COX-II Inhibition Assay

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Tradename: ABS Willow Bark Extract

Code: 10200

CAS #: 84650-64-6

Test Request Form #: 1037

Lot #: 36108

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

Test Performed:
COX-II Inhibition Assay

Introduction

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme exhibiting both COX and peroxidase activities. The COX component converts arachidonic acids to hydroperoxy-endoperoxide (PGG_2), and the peroxide component reduces endoperoxide to the corresponding alcohol (PGH_2), the precursor of prostaglandins (PGs), thromboxanes, and prostacyclins. It is well established that there are two isoforms of COX. COX-I is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. Stimuli, such as phorbol esters, lipopolysaccharides, and cytokines lead to the induced expression of the second isoform, COX-II. COX-II is responsible for the biosynthesis of inflammatory mediators. COX-II is also known to play a role in aging due to increased expression in aged and photodamaged skin. A cosmetic product that decreases COX-II is therefore a beneficial anti-aging weapon.

Accordingly, a COX-II Inhibition Assay was conducted to assess the ability of **ABS Willow Bark Extract** to inhibit COX-II *in chemico* and *in vitro*.

Assay Principle

The COX-II Inhibitor Screening Assay directly measures Prostaglandin F2α ($PGF_{2\alpha}$) by stannous chloride ($SnCl_2$) reduction of COX-derived PGH_2 produced in the COX reaction. Stannous chloride is used to reduce PGH_2 to a more stable prostaglandin, $PGH_{2\alpha}$. The $PGF_{2\alpha}$ is quantified via enzyme immunoassay (ELISA) which binds all major PG compounds using a specific antiserum. This assay is based on the competition between PGs and PG-acetylcholinesterase (AChE) conjugate for binding with antiserum-PG. The amount of PG-AChE conjugate will be held constant while the concentration of PGs varies depending on the sample, therefore, the concentration of sample PGs in the well will be inversely proportional to the amount of PG-AChE that is able to bind to the $PGF_{2\alpha}$ antibody. Ellman's Reagent is used to visualize the reaction of PG-AChE conjugate bound to antiserum-PG, which has a distinct yellow color that is quantitated through optical density (OD) readings on a microplate spectrometer. The standard curve, along with manufacturer specified calculations provides a reference from the OD readings for the amount of COX-II in each sample.

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Materials

A.	**Kit:**	COX-II Inhibitor Screen Assay Kit (Cayman Chemical; 560131)*
B.	**Incubation Conditions:**	37°C at 5% CO_2 and 95% relative humidity (RH)
C.	**Equipment:**	Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes
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)*; Phosphate Buffered Saline (PBS); Amphotericin (45 pg/mL)
F.	**Culture Plate:**	Falcon flat bottom 24-well tissue culture treated plates
G.	**Reagents:**	Dexamethasone (DEX)
H.	**Other:**	Sterile disposable pipette tips; wash bottles

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

Methods

Initially, undiluted **ABS Willow Bark Extract** and 10 mM (0.392%) DEX were tested directly to demonstrate the *in chemico* function of each test article. Next, human epidermal keratinocytes were seeded into 24-well tissue culture plates and allowed to grow to confluency in Complete Media. 1.0% **ABS Willow Bark Extract** and DEX were added to Complete Media and incubated with keratinocytes for 72 hours. DEX was utilized as a positive control at 1.0 µM (0.0000392%) to inhibit COX-II. Complete Media was used as an untreated control. After a 72-hour incubation, the supernatants were collected and utilized for the assay. Additionally, all test materials were utilized in the COX-II Inhibitor Screening Assay Kit according to the manufacturer instructions.

Briefly, prostaglandin standards were prepared in concentrations ranging from 0 pg/mL to 2000 pg/mL. 100 µL of ELISA buffer was added to the 'Non-Specific Binding' (NSB) wells and 50 µL was added to the 'Maximum Binding' wells. 50 µL of each sample, standard, and internal assay control was added in duplicate to appropriate wells. Next, 50 µL of PG-AChE conjugate was added to all wells. Finally, 50 µL of the PG Antiserum was added to all wells except the NSB wells and incubated at room temperature for 18 hours on an orbital shaker. After incubation, all wells were aspirated and rinsed with Wash Buffer five times. 200 µL of Ellman's Reagent was added to each well and 5 µL of PG-AChE conjugate was added to the '100% Activity' control wells. Following a 1-hour at room temperature protected from light on an orbital shaker, the absorbance was measured at 412 nm on the Synergy HT Microplate Reader.

