OECD 456:
H295R STEROIDOGENESIS ASSAY

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Tradename: PhytoCide Lichen

Code: M16008

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

Test Request Form #: 11521

Lot #: 9394500

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

Test Performed:
OECD 456: H295R Steroidogenesis Assay

Introduction

Endocrine disrupting agents are substances that interfere with the endocrine system and are associated with several negative health effects. Located throughout the body, endocrine glands release hormones into circulation and these signaling molecules are vital to maintaining homeostasis. Endocrine glands and hormones are extremely sensitive and small disruptions to the structure or function of any aspect of the endocrine system significantly alter physiological processes. Endocrine disruptors work through one or a combination of the following mechanisms: 1) mimicking, or partly mimicking, naturally occurring hormones, potentially leading to over production, 2) binding to a receptor and blocking a naturally occurring hormone from binding, and/or 3) interfering with or preventing the synthesis or utilization of natural hormones and/or receptors. The OECD 456: H295R Steroidogenesis Assay was developed as an *in vitro* test to screen for test article effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T).

A H295R Steroidogenesis Assay was conducted to determine the potential of PhytoCide Lichen to induce or inhibit the production of 17β-estradiol and testosterone.

Assay Principle

The *in vitro* H295R Steroidogenesis Assay utilizes a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 "*in vitro* assay, providing mechanistic data" to screen for test article effects on steroidogenesis, but does not aim to provide specific mechanistic information concerning the interaction of the test substance with the endocrine system. Moreover, the H295R assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic pituitary-gonadal axis. The objective of the H295R Steroidogenesis Assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone (T). The H295R assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions from cholesterol to the production of E2 and/or T. Human adreno-carcinoma H295R cells are utilized because the cell line expresses genes that encode all key steroidogenesis enzymes, enables the detection of both increases and decreases in the production of both E2 and T, and permits the direct assessment of the potential impact of a test article on cell viability/cytotoxicity. The purpose of the assay is the detection of substances that affect E2 and T production by distinguishing test articles that typically inhibit or induce key enzymes of the steroidogenesis pathway.

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The human adreno-carcinoma H295R cells are exposed to different concentrations of the test substance. After the incubation period, the medium is removed from each well and cellular viability is immediately analyzed. Next, concentrations of E2 and T in the medium are measured.

Materials

A. Kit:	Estradiol ELISA Kit (Cayman Chemical; 501890)*; DetectX® Testosterone ELISA Kit (Arbor Assays; K032-H)*; CyQUANT™ MTT Cell Viability Assay (ThermoFisher; V13154)*
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; Weigh Boats; Chemical Spatula; Analytical Balance
D. Cell Line:	Human Adreno-Carcinoma Cell (NCI-H295R [H295R]) (ATCC; CRL-2128)*
E. Media/Buffers:	DMEM: F-12 Medium (ATCC; 30-2006)*; ITS+ Premix Universal Culture Supplement (Corning®; 354352)*; Nu-Serum™ Growth Medium Supplement (Corning®; 355100)*; Dimethyl sulfoxide (DMSO); Deionized water
F. Reagents:	Forskolin (ThermoFisher; J63292.MF)*; Prochloraz (ThermoFisher; P21371G)* Phosphate Buffered Saline (PBS); Trypsin; Trypsin-EDTA; Methanol; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT Assay; ThermoFisher; V13154); SDS-HCl Solution (sodium dodecyl sulfate hydrochloride) (MTT Assay; ThermoFisher; V13154)
G. Culture Plate:	24 Well Tissue Culture Treated Microplates; Tissue Culture Treated Flasks
H. Software:	Excel Analysis ToolPak (Microsoft)
I. Other:	Sterile disposable pipette tips

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

Methods

One source vial of H295R cells were initiated from cryopreservation and expanded three passages to establish a lot for screening and cells were expanded five times before used for testing or frozen for storage. Cells cultured from the expanded stock did not exceed 10 passages. Cells were passaged and maintained in Complete Media (DMEM: F-12 Medium + 1.0% ITS+ Premix Universal Culture Supplement + 2.5% Nu-Serum™ Growth Medium Supplement). The maintenance, expansion, and cryopreservation standard operating procedures were adopted directly from the guideline specifications. A quality control test plate to establish acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement assays, and variability between replicate hormone measures of the same sample were performed and met criteria according to the guideline specifications.

