Intracellular Calcium Assay

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Tradename: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 8059

Lot #: 8061700

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principal Investigator: *Daniel Shill*

Test Performed:
Intracellular Calcium Assay

Introduction

Intracellular calcium concentrations, often linked to light exposure, play a key role in skin health and immune response as it can help reduce inflammation, with a direct effect on tissue repair and keratinocyte function. Calcium also helps maintain skin moisture levels and promotes the production of antioxidants.

Keratinocytes, making up 90-95% of the epidermis, are a physical and chemical barrier for the skin by minimizing UV radiation damage, water loss, and microbial, viral, and parasitic invasion. Accordingly, keratinocytes must progressively proliferate, differentiate, and migrate to maintain skin barrier integrity and homeostasis. Provided keratinocyte proliferation, differentiation, and migration are calcium dependent, intracellular calcium levels can serve as a surrogate for skin health.

A Fluo-4 Direct™ Calcium Assay was performed to assess changes in intracellular calcium levels in **ABS White Willow Bark Extract Powder**-treated human epidermal keratinocytes *in vitro*.

Assay Principle

The Fluo-4 Direct™ Calcium Assay Kit detects intracellular calcium by utilizing Fluo-4, a cell-permeant fluorescent dye. Upon entering the cell and binding to free calcium, Fluo-4 exhibits fluorescence signals when excited at 488 nm providing an indication of intracellular calcium. A suppression dye, which reduces non-intracellular fluorescence and inhibits extracellular transport, is included to eliminate background fluorescence from the complete media and prevent the movement of Fluo-4 out of the cell. An increase in fluorescent signal intensity generated by labeled calcium molecules is indicative of higher levels of intracellular calcium.

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Materials

- **A. Kit:** Fluo-4 Direct™ Calcium Assay Kit (ThermoFisher; F10471)*
- **B. Incubation Conditions:** 37°C, 5% CO_2, and 95% relative humidity (RH)
- **C. Equipment:** Forma Humidified Incubator; ESCO Biosafety Laminar Flow Hood; Synergy HT Microplate Reader; Pipettes; Light Microscope
- **D. Cell Line:** Normal Human Epidermal Keratinocytes (ATCC; PCS-200-011)*
- **E. Media/Buffers:** Keratinocyte Basal Medium (ATCC; PCS-200-030)*; Keratinocyte Growth Kit (ATCC; PCS-200-040)*
- **F. Reagents:** Calcium chloride ($CaCl_2$) (Carolina; 85-1800)*; Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (EMD Millipore; 32-462-6)*
- **G. Culture Plate:** Flat Bottom 96-well Tissue Culture Treated Microplates
- **H. Other:** Sterile disposable pipette tips

*Or suitable alternatives, subject to change without notice based off vendor availability

Methods

Human epidermal keratinocytes were seeded into a 96-well tissue culture plate and allowed to grow to confluency in complete media (CM). 0.01%, 0.1%, and 1.0% concentrations of **ABS White Willow Bark Extract Powder** were added to CM and incubated with keratinocytes. As experimental controls, CM containing 1 mM $CaCl_2$ was utilized as a positive control to increase intracellular calcium levels, and 2 mM EGTA in the presence of 1 mM $CaCl_2$ was utilized as a negative control to reduce the $CaCl_2$-induced increases in intracellular calcium. After a 60-minute incubation at 37°C, 50 µL of 2X Fluo-4 Direct™ calcium reagent loading solution was added to all wells and the plate was returned to 37°C for 60 minutes, followed by 30 minutes at room temperature. Next, fluorescence measurements (excitation 494 nm, emission 516 nm) were taken on a Synergy H1 Microplate Reader.

Three separate experiments were performed with conditions in duplicate and average values were recorded. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Intracellular Calcium results are shown as Relative Fluorescence Units (RFU) and expressed as percentage change, relative to untreated keratinocytes, calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{RFU_{Sample} - RFU_{CM}}{RFU_{CM}} \times 100$$

Results

The data obtained from this study met criteria for a valid assay and the experimental controls performed as anticipated. Compared to untreated keratinocytes, the addition of $CaCl_2$ to CM enhanced intracellular calcium levels, whereas the addition of EGTA to $CaCl_2$ and CM reduced the $CaCl_2$-induced increase in intracellular calcium levels. Keratinocytes treated with **ABS White Willow Bark Extract Powder** at 0.01%, 0.1%, and 1.0% augmented intracellular calcium levels compared to CM alone.

