

Tradename: AC WonderShroom

Code: 21029

CAS #: 84650-60-2 & 999999-99-4 (or) 68917-13-5 & 999999-99-4 (or) 68917-13-5 & N/A (or) 9057-02-7

Test Request Form #: 11685

Lot #: N240425D

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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 (PGG₂), and the peroxide component reduces endoperoxide to the corresponding alcohol (PGH₂), 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 **AC WonderShroom** to inhibit COX-II in epidermal keratinocytes. L-theanine, a standardized component in **AC WonderShroom**, was also tested to demonstrate the superior nature of **AC WonderShroom** compared to L-theanine alone.

Assay Principle

The COX-II Inhibitor Screening Assay directly measures Prostaglandin F_{2α} (PGF_{2α}) by stannous chloride (SnCl₂) reduction of COX-derived PGH₂ produced in the COX reaction. Stannous chloride is used to reduce PGH₂ to a more stable prostaglandin, PGH_{2α}. The PGF_{2α} 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_{2α} 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.

Materials

- A. **Kit:** COX-II Inhibitor Screen Assay Kit (Cayman Chemical; 560131)*
- 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 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

Human epidermal keratinocytes were seeded into 24-well tissue culture plates and allowed to grow to confluency in Complete Media. 0.01%, 0.05%, and 0.1% **AC WonderShroom** were added to Complete Media and incubated with keratinocytes for 72 hours. Additionally, a solution of 0.0025% L-theanine was prepared in CM and placed on cells. This concentration is equivalent to the maximum standardized amount of L-theanine in 0.1% **AC WonderShroom**. DEX was utilized as a positive control at 1.0 μM to inhibit COX-II. Complete Media was used as an untreated control. After a 72-hour incubation, the supernatants were collected and utilized in the COX-II Inhibitor Screening Assay Kit according to the manufacturer instructions.

Briefly, COX reactions were performed with all samples to be utilized in the ELISA. The Inhibitor Control was generated by inactivating the COX-II enzyme before the reaction. Next, prostaglandin standards were prepared in concentrations ranging from 0 pg/mL to 500 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 for 18 hours at 4°C. 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 Enzyme Activity Control wells. Following a 1.5-hour incubation at room temperature protected from light on an orbital shaker, the absorbance was measured at 410 nm on the Synergy HT Microplate Reader.

Assays were repeated three times with each sample run in duplicate. Duplicates for each replicate were averaged, 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 **AC WonderShroom** 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:

$$\text{Percent Inhibition (\%)} = \frac{(\text{COX - II Concentration})_{\text{Enzyme Activity Control}} - (\text{COX - II Concentration})_{\text{Sample}}}{(\text{COX - II Concentration})_{\text{Enzyme Activity Control}}} \times 100$$

Results

The data obtained met criteria for a valid assay and the positive control performed as anticipated. The keratinocytes treated with DEX, L-theanine, and **AC WonderShroom** inhibited COX-II compared to untreated keratinocytes.

