Alpin Heilmoor Extract (AHE) – a New Solution for Oral and Dental Care

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abstract

Alpin Heilmoor Extract (AHE), a natural (organic) active ingredient extracted from Austrian therapeutic Moor (Heilmoor) deposits at 500m above sea level, was investigated for its efficacy in maintaining and enhancing oral health. *In-vitro* studies were performed to determine the anti-bacterial, anti-inflammatory, anti-oxidant and other properties of AHE that promote healthy gums. We found that AHE enhances oral mucosa barrier, prevents gingivitis, and potentiate wound healing and tissue regeneration after injuries. AHE also prevents cell senescence which promotes healthy gums. Furthermore, AHE showed excellent improvement in oral hygiene by reducing tooth shade, dental plaque, and halitosis. AHE was also very well tolerated.

Introduction

The oral cavity is home to a number of bacteria species. Synergy and interaction of these variable oral microorganisms help human body against invasion of undesirable stimulation outside. However, an oral microbial dysbiosis, caused by abnormal proliferation of certain pathogenic bacteria, is responsible for oral inflammation, development of periodontal diseases and systemic diseases. Gingivitis and periodontitis are common oral infection that affects the tissues that surround and support teeth. The prevalence of periodontal disease was reported to range from 20% to 50% around the world and is considered a major health problem [1].

Periodontitis, a chronic inflammatory disease causes tooth loss and deterioration of gingiva, alveolar bone, and periodontal ligaments. Gingivitis, an inflammatory condition of the gingival tissue, characterized by gingival redness, swelling, bleeding, is a prerequisite for the development of periodontal disease. A keystone pathogen *Porphyromonas gingivalis* is strongly implicated in the pathogenesis of periodontitis [2]. This bacterium is a gram negative, rod-shaped, obligate anaerobe belonging to the 500 bacterial species living in the oral cavity [3]. It infects periodontal tissues as a secondary infection through interactions with commensal streptococci [4]. In addition, *P. gingivalis* induces impairment of oral epithelial barrier functions which potentiate susceptibility to microbial infections, leading to local tissue inflammation responsible for periodontium (tooth-supporting tissues such as gingiva and alveolar bone) and systemic diseases [5]. Recent study shows that *P. gingivalis* induces premature senescence, a cellular process defined as irreversible cell cycle arrest which contributes to aging and age-related diseases [6]. Antibiotics are used to target anaerobic bacteria, especially *P. gingivalis*. However, *P. gingivalis* strains show resistance levels which requires the development of new alternative and natural solutions presenting multiple benefits for an overall improvement in oral health and hygiene.

Our previous studies demonstrated multiple properties of AHE, including anti-inflammatory, antioxidant, skin barrier strengthening effects, for better maintenance of healthy skin. Here we found that AHE is equally effective in maintaining oral health.

Here we performed several *in vitro* and clinical studies to determine the benefits of AHE in maintaining oral health and hygiene.

Material and methods

The anti-bacterial properties of AHE formulated at 5% in a toothpaste, were evaluated using *P. gingivalis*. The colonies were counted after incubation with AHE 5% at 37°C for 5 min, 10 min and 24h. Due to the culture and growth conditions of the used test strains, the growth period was prolonged to allow CFU counting of the grown colonies. We determined the antibacterial effects of AHE by quantifying the six bacterial species in sub-gingival pockets before and after application of AHE using real-time PCR (Carpegen® Perio Diagnostik).

In vitro studies using HaCaT cells, Normal Human Dermal Fibroblasts (NHDF), RAW cells, and NIH-3T3 Fibroblasts were conducted to analyze barrier strengthening, senolytic, pro healing, anti-inflammatory, anti-glycation and antioxidant properties of AHE.