Assays were repeated three times with each sample run in duplicate. Duplicates for each replicate were averages, and the average of all three experiments is displayed. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. A standard curve was created by reducing the data and generating a 4-parameter logistic curve fit. The COX-II concentration of **ABS Willow Bark Extract** was determined by extrapolation from the standard curve and expressed in terms of percent inhibition. Percent inhibition of COX-II production is calculated by the following formula:

$$Percent\ Inhibition\ (\%) = \frac{(\%Bound/Max\ Bound)_{COX-II\ 100\%\ Activity} - (\%Bound/Max\ Bound)_{Sample}}{(\%Bound/Max\ Bound)_{COX-II\ 100\%\ Activity}} \times 100$$

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Results

The data obtained met criteria for a valid assay and the positive control performed as anticipated. The *in chemico* analysis demonstrated DEX and 100% **ABS Willow Bark Extract** inhibited COX-II compared to the Activity Control. Similarly, the *in vitro* analysis demonstrated keratinocytes treated with DEX and 1.0% **ABS Willow Bark Extract** inhibited COX-II compared to untreated keratinocytes.

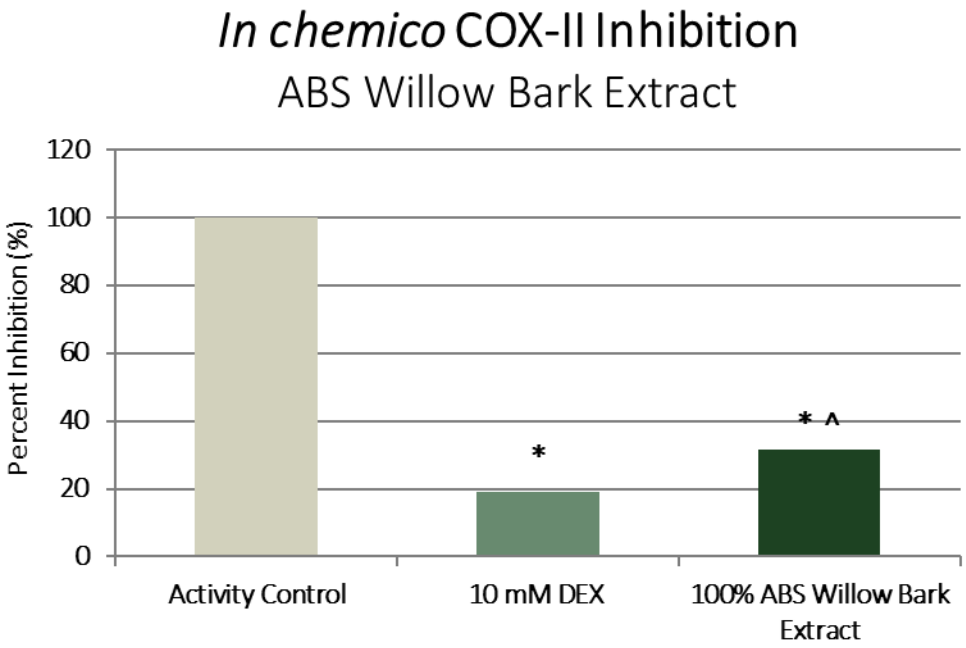

Figure 1. The *in chemico* effect of DEX and **ABS Willow Bark Extract** on COX-II Inhibition. * indicates significance ($p \leq 0.05$) compared to the COX-II Activity Control. ^ indicates significance ($p \leq 0.05$) between DEX and **ABS Willow Bark Extract**.

Table 1. Results from one-way ANOVA Statistical Analysis between *in chemico* conditions. * indicates significance ($p \leq 0.05$) compared to the COX-II Activity Control. ^ indicates significance ($p \leq 0.05$) between DEX and **ABS Willow Bark Extract**.

