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# **A method to quantify intracellular glycation in dermal fibroblasts using liquid chromatography coupled to fluorescence detection – Application to the selection of deglycation compounds of dermatological interest**

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4 **A method to quantify intracellular glycation in dermal fibroblasts using**  
5 **liquid chromatography coupled to fluorescence detection – Application to**  
6 **the selection of deglycation compounds of dermatological interest**  
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63 **Abstract**  
64

65 Glycation is a common non-enzymatic reaction between proteins and sugars, which  
66 gives rise in the human body to the formation of advanced glycation end products  
67 (AGEs). These modifications impacts both extra and intracellular proteins, leading to  
68 cells and tissues dysfunctions. In the skin, accumulation of AGEs leads to aesthetic  
69 consequences, wrinkles, dark spots and yellowish skin tone, as it can be seen in diabetic  
70 patients. Consequently, there is a growing dermatological interest to find compounds  
71 able to eliminate AGEs accumulated in skin.  
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74 In this context, a method has been developed to detect and quantify intracellular  
75 glycation in human dermal fibroblasts. After cultivation of fibroblasts, cell lysates were  
76 injected in an HPLC system coupled with a fluorescence detector in by-pass mode. The  
77 system allows the simultaneous measurement of global AGEs and particular  
78 pentosidine amounts using two sets of wavelengths in a single run of one minute. The  
79 immunocytochemistry approach was used to valid the HPLC analysis data.  
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82 The method developed was able to quantify changes in global AGEs and pentosidine  
83 content in cells in response to glyoxal treatment. Fibroblasts treated with 500  $\mu$ M of  
84 glyoxal for 48 hours showed a significant 2.3-fold and 2.6-fold increase in the content of  
85 AGEs and pentosidine respectively compared to control cells.  
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88 As an application, a screening of natural extracts have been done and the method  
89 allowed identifying extracts able to significantly reduce the amount of pentosidine in  
90 fibroblasts (- 32 %). These extracts act as deglycation agents of interest in the field of  
91 dermatology and cosmetology.  
92  
93

94 **Keywords:** Advanced glycation End product (AGEs), HPLC, Fluorescence, Human  
95 Dermal Fibroblasts, Immunocytochemistry, Deglycation  
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## 1. Introduction

The glycation process, discovered by Maillard in 1912 [1], is a non-enzymatic transformation involving reducing sugars and amino-acids residues of proteins, leading to the formation of a complex and heterogeneous group of compounds named advanced glycation end products (AGEs). More precisely, the process of glycation involves first the reaction of an ose (mainly glucose) with an amine group of a protein amino acid (like lysine or arginine), to form a Schiff base. An Amadori molecular rearrangement then leads to the irreversible formation of AGEs by successive cyclisation and oxidation reactions (Fig. 1.). Due to the various reactions leading to their creation, multiple AGEs have been detected in tissues, and can be divided into three categories: fluorescent AGEs forming reticulations between proteins (for example: pentosidine, crossline), non-fluorescent AGEs forming reticulations between proteins (for example: glyoxal-lysine dimer (GOLD), methylglyoxal-lysine dimer (MOLD)), and fluorescent or non-fluorescent AGEs forming adducts on proteins (for example: *N*- $\epsilon$ -carboxymethyllysine (CML), pyrrolaline)[2].

AGEs accumulate during the lifetime in whole body including blood plasma, extracellular fluids and cells [3]. Consequently, they are considered as the main causing agent of numerous age-related diseases [4] such as diabetic vascular complications [5], atherosclerosis [6] or Alzheimer's disease [3, 7]. Long lifespan proteins of the body like hemoglobin, collagen or elastin, are known targets of irreversible modifications due to AGEs formation [4]. Extracellular proteins cross-linking leads to cell growth inhibition, impaired cell adhesion and tissue dysfunction [8]. But AGEs are also formed inside cells, causing damages by generation of reactive oxygen species (ROS) [9]. They bond to the

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183 Receptor of Advanced Glycation End products (RAGE) [10, 11], thus increasing  
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185 inflammation mediator release via the NF- $\kappa$ B pathway [12]. Activated inflammatory and  
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187 oxidative cascades lead to the formation of new AGEs in cells. Moreover, excessive ROS  
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189 levels affect intracellular detoxification systems like the proteasome, as well as other  
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191 enzymes involved in cellular repairing. All this leads to a decrease in the antioxidative  
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193 and repair potential of cells [11]. The overall negative effects of AGEs in cells lead to  
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195 tissue and organ dysfunction [13].  
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201 The glycation process does not spare the skin. AGEs have been shown to accumulate in  
202  
203 the dermis and epidermis [14, 15]. Carboxymethyl-lysine (CML) and pentosidine are the  
204  
205 most common AGEs in the skin [15]. Aesthetic consequences due to glycation are  
206  
207 stiffening of the dermis leading mainly to collapsing of the skin, wrinkles formation, skin  
208  
209 tone yellowing and emergence of brown spots, as it can be seen in diabetic patients [16,  
210  
211 17]. Most compounds on the market act by preventing the binding between sugars and  
212  
213 proteins, or by reversing the first reversible steps of the glycation reaction. But there  
214  
215 are only a few compounds that can reduce the number of final AGEs accumulated in the  
216  
217 body [18]. Consequently, there is a growing dermatological, cosmetic and therapeutic  
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219 interest in finding compounds able to get rid of AGEs accumulated in the skin.  
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222 However, most of the methods reported in the literature were used to detect and  
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224 quantify glycation in food [19, 20], human urine, plasma or serum [21-24], or *in tubo*  
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226 formed AGEs by mixing a single protein with one reducing sugar [25, 26]. In the skin,  
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228 studies mainly focus on extracellular proteins modified by glycation [27, 28] and not  
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230 directly on the final AGEs formed at the end of the process. Few methods were reported  
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232 in the literature to quantify glycation in skin cells, which are suitable with the screening  
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234 of a very large number of drug candidates.  
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245 The goal of this work was to develop a reliable method able to detect and quantify  
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247 intracellular AGEs naturally formed and accumulated in human cells upon aging,  
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249 without using any AGE inducers. The level of AGEs was evaluated by measuring the  
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251 characteristic autofluorescence of many AGEs [21, 29] and of particular pentosidine  
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253 [30]. The developed method consist of the analysis of cell lysates using an HPLC system  
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255 coupled with a fluorescence detector, in by-pass mode to allow a direct measurement of  
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257 fluorescence using several wavelengths in the same time. The current article provides a  
258  
259 detailed description of the technique, and demonstrates how it can be used to screen a  
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261 large number of natural extracts in order to find new deglycation compounds capable of  
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263 reducing the intracellular glycation in human fibroblasts.  
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## 270 **2. Material and Methods**

