# ABS White Willow Bark Extract Powder

## Efficacy Data

<table>
<thead>
<tr>
<th>Type of Study</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular Renewal Assay</strong></td>
<td>ABS White Willow Bark Extract Powder provided better cellular renewal than the 1% Synthetic Salicylic Acid. ABS White Willow Bark Extract Powder showed a 26% increase, while the synthetic showed a 22% increase. ABS White Willow Bark Extract Powder is an excellent substitute for salicylic acid in cosmetics.</td>
</tr>
<tr>
<td><strong>High Resolution Skin-Imaging Assay</strong></td>
<td>ABS White Willow Bark Extract Powder improved skin density by 33.59%, after 24 hours and by 44.42% after 4 weeks when compared to the untreated control. When compared to the base cream ABS White Willow Bark Extract Powder improved skin density by 36.72% after 24 hours and after 4 weeks ABS White Willow Bark Extract Powder improved density by 32.41%.</td>
</tr>
<tr>
<td><strong>ORAC Assay</strong></td>
<td>Oxygen Radical Absorbance Capacity (ORAC) is a measure of a materials potential to protect against oxidative stress or reactive oxygen species (ROS). ABS White Willow Bark Extract Powder demonstrated significant antioxidant activity by reducing the presence of ROS compared with Trolox, the vitamin E analog.</td>
</tr>
<tr>
<td><strong>IL-6 ELISA Assay</strong></td>
<td>As expected, the changes in IL-6 production using ABS White Willow Bark Extract Powder appears to be dose dependent. This decrease in IL-6 production indicates a reduced inflammatory environment.</td>
</tr>
</tbody>
</table>

**Code:** 10229  
**INCI Name:** Salix Alba (Willow) Bark Extract  
**CAS #:** 84082-82-6  
**EINECS #:** 282-029-0
**Anti-microbial Assay**

The results of the antibacterial assay indicated significant activity against all of the gram-positive bacteria tested with minimum inhibitory concentrations ranging from 0.1 to 5.0 mg/ml. Methanolic extracts exhibited higher fungal growth inhibition, whereas the water extracts showed the least inhibition.

**NF-kb Activation**

Significant inhibition of UVB induced NK-kb activation in keratinocytes was observed when treated with 1µg of salicylic acid for 6 hours vs. 0µg and exposed with 0 – 25mJ/cm² UVB.

**HSP 70 Protein Determination**

Salicylic acid was found to significantly increase the induction of HSP70 Protein in keratinocytes when treated with different doses of salicylic acid (0-100mg/ml) for 24 hours.

**DNA Repair of UVB-Induced Thymine Dimers (TT-dimers)**

*in-vivo* studies conclude that salicylic acid significantly increases DNA repair (removal rate of DNA damage) when topically pretreated with 200 mg/ml of salicylic acid (in sterile H₂O) for 6 hours and then UVB irradiated at 0, 100, and 150 mJ/cm².
Abstract

ABS White Willow Bark Extract Powder was evaluated for its ability to accelerate cell renewal by means of a traditional Dansyl Chloride protocol.

Methods & Materials

A 5% Dansyl Chloride was prepared by dispersing Dansyl Chloride 95% (Sigma) in petrolatum. Approximately 0.2 g of the ointment was applied to three 2cm x 2cm locations on the volar forearm of 12 (M/F) subjects between the ages of 19 and 43. The material was allowed to remain in place for 24 hours at which time any excess ointment was removed.

Two products were tested, with the remaining untreated site serving as the biological control. The products were applied in a randomized fashion. Approximately 50 μl of product was applied to the appropriate test site once per day. The sites were then examined daily under ultraviolet light (SL-3660 Long Wave Ultra Violet, Black Light Eastern Corp., Westbury, Long Island, NY) for fluorescence. The test was continued until no fluorescence was detectable at any site. The values listed reflect the average time for each product.