Cells utilized for testing were seeded at a target density of 200,000 to 300,000 cells per mL of Complete Media resulting in approximately 50-60% confluency in the wells at 24 hours to achieve the optimal cell density for hormone production. After an acclimation period of 24 hours, cells were exposed for 48 hours to seven concentrations of PhytoCide Lichen, diluted with DMSO, in triplicate. Cells were also exposed to Complete Media and positive, negative, and solvent controls. Prepared in DMSO, Forskolin (10 µM) and Prochloraz (1.0 µM) were utilized as positive and negative controls as they are known inducers and inhibitors, respectively, of E2 and T synthesis. DMSO was utilized as the solvent control. At the end of the 48-hour exposure period, the media is removed from each well and stored in aliquots at -80°C until hormone analysis. Cell viability in each well is analyzed immediately after removal of media. Conclusions regarding the ability of a test article to affect steroidogenesis are based on at least two independent experimental runs.

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To determine cell viability, fresh Complete Media with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to all wells, except for the positive control wells which received 70% Methanol in Complete Media provided Methanol is a known cytotoxicity inducer. After a 4-hour incubation period at 37°C, the SDS-HCl solution was added to all wells and the plate was incubated again at 37°C for 4 hours after which the absorbance was read at 570 nm. Cell viability data is displayed as Mean Absorbance Units (MAU) and reflects averages of the triplicates for independent experimental runs. The results were analyzed utilizing a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Conditions demonstrating viability lower than 80% were not included in the final data analysis. The percentage of viable cells was calculated with the following equation:

$$Viable\ Cells\ (\%) = \frac{MAU_{Sample} - MAU_{Methanol}}{MAU_{DMSO} - MAU_{Methanol}} \times 100$$

After cellular viability was determined, 17β-estradiol (E2) and testosterone (T) concentration levels in the media were determined with an Estradiol ELISA Kit (Limit of Quantification 6.0 pg/mL) and DetectX® Testosterone ELISA Kit (Limit of Quantification 30.6 pg/mL), respectively. Frozen media aliquots were thawed at room temperature and both ELISAs were performed according to the manufacturers' protocols. Briefly, thawed media aliquots were loaded onto the plate along with prepared standards. Next, conjugates and antibodies were added to appropriate wells and allowed to incubate at room temperature. After the incubation, wells were washed, and antibody detection reagents were added to each well followed by stop solutions to stop the antibody detection reactions. Lastly, absorbance values were measured.

The criteria for a negative, positive, or equivocal run are provided in Diagram 1. A minimum of two independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at $\log_{10}$ intervals with 10^{-3} M being the maximum concentration. If the test article is soluble, and not cytotoxic at any of the tested concentrations, and the first run was negative for all concentrations, then it is to be confirmed in one more run using the same conditions as the first run was conducted (Table 1). If the results of the first run are equivocal or positive, the test should be repeated as indicated in Table 1 and footnote 'a' to Diagram 1, by refining the selected test concentrations. Test concentrations in runs two and three (if applicable) should be adjusted on the basis of the results of the initial run bracketing concentrations that elicited an effect using ½-log concentration spacing (e.g. if the original run of 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM resulted in inductions at 1 and 10 µM, the concentrations tested in the second run should be 0.1, 0.3, 1, 3, 10, 30, 100 µM), unless lower concentrations need to be employed to achieve a Lowest-Observed-Effect-Concentration (LOEC). In the latter case, at least five concentrations below the lowest concentration tested in the first run should be used in the second run using a ½-log scale. If the assay is equivocal or positive, the LOEC is reported. If the assay is negative, the highest concentration tested is reported as the No-Observed-Effect-Concentration (NOEC).