### 272 **2.1 Chemical and reagents**

273  
274 Primary normal human dermal fibroblasts (NHDF) derived from a 54-years-old woman  
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276 skin tissue were purchased from Promocell and cultured according to the supplier's  
277  
278 instructions in fibroblasts growth medium2 (Promocell GmbH, Heidelberg, Germany).  
279  
280 Glyoxal solution and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma  
281  
282 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Radioimmunoprecipitation assay  
283  
284 (RIPA) buffer and Phosphate Buffered Solution (PBS) were obtained from  
285  
286 ThermoFisher (ThermoFisher Scientific, Villebon sur Yvette, France). TritonX-100 was  
287  
288 from Euromedex (Euromedex France, Souffelweyersheim, France). Dimethyl sulfoxide  
289  
290 (DMSO) was obtained from Carlo Erba (Carlo Erba Reagents S.A.S, Val de Reuil, France).  
291  
292 Tween-20 was obtained from Acros Organics (Acros Organics, Geel, Belgium). Bovine  
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294 serum albumin (BSA) was obtained from Dutscher (Dominique Dutscher SAS, Brumath,  
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303 France). Paraformaldehyde was obtained from Electron Microscopy Science (EMS,  
304 Hartfield, PA, U.S.A). Mouse anti-CarboxyMethyl-Lysine (CML) antibody (KH011) was  
305 obtained from Cosmo Bio Co (Cosmo Bio Co LTD, Tokyo, Japan). Goat anti-mouse  
306 secondary antibody Alexa-488 conjugate (A11017) was obtained from Lifetechnologies  
307 (ThermoFisher Scientific, Villebon sur Yvette, France). Mounting medium with DAPI  
308 was obtained from Vector (Vector Laboratories Inc, Burlingame, CA, U.S.A). MilliQ water  
309 was obtained using a Millipore Synergy UV-R system (Merck-Millipore, Darmstadt,  
310 Germany).  
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## 323 **2.1 Natural extracts**

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325 A library of 269 ethyl acetate extracts of symbiotic microorganisms was used for this  
326 study [31, 32]. Their cytotoxicity have been tested against MRC-5, MDA-MB-435 cell  
327 lines. The extracts devoid of toxicity were then tested on primary dermal fibroblasts  
328 (NHDF) at three concentrations (10 µg/mL, 1 µg/mL and 0,1 µg/mL in DMSO; data  
329 available in Table S1 of supplementary material). The extracts having no toxic effect  
330 against NHDF at 1 µg/mL were used for the screening of their activity against  
331 intracellular AGEs.  
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## 342 **2.3 Cell culture**

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344 Normal human dermal fibroblasts (NHDF) were grown in a humidified atmosphere of 5  
345 % CO<sub>2</sub> at 37°C in fibroblasts growth medium<sup>2</sup>.  
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### 351 *2.3.1 Method development*

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353 The NHDF were seeded at the density of 7000 cells/cm<sup>2</sup> on T25 flasks at day 0.  
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363 At day 1, the culture medium was supplemented with glyoxal (final concentration: 250  
364  $\mu\text{M}$  and 500  $\mu\text{M}$ ) for 48 hours. The control cells were treated with DMSO only. On day 3,  
365  
366 at the end of treatment, culture medium was removed, and cells were washed with  
367  
368 HEPES-Buffered saline solution (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid)  
369  
370 before adding fresh culture medium. Cells were cultivated for another 48 hours before  
371  
372 being harvested on day 5 with Trypsine-EDTA solution. Cells were counted using an  
373  
374 Automated Cell counter TC20 (BioRad Laboratories, Hercules, CA, U.S.A) and their  
375  
376 pellets were kept at  $-20^{\circ}\text{C}$  until use. Experiments were done in duplicate.  
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### 382 383 *2.3.2 Application: Screening of natural extracts*

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385 The NHDF were seeded at the density of 7000 cells/cm<sup>2</sup> on 24-well-plates at day 0.  
386

387 At day 1, the extracts were added in the culture medium (final concentration: 1  $\mu\text{g}/\text{mL}$ )  
388  
389 for 24 hours. Each experiment was conducted in duplicate. The control cells were  
390  
391 treated with DMSO only. On day 2, after 24 hours of treatment, culture medium was  
392  
393 removed, and cells were washed with HEPES-BSS solution before adding fresh culture  
394  
395 medium. Cells were cultivated for an additional 48 hours before being harvested on day  
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397 4 with a Trypsine-EDTA solution. Cells were counted, freezed and stored at  $-20^{\circ}\text{C}$  until  
398  
399 use.  
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### 404 **2.4 Cell lysis**

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406 Cell pellets were lysed for 30 minutes on ice, using RIPA lysis buffer in which a PMSF  
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408 solution was added to prevent the action of proteases. After centrifugation at 14000 g,  
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410 the supernatants were stored at  $-20^{\circ}\text{C}$  until analysis.  
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### 415 **2.5 HPLC/Fluorescence method:**



### 2.5.1 Sample preparation for HPLC/fluorescence

The day of the analysis, cell lysates were diluted three times with PBS 1X, filtered, and put into an HPLC insert vial. HPLC vials were kept at 4°C within the HPLC autosampler during all the experiment.

### 2.5.2 HPLC/Fluorescence analysis

For the analysis of the cell lysates, a Waters 2690/S separation module HPLC system coupled with a Waters W2475 fluorescence detector was used (Waters Corporation, Guyancourt, France). Since no separation was required, a by-pass was set-up instead of the column. Wavelengths on the fluorescence detector were set at  $\lambda_{\text{ex}} = 370 \text{ nm}$  /  $\lambda_{\text{em}} = 445 \text{ nm}$  for measuring global AGEs amount, and at  $\lambda_{\text{ex}} = 335 \text{ nm}$  /  $\lambda_{\text{em}} = 385 \text{ nm}$  for measuring particular pentosidine amount, as previously described [21, 29, 30].

Mobile phase used was 100 % milliQ H<sub>2</sub>O at a flow rate of 0.5 mL/min. Run length was 1 minute. Cell lysates (6  $\mu\text{L}$ ) were injected in triplicate, and a blank (water) was injected every 8 runs to confirm that no fluorescence accumulated in pipes.

### 2.5.3 Results analysis

Fluorescence picks were automatically integrated using Empower software (Waters Corporation, Guyancourt, France). Picks areas were analyzed and correspond to the cell lysate fluorescence value expressed in arbitrary units.

To allow the comparison between treatments, the fluorescence values were normalized for 1000 cells.