Results:

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
<th>Days</th>
<th>% Cell Renewal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic Acid</td>
<td>1%</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Untreated Control</td>
<td>N/A</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>ABS White Willow Bark Extract Powder</td>
<td>2%</td>
<td>18</td>
<td>27</td>
</tr>
</tbody>
</table>
Discussion

The results indicate that **ABS White Willow Bark Extract Powder** is capable of increasing cellular renewal by 27% when compared to the untreated biological control.
Introduction

An in-vivo study was conducted over a period of four weeks to evaluate the effect on skin density of ABS White Willow Bark Extract Powder. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly improving skin density compared to the control.

Materials

A. Equipment: DermaLab Skin Combo (Ultrasound Probe)

Methods

High Resolution Ultrasound Skin imaging is based on measuring the acoustic response after an acoustic pulse is sent into the skin. The energy of the acoustic pulse is low and will not affect the skin in any way. When the acoustic pulse is emitted and hits different areas of the skin, part of the pulse will be reflected and part will be transmitted further into the skin. The reflected signal travels back and is picked up by the ultrasound transducer. After processing the signal, a cross-sectional image appears on the screen. This image represents an intensity, or amplitude, analysis of the signals.

The intensity of the signals that are received refer to a color scale. Dark colors represent areas of the skin with low reflection. This means that there are no changes or very small changes in density between the structures in the skin. Bright colors represent areas with strong reflections, signifying substantial changes in density between structures.
10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. The DermaLab ultrasound probe was used to determine the skin density of the subject’s volar forearms. Baseline elasticity readings were taken on day one of the study.

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for 4 weeks. The test material consisted of 2.0% ABS White Willow Bark Extract Powder in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

Results

ABS White Willow Bark Extract Powder showed improvements in skin density at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

Individual Raw Data:

<table>
<thead>
<tr>
<th></th>
<th>T = 24</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subject 1-Test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated Control</td>
<td>60</td>
<td>68</td>
<td>75</td>
<td>54</td>
<td>67</td>
</tr>
<tr>
<td>Base Lotion</td>
<td>53</td>
<td>54</td>
<td>50</td>
<td>42</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
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<td>41</td>
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<tr>
<td><strong>Subject 2-Test</strong></td>
<td>100</td>
<td>98</td>
<td>93</td>
<td>99</td>
<td>82</td>
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<td>68</td>
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<tr>
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<td>65</td>
<td>69</td>
<td>89</td>
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<td></td>
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<tr>
<td><strong>Subject 3-Test</strong></td>
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<tr>
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<td>62</td>
<td>79</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Subject 4-Test</strong></td>
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<td>93</td>
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<tr>
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<td>72</td>
<td>59</td>
<td>54</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>Base Lotion</td>
<td>50</td>
<td>70</td>
<td>74</td>
<td>52</td>
<td>67</td>
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<tr>
<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject 5-Test</strong></td>
<td>76</td>
<td>82</td>
<td>89</td>
<td>94</td>
<td>96</td>
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<tr>
<td>Untreated Control</td>
<td>46</td>
<td>53</td>
<td>43</td>
<td>50</td>
<td>58</td>
</tr>
<tr>
<td>Base Lotion</td>
<td>47</td>
<td>53</td>
<td>42</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subject 6-Test</strong></td>
<td>85</td>
<td>79</td>
<td>92</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
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<td>46</td>
<td>56</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Base Lotion</td>
<td>44</td>
<td>59</td>
<td>46</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
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</table>

Information contained in this technical literature is believed to be accurate and is offered in good faith for the benefit of the customer. The company, however, cannot assume any liability or risk involved in the use of its chemical products since the conditions of use are beyond our control. Statements concerning the possible use of our products are not intended as recommendations to use our products in the infringement of any patent. We make no warranty of any kind, expressed or implied, other than that the material conforms to the applicable standard specification.
### High Resolution Ultrasound Skin Imaging Assay

