Fighting glycation for rejuvenated skin

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ABSTRACT

In our ageing society, the increasing incidence of metabolic diseases such as diabetes poses substantial problems. The rising prevalence of diabetes is attributed to obesity linked with a sugar-rich diet. In the skin, a high presence of free sugar molecules causes glycation, a process where proteins and sugars are cross-linked to form advanced glycation end products (AGE). The formation of AGEs has a negative impact on the elasticity of the skin as cross-linked collagen and elastin fibers are stiffened. Furthermore, the yellow-brownish colour of AGEs leads to a change in skin tone towards a more yellow appearance. Additionally, AGEs lead to constant inflammatory processes which exacerbate the ageing effect of high sugar concentrations in the skin. Here we show a cosmetic approach with different testing methods to help prevent the formation of AGEs and to ameliorate the downstream effects of glycation in the skin using an extract of *Ziziphus spina-christi* leaves.

The prevalence of diabetes has doubled worldwide since the 1980s and continues to be on the rise.[1] One of the main reasons is the increased consumption of sugar and a general unhealthy lifestyle in the industrialised world. Apart from profound clinical implications such as high blood pressure, diabetes also affects the skin. Diabetic skin parameters are similar to prematurely aged skin: loss of elasticity, dry skin, decreased microcirculation, and a yellowish skin tone. The underlying reason is a process called glycation, which is caused by free sugar molecules in the bloodstream. The blood sugar levels are especially high for diabetes patients where sugar uptake into the cells is disrupted. Notably, also during the normal ageing process, glycation takes place in the skin tissue, a process that is exacerbated by UV irradiation.

During glycation, free amino groups from proteins and reducing sugars, for example glucose, are forming a covalent bond. This non-enzymatic process is an important part in the frying and bread baking process where it is responsible for the typical browning process. Due to the much lower temperatures in our bodies, the glycation process is much slower, but over many years the effects are also visible in the skin. Advanced glycation end products (AGEs) are formed as end products of the reaction between proteins and sugars. Oxidised lipids also play a role in this process and can also be crosslinked with proteins to form a covalent bond. The crosslinking of collagen and elastin leads to a stiffening of these normally elastic fibres, which reduces the skin's elasticity. As the initial products of the glycation reaction are highly reactive and susceptible to oxidation and fragmentation, a high level of glycation will lead to collagen fragmentation in the skin. These fragments can further react with other proteins and contribute to an accelerated AGE formation. The AGEs that are formed by the glycation reaction do not just hinder normal protein activity and cellular function. AGEs bind to the AGE receptor (RAGE) that is present on the surface of many cell types, including our skin cells. This binding of AGEs to RAGE causes an inflammation response.[2] AGEs also cause the formation of free radicals which lead to RAGE upregulation. This results in a vicious cycle of chronic inflammation and skin damage (Fig 1). Another hallmark of increased glycation is a yellow toned skin due to the yellow-brown colour of AGEs. It is therefore important to counteract the formation of AGEs in the skin. While glycation happens spontaneously, the removal of AGEs will require the help of an enzymatic machinery. Consequently, this machinery needs to be activated in order to recycle the AGEs already present in the skin for a rejuvenation effect.

Ziziphus spina-christi.