## 2.6 CML immunofluorescence

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482  
483 NHDF were plated on glass coverslips set into a 24-wells-plate and cultured in standard  
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485 conditions as described above. On day 4, the fibroblasts were fixed with 2 %  
486  
487 paraformaldehyde (500  $\mu$ L) for 12 minutes at room temperature, permeabilized with  
488  
489 0.2 % TritonX-100 (500  $\mu$ L) for 10 minutes and finally blocked with 5 % BSA, 0.2 %  
490  
491 Tween-20 (500  $\mu$ L) for further 10 minutes. Cells were then incubated with the primary  
492  
493 antibody anti-CML (mouse) at a concentration of 7  $\mu$ g/mL (30  $\mu$ L) at 4°C overnight,  
494  
495 followed by incubation with Alexa-488 anti-mouse secondary antibody diluted at 1:200  
496  
497 (30  $\mu$ L) for 2 hours at room temperature. After washing with 5 % BSA, 0.2 % Tween-20  
498  
499 (3x500  $\mu$ L), coverslips were mounted onto microscope slides using a DAPI containing  
500  
501 mounting medium, and observed under a confocal photonic microscope (Spinning-Disk  
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503 microscope, Roper/Nikon – parameters for DAPI: Laser 447/60 405 ; Gain : 3(4x) ;  
504  
505 Digitizer : 5MHz ; EM gain : 150 ; Exposition : 200 ms ; Laser power : 30% - parameters  
506  
507 for Alexa 488 : Laser 525/45 491 ; Gain : 3 (4x) ; Digitizer : 5MHz ; EM gain : 200 ;  
508  
509 Exposition : 300ms ; Laser power: 25%). Images are recorded using a Z stack, allowing  
510  
511 fluorescence to be recorded at different positions in the cell. Five images were recorded  
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513 by coverslips (ten pictures per treatment condition). All pictures were taken with the  
514  
515 same microscope parameters. Fluorescence quantification on the images was done  
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517 using ImageJ software.  
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## 524 **2.7 Statistical analysis**

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526 Data were presented as mean  $\pm$  standard error of the mean. The mean data were  
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528 analyzed with one-way analysis of variance (one way ANOVA) followed by Dunnett's  
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530 multiple comparison test. A  $P < 0.05$  was considered to be significant.  
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## 535 **2.8 Method validation**

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543 To determine the reproducibility of the method, we calculated the relative standard  
544 deviation (RSD) for triplicates of the same sample, and biological duplicates of the same  
545 treatment (data available in Table S2 of supplementary material).  
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### 551 552 **3. Results**

#### 553 *3.1 Method development*

554  
555 We first examined whether the proposed approach was able to detect and quantify the  
556 variations in the amount of AGEs and pentosidine in lysates of fibroblasts treated with  
557 250  $\mu$ M and 500  $\mu$ M of glyoxal for 48 hours.  
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561 Glyoxal is a dicarbonyl formed *in vivo* mainly by auto-oxidation of reducing sugars and  
562 by lipid peroxidation. Both highly reactive carbonyl groups of glyoxal react with the  
563 amino groups of proteins to form AGEs as previously reported [33-35].  
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567 Lysates of glyoxal treated cells and lysates of untreated controls were injected in  
568 triplicate in the HPLC system in by-pass mode, using MilliQ H<sub>2</sub>O as mobile phase, and  
569 the signal was recorded with a fluorescence detector using two sets of wavelengths for  
570 the simultaneous detection of AGEs ( $\lambda_{\text{ex}} = 370 \text{ nm} / \lambda_{\text{em}} = 445 \text{ nm}$ ) and pentosidine ( $\lambda_{\text{ex}}$   
571  $= 335 \text{ nm} / \lambda_{\text{em}} = 385 \text{ nm}$ ). The use of the HPLC system allows the analysis of small  
572 amounts of samples, with good reproducibility. The fluorescence spectra of pentosidine  
573 recorded in control cells lysate (triplicate) are presented in Figure 2. The fluorescence  
574 value corresponds to peak integration value. To allow a reliable comparison between  
575 the treatments, the number of cells harvested at the end of the cell culture was counted,  
576 and the fluorescence values were normalized for 1000 cells.  
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593 As shown in Table I, the treatment of fibroblasts with glyoxal increases significantly the  
594 fluorescence detected in cell lysates for both AGEs and pentosidine, in a dose-dependent  
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603 manner. A 1.3-fold and 2.2-fold increase in the amount of intracellular AGEs present in  
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605 cells treated respectively with 250  $\mu$ M and 500  $\mu$ M of glyoxal for 48 hours, compared to  
606  
607 controls was observed. For pentosidine, a 1.4-fold and 2.6-fold increase in cells treated  
608  
609 respectively with 250  $\mu$ M and 500  $\mu$ M of glyoxal was detected.  
610

611  
612 To valid these findings, we analyzed the level of carboxymethyl-lysine (CML) by  
613  
614 immunocytochemistry in cells treated with 500  $\mu$ M of glyoxal for 48 hours (Fig. 3.). CML  
615  
616 is an AGE forming adducts on proteins. Quantification of the immunofluorescence signal  
617  
618 on images taken by confocal photonic microscopy shows that the intracellular CML  
619  
620 content was indeed significantly higher in cells treated with glyoxal ( $1.57 \pm 0.07$   
621  
622 increase compared to non-treated control cells).  
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### 627 *3.2 Application: The effect of natural products on the amount of intracellular* 628 629 *pentosidine* 630

631 As the developed method was able to detect variations in the content of AGEs in cells  
632  
633 treated with different concentrations of glyoxal, we used it to screen eighty natural  
634  
635 extracts and evaluate their potential to reduce the quantity of intracellular AGEs and  
636  
637 pentosidine in human skin cells. Treatment of fibroblasts with extracts was done for 24  
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639 hours, in biological duplicate. The intracellular AGEs and pentosidine level was  
640  
641 recorded by HPLC with fluorescence detector as described above. Aminoguanidine, an  
642  
643 AGE inhibitor compound, was chosen as control. Table II show the results of twenty-  
644  
645 three of the extracts as the mean amount of pentosidine in cells compared to control.  
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651 The screening conducted in biological duplicates showed that aminoguanidine did not  
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653 reduce the intracellular amount of pentosidine. However, among the eighty natural  
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655 extracts, seven of them were shown to significantly reduce the intracellular content of  
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663 pentosidine in dermal fibroblasts. SNB-GTC2701 was the most active extract, reducing  
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665 intracellular pentosidine content by 32 % in fibroblasts after 24 hours of treatment at 1  
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667  $\mu\text{g/mL}$ .  
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669  
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### 671 672 *3.3 Reproducibility* 673

674 The reproducibility of the method was evaluated taking into account the triplicates of  
675  
676 the same sample and biological duplicates of the same treatment.  
677

678 The relative standard deviations of samples (RSD) between the triplicates of the same  
679  
680 sample ranged within 10 % (except for treatment SNB-CN76) for AGEs and for  
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682 pentosidine (Table S2). RSD calculated between biological duplicates showed good  
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684 reproducibility as well (< 10 %). According to these results, the method can be  
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686 considered as robust.  
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## 691 **4. Discussion** 692 693

