Skin ageing detox system reacts to cellular senescence

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Skin cells are constantly exposed to environmental stresses that will inexorably cause structural and biological damage. Oxidative reactions, DNA damage, metabolic dysregulations or even cell death are direct consequences of such environmental insults. Indirect effects, through the production of enzymes, cytokines and interleukines, may also occur and will eventually translate into the degradation of macromolecules, chronic inflammatory reactions, chemoattraction and immunosuppression. Depending upon the extent of damage they undergo, skin cells can take various decisional pathways.[1] If the extent of damage is relatively low, cells will trigger a repair process to fix any deteriorated molecules or impaired metabolic pathways before resuming their normal functions. If excessive damage is caused making it impossible (or too energy-consuming) to repair, cells will undergo organised cell death also called apoptosis. In that scenario cells will be eliminated. When the extent of damage is 'medium' – significant enough to overwhelm the repair machinery but not sufficient to command apoptosis – cells will become senescent. Upon entering senescence, cells will no longer divide into daughter cells. Senescence is thus a mechanism of protection or an adaptation to stress preventing cells from transmitting damage (genetic) to their progeny. However, unlike apoptotic cells, senescent cells remain alive, metabolically active and they accumulate in the skin as we age. The accumulation of senescent cells can cause a form of age-dependent endogeneous toxification of the skin. A likely link between cellular senescence and ageing and age-related conditions is acknowledged by the scientific community.[2,3]

Cellular senescence contributes to the ageing process

Cellular senescence is a mechanism of adaptation that can be induced by various stresses. DNA damage, radiation, chemicals, inflammation, cigarette smoke and oxidative stress are among the triggers that can induce cellular senescence (Fig 1). Skin cells become senescent by 'freezing themselves up' in a state of non-proliferation. Yet, senescent cells remain metabolically active. In fact, upon the onset of senescence, cells will start secreting pro-inflammatory cytokines and proteases which are collectively known as the senescence-associated secretory phenotype (SASP) factors.[4] The SASP is under the control of the synthesis regulator (transcription factor) called NF-κB that is itself rapidly activated as cells become senescent.[5] NF-κB activation occurs by the release and degradation of the inhibitor IκB that otherwise keeps NF-κB latent in normal cells. Once activated, NF-κB migrates to the cell nucleus and begins the synthesis of numerous SASP factors. Secreted by senescent cells in the skin extracellular environment, the SASP factors may in turn trigger chronic inflammatory reactions, oxidative damage and degradation of extracellular matrix components. Senescent cells accumulate in skin with age and because of the SASP factors are thought to actively contribute to the skin ageing process.

Various stress factors ( ) can force a cell to enter senescence (Fig 1).

One key reaction that is sparked upon senescence is the activation of NF-κB. This regulator (transcription factor) is kept latent ( ) and compartmentalised in the cytoplasm of normal cells. In its inactive configuration, the catalytic subunit ( ) is bound to the inhibitory subunit IκB ( ). NF-κB becomes activated by the release and degradation of IκB ( ). Freed from IκB, the active catalytic subunit of NF-κB ( ) translocates to the cell nucleus inducing the synthesis of proinflammatory cytokines, reactive oxygen species and degradative enzymes (MMPs) collectively known as SASP factors. The actions of SASP factors contributes to chronic inflammation, oxidative damage and the breakdown of extracellular matrix components thus accelerating tissue structure decay and the ageing process. Among SASP factors eotaxin, COX-2 (cyclooxygenase type 2) and iNOS (inducible nitric oxide synthase) have direct and indirect negative effects on skin homeostasis.