#### Results of Group:

<table>
<thead>
<tr>
<th></th>
<th>t = 24</th>
<th>1 week</th>
<th>2 week</th>
<th>3 week</th>
<th>4 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental (2.0% White Willow Bark Extract Powder in Base Lotion)</td>
<td>77.8</td>
<td>83.2</td>
<td>82.0</td>
<td>72.8</td>
<td>77.6</td>
</tr>
<tr>
<td>Untreated</td>
<td>58.2</td>
<td>56.2</td>
<td>56.7</td>
<td>51.9</td>
<td>53.8</td>
</tr>
<tr>
<td>Base Lotion Control</td>
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<td>58.3</td>
<td>58.5</td>
<td>57.4</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>t = 24</td>
<td>1 week</td>
<td>2 week</td>
<td>3 week</td>
<td>4 week</td>
</tr>
<tr>
<td>Experimental vs. Untreated Control</td>
<td>33.59%</td>
<td>48.04%</td>
<td>44.62%</td>
<td>40.27%</td>
<td>44.42%</td>
</tr>
<tr>
<td>Base Lotion vs. Untreated Control</td>
<td>-2.29%</td>
<td>3.74%</td>
<td>3.17%</td>
<td>10.60%</td>
<td>9.07%</td>
</tr>
<tr>
<td>Experimental vs. Base Lotion</td>
<td>36.72%</td>
<td>42.71%</td>
<td>40.17%</td>
<td>26.83%</td>
<td>32.41%</td>
</tr>
</tbody>
</table>

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Discussion

As evidenced in a 4 week efficacy study of **ABS White Willow Bark Extract Powder** on skin, skin density was improved by 33.59% after 24 hours and by 44.42% after 4 weeks when compared to the untreated control. When compared to the base cream **ABS White Willow Bark Extract Powder** improved skin density by 36.72% after 24 hours and after 4 weeks **ABS White Willow Bark Extract Powder** improved density by 32.41%. Results indicate that **ABS White Willow Bark Extract Powder** is capable of improving skin density when compared to both the untreated control as well as the base lotion.

**ABS White Willow Bark Extract Powder** has a strong positive effect on skin’s density when used at recommended use levels.
Introduction

Reactive oxygen species (ROS) are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS are detrimental to cellular structures and functional molecules (i.e DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. The oxygen radical absorbance capacity (ORAC) assay is a standard method used to assess antioxidant capacity of physiological fluids, foods, beverages, and natural products. The assay quantitatively measures a sample’s ability to quench free radicals that have the potential to react with and damage cellular components.

Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of **ABS White Willow Bark Extract Powder**.

Assay Principle

This assay is based upon the effect of peroxyl radicals generated from the thermal decomposition of 2, 2’-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The degree of change is indicative of the amount of radical damage and the presence of antioxidants results in an inhibition in the free radical damage to the fluorescein. The antioxidant protection of the sample can be calculated by comparing it to a set of known standards. Trolox®, a water soluble vitamin E analog, with known antioxidant capabilities is used in this ORAC assay as the standard for measuring the antioxidant capacity of unknown substances. ORAC values, expressed in µM of Trolox® equivalents (TE), are calculated using the area under the curves (AUC) of the test product, Trolox®, and the control materials. Trolox equivalency is used as the benchmark for antioxidant capacity of mixtures since it is difficult to measure individual components.
Oxygen Radical Absorbance Capacity (ORAC) Assay

Materials

A. Equipment: Synergy H1 Microplate reader (BioTek Instruments, Winooski, VT); Gen5 software (BioTek Instruments, Winooski, VT); Pipettes

B. Buffers: 75mM Potassium Phosphate (pH 7.4); Deionized H₂O

C. Reagents: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)

D. Preparation: Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.

E. Microtitre Plates: Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of ABS White Willow Bark Extract Powder and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared at a concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37°C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

\[ AUC = 0.5 + \frac{R_2}{R_1} + \frac{R_3}{R_1} + \frac{R_4}{R_1} + \cdots + \frac{R_n}{R_1} \]  
\[ \rightarrow \text{Where } R \text{ is fluorescence reading} \]

\[ \text{Net AUC} = AUC_{\text{sample}} - AUC_{\text{blank}} \]

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample’s Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents (µMTE), where 1 ORAC unit is equal to 1 µMTE.
Results

**ABS White Willow Bark Extract Powder** began exhibiting antioxidant activity at a 0.0005% concentration.

![Antioxidant capacities](image)

**Figure 1:** Antioxidant capacities

Discussion

As shown in figure 1, **ABS White Willow Bark Extract Powder** exhibited antioxidant activity comparable to 200μM Trolox®. The antioxidant capacity of **ABS White Willow Bark Extract Powder** increased as the concentration increased, as a result we can assure that its ability to minimize oxidative stress is dose dependent.