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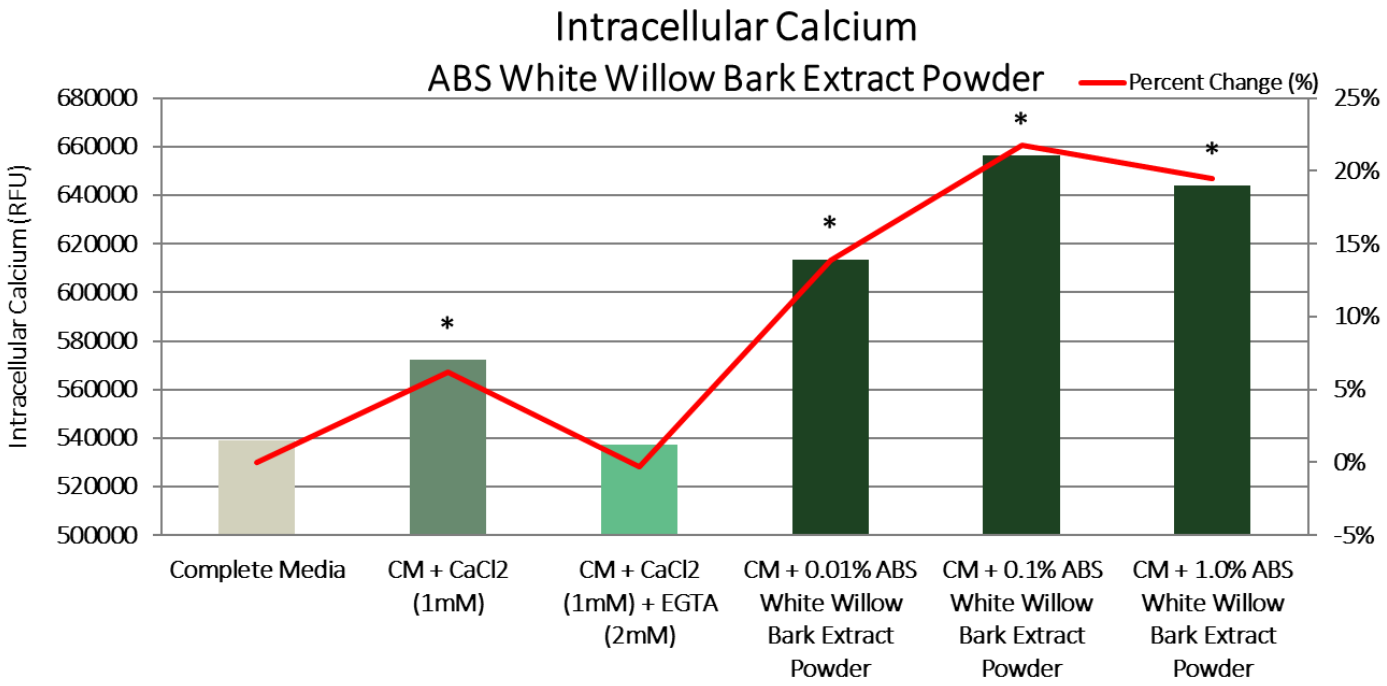

Figure 1. The effect of **ABS White Willow Bark Extract Powder** on keratinocyte intracellular calcium levels. * indicates significance ($p \leq 0.05$) compared to Complete Media (untreated fibroblasts).

Table 1. Results from one-way ANOVA Statistical Analysis Compared to Complete Media. * indicates significance ($p \leq 0.05$) compared to Complete Media (untreated fibroblasts).

	$CaCl_2$	$CaCl_2$ + EGTA	0.01% **ABS White Willow Bark Extract Powder**	0.1% **ABS White Willow Bark Extract Powder**	1.0% **ABS White Willow Bark Extract Powder**
P-value	0.021*	> 0.05	< 0.001*	< 0.001*	< 0.001*

Discussion

As shown in Figure 1, adding $CaCl_2$ to complete media increased keratinocyte intracellular calcium by 6% compared to untreated keratinocytes. Conversely, when EGTA was introduced to $CaCl_2$ and complete media, intracellular calcium was reduced by 6% compared to $CaCl_2$ alone. These data establishing the experimental model by demonstrating keratinocyte intracellular calcium levels are dynamic and can be manipulated with exogenous compounds.

Similarly, keratinocytes treated with **ABS White Willow Bark Extract Powder** at 0.01%, 0.1%, and 1.0% enhanced intracellular calcium levels by 14%, 22%, and 20% compared to complete media alone, respectively (Table 1). These data demonstrate **ABS White Willow Bark Extract Powder** augments keratinocyte intracellular calcium levels.

Collectively, intracellular calcium levels are an indicator of keratinocyte proliferation, differentiation, and migration, which contribute to the preservation of skin barrier integrity and homeostasis. These data indicate **ABS White Willow Bark Extract Powder** augments intracellular calcium levels, which may help to maintain overall skin health.