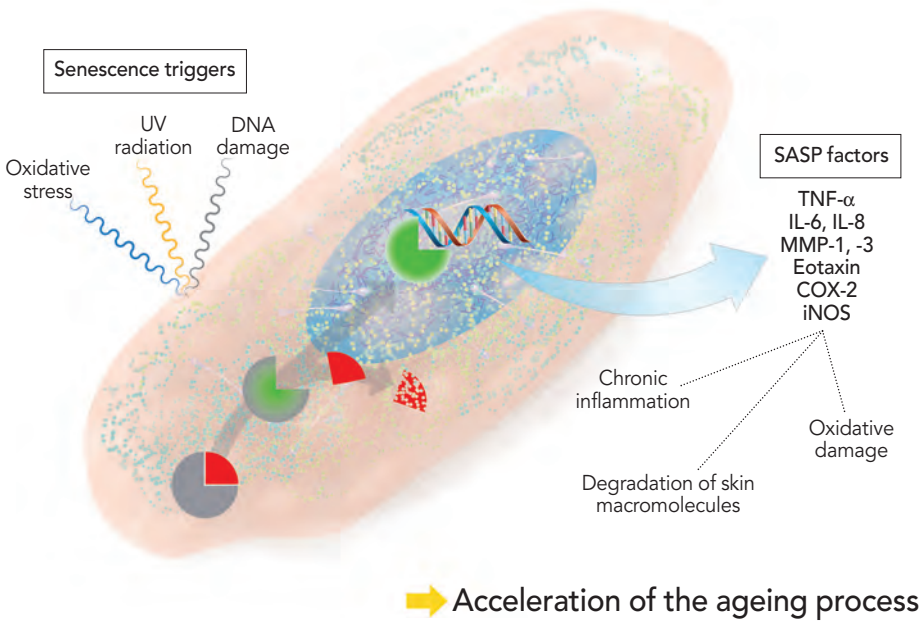

Figure 1: Activation of NF-κB and SASP pathways upon cellular senescence induction.