**ABS White Willow Bark Extract Powder** was designed for problem skin with exfoliation and antimicrobial properties. With the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing potent antioxidant benefits when added to cosmetic applications.
IL-6 ELISA Analysis

Tradename: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 258

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Erica Segura
Principle Investigator: Meghan Darley

Test Performed:
Interleukin (IL)-6 Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Interleukin-6 is a proinflammatory cytokine known to play an active role in inflammation, immunology, bone metabolism, reproduction, arthritis, neoplasia, and aging. IL-6 signals through the nuclear factor-kappa B (NF-κB) pathway that results in the transcription of inflammatory mediators, including matrix metalloproteinase-1 (MMP-1). MMP’s are responsible for breaking down the extracellular matrix and collagen in the skin leading to wrinkles, fine lines, and loss of skin elasticity. Reducing the level of IL-6 and other inflammatory mediators is believed to slow down degradation of the skin matrix and, possibly, stimulate its replenishment.

Interleukin-6 ELISA was conducted to assess the changes in IL-6 levels in ABS White Willow Bark Extract Powder-treated in vitro cultured human dermal fibroblasts.

Assay Principle

This ELISA utilizes a colorimetric reaction employing antibodies with antigen specificity to human IL-6. Monoclonal antibodies specific for IL-6 epitopes are coated on a microtiter plate. In positive samples, IL-6 will bind to these antibodies and are tagged a second time with another IL-6-specific antibody labeled with horseradish peroxidase (HRP). The addition of the chromagen solution, containing 3,3',5,5'-tetramethylbenzidine, provides the colorimetric reaction with HRP that is quantitated through optical density (OD) readings on a microplate spectrometer. The standard curve provides a reference from the OD readings for the amount of collagen in each sample.
IL-6 ELISA Analysis

info@activeconceptsllc.com • +1 (704)-276-7100 • Fax: +1 (704)-276-71

Materials

A. Kit: IL-6 ELISA Kit (Biosource; KAC1261)
B. Incubation Conditions: 37°C at 5% CO₂ and 95% relative humidity (RH)
C. Equipment: Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes
D. Cell Line: Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers: Dulbecco’s Modified Eagle Medium (DMEM); Penicillin-Streptomycin (50U-50mg/mL); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Amphotericin (45pg/mL)
F. Culture Plate: Falcon flat bottom 12-well tissue culture treated plates
G. Reagents: Lipopolysaccharide (LPS) (1µg/mL); Solution A (human plasma with preservatives); Solution B (buffer with preservative)
H. Other: Sterile disposable pipette tips; wash bottles

Methods

Human dermal fibroblasts were seeded into 12-well tissue culture plates and allowed to grow to confluency in complete DMEM. 1%, 0.1%, 0.01% concentrations of ABS White Willow Bark Extract Powder were added to complete DMEM containing 1µg/mL LPS and incubated with fibroblasts for 24 hours. Complete media containing 1µg/mL LPS was used as the positive controls and complete DMEM was used a negative control.

Standards were prepared in concentrations ranging from 0pg/mL to 2476pg/mL. 50µL of Solution B was added to wells for standards and assay controls and 50µL of Solution A was added to experiment wells. 100µL of standards, controls, and samples were added to appropriate wells. After a one hour incubation at room temperature and washing, 50µL Solution A and 100µL anti-IL-6 conjugate was added to all wells. Following a one hour incubation and washing, 100 µL chromagen solution was added for the colorimetric reaction. One-hundred µL stop solution was added to stop the reaction after 15 minutes. The optical density was read at 450nm on the Synergy HT Microplate Reader.

A standard curve was created by reducing the data and generating a linear curve fit. The IL-6 concentration of ABS White Willow Bark Extract Powder treated-fibroblasts was determined by extrapolation from the standard curve and expressed in pg/mL.
Results

The data obtained from this study met criteria for a valid assay and the positive and negative controls performed as anticipated.