Maintenance of oral mucosa barrier was determined by measuring E-cadherins expression using Western Blot technique. Anti-inflammatory effects of AHE, were evaluated by quantification of IL 1β-induced or LPS-induced proinflammatory mediators (IL 6, IL 8, Prostaglandin E2, IL 1β, TNF α, monocyte chemoattractant protein 1 (MCP1) and Matrix Metallopeptidase 9 (MMP9))

We determined the anti-glycation and antioxidant properties of AHE by measuring Regulator of calcineurin 1 (RCAN1), receptor for advanced glycation end-products (RAGE) and Advanced glycation endproduct (AGE) receptor 1 (AGE-R1) gene level expression using real-time quantitative PCR and by quantification of H_2O_2-induced ROS and LPS-induced 8 iso PGF 2α using FLUOStar Spectro-fluorometer and ELISA Kits (Cayman/Biomol, Germany), respectively. NO suppression was analyzed using Griess method by sodium nitrite standard curve. Protection against senescence was determined by using Senescence β-Galactosidase Activity Assay Kit (PerkinElmer Victor X5). NIH-3T3 fibroblast and Incucyte® Scratch Wound Assay were used to analyze the pro healing capacities of AHE, by measurement of relative wound density (RWD).

Clinical studies were performed to evaluate the benefits of AHE in improving oral health. Twenty participants completed the study and used toothpastes with AHE 5% twice daily for 28 days. Whitening effect was evaluated with VITA Bleachedguide 3D-MASTER® under dermatological and dental control. Other parameters were analyzed, including plaque index, gingival index, mean maximal gingiva index. The effectiveness of AHE toothpaste was also evaluated through a self-assessment questionnaire.

Results and discussion

Antibacterial activity of AHE

The oral cavity hosts a consortium of bacteria which differs greatly in healthy and diseased subjects. Patients with periodontal disease have a greater proportion of Gram-negative, proteolytic, bacteria such as *P. gingivalis* or *Tanneralla forsythia*, as well as species of *Prevotella*, *Fusobacterium*, and *Treponema* in their sub-gingival plaques while healthy adults are composed primarily of Gram-positive species belonging to the genera of *Actinomyces* and *Streptococcus*, and to the Gram-negative genus Veillonella [7]. *P. gingivalis* is known to be highly proteolytic, with primary activity from a class of endopeptidases known as gingipains that are critical virulence factors [8]. Proteolysis actively changes the environmental conditions; importantly, causing a pH increase, thus creating an environment more hospitable to many Gram-negative anaerobes which mediates periodontitis [9]. These bacteria are also responsible for the bad smell related to halitosis. AHE was investigated by quantifying, in gingiva probing samples, the 6 most important bacterial species indicative for periodontitis, peri-implantitis and halitosis before and after application. After 24 hours of contact time a base toothpaste with 5% AHE was shown to be 99% effective against P. *gingivalis*

(Figure 1). AHE showed excellent anti-bacterial activity against *P. gingivalis* and can be applied to prevent periodontal diseases and halitosis.

