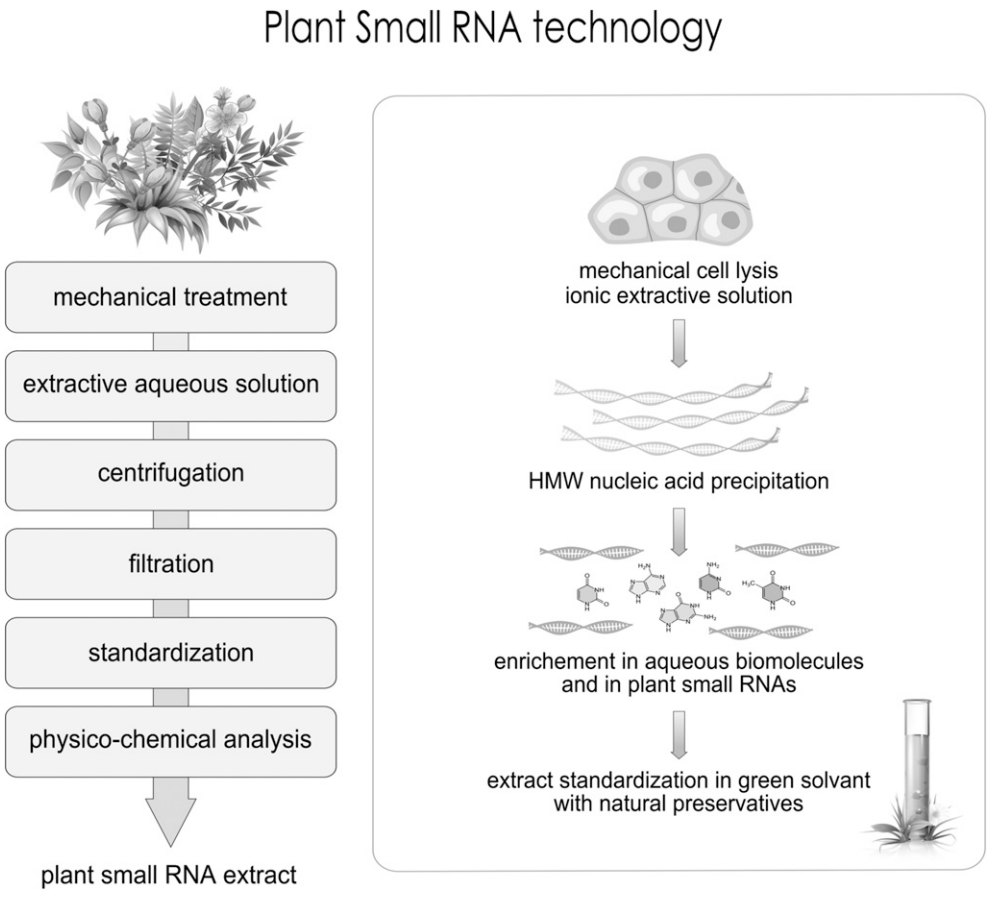


Figure 1. Flow chart of the PSR technology.

The small RNAs, a class of noncoding RNAs, play a role in the regulation of genomes and transcriptomes targeting chromatin or transcripts. They are considered epigenetic regulators. Epigenetics is the study of modifications that are not caused by changes in the DNA sequence; it allows us to perceive the influence of the environment on the transcriptional potential of a cell through modifying epigenetic marks (6). For example, baobab seeds must contend with harsh environmental conditions, such as drought and heat. Their resistance to these conditions is possible, thanks to the small RNAs that will regulate their gene expression. Small RNAs are also involved in efficient defense systems that provide resistance to environmental stresses, and scavenge reactive oxygen species (ROS).

Small RNAs include miRNAs, consisting of 22–24 ribonucleotides. miRNAs are involved in post-transcriptional regulation through interaction with messenger RNAs. In the cells of both humans and plants, they are involved in numerous processes such as development, metabolism, and reproduction, as well as in the response to biotic or abiotic stresses and diseases. Regulation by miRNAs accounts for more than a third of all gene expression (7).