**ABS White Willow Bark Extract Powder**, at a concentration of 1% was able to decrease IL-6 production compared to our positive control.

IL-6 levels are expressed by the following formula:

\[ \text{IL-6 levels} = \text{Average}_{IL-6\, \text{Concentrations}} \times \text{Dilution Factor} \]

![Figure 1: ABS White Willow Bark Extract Powder-treated fibroblasts IL-6 concentrations](image-url)
IL-6 production percent decrease is calculated by the following formula:

\[
\text{Percent Decrease} = \frac{\text{Positive Control Avg. Concentration} - \text{Sample Avg. Concentration}}{\text{Positive Control Avg. Concentration}} \times 100
\]

![Percent Decrease in IL-6 Production](chart)

**Figure 2:** Percent decrease in IL-6 production compared to positive control

**Discussion**

As shown in figure 1, **ABS White Willow Bark Extract Powder** exhibited anti-inflammatory effects on LPS-treated fibroblasts. As expected, the changes in IL-6 production using **ABS White Willow Bark Extract Powder** appears to be dose dependent. This decrease in IL-6 production indicates a reduced inflammatory environment which could decrease the signs of aging and reduce the formation of fine lines and wrinkles. For these reasons, we can assume **ABS White Willow Bark Extract Powder** is suitable for cosmetic applications designed to provide soothing and anti-aging properties.
Antimicrobial Efficacy Test
PCPC Section 20
Method 3
Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product
ABS White Willow Bark Extract Powder
Code: 10229
Lot #: 33482

Test Request #
903

Purpose
This study was initiated to determine the adequacy of 2% ABS White Willow Bark Extract Powder as a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates
The study was started on February 18th, 2014 and was completed on April 22nd, 2014.

Test Organisms
1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:
Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.
Test Method

Fifty grams of Generic Cream Formula containing 2% ABS White Willow Bark Extract Powder was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard, and further diluted to yield $10^4$ to $10^6$ microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the products composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test solution.

<table>
<thead>
<tr>
<th>Inoculum (initial)</th>
<th>Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU/ml</td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td>$8.5 \times 10^6$</td>
</tr>
<tr>
<td>Day 3*</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 7</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 14</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 21</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 28</td>
<td>&gt;99.999%</td>
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</table>

<table>
<thead>
<tr>
<th>Inoculum (re-inoculated)</th>
<th>CFU/ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$8.7 \times 10^6$</td>
</tr>
<tr>
<td>Day 31</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 35</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 42</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 49</td>
<td>&gt;99.999%</td>
</tr>
<tr>
<td>Day 56</td>
<td>&gt;99.999%</td>
</tr>
</tbody>
</table>

Table 1. Challenge Test results for Generic Cream Formula containing 2% ABS White Willow Bark Extract Powder inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.
Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of 2% ABS White Willow Bark Extract Powder as a cosmetic ingredient with antimicrobial properties in Generic Cream Formula. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

**Bacteria** – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

**Yeasts and Molds** – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by greater than 99.999% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by greater than 99.999%.
Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT’s goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer’s internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

The information contained in this report is provided by Active Micro Technologies after the exercise of all reasonable care and skill in its compilation, preparation, and issue. It is provided without liability regarding its subsequent application and use. This type of screening test will be conducted only for validation of the efficacy of the antimicrobial agent or preservative system in the specific formulation tested. It does not address the suitability of the overall formula, nor does it address the regulatory status of any component therein. This testing does not account for the possibility of environmental microorganisms and cannot be relied upon as sufficient to justify commercialization of the product tested. By submitting samples for testing, the customer acknowledges that they will not hold Active Micro Technologies responsible for products launched based solely on the support of these studies.
SALICYLIC ACID PROTECTS THE SKIN FROM UV DAMAGE

Thomas Mammone, Ph.D., David Gan, Earl Goyarts, and Daniel Maes, Ph.D.