If the second run does not confirm the first run, a third experiment is to be conducted using the original testing conditions. Equivocal results in the first run are considered negative if the observed effect could not be confirmed in any of the two subsequent runs. Equivocal results are considered as weak positive responses (effect) when the response can be confirmed in at least two more runs within a ± 1 concentration increment (Diagram 1; Table 1). Non monotonic concentration response patterns are rare but possible. If reproducible effects are seen at lower concentrations which then dissipate at greater concentrations and/or return at the top concentrations, this data should not be disregarded and should be included in the study report.

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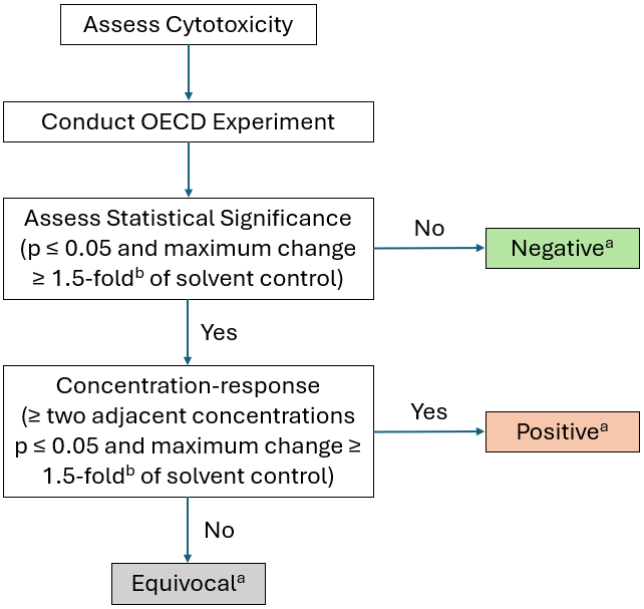


Diagram 1. Overview of the Data Interpretation Procedure within an Individual Run. Diagram from OECD 456 Test Guidelines.

[a] Conduct second or third confirmatory runs as specified in Table 1.

[b] 1.5-fold applies to both induction and inhibition of hormones.

Table 1. Decision Matrix for Possible Outcome Scenarios. Table adapted from OECD 456 Test Guidelines.

Run 1		Run 2		Run 3		Final Call
Scenario	*Decision*	*Scenario*	*Decision*	*Scenario*	*Decision*	
Negative	Confirm[a]	Negative	Stop			Negative
Negative	Confirm[a]	Positive/Equivocal[c]	Refine[b]	Negative	Stop	Negative
Negative	Confirm[a]	Positive	Refine[b]	Positive	Stop	Positive
Negative	Confirm[a]	Positive	Refine[b]	Equivocal[c]	Stop	Weak Positive
Negative	Confirm[a]	Equivocal[c]	Refine[b]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Negative	Stop	Negative
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Equivocal[c]	Confirm[a]	Equivocal[c]/Positive	Stop	Weak Positive
Equivocal[c]	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Negative	Confirm[a]	Positive	Stop	Positive

[a] Confirm previous run using the same experimental design/concentration-spacing.

[b] Re-run assay at ½ log concentration spacing (bracketing the concentration that tested significantly different in the preceding experiment). A smaller concentration-spacing in runs 2 and 3 is recommended given the chance of having only one concentration showing a significant response at a 10-fold increment. Applying a ½ log or even smaller spacing increment should result in at least two significant responses. If the confirmatory run at lower concentrations at the ½ log scale is positive, then the test article is positive. However, on rare occasions an "all-or-nothing" response may occur that only shows an effect at one concentration, which would be captured by this decision process.

[c] Fold-change at one concentration is statistically different from the solvent control.