To act on miRNA targets, in plants, this process is performed by a single RNase III enzyme, DCL1 (Dicer-like 1), in the nucleus (8). In animals, miRNAs follow a maturation process divided into two major steps. The primary miRNA is processed by the RNase III endoribonuclease Drosha to generate a shorter precursor miRNA (pre-miRNA). After export in the cytoplasm by the nucleoplasmic transport protein exportin-5, the pre-miRNA is cleaved by Dicer. Thereby, the miRNA is matured and assembled into the RNA-induced silencing complexes (RISC) and is ready to inhibit its mRNA targets (9). On senescent WI-38 human diploid fibroblasts from the lung, the expression of these two key enzymes has been shown to decrease, involving the dysregulation of 20% of miRNAs (10). Among the miRNAs dysregulated during cellular aging, miRNA-19b is described as a biomarker of cellular aging. Indeed, Hackl et al. showed a downregulation of this miRNA with aging, in different replicative cell and organismal aging models (11). In addition, this miRNA is part of small noncoding RNA signature in centenarians. Indeed, miRNA-19b expression was shown to be similar in the cells of both young and centenarian people (12).

On the basis of their functions, plant miRNAs could be considered as a new class of micronutrients, along with vitamins. Thus, an exclusive and novel extraction process maximizing the potential of PSRs was developed and named PSR technology. Tests on ex vivo skin and *in vitro* fibroblasts demonstrated the benefits of PSR plant presence in extract on maintaining skin homeostasis by stabilizing the maturation of the skin's miRNA machinery.

MATERIALS AND METHODS

PLANT MATERIAL

The seedcake of *Adansonia digitata* used in this study originated from Senegal. We appreciate the partnership of two complementary companies in obtaining the seedcake: Biomega, an Austrian society specializing in food technology; and Baonane, a Senegalese company and leading producer of baobab products, particularly baobab fruit valorization. The baobab fruits are matured 5–6 mo after flowering. During the harvest, they are

hand-picked, and the seeds are removed from the pulp and red fiber. Oil is obtained from the seeds by cold pressing. The coproduct of the oil extraction is the seedcake. The seedcake is micronized and was used to perform the extraction.

PREPARATION OF PLANT EXTRACT

Baobab seedcake was first ground with a blender to obtain a powder. Then, PSR technology patented process was applied to obtain the small RNA-enriched baobab extract and other phytochemicals. The extract was then diluted by 30% with either water or a cosmetic solvent such as glycerin. The extract was then transferred into sterile bottles and sterilized at low temperature. To obtain a plant extract placebo for the biological evaluation, another baobab extract that did not contain small RNAs was prepared. These two baobab extracts were comparatively studied for their effects on skin.

PHYTOCHEMICAL ANALYSIS OF THE EXTRACT

Phytochemical screening was performed on the baobab seedcake and on the final extract to determine the dry weight and the quantification of total protein and sugar. Polyphenol and amino acid content analysis was performed only on the final extract.

Dry weight of the raw material and of the final extract was determined by placing 2 g of baobab seedcake powder in a metal cup in duplicate for 2 h at 105°C in a ventilated oven (Memmert, Schwabach, Germany).

Protein content was determined by Lowry protein assay, which was used to quantify the total protein content of the extract (13). Protein measurement with the Folin phenol reagent was used to quantify the total protein content of the extract. The Lowry method is based on the reaction of Cu+, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent. The absorbance of the sample is read on the spectrophotometer at 550 nm. The protein content is determined using a BSA standard curve.

Sugar content was determined colorimetrically via an adaptation of the assay described by Dubois et al. (14). This analysis consisted of concentrated sulfuric acid, which was then reacted with phenol to form a colored complex. The absorbance of the complex was read on the spectrophotometer at 490 nm. The sugar content was determined using a glucose standard curve.

Polyphenolic compound quantity was determined using the Folin–Ciocalteu assay (15). Polyphenol compounds in the sample reacted with the Folin–Ciocalteu reagent, and the oxidation of the reagent turns the polyphenol compounds blue. The absorbance of the sample was read on the spectrophotometer at 760 nm. The content was expressed as gallic acid equivalents using a gallic acid standard curve.