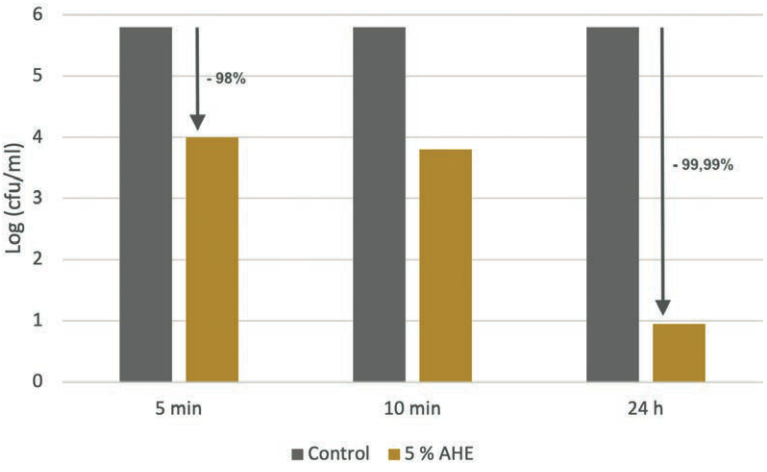

Fig.1 Immediate and long term Effect of 5% AHE on *P. gingivalis In-Vitro*

AHE reinforces oral epithelial barrier and protects against oral mucosa invasion

Epithelial cells are interconnected to each other by a number of specialized transmembrane molecular complexes, among them cell-cell junctions comprising tight junctions adherens junctions, gap junctions and desmosomes. They provide the first-line of defense in the oral mucosa. It is known that *P. gingivalis* impairs oral epithelial barrier partly through targeting grainyhead like 2 (GRHL2), an epithelial-specific transcription factor which regulates the expression of the junction proteins [5]. E cadherins are components of adherens junctions. These structures ensure intercellular adhesion amongst epithelial cells. They regulate a diverse range of other cellular processes next to adhesion, such as cell shape, division, growth, apoptosis, wound healing and barrier function. AHE was found to activate E Cad B1 expression by 57% and E Cad B2 by 25% in cultured HaCaT cells, hence counteracting the invasive properties of *P. gingivalis*. AHE therefore enhances oral mucosa barrier thus preventing injuries and penetration of noxious substances that can impair oral health and responsible for non-oral systemic diseases.

AHE protects against cell senescence

Recent study shows that *P. gingivalis* induces premature senescence in dendritic cells by direct cellular invasion leading to disruption of immune homeostasis in periodontitis [6]. Cellular senescence is an irreversible growth arrest evoked by various stimuli, including oxidative, epigenetic, and genotoxic stresses, telomere damage, and oncogene activation [10]. During the senescence process, senescent cells secrete senescence-associated phenotype (SASP) factors (including cytokines, proteases, growth factors, and matrix metalloproteases) that preclude senescent cells clearance by immune system, a process orchestrated by age-related immune system remodeling [11]. While accumulation of senescent cells have been demonstrated to play a causal role in driving aging and chronic diseases, their clearance has been shown to delay and reduce the aging phenotype in several tissues of premature and natural aged models. Defense against infection, improvement of wound healing and regenerative capability of tissues are also crucial parameters in preventing premature gingival ageing and bacteria invasion. Gingival fibroblasts are the primary cell type present in periodontal connective tissue and maintain gingival tissue integrity by regulating collagen and proteoglycan metabolism. However, in response to *P. gingivalis* LPS, gingival fibroblasts produce several proinflammatory cytokines such as interleukin IL 6 and IL 8 which mediates periodontitis [12]. AHE may therefore acts through various potential mechanisms involving gingival fibroblasts and senescent cells that play important roles in the pathogenesis of periodontitis and gingival ageing. Here we used senescence-associated β-galactosidase (SA β-gal), a common biomarker of cellular senescence s. AHE 1%, 2% and 5% was able to clear by 8%, 14 and 21% the SA β-gal positive cells, respectively, demonstrating that AHE exhibits significant dose-dependent senolytic properties.

AHE **(Figure 2)** can therefore be used to prevent premature aging and consequently to preserve the wound healing and regenerative properties of gums.