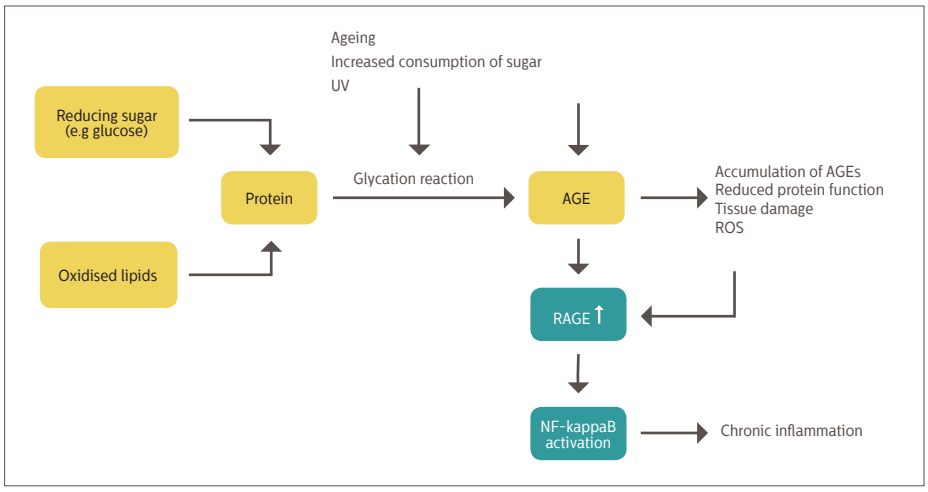

Figure 1: Schematic representation of the effects of AGEs in the skin.

In this publication, an extract of the leaves of the *Ziziphus spina-christi* tree was investigated for its anti-glycation properties. *Ziziphus spina-christi*, which is also known as Christ's thorn jujube, is a thorny evergreen shrub that can also grow into a tree measuring up to 20 m in height. It is cultivated for its fruits and also in order to provide shade in northern Africa, throughout the Middle East, and in northern India. *Ziziphus spina-christi* is very tolerant to high temperatures and grows in arid regions where dry seasons can last up to 10 months. This drought-resistant plant can even be found

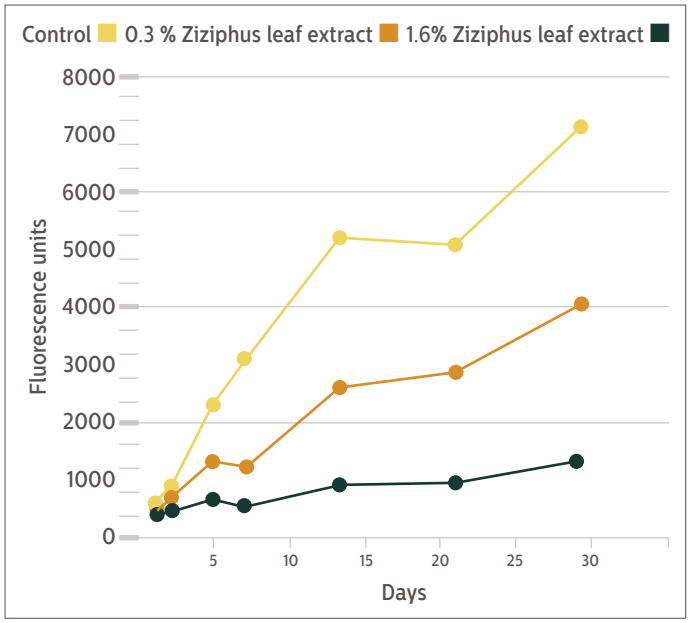

Figure 2: Concentration-dependent inhibition of AGE formation by a *Ziziphus spina-christi* leaf extract.

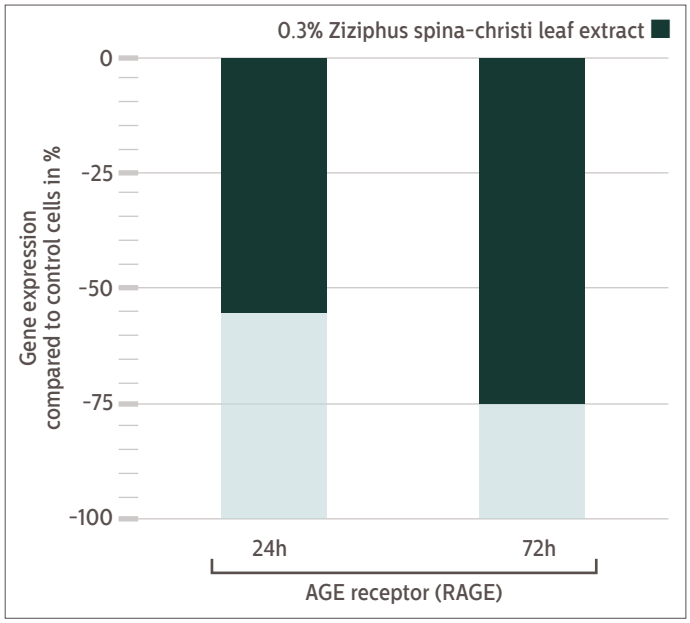

Figure 3: Influence of a *Ziziphus spina-christi* leaf extract on the expression of the AGE receptor RAGE in keratinocytes.