Estee Lauder Companies, Melville, NY

Abstract
Aspirin (acetyl salicylate) has long been used as an analgesic. Salicylic acid has previously been reported to have anti-inflammatory properties. These activities include inhibiting activity of Cox-1, Cox-2, and NF-kb. In addition, salicylic acid has also been shown in some systems to induce Hsp70. We have demonstrated that salicylic acid inhibits UVB-induced NF-kb activation in keratinocytes. In addition, salicylic acid was found to induce Hsp 70 in keratinocytes and increase keratinocyte survival to UVB toxicity. In living skin equivalents, salicylic acid was found to reduce UVB induced sunburn cell formation, as well as increase the removal of UVB induced TT dimer formation in living skin equivalents. Given these protective properties of salicylic acid, we propose the use of salicylic acid as a topical therapeutic to protect the skin from sun damage.

Introduction
Willow Bark has been used for centuries in Europe and China, and is still used today for its multiple medicinal properties. The medicinal ingredient, salicylic acid has long been used in modern medicine, initially as an analgesic. Today, aspirin (acetyl salicylate) is taken as an analgesic, anti-inflammatory, blood thinner, and as preventative medicine for heart disease. Salicylic acid has been reported as a Cox-2 inhibitor (Wu, 2003) as well as an inhibitor of NF-kb activity (Kwon, 2003, and Constantz, 2003), which would explain in part, its analgesic properties. In skin care, salicylic acid is used for acne treatment and for skin desquamation. However, it has also been reported that salicylic acid activates the binding of the HSF-1 transcription factor to the heat shock response element upstream of the Hsp70 gene in mammalian cells (Jurivich, 1992). Recently, it was reported that sodium salicylate was found to induce heat shock proteins in mammalian cells (Shimam, 2003). As heat shock proteins such as Hsp70 have been shown to be cytoprotective, it is likely that salicylic acid may be useful as a protective agent in skin.

Methods
NF-kb activation assay:
Normal Human Epidermal Keratinocytes (NHEK) were grown on 100mm plates to 50% confluence in the absence of hydrocortisone. These NHEK were then treated with 0, or 1mM salicylic acid for 6hrs. The keratinocytes were then treated with 0.25mL/cm2 UVB. NF-kb p65 was isolated from NHEKs with the Trans-AM NF-kb p65 kit (Active Motif). The kit contains an ELISA plate with oligonucleotides containing an NF-kb consensus-binding site for sequencing NF-kb present in NHEK nuclear extracts. Detection of the NF-kb protein is via a primary antibody and conjugated secondary antibody. Following the addition of substrate, the enzymatic reaction is allowed to proceed for up to 10 minutes before measuring the absorbance on a spectrophotometer at 450nm.

Hsp70 protein determination:
NHEK were grown to 75% confluence in 6 well plates before being treated with different doses of salicylic acid (0-100mg/ml). These treatments were carried out for 24 hours. Following the post incubation, the keratinocytes were harvested and pelleted. The Hsp70 ELISA kit from StressGen was used to quantify the levels of Hsp70 in the NHEK samples.

UVB Viability Assay:
NHEK were grown to 75% confluence in 6 well plates before being treated with different doses of salicylic acid (0-100mg/ml). Following a 6 hour pre-incubation, the NHEK were exposed to 30, 60, and 90mJ/cm2. The MTS survival assay was done 18hrs post UVB treatment.

