Title: PLANT EXTRACTS FOR IMPROVING SKIN BARRIER FUNCTION

Abstract: The present invention provides a formulation comprising one or more of: a first extract of fruit of Empetrum nigrum; and/or a second extract of Betula Alba bark.

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PLANT EXTRACTS FOR IMPROVING SKIN BARRIER FUNCTION

The present invention provides a formulation comprising a first extract of *Empetrum nigrum* and/or a second extract of *Betula Alba* bark for use in the treatment of skin disorders associated with reduced skin barrier function.

5 BACKGROUND OF INVENTION

The skin is composed of several morphologically distinct layers. The protection of the skin is provided primarily by the stratum corneum. Underlying the stratum corneum is the viable epidermis (50-100 µm thick), which is responsible for generation of the stratum corneum. The viable epidermis consists of various layers. From the inside to the outside, these layers of the viable epidermis are: the stratum basale, the stratum spinosum and the stratum granulosum. The epidermis is a dynamic, constantly self-renewing tissue, in which a loss of the cells from the surface of the stratum corneum (desquamation) is balanced by cell growth in the lower epidermis. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum they undergo several changes in both structure and composition. The keratinocytes synthesize and express numerous different structural proteins and lipids during their maturation. The final step in keratinocyte differentiation is associated with profound changes in their structure resulting in their transformation into corneocytes. The corneocytes are flat dead cells filled with keratin filaments and water, which are surrounded by densely crosslinked protein layers, the cell envelope. A liquid envelope is chemically linked to this densely packed cell envelope. This lipid monolayer serves as an interface between the hydrophilic corneocytes and the lipophilic extracellular non-polar lipids which are surrounding the corneocytes. Furthermore, corneodesmosomes are interconnecting the corneocytes and are important for the stratum corneum cohesion.

The most important functions of the skin (also known as ‘skin barrier function’) are the protection against water loss and the prevention of substances and bacteria penetrating the body are the most important functions (known as the ‘skin barrier function’) of the skin. This so-called ‘skin barrier function’ is the natural frontier between the inner organism and the environment and is primarily formed by the epidermis.

There is therefore a need for a cosmetic or pharmaceutical formulation for use to improve the skin barrier function of the skin of a user by one or more of: reducing water loss from the body and/or preventing substances and bacteria penetrating the body.

In particular, there is a need for a cosmetic or pharmaceutical formulation which is capable of inhibiting the production of kallikrein-5 (KLKS). By inhibiting the activity of kallikrein-5, the
formulation can improve skin barrier function by reducing and/or preventing cell shedding and the degradation of proteins which form the extracellular component of cell junctions in the stratum corneum.

STATEMENT OF INVENTION

According to a first aspect of the present invention, there is provided a formulation comprising one or more of:

a first extract of fruit of Empetrum nigrum; and/or

a second extract of Betula Alba bark.

According to a second aspect of the present invention, there is provided a use of a formulation as described herein for improving the skin barrier function of the skin of a user.

The term “improving the skin barrier function” is used herein to include: preventing or inhibiting of degradation of the stratum corneum and/or shedding; maintaining or restoring a healthy skin barrier; improving skin moisture within the skin; improving water balance, lipid composition and/or mechanical structure of the skin.

In one embodiment, the formulation is a cosmetic or pharmaceutical formulation, preferably a topical cosmetic or pharmaceutical formulation.

In one embodiment, the formulation is an inhibitor of kallikrein-5 (KLKS).

In one embodiment, the first extract of the formulation is an extract of fruit of Empetrum nigrum obtained by cold pressing in a non-denaturing condition stabilized with organic vegetal glycerine.

In one embodiment, the second extract of the formulation is a water/glycerine extract of Betula Alba bark.

In one embodiment, the first extract comprises at least one compound selected from: cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, delphinidin-3-arabinoside, or any combination thereof. Preferably, the first extract comprises at least one compound selected from: cyanidin-3-O-glucoside, malvidin-3-O-glucoside, delphinidin-3-arabinoside, or any combination thereof.

In one embodiment, the second extract comprises at least one compound selected from: catechin-7-O-beta-D-xylopyranoside, catechin, apiosylepirhododendrin, rhododendrin, apiosylepirhododendrin,
platyphylloside, (SS)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-apiofuranosyl-(1->6)-β-D-glucopyranoside, (3R)-1,7-Bis-(4-hydroxyphenyl)-3-heptanol-3-O-<2,6-bis-O-(β-D-apiofuranosyl)-β-D-glucopyranoside>, aceroside VIII, 5-hydroxy-3-platyphyllone, aceroside VII, centrolobol, acerogenin E, or any combination thereof.

Preferably, the formulation comprises a first extract and a second extract.

Preferably, the ratio by weight of the first extract to the second extract within the formulation is no more than 5:1, for example no more than 3:1. Preferably, the ratio by weight of the first extract to the second extract within the formulation is at least 0.5:1, for example at least 1:1. The ratio by weight of the first extract to the second extract within the formulation is preferably within the range of 0.5:1 and 5:1, preferably within the range of 0.5:1 and 3:1, preferably within the range of 1:1 and 5:1, for example within the range of 1:1 and 3:1.