in desert areas where there is only 100 mm rainfall annually. The leaves of *Ziziphus spina-christi* were used throughout history as herbal remedies against wounds, skin infections, insomnia, and diabetes.[3] In addition to the historic uses, modern medicine discovered antibacterial, antioxidative and, interestingly, also antidiabetic properties of *Ziziphus spina-christi* leaves.[4] This makes *Ziziphus spina-christi* an interesting candidate for a cosmetic anti-AGE ingredient.

Mibelle Biochemistry collaborated with a research group from the University of Basel to elucidate the composition of *Ziziphus spina-christi* leaves from different countries and a detailed phytochemical profiling was published in 2017 in the peer-reviewed journal "Phytochemistry".[5] From the leaf extract, a novel active ingredient (GlowAGE™; INCI: *Ziziphus Spina-Christi* Leaf Extract (and) Trehalose (and) Aqua / Water, from here on "Ziziphus active") was developed and tested for its efficacy to prevent the formation of AGEs, as well as to boost the cellular machinery to recycle AGEs that have already been formed.

Materials and methods

Anti-glycation assay *in tubo*

Glucose (500 mM) and Albumin (10 mg/mL) were incubated with or without (control) the test material (0.3% or 1.6% *Ziziphus spina-christi* extract) in buffer (200mM Na2HPO4 pH 7.4) at 37°C for four weeks. AGE formation was detected via fluorescence measurements (excitation: 350 nm, emission: 430 nm).

In vitro gene expression assay

Normal human epidermal keratinocytes (NHEK) were cultivated in a 24-well format for 24 h. The culture medium was exchanged for an assay medium which contained 0.3% *Ziziphus spina-christi* extract. Assay medium without extract served as a negative control. The cells were incubated at 37°C for 24 h or 72 h, washed in phosphate buffered saline (PBS) and frozen at -80°C. RNA was extracted with a TriPure Isolation Reagent (Roche Molecular Systems Inc.). The gene expression of selected target genes was analyzed via RT-qPCR ("LightCycler" System, Roche Molecular Systems Inc.).

Clinical study to assess various ageing parameters of the skin

A double-blind placebo controlled clinical study was carried out with 28 women (age 55-70). During a 28-day wash-out phase,

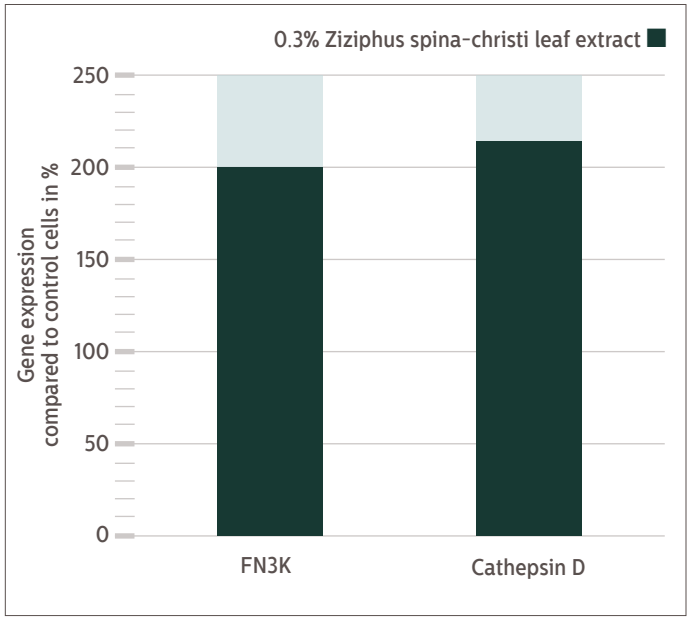

Figure 4: Influence of a *Ziziphus spina-christi* leaf extract on the expression of genes involved in AGE degradation in keratinocytes.