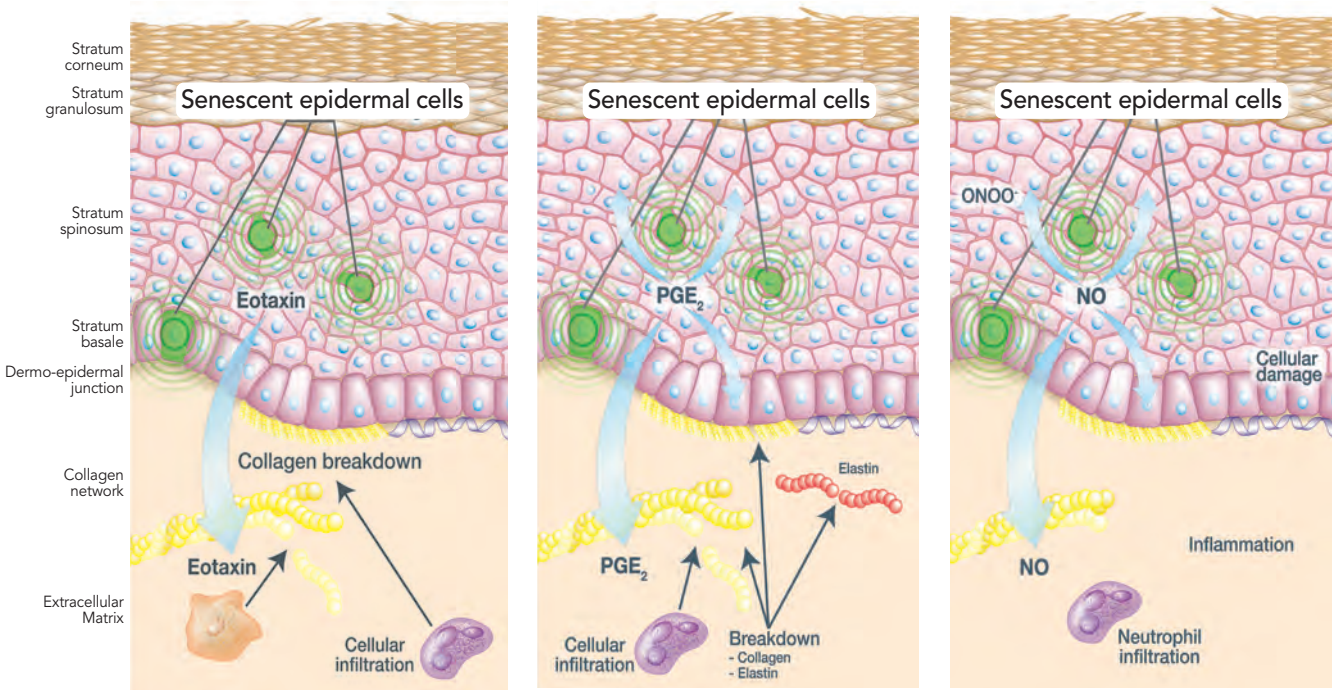

Figure 2: **A:** Skin reactions to the secretion of eotaxin by senescent epidermal cells. **B:** Skin reactions to the activation of COX-2 and the secretion of PGE2 by senescent epidermal cells. **C:** Skin reactions to the activation of iNOS and the secretion of NO by senescent epidermal cells.

Consequences of senescence - Actions of NF-κB-regulated SASP pathways

Upon the activation and the nuclear translocation of NF-κB, senescent epidermal cells initiate the secretion of the chemo-attractant eotaxin that triggers cellular infiltration in recruiting macrophages, eosinophils and mast cells (Fig 2A). Subsequently, those cells start secreting proteases, MMPs and cytokines. This results in the degradation of the extracellular matrix and dermo-epidermal junction components. The production of eotaxin increases with age and this is compatible with its association with cellular senescence.

Cyclooxygenase-2 (COX-2) in an enzyme responsible for the conversion of arachidonic acid into ultimately prostaglandin E2 (PGE2) – an important inflammatory mediator. Senescent epidermal cells secrete PGE2 (through activation of COX-2) that triggers inflammatory reactions in neighbouring cells and also recruits neutrophils (Fig 2B). The resulting production of elastase, MMP-9 and other cytokines causes the degradation of the extracellular matrix and dermo-epidermal junction components.

Nitric oxide (NO) produced by senescent epidermal cells can cause oxidative stress (Fig 2C). NO is itself under the control of the inducible nitric oxide synthase (iNOS) that is usually silent in normal cells but activated during senescence. NO can also generate a more reactive nitrogen species peroxinitrite ($ONOO^-$) that triggers deleterious effects directly in the epidermis. Furthermore, by diffusing to deeper layers, NO can attract neutrophils and induce chronic skin inflammation.

Cellular senescence, an adaptative cell fate in reaction to stress/damage, triggers various intracellular and extracellular pathways that can be deleterious for the skin integrity. Senescent cells accumulate in the skin as we age as more cells are susceptible to damage (intrinsic and extrinsic damage) and to enter senescence with time. Skin ageing and the accumulation of senescent cells are thus initiate a sequence of reciprocal causes and effects in which the SASP factors produced and secreted by senescent cells likely to further accelerate the skin ageing deterioration process. Time is definitively against us when it comes to skin ageing.

SASP factors and the master regulator NF-κB represent key targets in fighting side effects from cellular senescence.