694 The present method was developed to quantify the amount of intracellular AGEs and  
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696 pentosidine in human dermal fibroblasts lysates, using their characteristic  
697  
698 autofluorescent properties.  
699

700 To develop the method, we used glyoxal treated fibroblasts. Glyoxal is mainly produced  
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702 during the autooxidation of reducing sugars, and is a source of macromolecular damage  
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704 in cells. Treatment of fibroblasts with glyoxal was shown to induce a senescent  
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706 phenotype *in vitro*, and to lead to an increase of AGEs levels, thus providing a model of  
707  
708 accelerated cellular aging [33].  
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710 Results obtained with the developed method confirmed that glyoxal induces a change in  
711  
712 the AGE content in fibroblasts. Moreover, the HPLC-fluorescent method was efficient to  
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714 detect and quantify these changes.  
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723 Besides, by analyzing the results obtained during the development phase, we noticed  
724 that the glycation level of the non-treated dermal fibroblasts obtained from a 54 years  
725 old donor was high enough to be easily detected and quantified by the HPLC coupled  
726 with fluorescence method. In fact, AGEs accumulates *in vivo* during normal aging, and  
727 the detection of naturally occurring AGEs in fibroblasts with our method was of high  
728 interest for the next step of our project. This allowed us to screen the natural extracts  
729 library on cells that have not undergone glyoxal treatment, thus enabling the screening  
730 to be performed under conditions closest to the biological conditions *in vivo*.  
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732  
733  
734 During the screening, aminoguanidine was chosen as a control compound. This  
735 molecule works as a scavenger of reactive dicarbonyl intermediates in the Maillard  
736 reaction, therefore inhibiting AGE formation. Our results showed that aminoguanidine  
737 did not reduce the pentosidine level in fibroblasts. This finding is in line with published  
738 data on its mode of action preventing the condensation of reducing sugars with amino-  
739 acids residues, or inhibiting the first reversible reaction of the glycation process [36].  
740  
741 We provide new evidence that aminoguanidine is not able to target AGEs at their final  
742 stage.  
743

744  
745 Among eighty natural extracts tested, seven of them showed positive results on  
746 pentosidine, significantly reducing its content in dermal fibroblasts after 24 hours of  
747 treatment. Comparing these results with those of aminoguanidine, we hypothesize that  
748 the mechanism of action of the tested active extracts might be linked to a possible  
749 activation or inhibition of specific biological pathways. Literature shows that AGE  
750 receptor (AGER) subunit AGER-1 (OST-48) binds to AGEs, triggering endocytosis and  
751 elimination of AGEs by cells, while this receptor does not bind to early glycation  
752 products like Amadori products [37-39]. Pentosidine can be degraded through this  
753 mechanism. We observed by western blot using a specific AGER-1 antibody that AGER-1  
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783 was overexpressed in cells treated with three of the active extracts (Figure S1).  
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785 Although further investigations will be necessary, it is possible that the active extracts  
786 trigger a natural cell detoxification mechanism.  
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789 Due to the dermatological interest of finding compounds able to decrease the amount of  
790 formed AGEs in skin, an effort was made to make the assay more rapid and as simple as  
791 possible, allowing the screening of a large number of candidates in one time.  
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794 The screening of a large number of substances in a biological test requires cultivating  
795 cells in large amounts. But miniaturization of biological tests into multiwell plates lower  
796 the available quantity of samples to be analyzed. The main advantage of using an HPLC  
797 system is to benefit from the auto sampler allowing precise sampling of small amounts  
798 like few  $\mu\text{L}$ . This allowed us to miniaturize our biological screening into 24-wells plates,  
799 and so to decrease the amount of cells to be cultivated as well as the extract quantity to  
800 be applied as treatment.  
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## 811 812 813 **5. Conclusion**

814 The method described in this article allows detecting and quantifying intracellular  
815 levels of AGEs in human dermal fibroblasts. It is reliable, reproducible, simple and can  
816 be performed at rather low-cost. The use of HPLC coupled to fluorescence detection has  
817 many advantages. First, the fluorescence detection at two wavelengths at the same time  
818 reduces the quantity of sample required for analysis (6  $\mu\text{L}$  per injections), allowing  
819 culture of cells in 24-wells plates. Then, once the cells harvested, one requires only a  
820 few steps of sample preparation. Finally, the automatic injection of the samples in a  
821 short one-minute run, with good reproducibility between technical and biological  
822 replicates, makes it suitable for the rapid and reliable screening of a large number of  
823 samples.  
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843 Contrary to commonly used methods i.e. *in tubo* tests employing one reducing sugar  
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845 and one protein or methods targeting extracellular proteins modified by glycation, our  
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847 experimental approach allows detection and quantification of intracellular AGEs formed  
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849 and accumulated during aging in cells without induction of glycation.  
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852 Eventually, the method was applied to test natural extracts and revealed the potential of  
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854 some of them to reduce the amount of AGEs naturally accumulated in cells with aging,  
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856 thus suggesting that new deglycation compounds of dermatological and cosmetic  
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858 interest may be discovered. The mode of action of the active extracts needs to be further  
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860 characterized but seems to involve complex biological reactions. Thus, the molecules  
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862 responsible for the activity will be characterized after bioguided isolation.  
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## Conflict of Interest

The authors declare that they have no conflict of interest.

