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New targets in the battle against dandruff

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Summary

Dandruff is a scalp disorder characterized by flaking skin and itch of an excessive oily scalp skin. It affects 55% of the global youth and adult population. Seborrheic dermatitis is a similar scalp skin disorder with aggravated itchy rashes and flaking. Different factors are identified in the dandruff development: increased sebum production, uncontrolled fungal growth of *Malassezia* strains and individual reaction to pro-inflammatory environment, and the susceptibility to trigger an immunological response. Using *in vitro* and *ex vivo* models, we show that an *Epilobium angustifolium* extract dose dependently reduces lipid synthesis in sebocytes to a maximum of -43% (1% extract), and protects the epidermis from *Malassezia*-induced morphological changes. *Epilobium angustifolium* extract also acts through innovative mechanisms involving regulations of defensins (human beta-defensins [hBD2] and hBD3) and toll-like receptor 2 involved in the immunological response of the skin. The anti-dandruff and sebum-regulating efficacy of *E. angustifolium* extract (1.5%) was confirmed in a clinical study that mobilized 24 volunteers with dandruff and greasy scalp for 30 days. At the end of the study, nonadherent and adherent dandruffs were significantly ($p < 0.0001$) reduced in average by -54% and -48%, respectively. Using Sebumeter® measurements, scalp sebum production was inhibited by -67% ($p < 0.0001$) in average over baseline. In conclusion, *E. angustifolium* extract offers a new innovative approach to dandruff reduction through immunomodulation of the skin response to *Malassezia* invasion.

INTRODUCTION

Dandruff is a common chronic scalp disorder, affecting mostly postpubertal aged persons, but not clearly related to gender or ethnicity. It is characterized by an excessive production of scalp skin cells, accumulating into oily patches or flakes, as corneocytes retain their cohesion (1). Alterations concern the whole scalp as changes of the barrier function and inflammation can be detected deeper beneath the upper layers of the scalp (2). Flakes appear as greasier and inflammation surfaces in the form of erythema (3), which can spread to body parts with a high density of oil-producing glands. Seborrheic dermatitis (SD) is found in 3–5% of individuals, whereas dandruff concerns more than half of the population (3,4). Recent research focuses on three main factors for the origin of dandruff, namely, increased sebaceous gland activity, oil-feeding fungus *Malassezia* strains releasing free fatty acids, and individuals' predisposition to react with an immunological response to pro-inflammatory free fatty acids released by the fungal colonization (5).

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Sebum is constituted of oily components such as triglycerides and wax esters. Those are digested by *Malassezia* strains that converts them into free fatty acids such as oleic acid. This particular metabolite induces scalp flaking, pruritus, and inflammation (5,6). However, the relation between free fatty acids and inflammation remains unexplained.

Malassezia fungus naturally belongs to a healthy skin microbiota. Under particular conditions, their biology is dysregulated and consequently may contribute to various skin disorders (7,8). For the scalp, specific strains of *Malassezia* have been identified and linked with dandruff and/or SD, namely *M. furfur*, *M. globose*, and *M. restricta* (9,10). They possess a high lipase activity, since their survival is linked to their host's lipids (10).

Oleic acid resulting from *Malassezia* activity induces a skin inflammatory response. Toll-like receptor 2 (TLR2) promotes inflammatory response after binding to the fatty acid, and consequently releasing human beta-defensins (hBD) (11,12). These defensins are upregulated in the presence of *Malassezia* (13). In particular, hBD2 and hBD3 induce adaptive immune responses from keratinocyte which produce pro-inflammatory cytokines.

The resulting situation of those factors induces a disturbance of the scalp *stratum corneum* (SC) promoting dandruff. The epidermal turnover is dramatically increased which leads to the accumulation of parakeratotic cells with pyknotic nuclei (condensed chromatin) in the upper layers of the epidermis (14). This increased cell proliferation leads to a decreased cell differentiation rate. All together these factors disturb the SC structure.

Native from North America, *Epilobium angustifolium* has been used as a medicinal plant. Folk medicine indicates use of the fireweed juice to soothe skin irritation and burns.

MATERIAL AND METHODS

PLANT MATERIAL AND REAGENTS

The fireweed flower/leaf/stem aqueous extract (*E. angustifolium* extract) used in this study is rich in oenothein B (0.12–0.36%), an ellagitannin possessing anti-inflammatory and antioxidant activity.