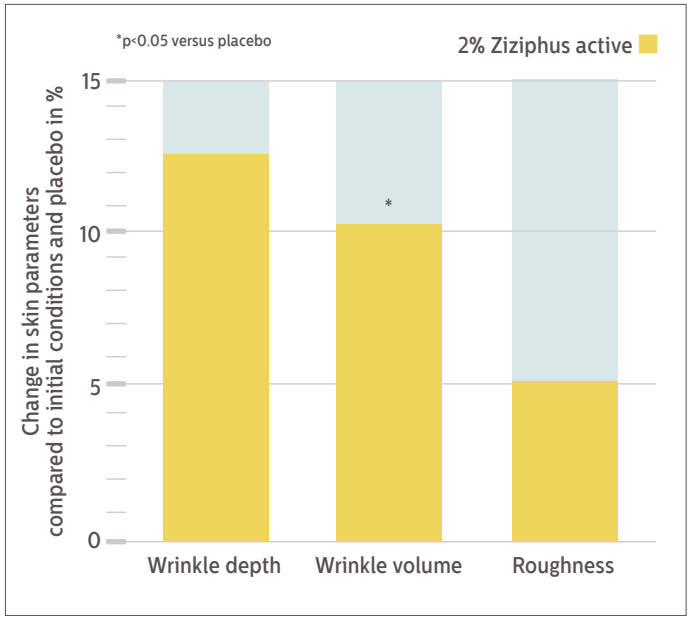

Figure 5: Decrease in crow's feet wrinkle parameters after 112 days treatment with 2% Ziziphus active.

Figure 6: Visible smoothing of the eye area after treatment with 2% Ziziphus active.

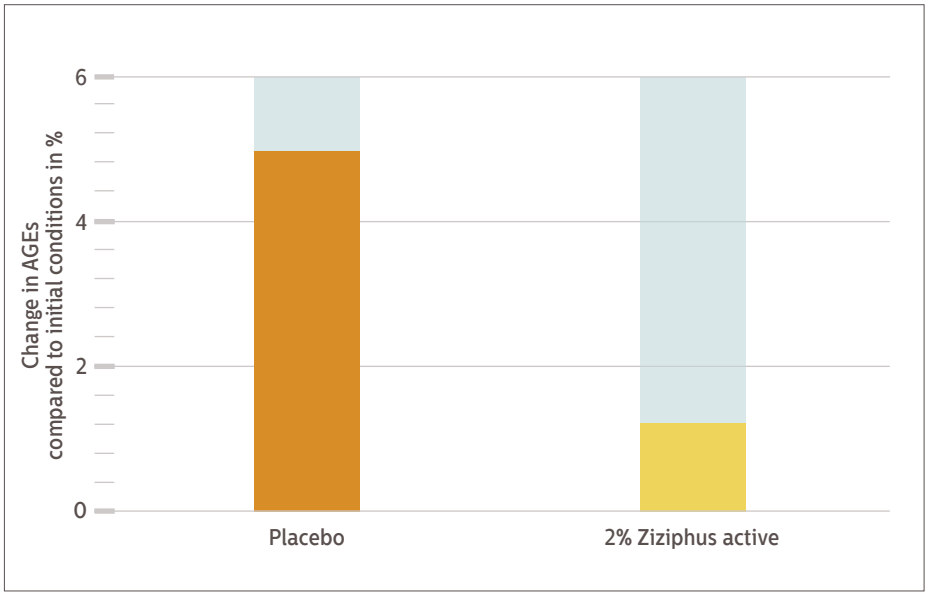

Figure 7: Reduction in AGE formation after treatment with 2% Ziziphus active.

the volunteers only applied a simple wash-out emulsion on their face without using any other face care products except for mild cleansing products. Afterwards, the volunteers applied a cream containing 2 % of the Ziziphus active on one half face and the corresponding placebo cream on the other half face twice daily for 112 days.