To address this issue, we have developed Bioptimized™ Guava as a skin

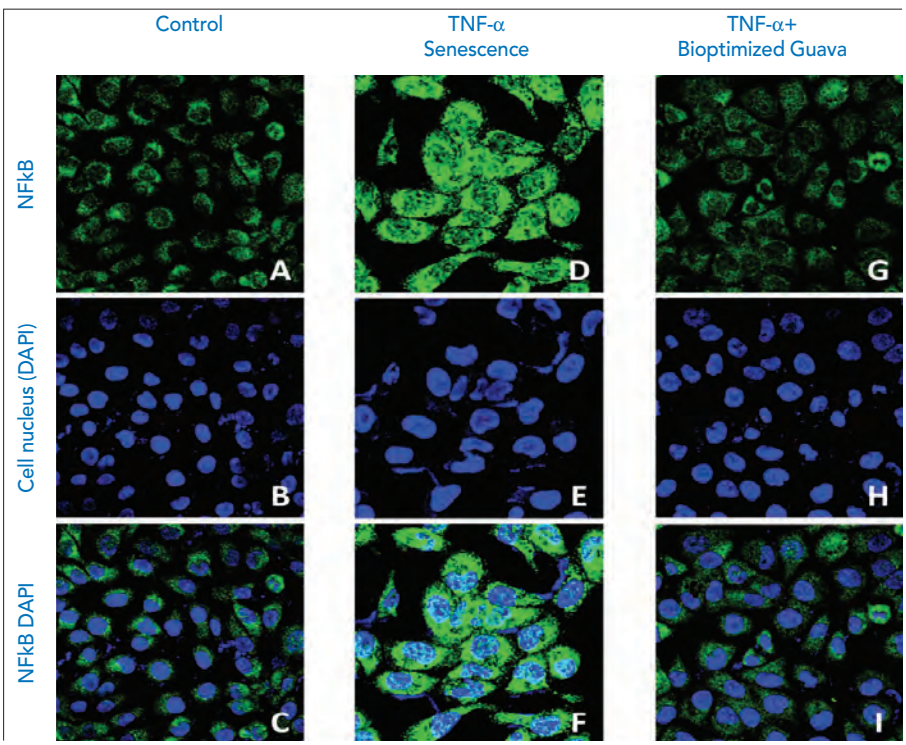

Figure 3: Activation and nuclear translocation of NF-κB in a TNF-α-induced senescence model – Inhibition by the Bioptimized Guava leaf active.

ageing detox system to selectively target skin cellular senescence. Bioptimized Guava is an extract of guava leaves 'biologically-optimised' with a *Saccharomyces* lysate to enhance its active biological properties. Bioptimized Guava is a unique biotechnology-derived ingredient that targets numerous pathways involved in skin cell senescence. It blocks the activation of the master regulator NF-κB by preventing the degradation of its inhibitor, IκB. Consequently, NF-κB is maintained inactive and does not translocate to the cell nucleus. Furthermore, the new ingredient inhibits the eotaxin, COX-2/PGE2 and iNOS/NO pathways all under the control of active NF-κB regulation. The guava leaf active is derived from *Psidium guajava* (guava) leaves harvested from a dedicated farm located on the shores of Jeju Island.

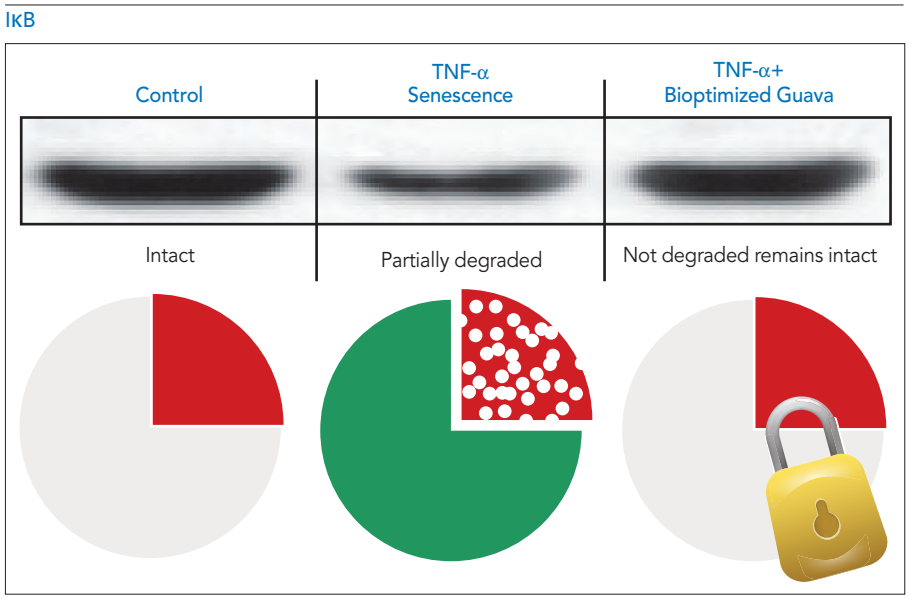

Figure 4: Inhibition of IκB (p39) degradation by the Bioptimized Guava leaf active.