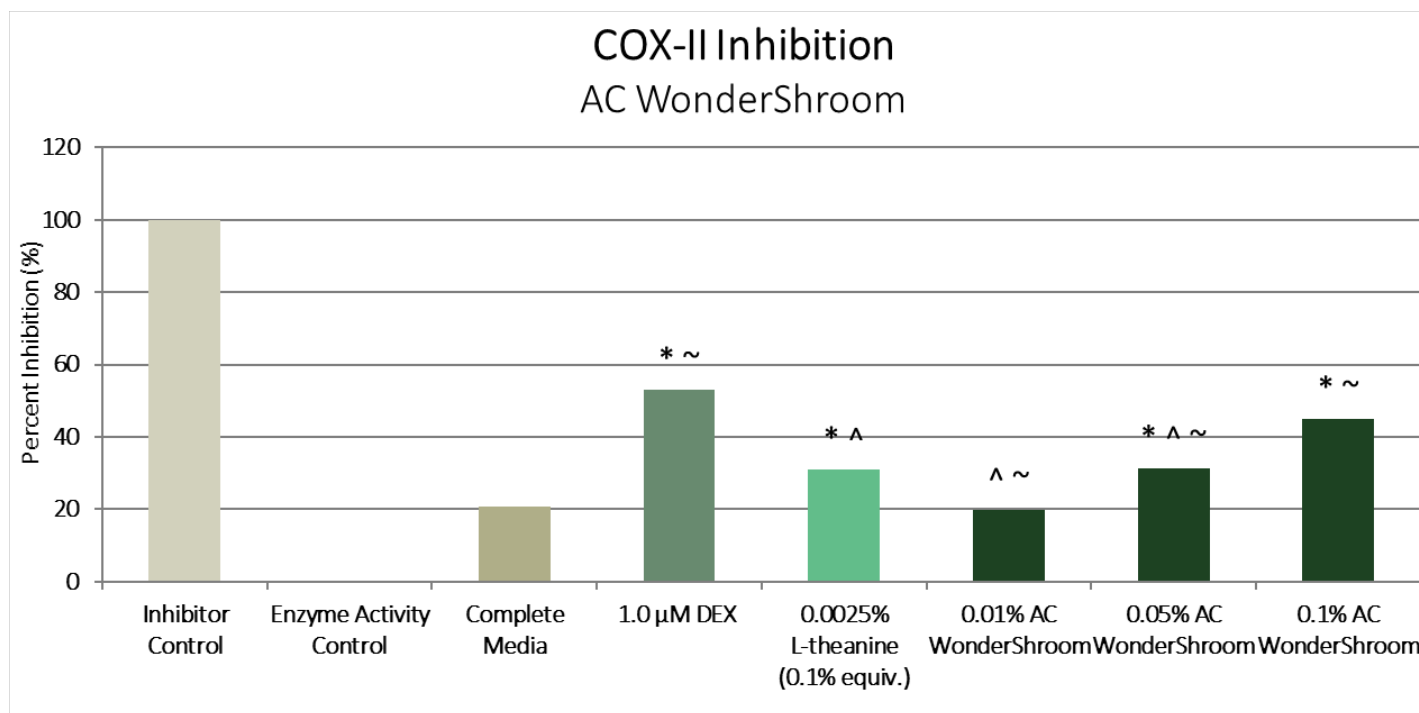


Figure 1. The effect of **AC WonderShroom** on COX-II Inhibition in Keratinocytes. * indicates significance ($p \leq 0.05$) compared to Complete Media. ^ indicates significance ($p \leq 0.05$) compared to DEX. ~ indicates significance ($p \leq 0.05$) compared to L-theanine. 0.0025% L-theanine is equivalent to the maximum standardized amount of L-theanine in 0.1% **AC WonderShroom**.

Table 1. Results from one-way ANOVA Statistical Analysis between conditions. * indicates significance ($p \leq 0.05$) compared to Complete Media. ^ indicates significance ($p \leq 0.05$) compared to DEX. ~ indicates significance ($p \leq 0.05$) compared to L-theanine.

	1.0 μM DEX	0.0025% L-theanine	0.01% AC WonderShroom	0.05% AC WonderShroom	0.1% AC WonderShroom
Complete Media	0.029*	0.001*	> 0.05	0.045*	< 0.001*
1.0 μM DEX	-----	0.004^	0.035^	0.031^	> 0.05
0.0025% L-theanine	0.004~	-----	0.046~	0.001~	< 0.001~

Discussion

As shown in Figure 1, keratinocytes incubated with Complete Media alone elicited a 20.7% inhibition in COX-II. Conversely, DEX exhibited a 53.0% inhibition in COX-II and significantly outperformed Complete Media alone (Table 1). These data demonstrate that COX-II in keratinocytes is dynamic and can be manipulated with exogenous compounds.

Similarly, keratinocytes treated with 0.01%, 0.05%, and 0.1% **AC WonderShroom** demonstrated a 19.9%, 31.4%, and 44.9% inhibition in COX-II, respectively, and significantly outperformed Complete Media alone (Table 1). Comparatively, L-theanine only inhibited COX-II by 30.9% compared to Complete Media and was significantly less effective than the equivalent 0.1% **AC WonderShroom** (Table 1). These data demonstrate **AC WonderShroom** inhibits COX-II in keratinocytes and highlight the superior nature of the product compared to the actives alone.

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