According to a third aspect of the present invention, there is provided the use of a formulation (for example a cosmetic or pharmaceutical formulation) as herein described in the treatment of skin disorders associated with reduced skin barrier function. The formulation is preferably topically applied to the skin of a user.

In one embodiment, the formulation is used to improve one or more of: skin hydration, skin hydration distribution, skin anisotropy, skin barrier resilience, skin barrier quality, skin cohesiveness, skin nourishing effect, skin barrier recovery, or any combination thereof.

Skin disorders associated with reduced skin barrier function include one or more of: atopic dermatitis, psoriasis, impaired desquamation, sensitive skin or any combination thereof.

The formulation has been found to be able to treat skin disorders associated with reduced skin barrier function, with improved efficacy compared to conventional medications.

The formulation of the present invention has been found to have improved anti-oxidant activity. The formulation of the present invention may therefore be used as an anti-oxidant.

The formulation of the present invention has been found to be capable of blocking the activity or inhibiting the production of kallikrein-5 (KLK5), which consequently reduces cell shedding and the degradation of proteins which form the extracellular component of cell junctions in the stratum corneum.

According to a further aspect of the present invention, there is provided a method for the production of a formulation as herein described, the method comprising:

- obtaining a first extract of fruit of Empetrum nigrum; and/or
obtaining a second extract of Betula Alba bark; and

forming a formulation with the first and/or second extract.

Preferably, the method comprises combining the first and second extract to provide a formulation.

Preferably, the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is no more than 5:1, for example no more than 3:1.

Preferably, the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is at least 0.5:1, for example at least 1:1.

Preferably, the first and second extracts are combined such that the ratio by weight of the first extract to the second extract within the formulation is within the range of 0.5:1 and 5:1, preferably within the range of 0.5:1 and 3:1, preferably within the range of 1:1 and 5:1, for example within the range of 1:1 and 3:1.

According to a still further aspect of the present invention, there is provided a kit for the production of a formulation as described herein, the kit comprising:

- a first extract of fruit of Empetrum nigrum; and
- a second extract of Betula Alba bark.

Embodiments of the present invention are described in detail with references to the accompanying Figures:

BRIEF DESCRIPTION OF FIGURES

Figure 1 illustrates the UHPLC/Q-ToF-MS chromatogram of a water/glycerine Betula Alba bark extract;

Figure 2 illustrates the UHPLC/Q-ToF-MS chromatogram of an extract of fruit of Empetrum nigrum obtained by cold pressing a non-denaturing condition stabilized with organic vegetal glycerine;

Figure 3 illustrates the colouration of different formulations containing an extract of fruit of Empetrum nigrum (2%) depending on pH;

Figure 4 illustrates the anti-oxidant activity of an extract of Betula Alba (Birch 1%) and an extract of fruit of Empetrum nigrum compared to vitamin C (VitC);

Figure 5 illustrates the percentage inhibition of the enzyme Kallikrein 5 by extracts of Betula Alba bark and fruit of Empetrum nigrum;
Figure 6 illustrates an isobole for 50% inhibition for an extract of *Betula Alba* bark and an extract of fruit of *Empetrum nigrum*;

Figure 7 illustrates the percentage inhibition of the enzyme Kallikrein 5 by a formulation comprising a first extract of fruit of *Empetrum nigrum* and a second extract of *Betula Alba*;

Figure 8 illustrates the percentage inhibition of the enzyme Hyaluronidase by extracts of *Betula Alba* bark and fruit of *Empetrum nigrum*;

Figure 9 illustrates the gene expression analysis for an extract of fruit of *Empetrum nigrum*;

Figure 10 illustrates the gene expression analysis for an extract of *Betula Alba* bark;

Figure 11 illustrates the gene expression of an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark;

Figure 12 illustrates the gene expression of niacinamide and retinol;

Figures 13A and 13B illustrate the effect of an extract of fruit of *Empetrum nigrum* and an extract of *Betula Alba* bark on protein expression of AQP3 and OCLN;

Figures 14A and 14B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on skin barrier;

Figures 15A and 15B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on skin barrier resilience and recovery;

Figures 16A and 16B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on cutaneous hydration index;

Figures 17A and 17B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on restructuring effect;

Figures 18A and 18B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on corneocytes cohesion (Desquamation index); and

Figures 18A and 18B illustrate the effects an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, a vehicle, and niacinamide on corneocytes cohesion (Squame surface).

**DETAILED DESCRIPTION**

Example 1 – Method of Extraction of *Betula Alba* bark
Betula Alba bark is extracted with a water/glycerine solvent (10% plant extract in a mixture of water/glycerine). The solvent is removed. The extract is an amber/dark amber liquid with a pH in the range of between 3.4 and 4.0.

Example 2 – Method of Extraction of fruit of Empetrum nigrum

The Empetrum nigrum fruit extract was obtained by cold pressing a non-denaturing condition stabilized with organic vegetal glycerine. The fruit extract is a translucent solution (with a slight precipitate) which is red to brown red in colour with a pH in the range of between 4.0 and 5.4.