	10 mM DEX	100% **ABS Willow Bark Extract**
Activity Control	0.033*	0.038*
10 mM DEX	-----	0.048^

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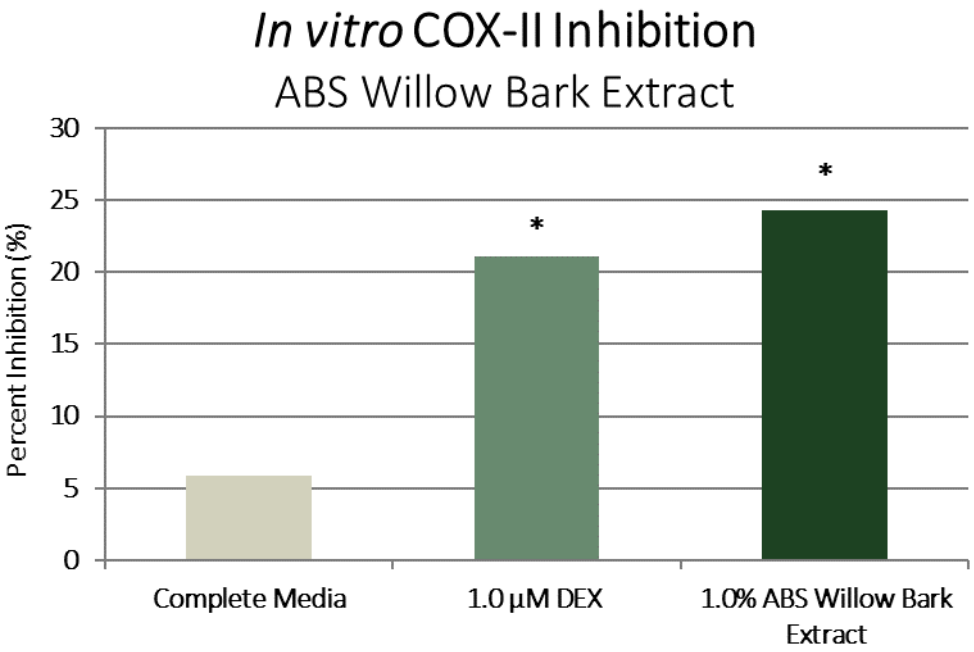

Figure 2. The *in vitro* effect of DEX and **ABS Willow Bark Extract** on COX-II Inhibition in Keratinocytes. * indicates significance ($p \leq 0.05$) compared to untreated keratinocytes (Complete Media).

Table 2. Results from one-way ANOVA Statistical Analysis between *in vitro* conditions. * indicates significance ($p \leq 0.05$) compared to untreated keratinocytes (Complete Media).

	1.0 µM DEX	1.0% **ABS Willow Bark Extract**
Complete Media	0.022*	0.002*
1.0 µM DEX	-----	> 0.05

Discussion

As shown in Figure 1, DEX, a known COX-II inhibitor, elicited a 19% inhibition in COX-II compared to the Activity Control when tested *in chemico*. Similarly, 100% **ABS Willow Bark Extract** elicited a 31% inhibition of COX-II compared to the Activity Control and was significantly more effective than DEX (Table 1). This data demonstrates **ABS Willow Bark Extract** has COX-II inhibiting properties *in chemico*.

Keratinocytes incubated with Complete Media alone elicited a 6% inhibition in COX-II (Figure 2). Conversely, DEX exhibited a 21% inhibition in COX-II and significantly outperformed Complete Media alone (Table 2). Similarly, keratinocytes treated with 1.0% **ABS Willow Bark Extract** demonstrated a 24% inhibition in COX-II and significantly outperformed Complete Media alone (Table 2). These data demonstrate **ABS Willow Bark Extract** inhibits COX-II *in vitro.*

Collectively, decreases in COX-II indicate a reduced inflammatory environment which can decrease the signs of aging and photodamage. These data indicate **ABS Willow Bark Extract** inhibits COX-II, which may help to attenuate characteristics of cellular aging due to inflammation.