Host-cell reactivation assay:
Fibroblasts from a 31 year-old donor (ATCC) were incubated with liposomes containing the pSEAP DNA reporter (SV40 promoter fused to an alkaline phosphatase reporter). Cells were plated in 24 well plates and grown for 24 hours prior to exposure with liposomes loaded with reporter DNA. Fibroblasts were treated for 24hrs prior to transfection and 48hrs after transfection with salicylic acid. The DNA reporter incorporated liposomes were then transfected into the fibroblasts. DNA damage was inflicted with UVB irradiation equivalent to 200, 400, 600, 800, 1000 or 1500 mJ/cm2. The positive control cells were treated with liposomes containing reporter DNA, but not exposed to UVB. Untreated cells did not receive reporter DNA. The vector-encoded alkaline phosphatase is heat stable at 65°C. To each well, 97 ml of assay buffer was added and incubated for 5 min at room temperature prior to adding 3 ml of 1mM MUP. The plate was incubated for 60 min in the dark at room temperature before measuring the fluorescence (excitation: 360 nm and emission: 460 nm).
UVB-induced Sunburn cell formation:
Excised portions (8mm) were taken from living skin equivalents (Organogenesis) and cultured over transwell membrane plates. These excised portions were pretreated topically with either distilled water, or salicylic acid (200μg/ml). After the post-incubation, these excised portions were UVB irradiated at 0, 100 and 150mJ/cm². Following a 24-hour post-incubation, these skin equivalents were fixed in formalin and stored at -4°C. These samples were then sent to Paragon Biotech for H&E staining. Sections were then evaluated using a microscope at 400X magnification. A section was selected from each sample and counts of sunburn cells were made. Sunburn cell levels were determined by %, (number of sunburn cells in a representative field divided by total number of cells in a representative field).

DNA repair of UVB-induced thymine dimers (TT-dimers):
Excised portions (8mm) were taken from living skin equivalents (Organogenesis) and cultured over transwell membrane plates. Excised portions (8mm) were taken from living skin equivalents (Organogenesis) and cultured over transwell membrane plates. These excised portions were pre-treated topically with salicylic acid 200μg/ml (in sterile H2O) for 6 hours. After the post-incubation, these excised portions were UVB irradiated at 0, 100, and 150 mJ/cm². Following a 24-hour post-incubation, these LSEs were fixed in formalin and stored at -4°C. These samples were then sent to Paragon Biotech for immunostaining of TT-dimers. Sections were then evaluated using a microscope at 400X magnification. Representative sections were selected from each sample and counts of cells at 400X magnification expressing TT-dimer staining were calculated. The TT-dimer levels of each section were expressed as number of cells expressing the immunofluorescent TT-dimer antibody tagged stain in that section.

Conclusions
Salicylic acid was found to have anti-inflammatory effect on normal human epidermal keratinocytes. We observed a significant inhibition of UVB induced NF-kB activation in keratinocytes. In addition, salicylic acid was found to significantly increase the induction of Hsp70 protein in keratinocytes. Salicylic acid was also found to increase keratinocyte cell viability to UVB toxicity, as well as increase DNA repair function of normal human dermal fibroblasts using the host cell reactivation assay. In living skin equivalents, salicylic acid significantly reduce UVB-induced sunburn cell formation and increase DNA repair (removal rate of DNA damage). These results suggest that salicylic acid which has previously been described to have anti-inflammatory properties as well as induce Hsp70 in mammalian cells may have benefits if used topically on the skin. Salicylic acid can act as an anti-inflammatory in the skin as well as increase DNA repair function in skin and may be a valuable topical agent to protect skin from sun damage.

References:
An extract of Salix nigra: an efficacious, safe remedy for problem skin.
(extract from black willow bark shows promise as cosmetic ingredient)(includes 
formulation for textured skin lotion)

Drug & Cosmetic Industry
03-01-1997

Introduction

Current research has shown that in their daily fight for survival, plants employ 
a host of defense mechanisms. An: important facet of their immune response 
involves endogenous signal molecules, many of which have been identified. 
Salicylic acid has been identified as one of these molecules. It functions 
directly in the plant defense response to pathogens.

Salix nigra, or Black Willow, is a tree found throughout North America. The bark 
of this tree is a source of salicylic acid-like ingredients. When added to 
cosmetic formulations, the extract can increase cell renewal and boost the 
anti-microbial capabilities of the formulation. Even though the extract is a 
source of salicylic acid-like ingredients and is able to contribute effects 
similar to those seen from salicylic acid, it has none of the drawbacks 
associated with salicylic acid--mainly irritation. The extract is a safe way to 
get the benefits of a b-hydroxy acid without the risk of irritation.

Materials and Methods

The cell renewal capabilities of the Willowbark Extract and the liposomal 
Willowbark Extract were tested versus the cell renewal capabilities of 1% 
salicylic acid. The concentration of the Extract was 10%, which corresponds to a 
1% concentration of salicylic acid. A dansyl chloride protocol was followed 
using twenty female panelists. Results are shown in Figure I.