The extracts contains anthocyan, this the formulation may change colour depending on pH.

The pH is an important factor in the colour change of anthocyan. The pH-dependent variation in the structure of anthocyan is a particular feature of these molecules. Visual inspection of an aqueous solution of anthocyan shows that the solution turns deep red at a highly acidic pH. The solution becomes paler as the pH increased towards neutrality. A neutral solution of freshly prepared anthocyan is blue but quickly changes colour. The colous changes are due to chemical balances between the various forms of anthocyan (Brouillard and Delaporte, 1977; Brouillard, 1982).

An Empetrum nigrum fruit extract having a pH of 4.5 is pale pink in colour (the pink colouration becomes more intensified with a more acidic pH). An Empetrum nigrum fruit extract having a pH of beyond 6 is green in colour.

Figure 3 illustrates the colouration of formulations containing 2% Empetrum nigrum fruit extract as a factor of pH.

Example 3 – Characterization of extracts of Betula Alba bark and Empetrum nigrum fruit using UHPLC/Q-ToF-MS

Betula Alba bark extract was diluted 10 times in 95:5 H₂O:ANC and filtered through a 0.2 μm syringe filter prior to LC/MS analysis.

The Empetrum nigrum fruit extract was used without dilution and was filtered through a 0.2 μm syringe filter prior to LC/MS analysis.

The extract or extract solutions were analysed by an Agilent UHPLC/Q-ToF (1290 Infinity UHPLC and 6520 A-ToF) using the following settings.

Column: Zorbax extend C₁₈ 2.1 x 150 mm, 1.8 μm
Column temp: 35°C
Flow rate: 0.25 mL/min
Injection volume: 20 μL

The UHPLC gradient results are illustrated in Table 1:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A% (H₂O + 0.1% FA)</th>
<th>B% (ACN + 0.1% FA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>70</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>90</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>95</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>96</td>
<td>98</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1

Q-ToF ion source: -ESI and +ESI
Gas temp: 350°C
Drying gas: 8 L/min
Nebulizer: 40 psig
Vcap: 3500 V

The chromatogram of Betula Alba bark extract solution in the -ESI mode is shown in Figures 1. The chromatogram of Empetrum nigrum fruit extract Figure 9 illustrates the gene expression analysis for an extract of Empetrum nigrum in the +ESI mode is shown in Figure 2.

The compounds found to be present within the Betula Alba bark extract are shown in Table 2:

<table>
<thead>
<tr>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin-7-O-beta-D-xylopyranoside</td>
</tr>
<tr>
<td>Catechin</td>
</tr>
<tr>
<td>Apiosylepirhododendrin</td>
</tr>
<tr>
<td>Rhododendrin</td>
</tr>
<tr>
<td>Apiosylepirhododendrin</td>
</tr>
</tbody>
</table>
Table 2 – Compounds present within *Betula Alba* bark extract

The compounds found to be present within the *Empetrum nigrum* fruit extract are shown in Table 3:

<table>
<thead>
<tr>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cynadin-3-O-glucoside</td>
</tr>
<tr>
<td>Petunidin-3-O-glucoside</td>
</tr>
<tr>
<td>Peonidin-3-O-glucoside</td>
</tr>
<tr>
<td>Malvidin-3-O-glucoside</td>
</tr>
<tr>
<td>Delphinidin-3-O-glucoside</td>
</tr>
</tbody>
</table>

Table 3

Example 4 – Preparation of oil-in-water formulations comprising an *Empetrum nigrum* fruit extract and a *Betula Alba* bark extract