Amino acids were quantified using a protocol published by Moore and Stein (16). The free amino acid content of the baobab extract was assessed by the formation of a colored complex, following the rupture of the amine and carboxylic functions by the reagent ninhydrin. The absorbance of the complex was read on the spectrophotometer at 570 nm. The total amino acids content was determined using a standard curve of amino acids pool.

For the analysis of quality and integrity of the small RNAs present in our baobab extract, the Agilent 2100 Bioanalyzer system was used, with a small RNA Chip Kit according to the manufacturer's instructions (Agilent, Santa Clara, CA).

CELL CULTURE

Fibroblasts were extracted from different normal human skin samples obtained from donors and were grown in Dulbecco's modified eagle medium (DMEM) 1 g/L glucose (Lonza, Verviers, Belgium) supplemented with 10% of FBS (Lonza), 2 mM of L-Glutamine (Lonza), and 100 µg/ml of Primocin (InvivoGen, San Diego, CA). Cell cultures were maintained at 37°C in a 5% CO_2-humidified atmosphere. In the model of replicative senescence, fibroblasts were cultured over a 3-mo period. At each passage, when cells reached confluence, a part of these cells were frozen and the other part was maintained in culture. After different passages (P-X), the cells were thawed and treated, or not (control condition), overnight or for a period of 24 or 48 h with 1% PSR baobab extract or with 1% placebo (baobab extract without small RNAs) (twice a day).

HUMAN SKIN SAMPLES

Ex vivo skin samples were obtained from donors after undergoing abdominal plastic surgery. On skin biopsies of 6 mm in diameter, a topical application of 20 µl of either 1X phosphate-buffered saline (PBS) (control) or 1% PSR baobab extract or 1% placebo diluted in 1X PBS (baobab extract without small RNAs) was applied twice a day for 48 h. Then, tissues were fixed in formalin and embedded in molten paraffin wax.

qPCR

After treatment, the culture medium was removed and the cells were rinsed with cold PBS. The mirVana miRNA Isolation Kit (Ambion, Naugatuck, CT) was used to extract total RNAs. Then, these RNAs were reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitors (Applied Biosystems, Foster City, CA). The reverse transcription was performed on 0.25 µg of total RNA using a MiniCycler thermocycler (MJ Research, Waltham, MA). Finally, real-time PCR was performed on a StepOnePlus thermocycler (Applied Biosystems). Collagen I, miRNA-19b or Drosha TaqMan Gene Expression Assay (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) were involved. RNU44 TaqMan Gene Expression Assay was used as an endogenous control to miRNA expression studies and 18S TaqMan Gene Expression Assay to Collagen I and Drosha expression study.

The comparative Ct method was used for relative quantification of target expressions (17), and the StepOne* Software (Applied Biosystems) was used for data treatment.

SENESCENCE-ASSOCIATED (SA) β-GALACTOSIDASE STAINING

Fibroblasts were washed in PBS, fixed for 3 min in 2% formaldehyde and 0.2% glutaraldehyde solution, washed, and incubated overnight at 37°C with SA β-galactosidase stain solution at 1 mg/ml [$MgCl_2$ at 2 mM, $K_3Fe(CN)_6$ at 5 mM, $K_4Fe(CN)_6$ at 5 mM, NaCl at 150 mM, citric acid at 40 mM, and X-gal (Qiagen, Hilden, Germany)]. The slides were rinsed and mounted in Aquatex (Merck, Kenilworth, NJ). Detection was managed and examined using a Nikon Eclipse E600 microscope with a ×20 objective. Photos were taken with a QImaging* EXi blue camera and processed by using the Q-Capture Pro 7 (QImaging, Survey, BC, Canada) acquisition software.

For image quantification, three images per condition were analyzed. Photoshop Elements 11 software (Adobe, San Jose, CA) allows users to convert each picture into an inverted image, using an intensity threshold to eliminate the background noise. These inverted images were then analyzed with Volocity image analysis software (Improvision), which generated the sum of red pixel intensities. The sum obtained was normalized by taking into account the total number of cells.