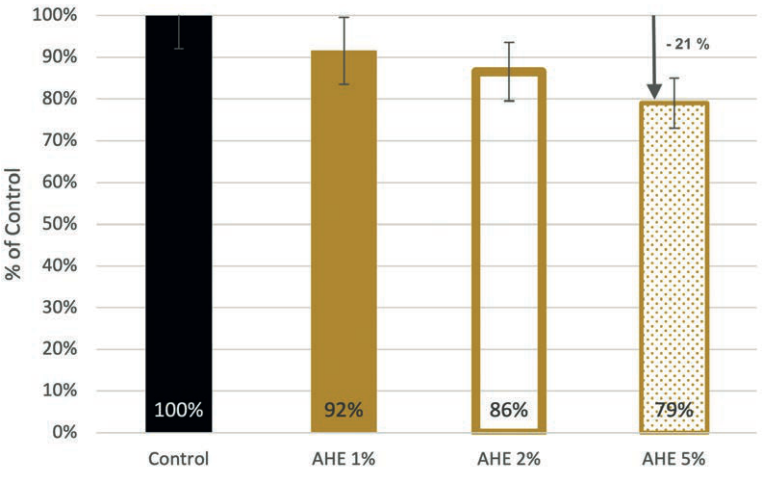

Fig.2 Dose-dependent reduction of senescent cells by Alpin Heilmoor Extract

AHE potentiates wound healing and regenerative capacity

Previously, we have shown the senolytic effects of AHE suggesting its ability to maintain the self-renewal of cells, essential for regeneration and wound healing of tissues. Wound healing and regenerative capacity are key properties for a healthy oral mucosa. After injury to periodontal tissues, a sequentially phased healing response is initiated that enables wound closure and partial restoration of tissue structure and function. Fibroblasts, which synthesize and organize the collagen fibers that link alveolar bone and gingiva to the cementum covering the tooth root, play a critical role during periodontal wound healing [13]. Importantly, regeneration of connective tissues involves different cellular activities driven by fibroblasts populations. These include the cellular proliferation, migration and the secretion of matrix molecules, the organization of these matrix components into functionally active fibers that finally restore the periodontium. A real-time wound healing assay with NIH-3T3 fibroblasts which followed the wound healing process by life imaging over 48 hours showed that AHE significantly accelerated wound healing process **(Figure 3)**, demonstrating its pro-healing properties and regenerative capacity.

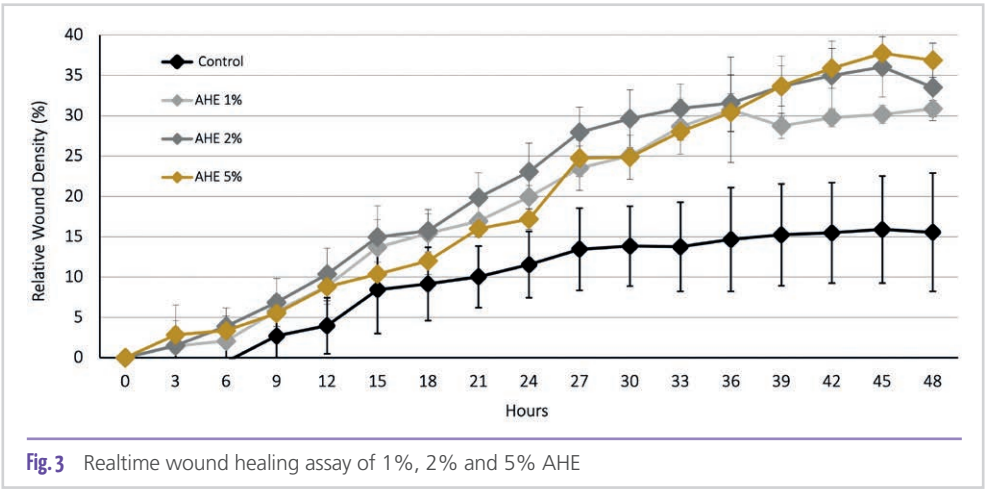

Fig. 3 Realtime wound healing assay of 1%, 2% and 5% AHE

AHE controls inflammation by reducing lps induced proinflammatory mediators release

Chronic inflammation of the gingiva, supporting connective tissues, and the alveolar bone causes periodontal diseases [14]. Thus, preventing inflammation is critical in preventing periodontal diseases. *P. gingivalis* is recognized as a keystone pathogen of the disease-provoking periodontal microbiota [15]. *P. gingivalis* contributes to the pathogenesis of aggressive periodontitis by inducing high levels of proinflammatory cytokines, such as IL 1β and IL 6 by peripheral CD4+ T helper cells [16]. *P. gingivalis* lipopolysaccharide (LPS) plays a key role in this process. First of all, AHE 1% and 2% reduced by 13%, and 65%, respectively, the release of prostaglandin E2 (PGE2), a proinflammatory lipid mediator, induced by LPS treatment in Raw cells. In addition, the anti-inflammatory of AHE was confirmed thanks to IL-1β or LPS-induced inflammatory responses in monocytes. AHE 2% reduced the release of several pro-inflammatory mediators including IL 6, IL 8, Isoprostan **(Figure 4)**.