Two different oil-in-water placebo bases were evaluated to determine effects on stability of the *Empetrum nigrum* fruit extract and *Betula Alba* bark extract. The two different bases were as shown in Tables 4 and 5:
<table>
<thead>
<tr>
<th>Component Part</th>
<th>Long Description</th>
<th>Weight Share (%)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>AQUA</td>
<td>70.1</td>
<td>SOLVENT</td>
</tr>
<tr>
<td>306</td>
<td>BUTYLENE GLYCOL</td>
<td>1</td>
<td>HUMECTANT</td>
</tr>
<tr>
<td>404</td>
<td>CAPRYLIC/CAPRIC TRIGLYCERIDE</td>
<td>21.5</td>
<td>MASKING</td>
</tr>
<tr>
<td>508</td>
<td>CETETH-20</td>
<td>0.3</td>
<td>SURFACTANT</td>
</tr>
<tr>
<td>520</td>
<td>CETYL ALCOHOL</td>
<td>1.35</td>
<td>EMULSIFYING</td>
</tr>
<tr>
<td>712</td>
<td>CAPRYLYL GLYCOL</td>
<td>0.35</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td>961</td>
<td>CITRIC ACID</td>
<td>0.1</td>
<td>BUFFERING</td>
</tr>
<tr>
<td>1103</td>
<td>POLYACRILATE CROSSPOLYMER-6</td>
<td>0.75</td>
<td>EMULSION</td>
</tr>
<tr>
<td>1304</td>
<td>GLYCERYL STEARATE</td>
<td>1.35</td>
<td>STABILISING</td>
</tr>
<tr>
<td>2361</td>
<td>SODIUM BENZOATE</td>
<td>0.2</td>
<td>PRESERVATIVE</td>
</tr>
<tr>
<td>2526</td>
<td>STEARETH-20</td>
<td>0.3</td>
<td>EMULSIFYING</td>
</tr>
<tr>
<td>2987</td>
<td>PEG-75 STEARATE</td>
<td>0.7</td>
<td>SURFACTANT</td>
</tr>
<tr>
<td>23832</td>
<td>EXTRACT BIRCH BARK ORGANIC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>23833</td>
<td>CAMADERM</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Component Part</th>
<th>Long Description</th>
<th>Weight Share (%)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>AQUA</td>
<td>84.55</td>
<td>SOLVENT</td>
</tr>
<tr>
<td>273</td>
<td>ETHYLHEXYLGLYKERIN</td>
<td>0.5</td>
<td>SKIN CONDITIONING</td>
</tr>
<tr>
<td>306</td>
<td>BUTYLENE GLYCOL</td>
<td>2</td>
<td>HUMECTANT</td>
</tr>
<tr>
<td>344</td>
<td>C12-15 ALKYL BENZOATE</td>
<td>2</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td>361</td>
<td>PPG-3 BENZYL ETHER MYRISTATE</td>
<td>0.9995</td>
<td>PLASTICISER</td>
</tr>
<tr>
<td>404</td>
<td>CAPRYLIC/CAPRIC TRIGLYCERIDE</td>
<td>3</td>
<td>MASKING</td>
</tr>
<tr>
<td>520</td>
<td>CETYL ALCOHOL</td>
<td>0.5</td>
<td>EMULSIFYING</td>
</tr>
<tr>
<td>696</td>
<td>CAPRYLHYDROXAMIC ACID</td>
<td>0.075</td>
<td>CHELATING</td>
</tr>
<tr>
<td>1052</td>
<td>CYCLOPENTASILOXANE</td>
<td>1</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td>1103</td>
<td>POLYACRILATE CROSSPOLYMER-6</td>
<td>0.9</td>
<td>EMULSION</td>
</tr>
</tbody>
</table>

9
<table>
<thead>
<tr>
<th></th>
<th>1143 DISODIUM EDTA</th>
<th>0.1</th>
<th>CHELATING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1281 GLYCERIN</td>
<td>0.105</td>
<td>HUMECTANT</td>
</tr>
<tr>
<td></td>
<td>1287 GLYCERYL CAPRYLATE</td>
<td>0.57</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td></td>
<td>1304 GLYCERYL STEARATE</td>
<td>0.5</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td></td>
<td>1911 PEG-100 STEARATE</td>
<td>0.5</td>
<td>SURFACTANT</td>
</tr>
<tr>
<td></td>
<td>2533 STEARYL ALCOHOL</td>
<td>0.5</td>
<td>EMOLLIENT</td>
</tr>
<tr>
<td></td>
<td>2395 SODIUM HYDROXIDE</td>
<td>0.0058</td>
<td>pH ADJUSTMENT</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------</td>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>116 PENTAERYTHRITYL TETRA-DI-T-BUTYL HYDROXYHYDROCINNAMATE</td>
<td>0.0005</td>
<td>ANTIoxidANT</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------</td>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>2700 XANTHAN GUM</td>
<td>0.2</td>
<td>VISCOSITY CONTROLLING</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------</td>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>23832 EXTRACT BIRCH BARK ORGANIC HYDROGLYCERINED EXTRACT (SB)</td>
<td>410017</td>
<td>1</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------</td>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>23833 CAMADERM</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5

No difficulties in terms of incorporation or incompatibility of the extracts within the formulations were determined. It was found that the extracts can both be added towards the end of the manufacturing process, after emulsification in terms of emulsions. Preferably, the extracts are incorporated into the formulation during cooling from 45 °C.

Example 5 – Anti-Oxidant activity testing

2,2-Diphenyl-1-picrylhydrazy radical (DPPH) is a stable free radical which can be used to assess the radical scavenging activity of plant materials. At radical state, the methanolic solution of this compound is purple (absorbs light at a wavelength of 516 nm) which when reacted with an antioxidant is reduced to the molecular form (DPPHH) which is yellow with no absorbance at 516 nm.
DPPH assay principle

The primary screening assays for anti-oxidant activity are performed according to the validated standard protocols (i.e. SP-SR-107-v4) for DPPH Assay.

The results of anti-oxidant activity screening for Betula Alba bark extract and Empetrum nigrum fruit extract are shown in Figure 4.

It can be seen that the extracts of Betula Alba bark and Empetrum nigrum fruit both display good antioxidant activity. The Betula Alba bark extract was found to have a 57.5% radical inhibition and the Empetrum nigrum fruit extract was found to have a 69.3% radical inhibition.

IC$_{50}$ Betula alba bark extract = 0.60 % ± 0.02 % (n=3)
IC$_{50}$ Empetrum nigrum fruit extract = 0.42 % ± 0.03 % (n=3)

The anti-oxidant activity of the Betula alba bark extract and Empetrum nigrum extract are comparable to that of Vitamin C. It is known that anti-oxidants support physiological mechanisms to maintain or restore a healthy skin barrier.