The following parameters were measured on the face: wrinkle depth, volume and roughness (PRIMOS lite, GfMesstechnik, Germany), collagen fragmentation with confocal microscopy (on five volunteers on the active ingredient face side; VivaScope 1500, MAVIG GmbH, Germany) and AGEs (AGE reader, Diagnoptics Technologies B.V., Netherlands). Photographs of the face of the volunteers were also taken (VisioFace® 1000, Courage & Khazaka, Germany) to document visible changes.

Results and discussion

Ziziphus spina-christi extract inhibits the glycation reaction

To investigate whether the components of *Ziziphus spina-christi* are able to slow down or inhibit the glycation reaction, different concentrations of the leaf extract were added to a mixture of glucose and albumin. After a few days at 37°C, protein-sugar complexes formed through the glycation reaction,

which could be detected via fluorescence measurement (Fig 2). The glycation process could be efficiently inhibited by the *Ziziphus spina-christi* extract in a concentration-dependent manner. The extract is therefore able to efficiently prevent AGE formation.

Ziziphus spina-christi extract affects expression of the AGE receptor and AGE recycling machinery

Keratinocytes were treated for 24 h or 72h with 0.3% *Ziziphus spina-christi* leaf extract and the gene expression of different factors involved in AGE degradation and downstream signaling was investigated. *Ziziphus spina-christi* leaf extract treatment led to the downregulation of the AGE receptor RAGE (Fig 3). This receptor binds AGEs and induces inflammation reactions.[2] Additionally, *Ziziphus spina-christi* extract induces gene expression of FN3K, a kinase that phosphorylates protein-bound sugars which leads to AGE degradation.[6] Further, treatment with *Ziziphus spina-christi* extract upregulated the expression of Cathepsin D (Fig 4), a proteinase in the lysosome that is responsible for protein recycling. In particular, it is responsible for degrading AGEs and it has been shown to be downregulated in photoaged skin.[7] Consequently, the *Ziziphus spina-christi* extract could help to remove AGEs from skin tissues and at the same time reduce inflammation caused by high AGE concentration in the skin.

Reduction of wrinkles and other anti-ageing effects after treatment with Ziziphus active

In a double-blind placebo controlled clinical study, the influence of the Ziziphus active on skin ageing parameters was investigated. After 112 days, a decrease of –12.55% in the depth of the wrinkles, –10.25% in the volume of the crow's feet area and – 5.1% in the roughness of the crow's feet area was observed compared to the placebo treatment (Fig 5). The smoothing effect was also visible in pictures taken of the eye area of the volunteers (Fig 6).

The cross-linking of collagen fibres with sugar molecules leads to a fragmentation of the collagen network. Fibroblasts cannot efficiently bind fragmented collagen and thus produce less collagen and more collagen degrading enzymes (MMPs). These enzymes however are not efficient at removing these crosslinks which leads to an even higher degree of collagen fragmentation.[8] Treatment with 2% Ziziphus active led to an 8% reduction of collagen fragmentation *in vivo* after 112 days as measured with confocal microscopy.

AGEs can be measured in the skin with an AGE reader, which detects the auto-fluorescence of protein-sugar-crosslinks.[9] After 112 days of treatment, AGEs increased by almost 5% for the placebo whereas the increase was much less after treatment with 2% Ziziphus active (Fig 7). These results show that the Ziziphus active is able to reduce the AGE formation in the skin.