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1083 Legends to figures:  
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1085 **Figure 1:** Glycation reaction of proteins with glucose leading to the formation of AGEs,  
1086 and structure of Pentosidine crosslink.  
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1089 **Figure 2:** Example of fluorescence signal recorded for pentosidine ( $\lambda_{ex} = 335 \text{ nm} / \lambda_{em} =$   
1090  $385 \text{ nm}$ ) in lysate of control cells. Injections were done in triplicate. Peak integration  
1091 (area) was done with Empower software (Waters).  
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1094 **Figure 3:** Confocal microscopy images (X40) of human dermal fibroblasts treated for 48  
1095 hours with (a) DMSO as control and (b) 500  $\mu\text{M}$  of glyoxal. Nucleus labeling by DAPI  
1096 (blue) ; Immunological labeling of CML by Alexa 488 (green).  
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1107 Tables:  
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1109 **Table I.** AGEs ( $\lambda_{ex} = 370 \text{ nm} / \lambda_{em} = 445 \text{ nm}$ ) and pentosidine expression ( $\lambda_{ex} = 335 \text{ nm} /$   
1110  $\lambda_{em} = 385 \text{ nm}$ ) in NHDF treated with 250  $\mu\text{M}$  and 500  $\mu\text{M}$  glyoxal for 48 hours.  
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1112 Concentrations have been normalized relatively to the negative control, for 1000 cells.  
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1114 Significance levels when compared to negative control were calculated by one-way  
1115 ANOVA followed by Dunett's multiple comparison test \*\*\*  $P < 0.001$  compared to control.  
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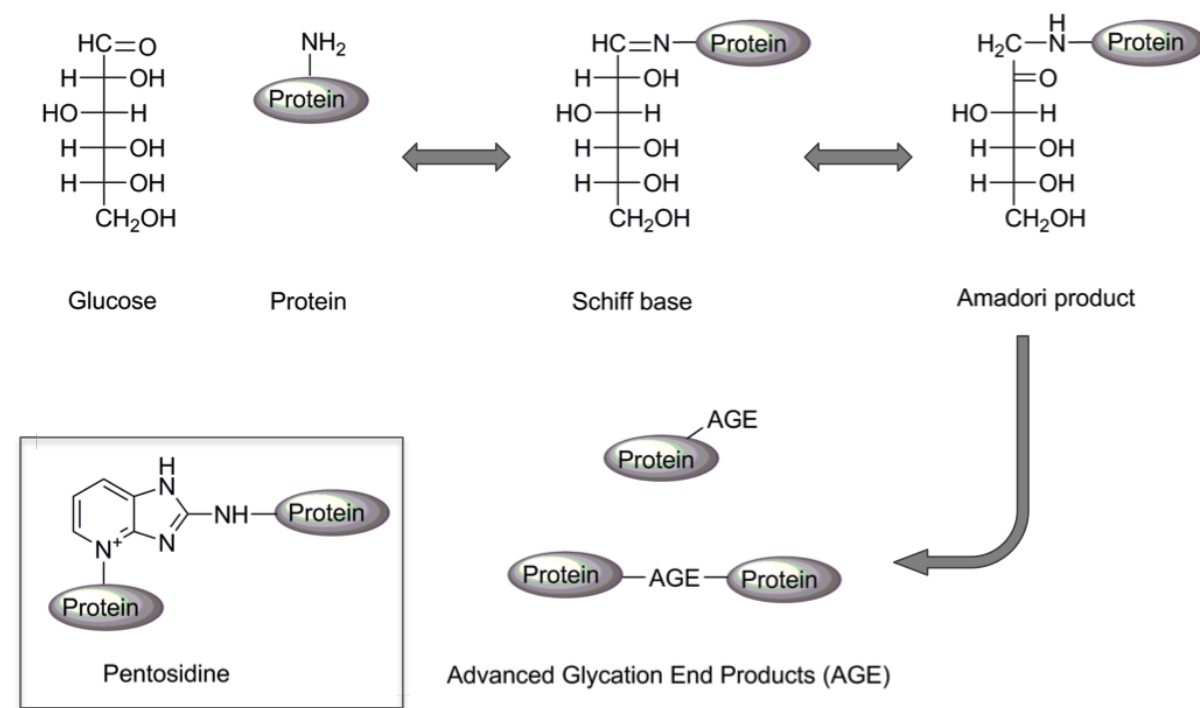
Treatment	AGEs expression $\pm$ SD	Pentosidine $\pm$ SD
GO 250 $\mu\text{M}$	1.32 $\pm$ 0.16 ***	1.38 $\pm$ 0.03 ***
GO 500 $\mu\text{M}$	2.16 $\pm$ 0.07 ***	2.61 $\pm$ 0.02 ***

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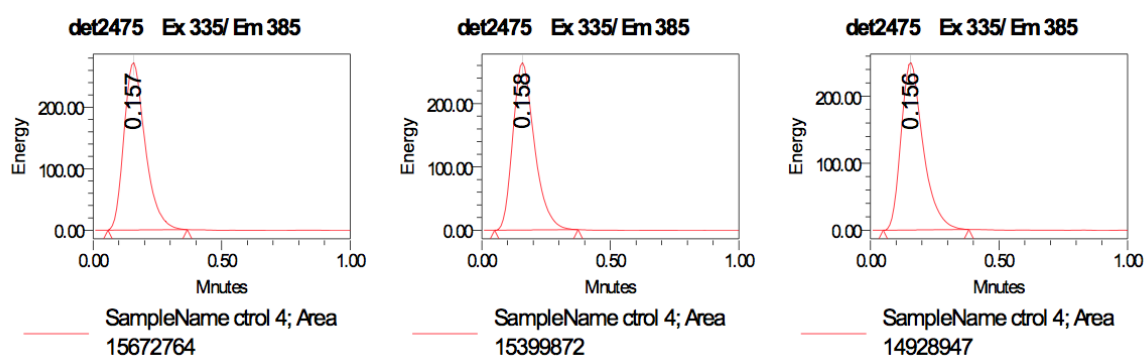
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 1146 **Table II.** Effect of 23 different microbial extracts and aminoguanidine on pentosidine  
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 1148 expression compared to control, measured by the HPLC/fluorescence method in human  
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 1150 dermal fibroblasts. Concentrations have been normalized relatively to the negative  
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 1152 control, for 1000 cells. Significance levels when compared to negative control were  
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 1154 calculated by one-way ANOVA followed by Dunett's multiple comparison test; \*P < 0.05;  
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 1156 \*\*P < 0.01; \*\*\*P < 0.001 compared to control, ns = not significant.  
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Treatment	Pentosidine amount $\pm$ SD
Control (untreated)	1.00 $\pm$ 0.05
Aminoguanidine	1.13 $\pm$ 0.18 <sup>ns</sup>
SNB-GTC2701	0.68 $\pm$ 0.07 <sup>***</sup>
SNB-CN20	0.71 $\pm$ 0.01 <sup>**</sup>
SNB-CN102	0.76 $\pm$ 0.03 <sup>**</sup>
SNB-CN55	0.79 $\pm$ 0.01 <sup>**</sup>
SNB-CN76	0.80 $\pm$ 0.03 <sup>***</sup>
SNB-GSS07	0.85 $\pm$ 0.04 <sup>ns</sup>
SNB-CN54	0.87 $\pm$ 0.05 <sup>*</sup>
SNB-CN79	0.87 $\pm$ 0.07 <sup>ns</sup>
SNB-GSS04	0.88 $\pm$ 0.01 <sup>**</sup>
SNB-CN13	0.89 $\pm$ 0.09 <sup>ns</sup>
SNB-CN59	0.89 $\pm$ 0.04 <sup>ns</sup>
SNB-GTC2810	0.91 $\pm$ 0.10 <sup>ns</sup>
SNB-CN104	0.93 $\pm$ 0.04 <sup>ns</sup>
SNB-CN66	0.93 $\pm$ 0.01 <sup>ns</sup>
SNB-CN27	0.94 $\pm$ 0.10 <sup>ns</sup>
SNB-CN69	0.97 $\pm$ 0.11 <sup>ns</sup>
SNB-CN67	0.97 $\pm$ 0.05 <sup>ns</sup>
SNB-CN75	0.98 $\pm$ 0.05 <sup>ns</sup>
SNB-LD 9.2	1.02 $\pm$ 0.10 <sup>ns</sup>
SNB-CN98	1.14 $\pm$ 0.03 <sup>ns</sup>
SNB-CN1	1.16 $\pm$ 0.09 <sup>*</sup>
SNB-CN85	1.18 $\pm$ 0.04 <sup>ns</sup>
SNB-CN10	1.58 $\pm$ 0.27 <sup>**</sup>