Example 6 – Kallikrein 5 Inhibition Assay

Human tissue Kallikrein 5 (KLK5 or KKS), also known as stratum corneum tryptic enzyme) is a serine protease expressed in the epidermis. KLK5 regulate cell shedding (desquamation) in conjunction with KLK7 and KLK14 given its ability to degrade proteins which form the extracellular component of cell junctions in the stratum corneum.

The activity of the enzyme KLK5 is measured by its ability to cleave the fluorogenic peptide substrate Boc-VPR-AMC. The assay measures the formation of AMC that is a highly fluorescent group ($\lambda_{\text{exc}} = 380\text{nm}; \lambda_{\text{em}} = 460\text{nm}$)
The primary screening assays are performed according to the validated standard protocol i.e. SP-SR-201-v3 for KLK5 enzyme inhibition Assay. The activity of the combinations of active components within a formulation is performed according to the validated standard protocol i.e. SP-SR-234 for Isobologram Analysis.

Figure 5 illustrates the % inhibition of Kallikrein 5 for extracts of *Betula Alba* bark and *Empetrum nigrum* fruit. The IC\(_{50}\) of each of these active components and extracts were determined and the results are shown in Table 6.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Betula Alba</em></td>
<td>0.06 %</td>
</tr>
<tr>
<td><em>Empetrum nigrum</em></td>
<td>0.51%</td>
</tr>
</tbody>
</table>

Table 6

It can be seen from Figure 5 and from Table 6 that the extracts of *Betula Alba* bark and *Empetrum nigrum* fruit show good KLK-5 inhibition activity. By inhibiting the production of or activity of KLK-5, the extracts of the present invention prevent or inhibit the degradation of proteins which form the extracellular component of cell junctions in the stratum corneum. As a result, the extracts of *Betula Alba* bark and *Empetrum nigrum* fruit (and the formulations of the present invention containing these extracts) can improve skin barrier function of the skin of a user by preventing or inhibiting the degradation of the stratum corneum and/or cell shedding.

In order to determine if the combination a synergistic effect of the two active components within formulations of the present invention, isobolograms analysis is performed according to the validated standard protocol i.e. SP-SR-234 for Isobologram Analysis.

The first step is to determine the IC\(_{50}\)s of each of the individual active components (i.e. active component A and active component B) within the formulation of the present invention, Table 6. The additive isobole for 50% inhibition is then traced on Graph Pad Prism (Figure 6).

The concentration of active component B that will give 50% inhibition is interpolated with a chosen concentration of active component A. The combination of the two active components A and B at these concentrations needs to then be screened.

•If the results show 50% inhibition, it means that there is an additive effect between the two active components of the formulation at those concentration; or
If the results show <50% inhibition, it means that the formulation needs more concentrated active components to be present in order to achieve 50% inhibition, i.e. that the combination of the active components has an antagonist effect; or

If the results >50% inhibition, it means that the formulation needs less concentrated active components to be present within the formulation to achieve 50% inhibition, i.e. that the combination of the active components has a synergistic effect.

Example 7- Formulation comprising a first extract of fruit of *Empetrum nigrum* and a second extract of *Betula Alba* bark.

A formulation was prepared comprising a first extract of fruit of *Empetrum nigrum* and a second extract of *Betula Alba* bark.

The first extract was present within the formulation in an amount of 0.073% by weight. The second extract was present within the formulation in an amount of 0.025% by weight. The results of the KLKS enzyme inhibition assay for this formulation are shown in Figure 7. The percentage (%) inhibition for the formulation is greater than the additive percentage (%) inhibition for each of the extracts. The formulation comprising the first and second extracts demonstrates a synergistic effect on the resultant KLKS enzyme inhibition.

It can therefore be seen that a formulation comprising a combination of the extracts of *Betula Alba* bark and *Empetrum nigrum* fruit can have a synergistic effect on the skin barrier function of the skin of a user by preventing or inhibiting the degradation of the stratum corneum and/or cell shedding.

Example 8 – Hyaluronidase Inhibition Assay

A key molecule involved in skin moisture is hyaluronic acid (HA). Hyaluronic acid is a nonsulfated glycosaminoglycan composed of alternating residues of the monosaccharide’s glucuronic acid and glucosamine. Hyaluronic acid is a natural compound found in the human body and is degraded and synthesized every day. Hyaluronic acid is degraded into fragments of varying sizes by hyaluronidase (HYAL) by hydrolysis.