IMMUNOFLUORESCENCE STAINING OF COLLAGEN I

Sections were deparaffinized and rehydrated with several successive xylene, alcohol, and water baths. Then, an unmasking protocol was performed that included both microwave exposure at 600 W in citrate buffer 0.01 M, pH 6 (Sigma, Saint Louis, MO) until boiling and 0.05% trypsin (Zymed, Invitrogen, Frederick, MD) digestion for 15 min at 37°C. The saturation of unspecific sites was performed with a solution of 5% bovine serum albumin (BSA) (Sigma) for 30 min. Depending on the experiment, the primary antibody used corresponded to polyclonal anti-collagen I (Rockland at 1:100 dilution in 1X PBS). After rinsing the slides with PBS, the secondary antibody was applied in the dark and under agitation at room temperature in a damp room. Finally, the sections were mounted in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA). Detection was managed and examined using a Zeiss Axiovert 200M microscope with a ×20 objective. Photos were taken with a QImaging EXi blue camera coupled to Volocity acquisition software (Improvision).

STATISTICAL ANALYSIS

Statistical analyses were performed using JMP* 10 software (SAS, Carry, NC) and Student's t test for independent samples with one-tailed direction of rejection. $p \leq 0.05$ was considered as significant, $p \leq 0.01$ as very significant and $p \leq 0.005$ as highly significant.

RESULTS/DISCUSSION

COMPOSITIONAL ANALYSIS OF BAOBAB SEEDCAKE

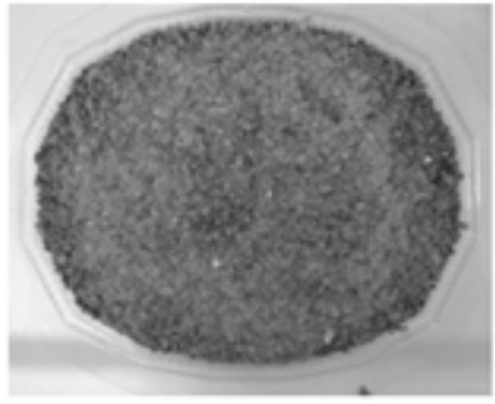
The baobab seedcake was ground before raw material analysis to obtain a powder around 2,000 µm in diameter (Figure 2B). Then, the chemical composition of the baobab seedcake was studied. Phytochemical analysis of the baobab seedcake revealed that the seedcake was very rich in different classes of chemical molecules (Figure 2C). This confirms its potential for use as a raw material on which to perform an aqueous extraction and obtain a biofunctional ingredient for use in the cosmetic field.

COMPOSITIONAL ANALYSIS OF BAOBAB SEEDCAKE EXTRACTS

The composition of the baobab seedcake extracts was studied. The small RNA content in both the target extract and the placebo extract was determined by bioanalyzer analysis (Figure 3). A high amount of small RNAs was found in the extract obtained by the specific patented process intended to enrich the extract in small RNAs, around 60 mg/l before dilution. By contrast, the extract obtained without PSR technology process did not contain small RNAs at all.

A

B



C

Odor	characteristic (sweet like honey)
Color	brown amber
Aspect	very dry in cluster
Dry weight (g/kg)	93
Humidity (%)	7
Nitrogen content (%)	4.2
Protein content (%)	26
Sugar content (%)	31

Figure 2. Observation of the baobab seedcake raw material and phytochemical analysis of the raw material. (A) Seedcake before grinding. (B) Seedcake after grinding. (C) Phytochemical analysis.