AHE protects against oxidative stress and consequences of glycation

Oxidative stress occurs as a state of disturbance between free radical produced and the capability of antioxidant system to counteract such. It is characterized by an accumulation of reactive oxygen species (ROS) and plays a key role in the progression of inflammatory diseases including periodontal diseases [17]. LPS from *P. gingivalis* as well as hypoxia induces a NOX4-dependent increase in H_2O_2 release in periodontal ligament fibroblasts which may contribute to the development and progression of periodontal diseases in the absence of antioxidative systems [18]. *P. gingivalis* also induces ROS that activate FOXO transcription factors through JNK signaling, which subsequently controls oxidative stress responses, inflammatory cytokine production and cell survival relevant to dysbiotic host responses in periodontal diseases [19]. Maillard advanced glycation end products (AGEs) are responsible for inducing low intensity chronic inflammation and thereby, for initiating and/or aggravating chronic diseases. Nowadays, research has demonstrated a significant association between AGEs and dental or periodontal pathology. Thus, we evaluated the effect of AHE on various oxidative stress and glycation markers. We demonstrated that AHE 1% neu-

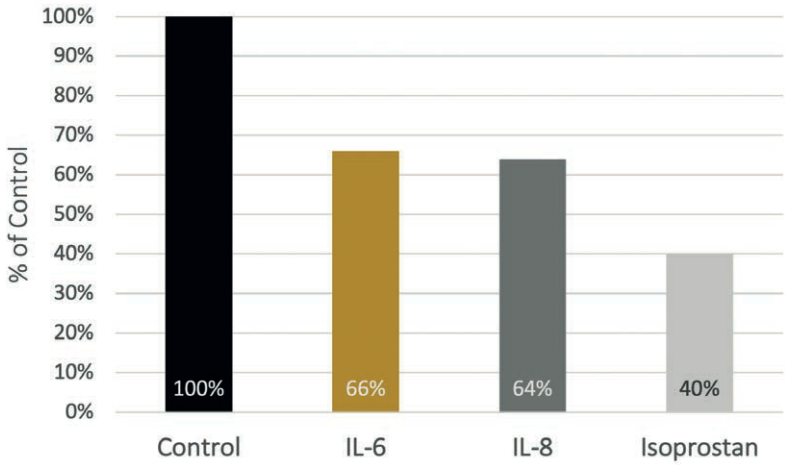

Fig. 4 LPS-induced inflammatory responses in monocytes by Alpin Heilmoor Extract

tralizes H_2O_2-induced ROS by 43% and was more effective than Trolox at 50 µg/ml, a potent antioxidant molecule. AHE also inhibited IL-1β-induced RCAN1 expression, LPS-induced NO and 8 Iso PGF2α production, a key biomarker of oxidative stress. Importantly, AHE increases AGE-R1 expression, an oxidative stress suppressor and a negative regulator of the inflammatory response to AGE. This makes AHE a potent antioxidant, a property that prolongs cell survival for healthy gums. Finally, we showed that AHE 1% strongly inhibited IL-1β-induced RAGE expression. This data suggests that AHE can inhibit the proinflammatory and pro oxidative effects of AGEs and can be considered as an anti-glycation active ingredient.

AHE reduces tooth shade

Bright teeth are cosmetically attractive. AHE was investigated for its ability to reduce tooth shade. The most prominent shade of the frontal teeth was determined on every subject before and after the period of product application, using 15 grades of the VITA Bleachedguide 3D-MASTER scale. in addition to demonstrating perfect tolerance, the volunteers saw an average 15% reduction in the tooth shade demonstrating the whitening effect of AHE **(Figure 5)**.