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To evaluate the relative increase/decrease in test article altered hormone production, the results are expressed as the average fold change relative to the solvent control of each test plate. Relative changes were calculated as follows:

$$Relative\ Change = \frac{Hormone\ Concentration_{Sample}}{Hormone\ Concentration_{DMSO}}$$

A test article is judged to be positive if the fold induction or inhibition is statistically different ($p \leq 0.05$) and above or below the 1.5-fold induction or inhibition threshold from the solvent control at two adjacent concentrations in at least two independent runs (Table 1). The 1.5-fold threshold applies for both increase and decrease of hormone concentrations, i.e. above 150% of the solvent control and below 66.7% of the solvent control (where cytotoxicity ≥ 80%). A test article is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in Table 1, the experimental results are not interpretable. Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity were evaluated and passed. Statistical significance was accepted at $p \leq 0.05$.

Results

The data obtained from this study met criteria for valid assays and the positive, negative, and solvent controls performed as anticipated. PhytoCide Lichen was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

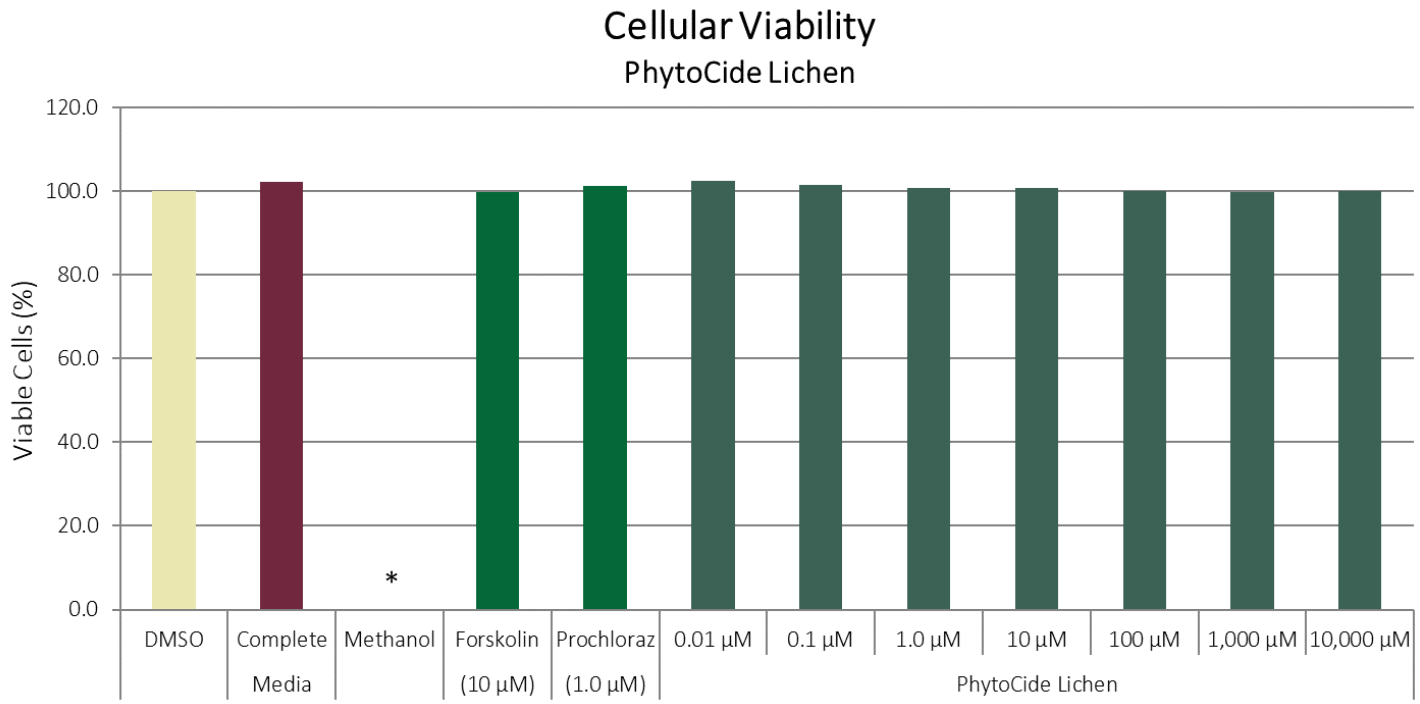


Figure 1. Cellular viability. * indicates significance ($p \leq 0.05$) compared to DMSO.