In addition, the quantity of each phytoconstituent was determined and was summarized in Table I. The dry matter of PSR baobab extract was 10–12 g/kg, comprising major compounds 3–4 g/kg of protein and 3–4 g/kg of sugar and also containing other interesting

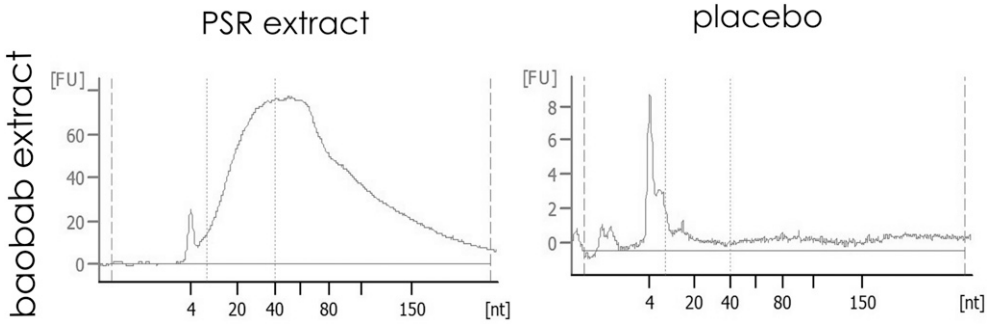


Figure 3. Characterization of PSR baobab extract and placebo baobab extracts by bioanalyzer.

molecules such as amino acids and polyphenols. Each of these molecules may be responsible for various beneficial effects for skin, particularly small RNAs.

BIOLOGICAL ACTIVITY OF PSR BAOBAB EXTRACT

To evaluate the biological effect of PSR baobab extract on skin, the expression of collagen I was investigated. On ex vivo skin biopsies, the baobab extracts which either did or did not include small RNAs (PSR baobab extract or baobab placebo) were applied at 1% for 48 h, collagen I expression was evaluated by immunostaining (Figure 4A). Collagen I mRNA expression was also evaluated by RT-qPCR in fibroblasts treated with the same baobab extracts at 1% for 24 h (Figure 4B). In ex vivo skin, only biopsies that were treated with PSR baobab extract showed an increase in collagen I expression. The collagen I mRNA level was observed to have increased in fibroblasts that were treated with baobab extracts, with a stronger efficacy for PSR extract (+72%) than for baobab placebo (+14%). Chung et al. demonstrated, *in vivo*, a perturbation in the balance between collagen synthesis and its degradation via matrix metalloproteinases, leading to decrease in collagen in aged skin (18). This first biological result showed the potential of the presence of PSRs to enhance collagen synthesis in ex vivo skin.

The model of fibroblasts that was aged by replicative senescence was used in the next experiment. The machinery of miRNA maturation via study of the expression of the enzyme Drosha was performed in these fibroblasts by RT-qPCR (Figure 5A). Our results showed that Drosha expression was found to have decreased in senescent fibroblasts, confirming previous observations (10). Indeed, the level of Drosha was maintained closer to the basal level found in young cells, but only in senescent cells that were treated with PSR baobab extract. The study of miRNA-19b completed this investigation (Figure 5B):

Table I
Phytochemical analysis of PSR baobab extract and conventional baobab extract

Process	Dry matter (g/kg)	Total protein (g/l)	Total sugar (g/l)	Total polyphenol (mg/l)	Total free amino acids (mg/l)	Small RNA (mg/l)
PSR baobab extract	12	2.7	3.3	274	380	32
Conventional baobab extract	12.5	3.3	5	247	310	0