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1206 **Figures:**  
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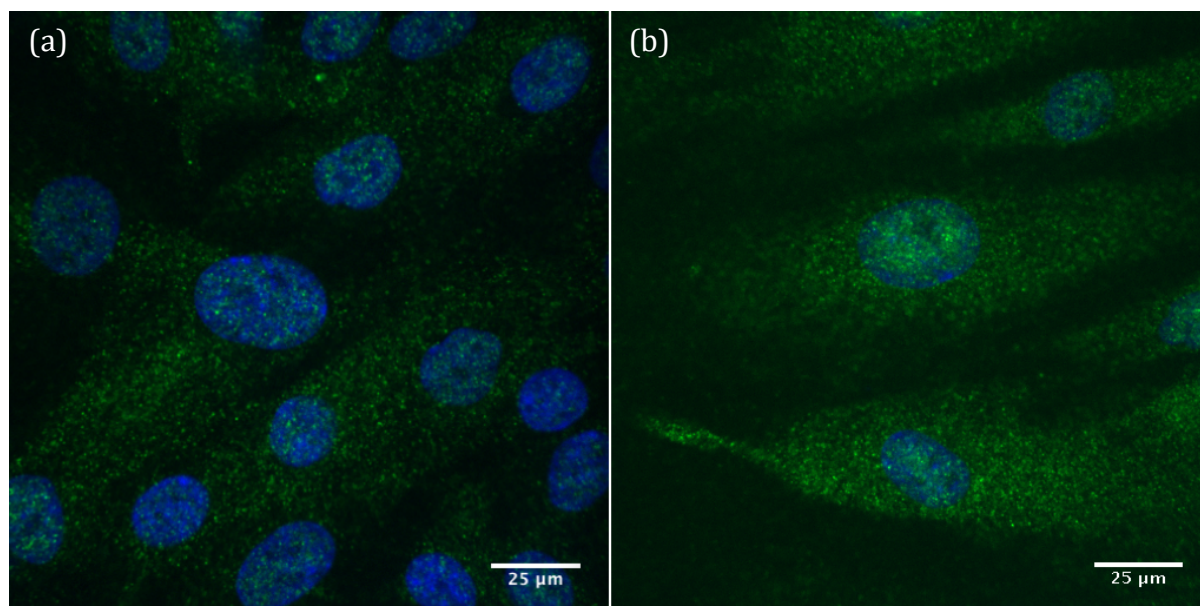


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1230 **Figure 1.** Glycation reaction of proteins with glucose leading to the formation of AGEs,  
1231 and structure of Pentosidine crosslink.  
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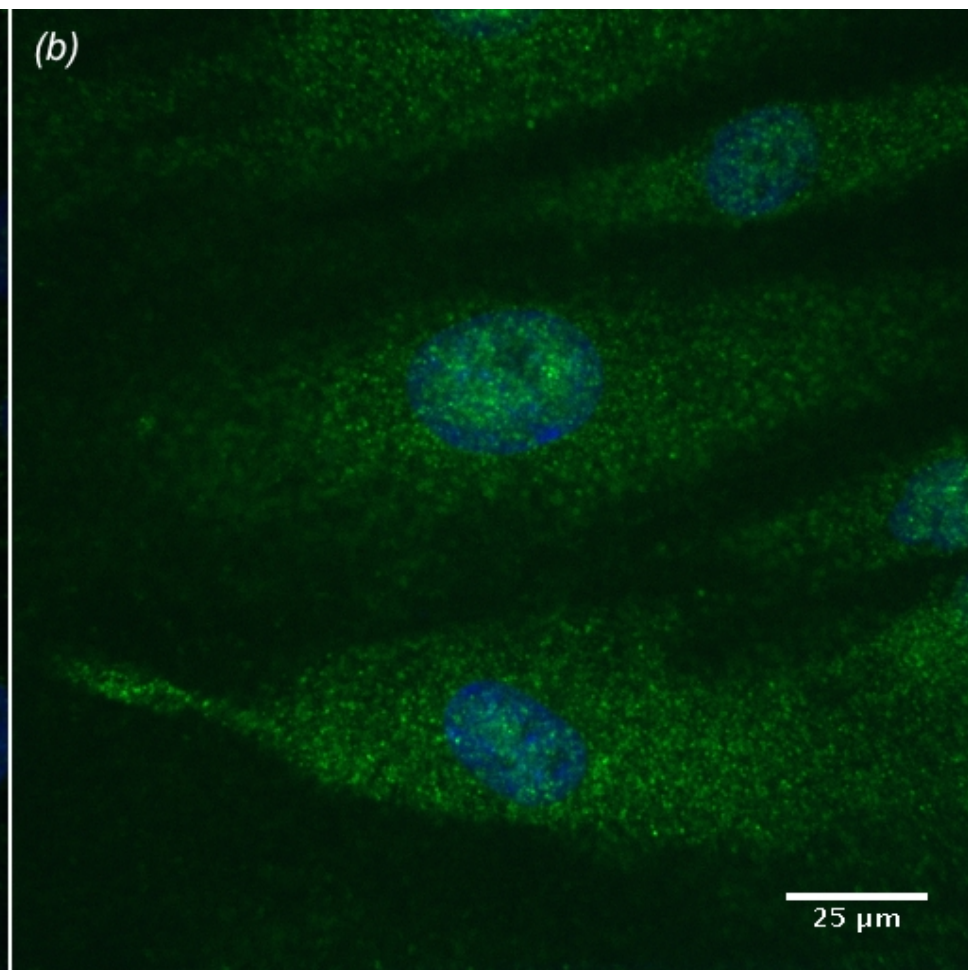
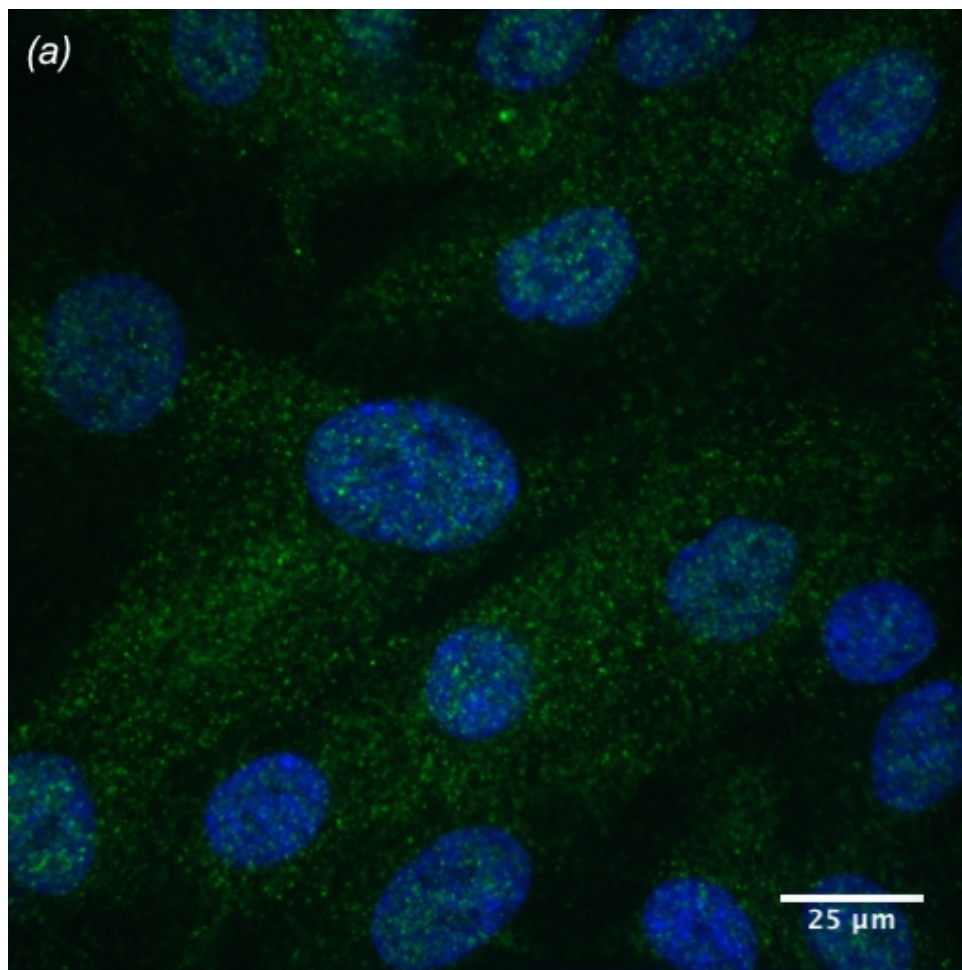
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1251 385 nm) in lysate of control cells. Injections were done in triplicate. Peak integration  
1252 (area) was done with Empower software (Waters).  
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**Figure 3.** Confocal microscopy images (X40) of human dermal fibroblasts treated for 48 hours with (a) DMSO as control and (b) 500  $\mu\text{M}$  of glyoxal. Nucleus labeling by DAPI (blue) ; Immunological labeling of CML by Alexa 488 (green).