The antimicrobial activity of the Willowbark Extract and the liposomal 
Willowbark Extract was tested and compared to the activity of salicylic acid. 
The extract was tested at 100% concentration and the salicylic acid was at 10% 
concentration. Zone of Inhibition protocol was followed where the organism was 
streaked onto agar and allowed to grow to confluence. A sterile blank paper disk 
was placed on the agar and the test material was dispensed onto the disk. The 
agar plates were incubated and after the appropriate time, the zone of clearance 
around the paper disks was measured in millimeters. Organisms tested were 
Staphylococcus aureus and Propionibacterium acnes, two of the skin flora 
implicated in the formation of acne. Results versus Staphylococcus aureus are 
shown in Figure II. Results versus Propionibacterium acnes are shown in Figure 
III.

The extract was also safety tested using a variety of in vivo and in vitro 
protocols. The CAMVA was used to determine irritancy. This in vitro assay 
determines the irritancy of a test compound based on its ability to induce 
hemorrhage on the chorioallantoic membrane of a chicken egg. Two other in vitro 
tests were run on Willowbark Extract--EpiDerm and EpiOcular. EpiDerm is a
three-dimensional system composed of human epithelial cells to which the test compound is applied. After incubation, the number of viable cells is measured using the MTT conversion assay. An \( \text{ET}_{50} \) is determined, which gives an idea of potential skin toxicity. EpiOcular is a three-dimensional system composed of stratified human keratinocytes to which the test material is applied. After incubation, the number of viable cells is measured using the MTT conversion assay. An \( \text{ET}_{50} \) is determined, which gives an idea of possible ocular irritation. Results are shown in Figure IV.

A fifty-person RIPT was run on Willowbark Extract to assess its ability to induce skin irritation and sensitization. The method is modified from the 200 person methodology cited in the reference Appraisal of the Safety of Chemicals in Food Drugs and Cosmetics. The material was tested at 100% concentration and underwent nine inductive patchings.

Results

Results from the cell renewal testing are found in Figure I. The Willowbark Extract was found to increase stratum corneum turnover more so than salicylic acid—24 percent as opposed to 22 percent. The liposomal form of the Willowbark Extract gave a 26.1 percent increase. Figure II gives results on the antimicrobial activity against Staphylococcus aureus. Willowbark Extract and liposomal Willowbark Extract performed the best, giving zones of clearance of 11mm and 13mm respectively, as compared to a 6mm zone of clearance for salicylic acid. Against Propionibacterium acnes (shown in Figure III), Willowbark Extract gives a zone of 4mm, liposomal Willowbark Extract is 6mm, and salicylic acid is 3mm.

The CAMVA gave an \( \text{RC}_{50} \) value of 28 percent. This value is indicative of a material that is not a primary irritant. The results for EpiDerm and EpiOcular are detailed in Figure IV. For Willowbark Extract, the \( \text{ET}_{50} \) for the EpiDerm was >24 hours and for the EpiOcular it was >240 minutes. In comparison, salicylic acid yielded \( \text{ET}_{50} \) values of 19.3 hours for EpiDerm and 14.8 minutes for EpiOcular. The Willowbark Extract gave scores similar to the scores for glycerin, whereas salicylic acid scored more closely to Triton X-100, the positive control for the system.

Discussion

The efficacy results given above indicate a material that has cell renewal and antimicrobial activities that are better than salicylic acid. Willowbark Extract is better able to increase turnover of the stratum corneum and also has more in vitro antimicrobial activity against Staphylococcus aureus and Propionibacterium acnes. Coupled with this increased efficacy, the Willowbark Extract has less irritation potential than salicylic acid. The safety testing done on the Willowbark Extract clearly shows this. The EpiDerm and EpiOcular Assays made actual comparisons between Willowbark Extract and salicylic acid, and the natural extract proved to be much less irritating.

Conclusion

Willowbark Extract is a safe, efficacious natural extract for use in a variety of cosmetic formulations.

[TABULAR DATA OMITTED]