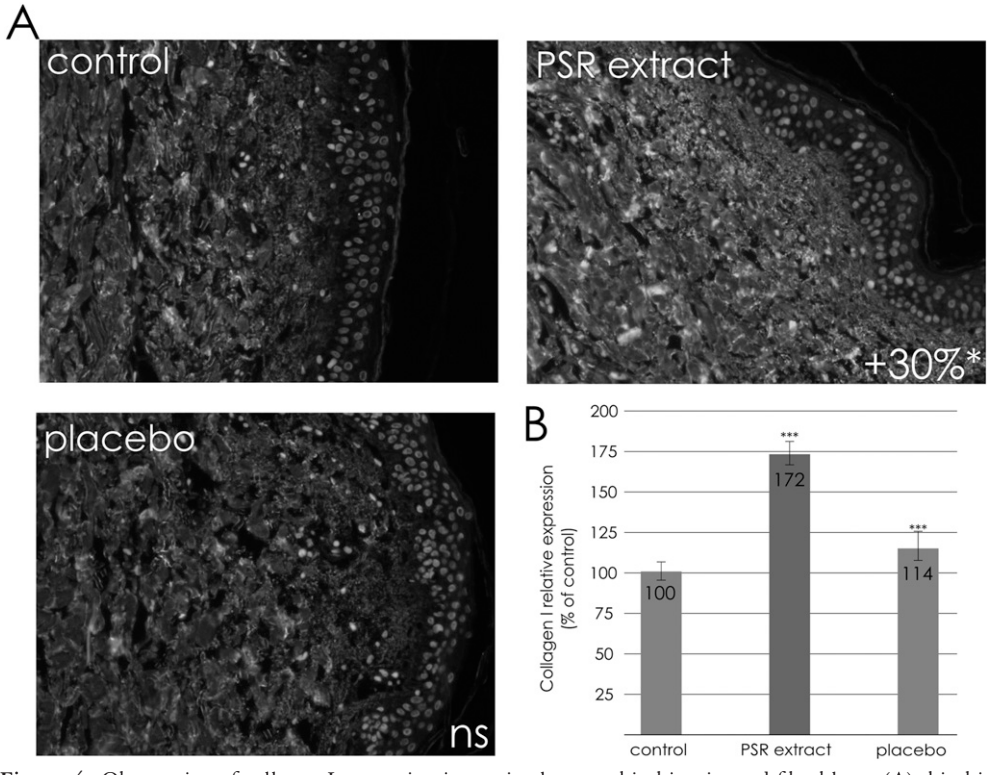


Figure 4. Observation of collagen I expression in ex vivo human skin biopsies and fibroblasts: (A) skin biopsies were treated with 1% PSR baobab extract or with 1% placebo (baobab extract without small RNA) for 48 h (twice a day) (˝20). Collagen I immunohistological staining in green and nuclei counter-stained in blue with DAPI (on the original pictures) (˝20). Quantification of immunohistological staining of collagen I (intensity in % adjusted by considering the area of the examined dermis zone; ns not significant; * significant compared with control, with Student's *t* test, $n = 3$). (B) Expression of collagen I mRNA in fibroblasts treated with 1% baobab PSR or placebo extracts for 24 h (twice a day). Error bars corresponded to the calculated RQmin and RQmax values, based on standard deviation of the mean expression level ***: highly significant compared with young control cells, compared with senescent control cells (indicated by an arrow) with Student's *t* test, $n = 3$ replicates.

this miRNA decreased with senescence. In senescent cells, after treatment with PSR baobab extract, the level of miRNA-19b exceeded the basal level of young cells. MiRNA-19b level was previously shown to be associated with senescence and with the quality of collagen (19).

The induction of senescence was verified by SA β-galactosidase staining at different passages. As expected, an increase in SA β-galactosidase activity was observed in *in vitro* senescent cells (Figure 6).

The image quantification of SA β-galactosidase activity allowed us to observe a decrease in this marker in senescent fibroblasts that were treated with PSR baobab extract; this observed decrease was more pronounced than with the placebo treatment.

In summary, these results allowed us to compare the effect of both PSR and placebo baobab extracts. We noted a better efficiency of PSR extract on the modulation of certain aging markers: an increase in collagen expression in ex vivo skin, an increase in miRNA-19b expression, stability in Drosha expression, and a decrease in SA β-galactosidase activity in