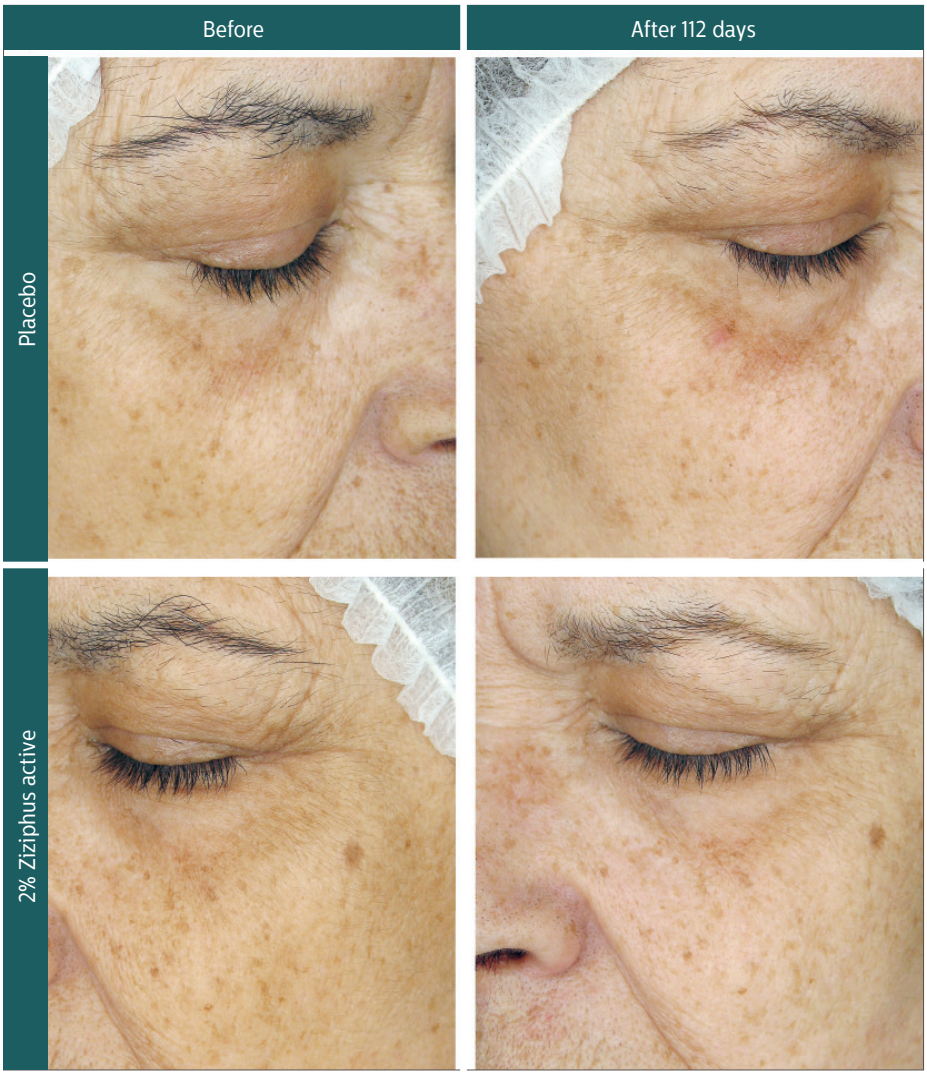

Figure 8: Visible anti-yellowing effect after treatment with 2% Ziziphus active.

As the glycation reaction in the epidermis leads to a change in the skin colour towards a more yellowish hue, pictures were taken of the volunteers' face to observe a potential anti-yellowing effect. After 112 days of application, a visible reduction in yellow skin tone was observed on the side of the face treated with 2% Ziziphus active on the volunteer shown in Figure 8, whereas the effect was not visible on the placebo side of the face.

Conclusion

Altogether, the positive effect of *Ziziphus spina-christi* on the prevention of AGE formation and the upregulation of the cellular AGE recycling machinery could be shown *in vitro*. A clinical study confirmed a reduction in AGE formation and demonstrated a decrease in collagen fragmentation, as well as wrinkle reduction and an anti-yellowing effect in the skin. An extract of *Ziziphus spina-christi* leaves is therefore a promising new cosmetic ingredient to prevent glycation and to protect from the harmful downstream effect of AGEs. **PC**

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