The principle of the hyaluronidase inhibition assay is to measure the turbidity at 600 nm based on the following reaction:

```
HA + Hyaluronidase → Di- and Monosaccharides + smaller HA fragments
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The addition of acidic albumin solution makes the remaining hyaluronic acid within the solution precipitate. Therefore, the absorbance of the control (hyaluronic acid with no enzyme) is equivalent
to 100% inhibition) results in the maximum absorbance. The maximum activity (i.e. hyaluronic acid plus hyaluronidase is equivalent to 0% inhibition) results in the minimum absorbance. The presence of a hyaluronidase inhibitor results in a greater inhibition resulting in a higher absorbance.

The primary screening assays are performed according to the validated standard protocols (i.e. SP-SR-197-v2) for Hyaluronidase Inhibition Assay. The results are shown in Figure 8. It can be seen that the extract of *Empetrum nigrum* fruit is a medium hyaluronidase inhibitor and the extract of *Betula Alba* bark is a good hyaluronidase inhibitor.

The extracts and formulations of the present invention (comprising one or more of extracts of *Empetrum nigrum* fruit and *Betula Alba* bark)) shows good inhibition of hyaluronidase and as such can reduce the degradation of hyaluronic acid. The extracts and formulation of the present invention can therefore improve skin barrier function by improving the skin moisture within the skin.

Example 9 – *Gene expression analysis of barrier genes in keratinocytes*

The effects of an extract of fruit of *Empetrum nigrum* and an extract of *Betula Alba* bark on 10 selected skin barrier genes of interest were analysed. The ten selected skin barrier genes were OCLN, AQP3, CLDN1, IVL, CASP14, KRT1, KRT10, FLG, PNPLA and TJP1.

A membrane transporter of water and glycerol expressed in the basal layer keratinocytes of epidermis in normal skin. Important for water content and elasticity of the skin.

20 OCLN, Occludin
Together with Claudin and TJP1, the main component of the tight junctions.

TJP1, Tight junction protein 1
Together with Claudin and OCLN, the main component of the tight junctions.

25 CLDN1, Claudin 1
Together with TJP1 and OCLN, the main component of the tight junctions.

FLG, Filaggrin

Filaggrin is essential for the regulation of epidermal homeostasis. Filaggrin monomers can become incorporated into the lipid envelope, which is responsible for the skin barrier function.
CASP14, Caspase 14
Caspase-14 is required for the degradation of Filaggrin into natural moisturizing factors (NMFs) in the skin. Blocking the filaggrin processing done by Caspase-14, results in defects in water retention.

KRT1 and KRT10, Keratin 1 and 10
Differentiation of keratinocytes from the basal to the spinous layer is characterized by a shift to Keratins 1 and 10. The primary function of the keratin intermediate filament cytoskeleton is to provide cells with structural resilience against mechanical trauma.

IVL, Involucrin
Cornified envelope protein, together with keratins responsible for the mechanical stability of the corneocytes. Involucrin binds covalently to ceramides, forming a backbone for the subsequent attachment of free ceramides.

PNPLA1, Patatin-like phospholipase domain-containing 1
PNPLA1, an enzyme expressed in differentiated keratinocytes, plays a crucial role in the biosynthesis of ω-O-acylceramide, a lipid component essential for skin barrier.

qPCR was used to analyze the gene expression of barrier genes. Human epidermal keratinocytes (KC) were cultured in 48-well plates and treated for 24h with actives before RNA extraction followed by cDNA synthesis and then qPCR was performed for the selected genes.

It was found that an extract of fruit of Empetrum nigrum significantly upregulated seven out of the ten genes (ACP3, OCLN, KRT1, KRT10, PNPLA1, IVL and Casp 14) as shown in Figure 9.

It was also found that the extract of Betula Alba bark significantly upregulated six out of the ten genes (OCLN, KRT1, KRT10, PNPLA1, IVL and Casp 14) as shown in Figure 10.

A combination of 0.5% of an extract of fruit of Empetrum nigrum together with 0.5% of an extract of Betula Alba bark was found to significantly upregulate eight of the ten genes (IVL, AQP3, OCLN, FLG, K1, K10, CASP14, CLDN1) as shown in Figure 11. It was found that the upregulation provided by the combination of 0.5% of an extract of Empetrum nigrum together with 0.5% of an extract of Betula Alba bark was greater than the upregulation provided by each extract alone.

Known agents for improving skin barrier function are niacinamide and retinol. The gene expression of barrier genes, as a result of being treated with niacinamide or retinol, was analyzed, and the results are shown in Figure 12.
From Figure 12, it can be seen that niacinamide and retinol did not show significant upregulation of the genes. Retinol was found to increase the gene expression of AQP3 while it downloaded most other genes.

The extracts of fruit of *Empetrum nigrum* and *Betula Alba* bark were found to provide good upregulation of several skin-barrier genes *in vitro*. These genes are involved in different aspects of skin barrier function such as water balance, lipid composition and mechanical structure. The extracts and formulations of the present invention can therefore be used to improve skin barrier function, and in particular to improve water balance, lipid composition and mechanical structure of the skin barrier.

**Example 10 – Detection of proteins using flow cytometry *in vitro***

The effect of the extracts of fruit of *Empetrum nigrum* and *Betula Alba* bark on protein expression of AQP3 and OCLN were investigated.

Human epidermal keratinocytes were treated with an extract of fruit of *Empetrum nigrum*, an extract of *Betula Alba* bark, an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark, or niacinamide (control).

Cells were treated with the actives for 48h before they were stained with antibodies for AQP3 and OCLN conjugated to Alexa fluor 488 and Alexa fluor 647 respectively. The stained cells were run through the flow cytometer where the intensity of fluorescent was analysed.