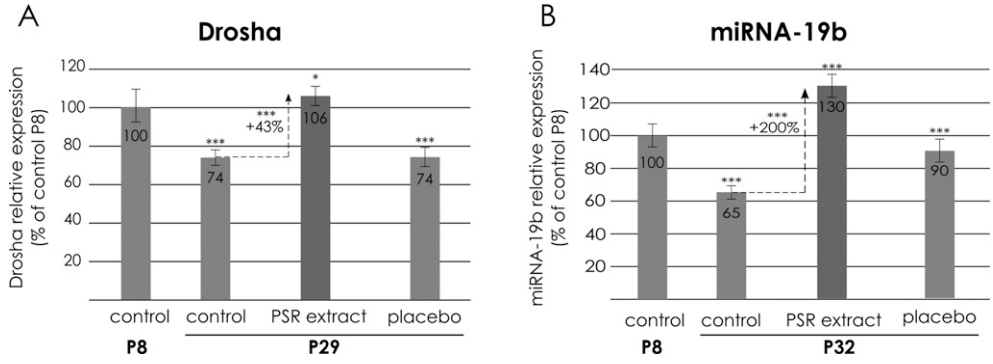


Figure 5. Evaluation of Drosha mRNA (A) and miRNA-19b (B) levels in young (P8) and senescent (P32) fibroblasts treated with 1% PSR baobab extract or with 1% placebo (baobab extract without small RNA) for 24 h (twice a day). Error bars corresponded to the calculated RQmin and RQmax values, based on standard deviation of the mean expression level ***: highly significant compared with young control cells, compared with senescent control cells (indicated by an arrow) with Student's *t* test, $n = 3$ replicates.

aged skin fibroblasts. These results allowed us to conclude that application of the small RNA-rich Baobab extract helps skin cells to counter the visible signs of skin aging via the modulation of essential markers of senescence.

CONCLUSION

Although there are many antiaging cosmetic products on the consumer market offering a wide range of skin benefits, there remains a need for effective topically applied cosmetic compositions that provide antiaging or rejuvenating benefits to the skin, hair, and/or nails using natural ingredients as active agents. In this article, we present an innovative extraction technology that allows for the generation of new botanical extracts containing small RNAs, which can be used as natural cosmetic ingredients. PSR technology appears to be well adapted to plant species such as baobab. Indeed, baobab trees are well-known for their capacity for survival and longevity, which is supported in particular by its small RNAs that can rapidly regulate gene expression and enable adaptation to stressful environment. By addressing the maintenance of miRNA maturation machinery in the skin,

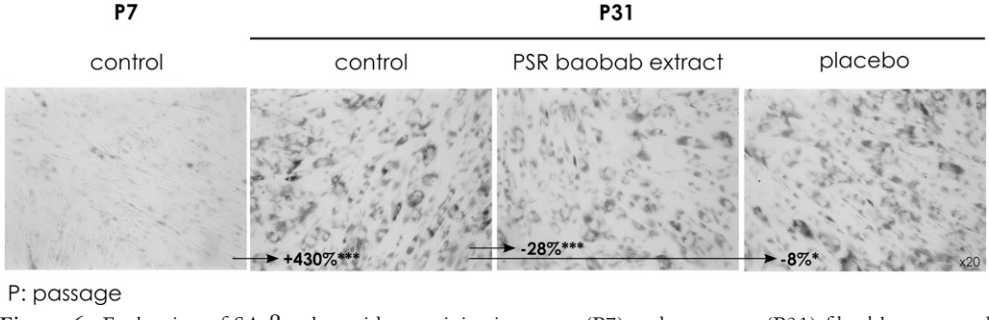


Figure 6. Evaluation of SA β-galactosidase activity in young (P7) and senescent (P31) fibroblasts treated with 1% PSR baobab extract or with 1% placebo (baobab extract without small RNA) for 48 h (twice a day) (×20). Quantification of blue staining (intensity in % adjusted by considering the cell number); ***: highly significant, *: significant compared with young control cells or compared with senescent control cells (indicated with an arrow) with Student's *t* test, $n = 3$ replicates.

PSR baobab extract evaluated in these experiments appears to be associated with an observed reduction in certain visible signs of skin aging.

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AUTHOR CONTRIBUTIONS

Elodie Oger, Ludivine Mur, Alexia Lebleu, Laurine Bergeron, and Catherine Gondran conceived and designed the experiments; Elodie Oger, Ludivine Mur, Alexia Lebleu, and Laurine Bergeron performed the experiments; Elodie Oger, Ludivine Mur, and Catherine Gondran analyzed the data; Elodie Oger and Ludivine Mur wrote the article; and Catherine Gondran revising it critically. Karine Cucumel supervised the study. All authors read and approved the final manuscript.

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