Preventing activation and nuclear translocation of NF-κB

As NF-κB is a master regulator for the production of SASP factors, we verified the effect of the guava leaf active on the activation and nuclear translocation of NF-κB. The active subunit of NF-κB (p65) can be observed in control human keratinocytes but at a relatively low level (Fig 3A). Most of the active NF-κB subunit is bound to its inhibitor IκB and remains – inactive – in the cytoplasm, outside of the cell nucleus (Fig 3C). As TNF-α has been used in other cell types to induce senescence,[6,7] we used it in our cytokine-induced keratinocyte cellular senescence model. As observed by a massive intensification in the p65 immunofluorescence, NF-κB is rapidly activated upon senescence induction (Fig 3D). Furthermore, the NF-κB activation coincides with the nuclear translocation of p65 (Fig 3F). In migrating to the cell nucleus, the active subunit of NF-κB will be in a position to initiate the synthesis of the SASP factors. Interestingly, the presence of the guava leaf active appears to completely inhibit the activation and the nuclear translocation of NF-κB (Fig 3G, I).

Materials and methods

Cultures of control human keratinocytes were used as is (Control) or incubated in the presence of 20 ng/ml TNF-α in the absence (Senescence) or in the presence of 50 µg/ml guava leaf active. Immunolocalisation of NF-κB active subunit (p65) was achieved with a monoclonal antibody anti-p65 and with DyLight488-conjugated 2nd antibody (green fluorescence, A, D and G). Cell nuclei were stained with the DNA-specific fluorescent dye DAPI (blue fluorescence, B, E and H). C, F and I are superimpositions of green and blue fluorescence signals. Fluorescence was revealed using a confocal microscopy (FV500, Olympus) at a 40x magnification with zoom factor of 1.5.

Locking NF-κB into an inactive configuration

To deepen the mechanism of action of the guava leaf active in preventing the activation and the subsequent nuclear migration of NF-κB, we have examined its