14 of the 20 subjects, who did not apply the placebo, displayed a decrease of tooth shade to more bright teeth with a mean reduction of 21%. The other 6 subjects as well as the subjects, who applied placebo, did not exhibit a change in tooth shade. Regarding all 20 subjects, who did not apply placebo, the tooth shade was reduced in mean by 15%. AHE can therefore be used to brighten teeth.

AHE reduces plaque intensity

Oral hygiene can be assessed by determining various indices which record, for example, the incidence of plaque and signs of inflammation of the gingiva. Plaque is a complex biofilm, that consists of protein, carbohydrates, phosphates and microorganisms. Plaque formation predisposes to dental caries and periodontal diseases [20]. Thus, the effective removal of dental plaque is important for maintaining periodontal and oral health. Here we determined the role of AHE in improving oral hygiene by reducing plaque intensity. Plaque can be visualized and assessed using a staining method (Mira-2-Ton®, Hager & Werken GmbH & Co.KG; plaque staining tablets).

The plaque index was determined after corresponding staining and flushing with water. The inner and outer area of every tooth was analyzed concerning the intensity of plaque. In the mean, we found that, the plaque index of the 20 subjects, was reduce by 75% (**Figure 6a** and **Figure 6b**), This shows that AHE improves oral hygiene.

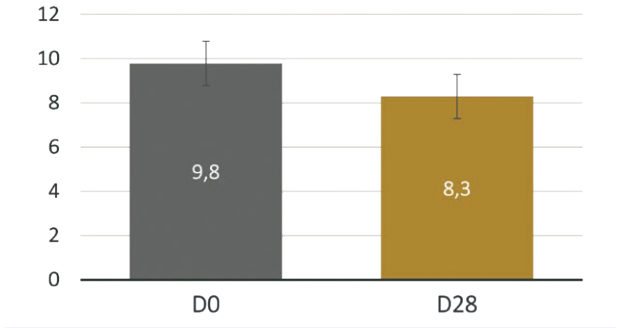

Fig. 5 Toothshade reduction of AHE with VITA Bleachedguide 3D-MASTER scale.

