Reactive Oxygen Species Scavenging Assay

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Tradename: PhytoCide Lichen

Code: M16008

CAS #: 513-85-9 & 92346-91-3

Test Request Form #: 9398

Lot #: N220621I

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Study Director: *Maureen Danaher*
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Test Performed:
Reactive Oxygen Species Scavenging Assay

Introduction

Low levels of intracellular oxidative stress are produced during normal physiological functions. However, UV irradiation, pollutants, foreign substances, and aging elicit unrestricted increases in reactive oxygen species (ROS). These deregulated augmentations in oxidative stress lead to an acceleration of DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation. Moreover, when intrinsic antioxidant capacities are reduced, such as during aging, an imbalance between pro- and anti-oxidant systems further accentuates these hallmarks of cellular aging.

Accordingly, a ROS Scavenging Assay was conducted to assess the *in vitro* effect of **PhytoCide Lichen** to scavenge unnecessary oxidative stress in dermal fibroblasts. Attenuating excessive ROS preserves cellular homeostasis and blunts intrinsic and extrinsic age-related declines in skin cell function.

Assay Principle

Two cell-permeant dyes, CellROX™ Orange Reagent and Hoechst, were utilized in conjunction to provide a specific and quantitative method for determining ROS levels. CellROX™ Orange Reagent fluoresces brightly when bound to ROS indicating oxidative stress, and Hoechst fluoresces when bound to nuclear DNA to indicate cellular nuclei. By displaying the relative fluorescent units (RFU) from the CellROX™ Orange Reagent (ROS Signal) as a function of Hoechst (Nuclear Signal), ROS can be quantified and normalized at the cellular level. To elicit supraphysiological mitochondrial- and non-mitochondrial-derived levels of oxidative stress, the cells were exposed to Antimycin A, a complex III inhibitor of the mitochondrial electron transport chain.

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Materials

A. Kit:	CellROX™ Orange Reagent (ThermoFisher Scientific, C10443)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% Relative Humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Synergy HT Microplate Reader; Pipettes
D. Cell Line:	Normal Human Neo-Natal Dermal Primary Fibroblasts (ATCC PCS-201-010)
E. Media/Buffers:	Fibroblast Basal Medium (PCS-201-030); Fibroblast Growth Kit (PCS-201-041); Ethanol; Phosphate Buffered Saline (PBS)
F. Reagents:	Hoechst 33342 (ThermoFisher Scientific, 62249); Antimycin A (Sigma Aldrich, A8674)
G. Culture Plate:	96 Well Black Side/Clear Bottom Tissue Culture Treated Microplates
H. Other:	Sterile disposable pipette tips

Methods

Human dermal fibroblasts were seeded into a 96-well tissue culture microplate and grown to 80%-90% confluency in complete media (CM). 0.01%, 0.1% and 1.0% concentrations of **PhytoCide Lichen** in CM were added to cells and placed at 37°C. Control wells were incubated with CM only. Following an 18-hour incubation, the media in all wells was removed and cells were washed once with PBS. Hoechst and CellROX™ Orange were diluted in CM, and added to all wells at final concentrations of 20 µM and 5 µM, respectively. Following a 30-minute incubation at 37°C, the Hoechst and CellROX™ Orange solution was removed and cells were washed once with PBS. Next, 200 pM of Antimycin A (AntA), initially dissolved in ethanol and further diluted in CM, was added to all wells, except control wells that received CM. Following another 30-minute incubation at 37°C, the AntA and CM was removed, CM was added to all wells, and fluorescence measurements were taken with the following wavelengths (excitation / emission): Hoechst (361 nm / 486 nm) and CellROX™ Orange (545 nm / 565 nm).

To account for differences in cell counts, ROS levels are expressed as the ROS Signal (CellROX™ Orange) divided by the Nuclear Signal (Hoechst), as calculated by the following equation:

$$ROS\ Levels = \frac{ROS\ Signal}{Nuclear\ Signal}$$

Percent change is expressed relative to AntA and calculated by the following equation:

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

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Results

The data obtained from this study met criteria for a valid assay and the positive control performed as anticipated. Compared to untreated fibroblasts, AntA (200 pM) increased ROS levels. Fibroblasts treated with **PhytoCide Lichen** at 0.01%, 0.1%, and 1.0% exhibited a reduction in oxidative stress levels compared to fibroblasts exposed to AntA.