The highest concentration of the extracts used was 0.25% of both extracts of fruit of *Empetrum nigrum* and *Betula Alba* bark.

It was found that 0.25% an extract of fruit of *Empetrum nigrum* did not show any upregulation of AQP3 and OCLN proteins after 48 hours. In contrast, 0.25% of an extract of *Betula Alba* bark showed significant upregulation of both AQP3 and OCLN proteins as shown in Figures 13A and 13B. Furthermore, 0.25% of an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark showed significant upregulation of both AQP3 and OCLN proteins. In comparison, niacinamide did not show any significant upregulation of AQP3 or OCLN proteins.

**Example 11 – Consumer/Clinical Testing**

A clinical study was carried out in September/October 2020 in France, on two groups of female volunteers.

In the first group, each of the volunteers used both the vehicle and the vehicle containing an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark at a concentration of 1%.
In the second group, each of the volunteers used both the vehicle containing an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark at a concentration of 1% and the vehicle containing Niacinamide at a concentration of 3%.

The study has been conducted using with a double-blinded set up with a CRO, meaning that none of the volunteers, investigators, local project manager and statisticians were aware of the nature of the products applied.

The Study population information is shown in Table 7:

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<tr>
<th>Number of volunteers</th>
<th>35 (group 1) and 36 (group 2) female volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteers age</td>
<td>18 to 65 years</td>
</tr>
<tr>
<td></td>
<td>(Mean age group 1 = 47 years, Mean age group 2 = 49 years)</td>
</tr>
<tr>
<td>Study location</td>
<td>Lyon, France</td>
</tr>
<tr>
<td>Investigation site</td>
<td>Face (Temple &amp; Forehead)</td>
</tr>
</tbody>
</table>
| Product application  | Split face: group 1 with one side for the vehicle, opposite side with vehicle + extracts of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark. Group 2 with one side for the vehicle + *Empetrum nigrum* together with an extract of *Betula Alba* bark at 1%, opposite side with vehicle + Niacinamide at 3%.
|                      | Double-blinded                                 |
|                      | Randomised                                     |
|                      | Application twice daily: morning & evening     |
| Wash out             | 2 weeks with the use of 30558 alt 78 only as Moisturiser |
| Timepoints           | Baseline, 1 month                              |

Table 7

The parameters measured during the study are shown in Table 8:
Skin barrier (TEWL)  
Skin barrier resilience (TEWL)  
Skin barrier resilience (TEWL)  
Cutaneous hydration index  
Restructuring effect (protein content removed)  
Corneocytes cohesion (Desquamation index)  
Corneocytes cohesion (Squame surface)  

Table 8

Skin barrier (TEWL)

The results of the treatments on the skin barrier for each group are shown in Figures 14A and 14B. It can be seen that there was no significant changes observed (versus baseline) as a result of being treated by the vehicle formulation or by the extract of fruit of Empetrum nigrum together with an extract of Betula Alba bark (Figure 14A).

There was however a significant difference between the formulation containing an extract of fruit of Empetrum nigrum together with an extract of Betula Alba bark and the formulation containing Niacinamide (Figure 14B). It can be seen that there was significant skin barrier improvement when treated with an extract of fruit of Empetrum nigrum together with an extract of Betula Alba bark in comparison to the niacinamide treated side.
Skin barrier resilience and recovery (TEWL)

The results of the treatments on the skin barrier resilience and recovery for each group are shown in Figures 15A and 15B. It can be seen that there was no significant changes or differences observed as a result of being treated by the vehicle formulation or the extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark. Furthermore, it can be seen that there was no significant changes or differences observed as a result of being treated by niacinamide or the extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark. Skin barrier recovery data remains inconclusive, irrespective of the group.

Cutaneous hydration index

The results of the treatments on the cutaneous hydration index for each group are shown in Figures 16A and 16B. It can be seen that there was a near significant improvement (versus baseline) observed for those treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark in the first group (p = 0.06). Furthermore, a significant improvement was seen for those treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark in the second group.

The improvement achieved by being treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark outperformed the improvement achieved by being treated with niacinamide following a month of use.

Restructuring effect

The results of the treatments on the restructuring effect are shown in Figures 17A and 17B. It can be seen that there was a significant improvement observed for those treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark when compared to those treated with the vehicle. Furthermore, there was a tendency of a better restructuring effect observed for those treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark compared to those treated with niacinamide (p=0.10).

Corneocytes cohesion (Desquamation index & Squame surface)

The results of the treatments on the corneocytes cohesion are shown in Figures 18A and 18B (Desquamation index) and in Figures 19A and 19B (Squame surface). It can be seen that a significant worsening (versus baseline) was observed for those treated with the vehicle formulation after 1 month. It can however also be seen that there was an improvement observed for those treated with