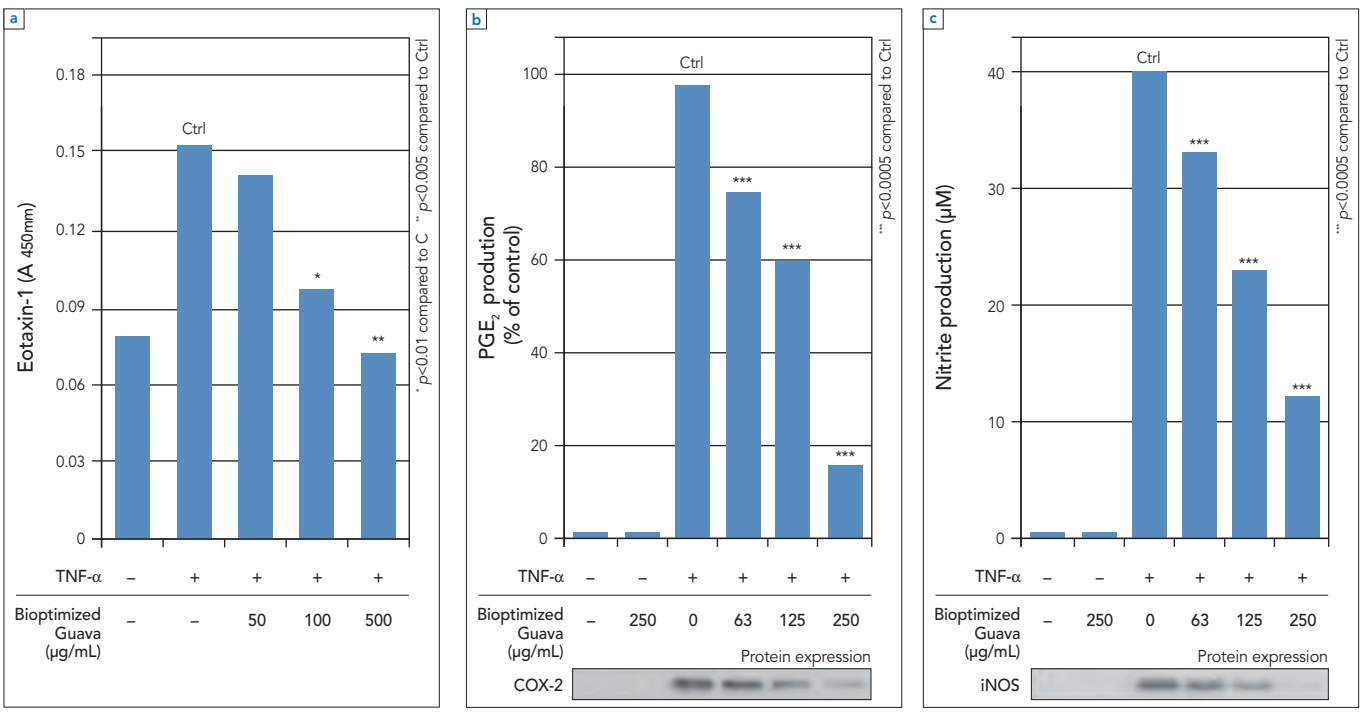

Figure 5: Effects of the guava leaf active on the production of selective SASP factors.

effect on the degradation of the inhibitor IκB (p39). As expected, the addition of TNF-α to the cell culture induces the degradation of IκB as revealed by a reduction of its immunostaining (Fig 4). This will set free the NF-κB active subunit. However, the presence of the guava leaf active prevents the degradation of IκB most likely keeping it bound to the active subunit to lock it in an inactive configuration.

Materials and methods

Cultures of control human keratinocytes were used as is (Control) or incubated in the presence of 20 ng/ml TNF-α in the absence (Senescence) or in the presence of 50 µg/ml guava leaf active. Cell lysates were micro analysed in Western blots probed with a monoclonal antibody anti-IκB (p39). Intact, latent NF-κB bound to the inhibitor IκB. ( ). Active catalytic NF-κB subunit ( ). Degraded IκB ( ).

Action of the guava leaf active on selective SASP factors

The guava leaf active prevents NF-κB activation and the migration of its active subunit to the cell nucleus. Inhibiting this pathway should also prevent the synthesis of SASP factors under the control of active NF-κB. We have verified the effect of the guava leaf active on the synthesis of eotaxin, COX-2/PGE2 and iNOS/NO pathways.

The guava leaf active significantly inhibited the TNF-α/senescence-induced up-regulation of eotaxin (Fig 5A), PGE2 (Fig 5B) and NO (as nitrite) (Fig 5C). Moreover, the inhibition of the secretion of PGE2 and NO paralleled the reduction of their synthesis enzymes COX-2 and iNOS, respectively (Fig 5B and C).

Materials and methods

Cultures of control human keratinocytes were used as is (Ctrl) or incubated in the presence of 20 ng/ml TNF-α in the absence (Induced-senescence) or in the presence of indicated concentrations of the guava leaf active.

The concentration of eotaxin (A) or PGE2 (B) in the culture medium was quantified by ELISA according to the manufacturer's instructions. The amount of nitrite, which is a stable physiological reservoir of NO, was determined by a colorimetric assay by extrapolation from a sodium nitrite standard curve. COX-2 or iNOS protein expression synthesis was determined by Western blot immunodetection using specific antibodies. Data are presented as means SD from three independent experiments.