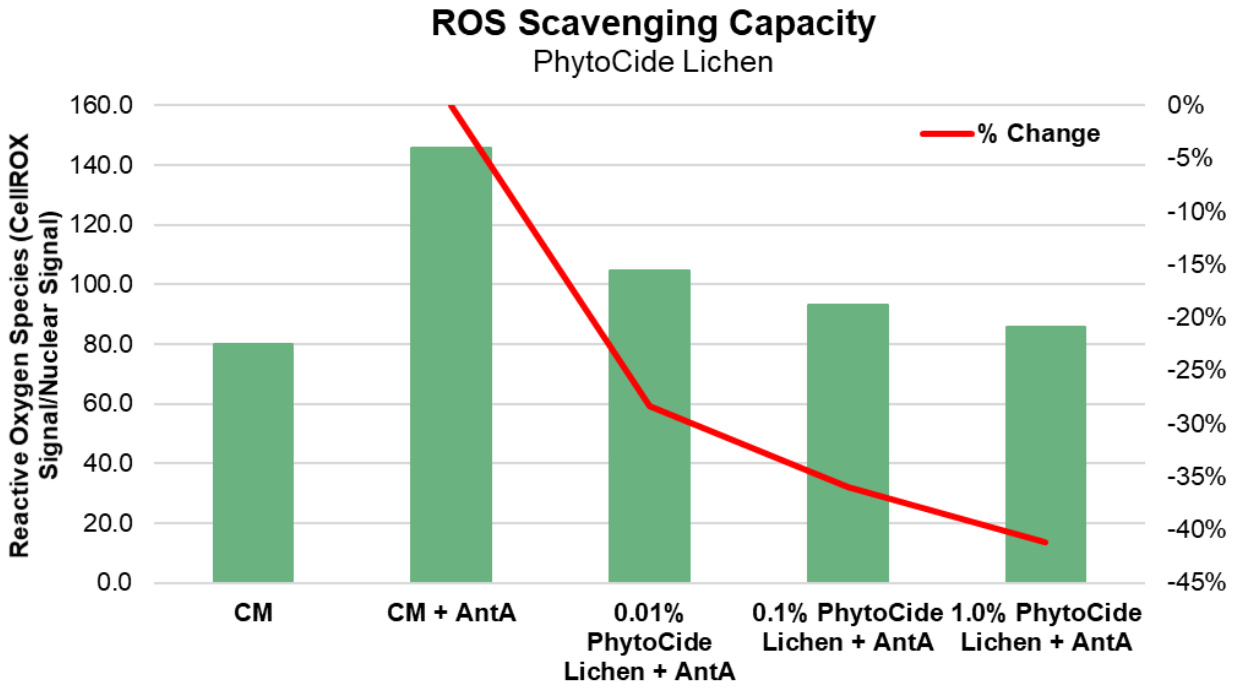


Figure 1: The effect of **PhytoCide Lichen** on ROS scavenging.

Discussion

As shown in Figure 1, fibroblasts incubated with AntA, a known inducer of oxidative stress, elicited an 82% increase in ROS levels, compared to untreated fibroblasts. These data demonstrate the supraphysiologic level of ROS induced by AntA and the magnitude of ROS in fibroblasts is dynamic.

Conversely, fibroblasts treated with **PhytoCide Lichen** at 0.01%, 0.1%, and 1.0% demonstrated 28%, 36%, and 41% reductions in ROS levels compared to fibroblasts treated with AntA, respectively. These data demonstrate **PhytoCide Lichen** attenuates excessive oxidative stress.

Collectively, intrinsic and extrinsic factors perturb skin homeostasis by stimulating abundant levels of ROS that amplify DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation. These data indicate **PhytoCide Lichen** scavenges unnecessary ROS, which may help to attenuate characteristics of cellular aging.

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