**Table S1. Viability of NHDF treated for 24 heures with ethyl acetate natural extracts at 10 µg/mL, 1 µg/mL and 0,1 µg/mL in DMSO.**

Extracts SNB n°	% Viability at 10 µg/mL	% Viability at 1 µg/mL	% Viability at 0,1 µg/mL	Extracts SNB n°	% Viability at 10 µg/mL	% Viability at 1 µg/mL	% Viability at 0,1 µg/mL
CN1	10,3	65,1	94,4	CN111	11,3	95,6	91,4
CN10	10,5	62,1	90,1	CN112	9,9	89,0	91,5
CN100	79,4	92,8	98,3	CN16	89,1	94,0	90,8
CN11	10,6	58,5	86,2	CN17	88,6	92,3	90,4
CN13	54,5	91,0	94,8	CN29	44,9	86,8	85,4
CN14	10,6	55,9	91,6	CN37B	106,6	92,4	92,9
CN15	<5,0	91,0	92,7	CN55	9,6	86,4	93,4
CN2	10,7	92,1	94,2	CN56	18,4	94,2	94,1
CN20	10,1	86,6	93,2	CN57	11,1	79,7	91,9
CN27	98,4	93,0	92,6	CN60	28,2	97,8	96,7
CN28	98,3	97,4	91,3	CN60bis	9,9	90,0	89,6
CN3	10,5	59,1	90,2	CN65	19,3	89,4	88,7
CN4	13,1	90,5	88,9	CN70	<5,0	ND	87,0
CN52	112,2	91,6	89,3	CN36A	129,7	ND	89,0
CN61	96,7	88,7	89,1	CN71	11,1	120,0	102,3
CN69	15,2	86,6	90,4	CN72	10,0	115,6	92,9
CN78	49,5	93,3	89,2	CN73	50,6	107,1	93,9
CN8	73,1	93,8	90,3	CN74	14,0	97,5	95,4
CN88	71,6	88,4	89,9	CN 82	80,3	92,7	92,9
CN89	<5,0	92,7	92,5	CN84	16,9	87,4	88,4
CN90	72,9	92,0	93,8	CN85	34,9	83,5	84,1
CN91	92,0	97,3	91,3	CN87	11,9	83,6	90,8
CN92	13,4	94,4	96,2	GSS11	28,1	85,0	87,2
CN94	101,1	96,1	92,3	GSS15	49,5	89,2	83,8
CN95	102,8	96,4	92,2	GTC0202	12,7	88,2	88,5
CN96	9,7	97,3	91,5	GTC2401	21,5	88,5	88,4
CN97	23,6	99,9	100,2	GTC2701	18,3	89,8	87,3
CN98	64,9	90,2	88,9	GTC2809	10,1	93,8	93,3
CN99	100,0	91,2	88,2	CN75	10,8	91,9	101,2
CN102	9,8	90,2	90,5	CN76	98,3	101,3	97,8
CN103	9,9	89,4	91,3	GTC2810	28,7	85,7	91,3
CN104	100,5	90,4	87,1	GTC2822	23,9	89,0	91,2
CN109	10,3	93,7	91,8	GTC3002	63,4	92,4	92,5
CN110	15,2	92,4	88,7	GSS07	11,0	91,7	91,6
LD2.10.2	51,9	84,5	97,7	GSS04	49,9	91,8	86,8
LD2.13	16,1	89,5	87,5	LD8.9	30,0	96,0	96,0
LD3.4	30,1	89,3	94,6	CN67	84,7	95,1	93,3
LD6.5.2	17,4	87,5	98,6	CN68	<5,0	ND	ND
LD7.1	9,9	91,6	98,3	Levure rose	<5,0	74,4	77,9

LD8.6	9,8	102,4	108,2	CN113	10,1	79,5	81,6
CN79	100,9	82,5	85,3	CN64	10,1	92,9	89,0
LD9.2	<5,0	88,7	89,1	CN66	98,6	90,0	92,3
CN59	12,7	86,5	92,4	CN54	90,4	89,9	90,2
CN62	39,2	93,6	88,0				