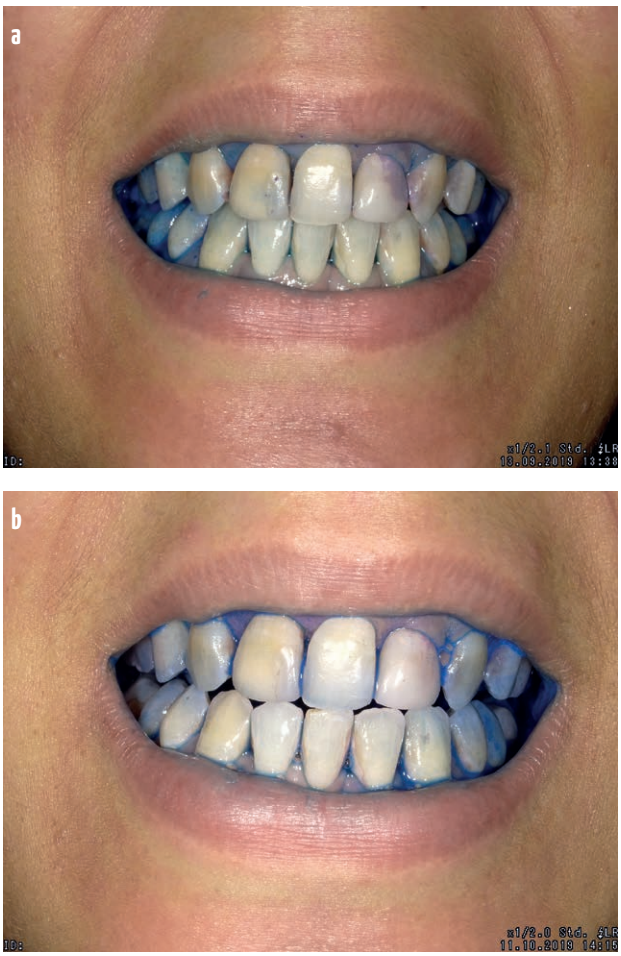

Fig. 6 **(a)** Plaque reduction T0, **(b)** Plaque reduction T28

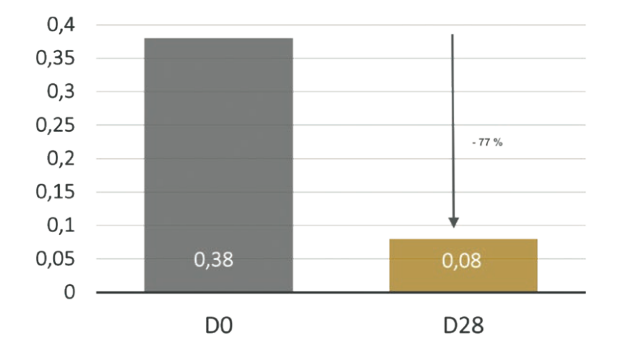

Fig. 7 Reduction of mean gingiva index by Alpin Heilmoor extract

AHE reduces gingivitis

The degree of inflammation of the gingiva was assessed by determining the gingiva index. The gingiva index, corresponding to the intensity of bleeding at gingival margin following probing, was determined on the inner as well as outer area of every tooth. In the mean the gingiva index of the 20 subjects, was reduce by 77% **(Figure 7)**. This shows that AHE reduces gingivitis.

Expected efficies of toothpaste

Within a master thesis- "No pain, no gain": About the attempt to exploit a negative sensory stimulus [21] – where 121 subjects were asked to rate the expected efficacy of toothpastes shows that consumers expected a BLACK toothpaste to be of higher quality and more effective concerning different qualities, properties and efficacies than a WHITE version **(Figure 8)**.

Conclusion

Dental and oral health is an essential part of overall health and well-being. An imbalance in oral microbial community can cause inflammatory lesions of the tooth-supporting soft tissues and poor oral hygiene such as halitosis. Our investigation found that AHE enhances oral epithelial barrier, prevents gingivitis, and potentiates wound healing and tissue regeneration after minor injuries to the oral mucosa. AHE also prevents cell senescence which promotes healthy gums thanks to the maintenance of cell proliferation and self-renewal. At clinical level, AHE showed excellent improvement in oral hygiene by reducing tooth shade, dental plaque, and halitosis. Moreover, no relevant skin, gingiva or mucosa reactions suggestive of irritation or allergy was detected. AHE was therefore very well tolerated. Thanks to its multiple actions and biological properties, demonstrated above, AHE is highly beneficial to oral health.

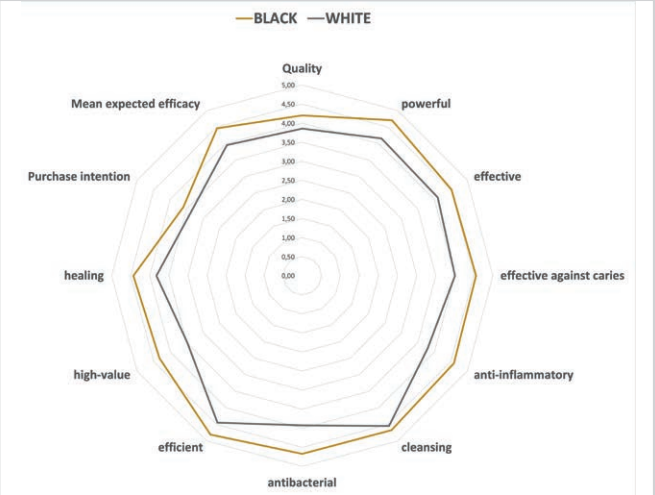

Fig. 8 Expected efficacy of black and white toothpastes

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