an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark after 1 month of use (Figures 18A and 19A), to a significant extent when compared to the vehicle treated side.

In the second group, a worsening versus baseline was observed for those treated with an extract of fruit of *Empetrum nigrum* together with an extract of *Betula Alba* bark and for those treated with niacinamide (Figures 18B and 19B).

The results show that the extracts of the present invention demonstrate efficacy in several parameters which relate to skin barrier function and hydration. In particular, the extracts of the present invention have been found to improve decrease protein content removed by tape stripping (restructuring effect), the desquamation index and squame surface (corneocytes cohesiveness). The extracts of the present invention have also been shown to significantly improve the cutaneous hydration index (compared to the baseline). The extracts of the present invention have also been shown to produce a significantly higher hydrating effect than that producing by the use of a niacinamide formulation. Differences in transepidermal water loss were observed after 4 weeks, with an observed decrease for those treated with extracts of the present invention compared to an increase in water loss for those treated with niacinamide.
CLAIMS

1. A formulation comprising one or more of:
   a first extract of fruit of *Empetrum nigrum*; and/or
   a second extract of *Betula Alba* bark.

2. A formulation as claimed in claim 1, in which the second extract is a water/glycerine extract of *Betula Alba* bark.

3. A formulation as claimed in either of claims 1 and 2, in which the first extract comprises at least one compound selected from: cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, delphinidin-3-arabinoside, or any combination thereof.

4. A formulation as claimed in any one of claims 1 to 3, in which the second extract comprises at least one compound selected from: catechin-7-O-beta-D-xylopyranoside, catechin, apiosylrhododendrin, rhododendrin, apiosylepirrhododendrin, platyphylloside, (5S)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-beta-D-apiofuranosyl-(1->6)-beta-D-glucopyranoside, (3R)-1,7-Bis-(4-hydroxyphenyl)-3-heptanol-3-O-<2,6-bis-O-(beta-D-apiofuranosyl)-beta-D-glucopyranoside>, aceroside VIII, 5-hydroxy-3-platyphyllone, aceroside VII, centrolobol, acerogenin E, or any combination thereof.

5. A formulation as claimed in any one of claims 1 to 4, in which the ratio by weight of the first extract to the second extract within the formulation is no more than 5:1.

6. A formulation as claimed in any one of claims 1 to 4, in which ratio by weight of the first extract to the second extract within the formulation is no more than 3:1.

7. A formulation as claimed in any preceding claim, in which the ratio by weight of the first extract to the second extract within the formulation is at least 0.5:1.

8. A formulation as claimed in any preceding claim, in which the ratio by weight of the first extract to the second extract within the formulation is at least 1:1.

9. A formulation as claimed in any preceding claim, in which the formulation is an inhibitor of kallikrein-5 (KLKS).

10. Use of a formulation as claimed in any one of claims 1 to 9, in the treatment of skin disorders associated with reduced skin barrier function.

11. Use as claimed in claim 10, in which the skin disorder(s) is selected from one or more of: atopic dermatitis, psoriasis, impaired desquamation, sensitive skin or any combination thereof.

12. Use of a formulation as claimed in any one of claims 1 to 9, for improving one or more of: skin hydration, skin hydration distribution, skin anisotropy, skin barrier resilience, skin barrier...
quality, skin cohesiveness, skin nourishing effect, skin barrier recovery, or any combination thereof, of the skin of a user.

13. A method for the production of a formulation as herein described, the method comprising:
   obtaining a first extract of fruit of *Empetrum nigrum*; and/or
   obtaining a second extract of *Betula Alba* bark; and
   forming a formulation with the first and/or second extract.

14. A method as claimed in claim 13, in which the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is no more than 5:1.

15. A method as claimed in claim 13, in which the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is no more than 3:1.

16. A method as claimed in any one of claims 13 to 15, in which the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is at least 0.5:1.

17. A method as claimed in any one of claims 13 to 16, in which the first and second extracts are combined such that the ratio of the first extract to the second extract in a ratio (by weight) within the formulation is at least 1:1.
Antioxidant Activity by DPPH Assay

n=3

EXP-20-HR5713

FIG. 4
Hyaluronidase Inhibition Assay

![Graph showing inhibition percentages for different treatments.](Image)

**FIG. 8**

Combination Betula Alba Birch extract and Empetrum Nigrum Fruit extract in the KLK5 Enzyme Inhibition Assay

![Graph showing inhibition percentages for different treatments.](Image)

**FIG. 7**
Gene expression analysis
Empertrum nigrum fruit extract 1%
keratinocytes, 24h treatment
n=5

FIG. 9

Gene expression analysis
Betula alba bark extract 1%
keratinocytes, 24h treatment
n=5

FIG. 10
Gene expression
24h stimulation
n=3

Fold Change

0.5% Empetrum nigrum fruit extract
0.5% Betula alba bark extract
0.5% Empetrum nigrum fruit extract + 0.5% Betula alba bark extract

Gene

FIG. 11

Gene Expression
24h Stimulation
n=3

Fold Change

0.1% Niacinamide
0.01% Niacinamide
10^{-4}\% Retinol
10^{-5}\% Retinol

Gene

FIG. 12

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### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>DATABASE GNPD [Online] MINTEL; 22 October 2020 (2020-10-22), anonymous: &quot;Intensive Serum&quot;, XP055984808, Database accession no. 8207073 abstract -----</td>
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  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed
  - "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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