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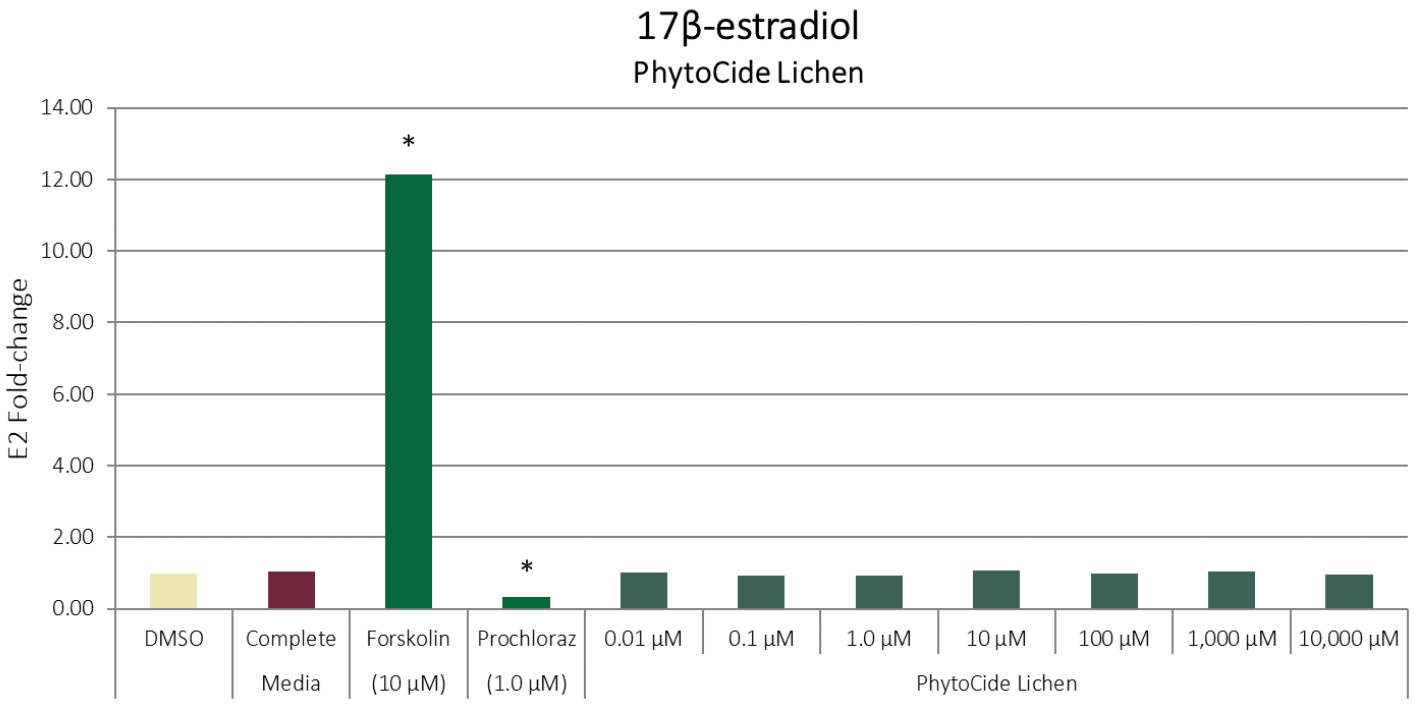


Figure 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		1.03
Forskolin		12.15*
Prochloraz		0.33*
PhytoCide Lichen	0.01 µM	1.01
	0.1 µM	0.92
	1.0 µM	0.93
	10 µM	1.07
	100 µM	0.98
	1,000 µM	1.03
	10,000 µM	0.95