*ND = Not determined.*

**Table S2. Reproducibility expressed as Relative Standard Deviation (RSD) between triplicates of the same sample, and between biological duplicates of the same treatment.**

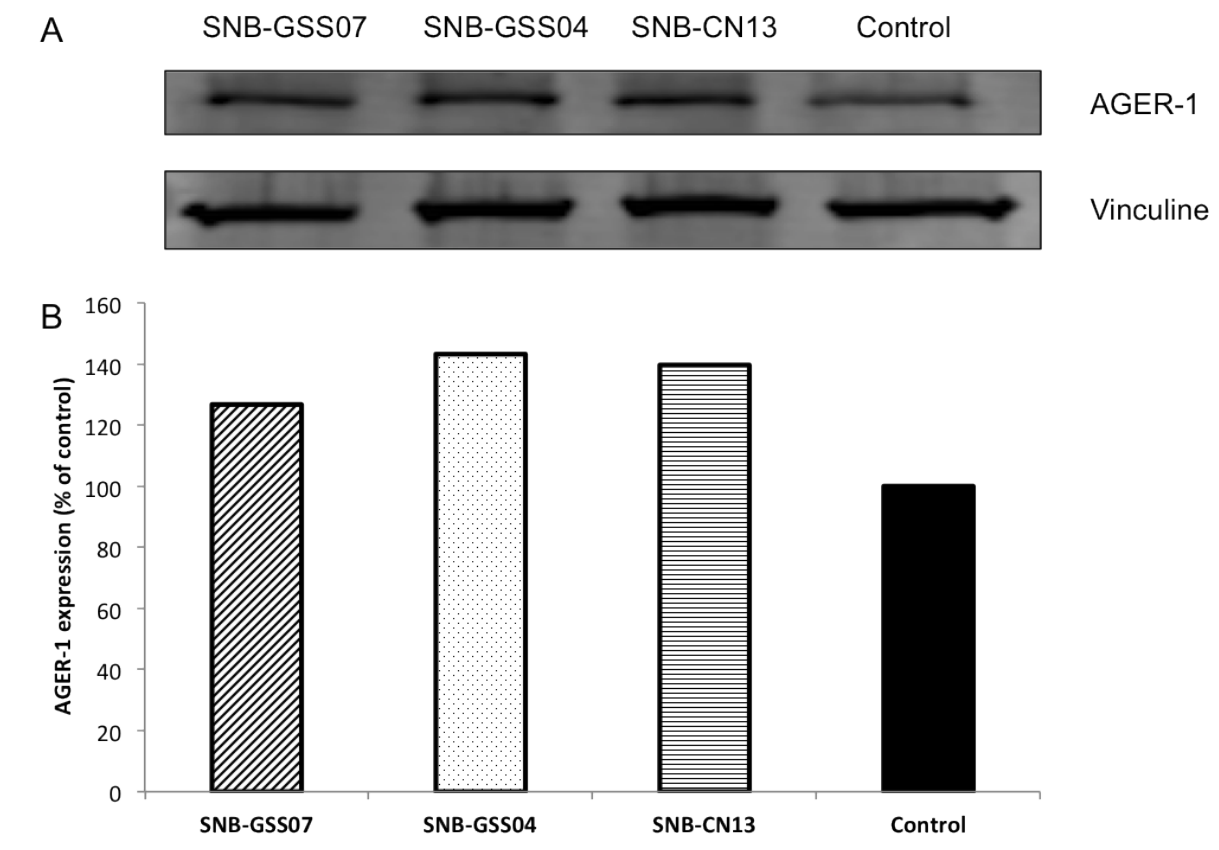
Reproducibility between triplicates			Reproducibility: between biological duplicates		
Extracts SNB n°	RSD for AGEs measurement (%)	RSD for Pentosidine measurement (%)	Extracts SNB n°	RSD for AGEs measurement (%)	RSD for Pentosidine measurement (%)
Control	4.0	2.4	Control	12.0	2.9
CN59	4.7	2.2	CN59	3.9	1.5
CN79	3.7	3.8	CN79	3.7	4.2
CN76	15.3	6.5	CN76	16.5	6.1
GSS04	4.5	4.6	GSS04	3.5	3.1
CN75	2.8	0.2	CN75	4.4	0.1
CN67	5.5	2.8	CN67	5.6	2.4
LD 9.2	4.1	19.3	LD 9.2	6.4	16.4
GSS07	1.5	0.7	GSS07	2.4	4.4
CN66	2.0	3.4	CN66	4.1	3.1
CN85	11.8	0.4	CN85	7.5	3.3
CN55	2.7	1.5	CN55	10.7	9.0
CN1	2.4	1.6	CN1	1.8	1.3
CN102	7.9	2.6	CN102	5.6	2.1
CN69	2.3	0.5	CN69	3.6	4.1
CN27	6.4	2.5	CN27	5.6	12.6
CN13	9.0	0.5	CN13	6.9	2.4
CN20	5.9	1.6	CN20	7.0	6.5
GTC2810	3.5	0.8	GTC2810	9.8	1.2
CN10	4.5	8.5	CN10	18.3	6.13
CN104	3.3	1.4	CN104	4.32	1.1
CN98	6.1	5.1	CN98	14.6	10.6
CN54	6.7	0.6	CN54	9.3	2.3
GTC2701	1.0	0.9	GTC2701	4.7	2.1
Amino- guanidine	3.9	1.2	Amino- guanidine	4.9	8.6

**Overexpression of AGER-1 in fibroblasts treated with extracts SNB-GSS07, SNB-GSS04 and SNB-CN13 at 1µg/mL during 24 hours.**

Protocol:

Cells were collected and solubilized in RIPA lysis buffer. Protein concentrations were determined using the BCA method. Equal amount of proteins (26 µg) were separated on SDS-PAGE and transferred on 0.45 µm PVDF membranes. The primary antibodies used were: monoclonal anti-vinculin antibody produced in mouse (Sigma-Aldrich #V9131), anti-DDOST (AGER-1) antibody produced in rabbit (Sigma-Aldrich #D6820). The following secondary antibodies were used: anti-mouse IRDye 800CW and anti-rabbit IRDye 680LT (LI-COR). Membranes were scanned with an Odyssey Imaging System (LI-COR). Quantification was performed using ImageJ software. Unnecessary lanes were cut off and demarcated using black line in the following figure.

Results:



**Figure S1.** Overexpression of AGER-1 in fibroblasts treated with extracts SNB-GSS07, SNB-GSS04 and SNB-CN13 at 1µg/mL during 24 hours. (A) Total protein extracts of treated and control fibroblasts were analysed by western blotting using anti-AGER-1 antibody. Vinculin was used as loading control. (B) Quantification of AGER-1 expression in cells (data shown in A) and expressed as percentage of control.