IN VITRO LIPID SYNTHESIS INHIBITION IN SEBOCYTES

Primary human sebocytes were obtained from a 66-year-old female volunteer, presenting a normal body mass index of 23.4. Cells were seeded in 96-black-well/clear-bottom plates, in sebocyte medium. Confluent cells (fourth passage) were treated with various concentrations of *E. angustifolium* extract (0.0125%, 0.05%, and 0.1%) for 3 days. The diacylglycerol acyltransferase (DGAT) inhibitor A922500 (2 μM) was used as a control. Intracellular lipid accumulation in cells was evaluated with Nile red staining. Relative fluorescence intensity was measured at ex/em 540 nm/620 nm (no cutoff) for total lipids and 485 nm/555 nm (515 cutoff) for neutral lipids.

EX VIVO MALASSEZIA-INDUCED ALTERATIONS OF SKIN MORPHOLOGY AND IMMUNE MARKERS IN SKIN EXPLANTS

Full thickness skin explants were obtained from a 47-year-old Caucasian woman. A 1:1:1 mixture of *M. furfur*, *M. globosa*, and *M. restricta* was prepared, for a total of 12.4×10^6

CFU/ml. On D0, a filter paper disk presoaked in 90 µl of the yeast mixture was applied (or not) for 24 h at the surface of skin explants, for a final concentration of 1.1×10^6 CFU/cm^2. On D1, D2, and D4, explants were treated (or not) with a solution of 1.5% *E. angustifolium* extract (2µl/cm^2). On D5, explants were collected. Observation of skin general morphology by optical **microscopy** was performed following staining of paraffinized sections according to Masson's trichrome Goldner variant. Immunostaining of hBD2 and hBD3 as well as TLR2 was done using antibodies specific for each proteins whose presence was subsequently revealed with secondary FITC-conjugate antibodies giving a green fluorescence. Cells nuclei were stained with propidium iodide.

CLINICAL EVALUATION OF *E. ANGUSTIFOLIUM* EXTRACT

A total of 24 volunteers (men and women, 20–61 years of age) with dandruff and greasy hair participated in the study. Following a 15 days wash-out period using a neutral shampoo, participants were instructed to wash their hair and scalp every 3 days for 30 days, with the same neutral shampoo to which was added 1.5% *E. angustifolium* extract. The presence of dandruff was estimated on a scale of 0–5 by a trained technician, on D0, D3, D9, D15, and D30 (before shampooing). For this evaluation, the head was divided into four equal parts with a comb. The final grade for dandruff presence on scalp was calculated as the mean of all four head part scores. Adherent and nonadherent dandruff were estimated separately. The presence of sebum on scalp was measured with the Sebumeter® SM 810 (Courage + Khazaka, Cologne, Germany) on D0, D15, and D30. Macrophotographs of scalp were additionally taken in standardized conditions on one preselected zone, at D0, D3, and D30. A self-assessment form was also filled out by all subjects on D30 to subjectively evaluate the properties of the study product (efficacy, tolerance, and future use).

RESULTS AND DISCUSSION

Using a normal human sebocyte culture model and Nile red staining, the effect of *E. angustifolium* extract on lipid synthesis is presented in Figure 1, as percentages of relative fluorescence units in comparison with DGAT (taken as 100%), a known inhibitor of lipid synthesis. As can be seen, *E. angustifolium* extract dose dependently inhibited sebum production. A substantial reduction by -43% was achieved with a 0.1% concentration of

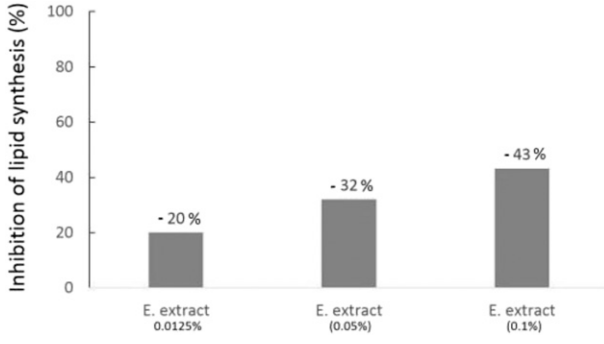

Figure 1. *Epilobium angustifolium* extract inhibits lipid synthesis in sebocytes.