The guava leaf active is a unique biotechnology-derived ingredient that targets numerous pathways involved in skin cell senescence (Fig. 6). It blocks the activation of the master regulator NF-κB by 'locking' it into an inactive, latent state. Consequently, NF-κB can no longer migrate to the cell nucleus to activate the synthesis of SASP factors. Besides, the guava leaf active directly inhibits eotaxin, PGE2 and NO pathways all under the control of active NF-κB regulation.

The guava leaf active inhibits the senescence-associated pathways preventing excessive degradation of the extracellular matrix components.

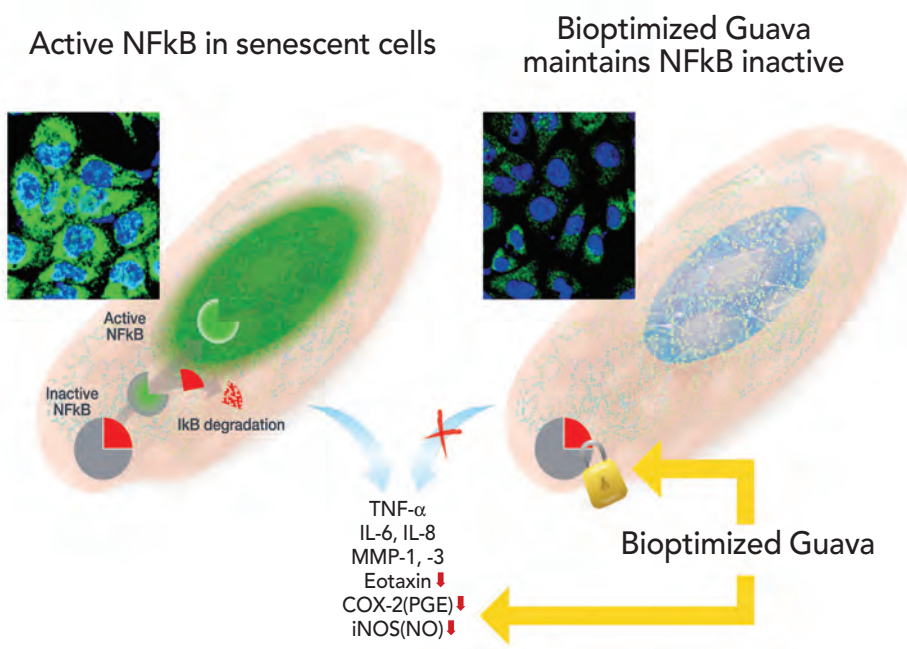

Figure 6: Summary of the guava leaf active actions in a cellular senescence model.

Conclusion

Bioptimized Guava is an extract of guava leaves 'biologically-optimised' with a *Saccharomyces* lysate to enhance its biological properties. It acts as a skin-ageing detox system specifically targeting SASP factors secreted by senescent epidermal cells that accumulate in the skin as we age. Formulators and marketers can take advantage of the Bioptimized™ concept to differentiate their products from competition. Bioptimized Guava is derived from leaves harvested in a dedicated greenhouse located on the shores of the volcanic Jeju Island. Its humid subtropical climate makes it an exceptional habitat for various plant species endowed with unique properties. Guava leaf extracts have been part of traditional medicines for many years in Asia and have be used, as is, on skin wounds to improve healing or also brewed for the preparation of infusions or teas. Anti-ageing formulations typically contain ingredients that address wrinkles, hydration, skin tone and oxidation reactions.

Bioptimized Guava is a novel ingredient that specifically targets senescence-induced SASP pathways that act as an insidious cause of the acceleration of the ageing-associated extracellular matrix disorganisation. Bioptimized Guava demonstrated a multifunctional action toward several pathways induced by cellular senescence and becomes a key ingredient in the armamentarium of formulators wishing to achieve complete and efficacious anti-ageing products. PC

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