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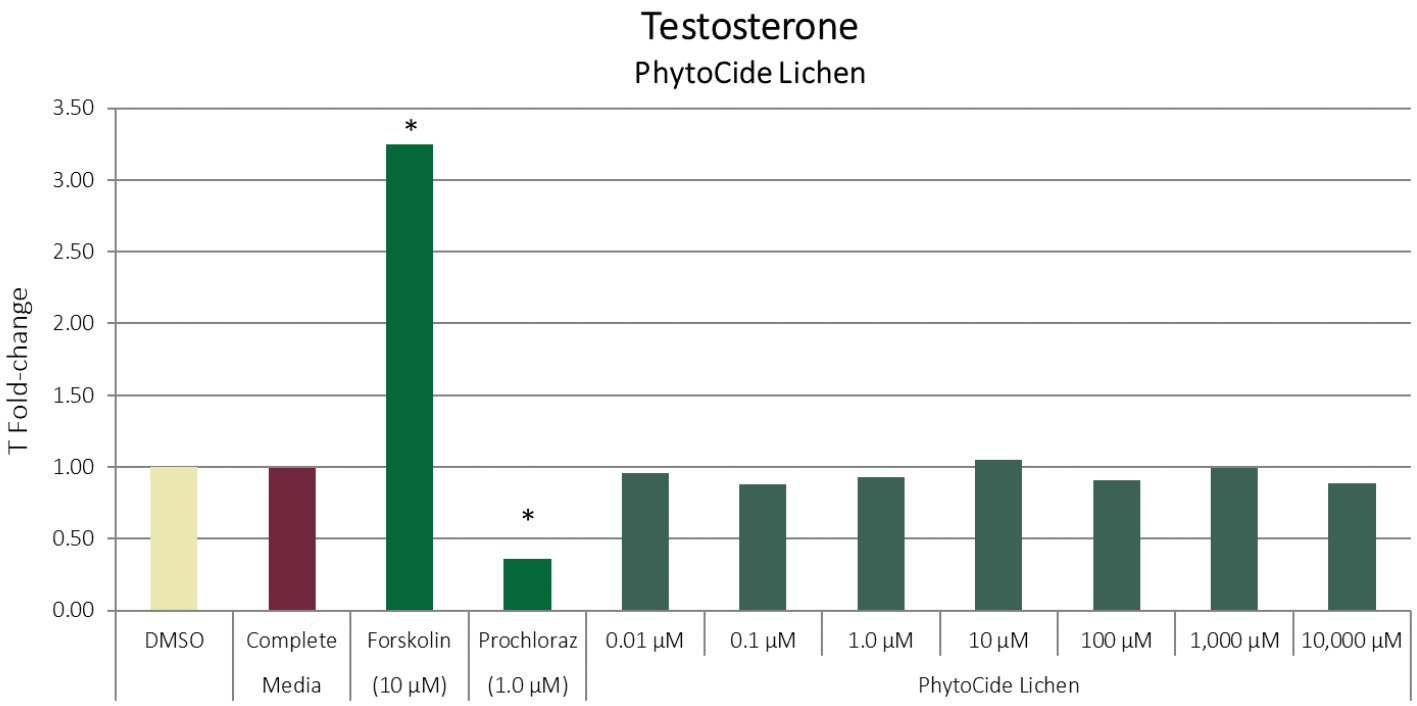


Figure 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		0.99
Forskolin		3.25*
Prochloraz		0.36*
PhytoCide Lichen	0.01 µM	0.96
	0.1 µM	0.88
	1.0 µM	0.93
	10 µM	1.05
	100 µM	0.91
	1,000 µM	0.99
	10,000 µM	0.89

Discussion

The objective of the H295R Steroidogenesis Assay is to screen for test article effects on cellular viability and steroidogenesis by detecting substances that affect 17β-estradiol and testosterone production. Collectively, the results demonstrate **PhytoCide Lichen** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

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