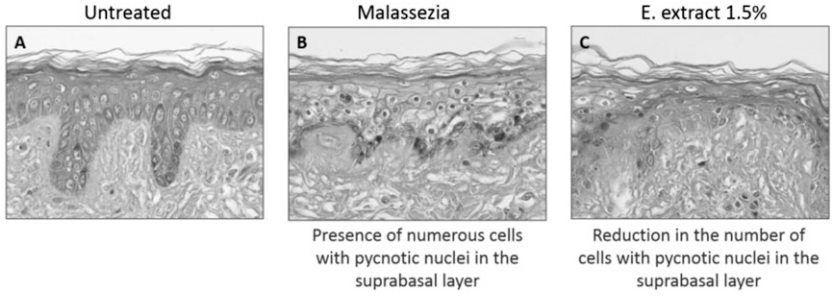

Figure 2. *Epilobium angustifolium* extract protects skin explants from Malassezia-induced morphological changes. (A) Unchallenged skin. (B) Presence of numerous cells with pyknotic nuclei in the suprabasal layer. (C) Reduction in the number of cells with pyknotic nuclei in the suprabasal layer.

E. angustifolium extract. Thus, *E. angustifolium* extract can act directly on sebocytes to reduce sebum production.

As seen in Figure 2, when skin explants were exposed to a mixture of *Malassezia* strains (*M. furfur*, *M. globosa*, and *M. restricta*) for 24 h, morphological alterations appeared after 5 days following exposure. Using histological techniques, we consequently observed an important number of cells showing pyknotic nuclei in the suprabasal layer of the epidermis (Figure 2B) compared to unchallenged skin (Figure 2A). Treatment of unchallenged skin with *E. angustifolium* extract (1.5%) had no effect (not shown). Treatment of yeast-challenged skin explants with the same concentration extract had a positive impact on protecting epidermis morphology, as a clear reduction of the number of cells undergoing pyknosis was observed (Figure 2C).

Skin explants challenged in the same fungal mixture and method described earlier were stained for hBD2, hBD3, and TLR2. Expressions of all three immunological response markers increased noticeably (middle column) compared to unchallenged skin explant (left column). As seen in Figure 3, TLR2 was very lightly expressed in normal skin (Figure 3A). When challenged with *Malassezia* mixture, the receptor was moderately expressed in superficial epidermal layers (Figure 3B) and inhibited when yeast-challenged explants were treated with *E. angustifolium* extract (1.5%). Same results were observed concerning hBD2 (Figure 3D–F). Finally, hBD3 was slightly expressed in normal skin (Figure 3G).

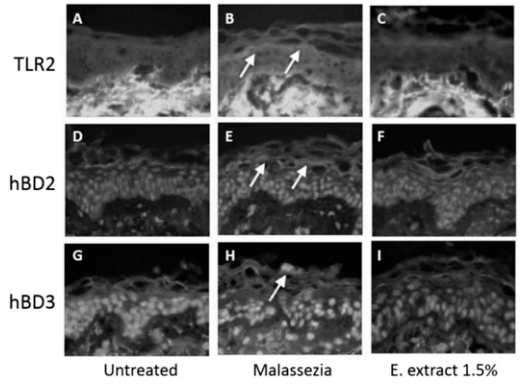

Figure 3. *Epilobium angustifolium* extract modulates TLR2, hBD2, and hBD3 expression in skin explants.

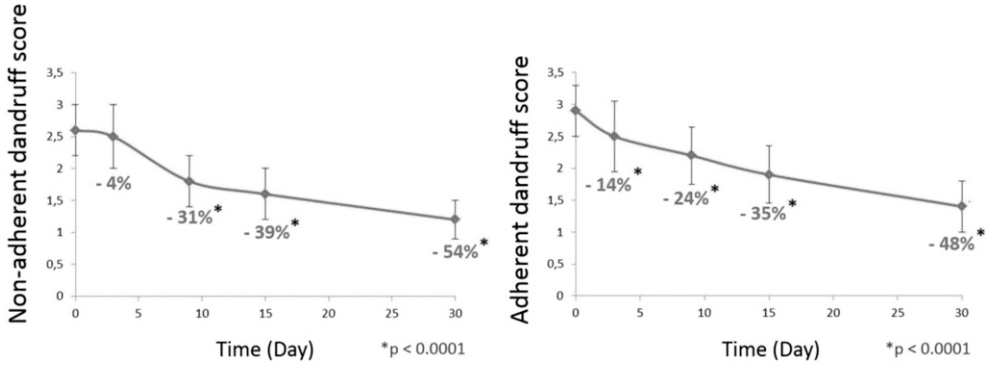

Figure 4. *Epilobium angustifolium* extract inhibits formation of adherent and non-adherent dandruff *in vivo.*

Although challenge with the *Malassezia* mixture induced a clear overexpression of the protein in the superficial epidermal layers (Figure 3H), treatment of the challenged skin with *E. angustifolium* clearly inhibited yeast-induced overexpression of hBD3 (Figure 3I). In this aspect, *E. angustifolium* extract treatment helps keeping a tight control of beta-defensins expression.

CLINICAL STUDIES

The Adherent Scalp Flaking Score (ASFS) method is a reliable and relevant clinical scoring protocol to quantitate dandruff severity (15). Accordingly, in our study, ASFS was performed by qualified technicians to measure the efficacy of antidandruff shampoos containing our extract (1.5%). As shown in Figure 4, both adherent and nonadherent dandruff scorings were significantly reduced ($p < 0.0001$) after the third treatment shampooing (D9) compared to initial scoring (D0). Number of both adherent and nonadherent dandruff scoring continued to decrease over the length of the study (D30) to reach a final reduction of -54% and -48%, respectively. Results indicate that *E. angustifolium* extract treatment was effective in controlling both types of dandruff flakes in real-life conditions.

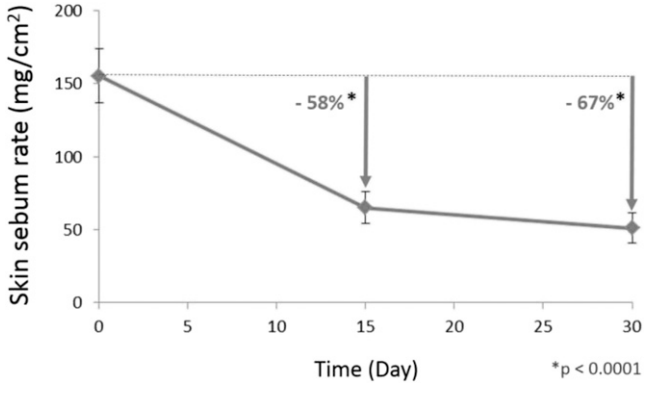

Figure 5. *Epilobium angustifolium* extract improves sebum regulation *in vivo*.

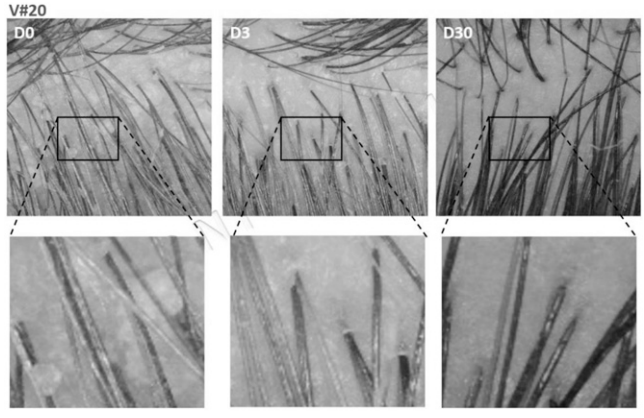

Figure 6. *Epilobium angustifolium* extract improves scalp appearance.

In a same manner, sebum production was evaluated with the Sebumeter® technology. With this photometric device, the total mass of lipids excreted by surface unit (in μg/cm²) can be quantified by reflectometry (Figure 5). At D15, there was a significant ($p < 0.0001$) decrease in sebum production on treated scalp by -58% compared to baseline (D0). The decrease in sebum production was continuous until the end of the study to reach a final -67% ($p < 0.0001$) compared to baseline (D0).

Visual improvement of the scalp, both for quantity of flakes and sebum production, was clearly noticeable, as shown by macrophotography (Figure 6). Photos of scalp taken at D0 D3 and D30 on volunteer 20 displayed less adherent flakes on hair and scalp, and less greasy appearance at the end of the study (D30) before treatment (D0).

CONCLUSION

The study reported here identifies *E. angustifolium* extract as an efficient antidandruff and antisebum cosmetic active ingredient targeting the three main characteristics of the skin disorder (sebum production, SC disruption, and skin inflammation). We propose that one important molecular mechanism underlying *E. angustifolium* extract efficacy involves normalization of defensins (hBD2 and hBD3) and TLR2 expression to limit inflammatory skin response to *Malassezia* infection. To our knowledge, this is the first time that an antidandruff active is associated with immunomodulation of the skin response to *Malassezia* invasion.

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