Natural Immune Systems Inc

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Report for:

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Report 1: Exploratory pilot testing of antioxidant and immune modulating effects in vitro.

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Report 1: Exploratory pilot testing of antioxidant and immune modulating effects in vitro.

Purpose

The purpose of this study is to compare specific aspects of biological properties of a nutritional product, Sterol 117™.

Proposed work

The following strategy for initial exploratory testing ***in vitro*** was performed:

1. CAP-e: Antioxidant protection from cellular damage.
2. Pilot test on activation of Natural Killer (NK) cells.

Background

The project involved a nutritional product, Sterol 117™, containing a blend of antioxidants, essential fatty acids, and anti-inflammatory compounds, and which is marketed as support of immune function, and recovery from fatigue syndromes.

The ingredients include multiple aqueous and non-aqueous sources of antioxidants with various other properties, including immune activating and anti-inflammatory properties.

Celt Corp needs to build onto the portfolio of data on the blend of these ingredients. A sequential strategy has been discussed, starting with selected bioassays, and moving towards a human clinical pilot study.

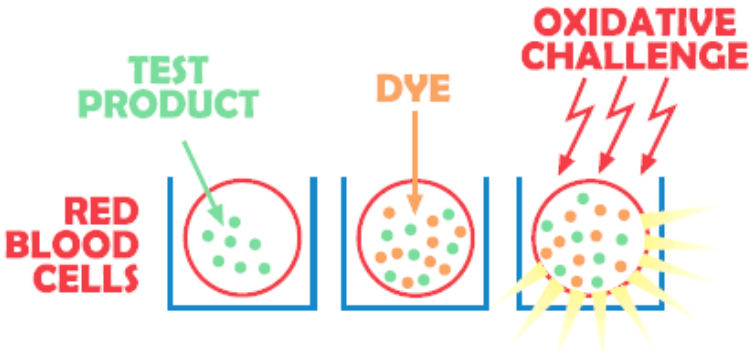
Based on the broad spectrum of potent antioxidants in the blend, the CAP-e antioxidant bioassay was performed as part of an initial foundation to gain insight into the antioxidant availability to living cells. This data also serves as a valuable baseline for interpreting anti-inflammatory data in subsequent studies.

In addition, a pilot test on a specific aspect of immune activity – namely effects on NK cells – was performed.

Results

1. Cell-based Antioxidant Protection assay CAP-e

The rationale behind the method that we use is important: It allows assessment of anti-oxidant potential in a method that is comparable to the ORAC test, but only allows measurement of anti-oxidants that are able to cross the lipid bilayer cell membrane. As a model cell type, we use the red blood cell (RBC). This is an inert cell type, in contrast to other cell types such as PMN cells, where pro-inflammatory compounds may induce the reactive oxidative burst, or anti-inflammatory compounds may perform cellular signaling and change the behavior of the PMN cell, at doses many times below levels of detection for antioxidants. We developed this assay particularly to be able to assess antioxidants from complex natural products in a cell-based system.



Aqueous and ethanol extracts were prepared in parallel, to allow testing of both water-soluble and non-polar compounds in the test product.

Human RBC were washed repeatedly in physiological saline (PBS)*, and then exposed to the test products. During the incubation with a test product, any antioxidant compounds able to cross the cell membrane can enter the interior of the RBC. Then the RBC were washed to remove compounds that were not absorbed by the cells, loaded with the DCF-DA dye, which turns fluorescent upon exposure to reactive oxygen species. Oxidation was triggered by addition of the peroxyl free radical generator AAPH. The fluorescence intensity was evaluated. The low fluorescence intensity of untreated control cells serves as a baseline, and RBC treated with AAPH alone serve as a positive control for maximum oxidative damage. If we observe a reduced fluorescence intensity of RBC exposed to a test product and subsequently exposed to AAPH, this indicates that the test product contains antioxidants available to penetrate into the cells and protect these from oxidative damage.

** PBS is a water-based physiological saline solution used with cells to preserve the correct osmotic conditions.*

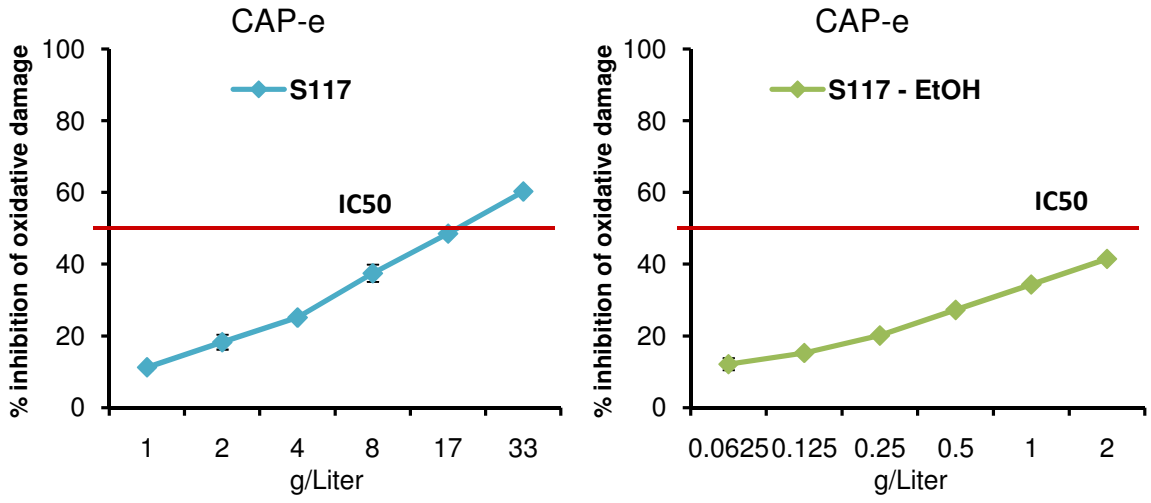
The graphs below show the results of the CAP-e test on both the PBS and the ethanol extracts of Sterol 117™. Cells cannot endure more than 2% ethanol, therefore the ethanol extract started at a lower concentration than the PBS extract and both were serially diluted 2-fold.

Both extracts showed a dose-dependent antioxidant protection of red blood cells. Thus, we can conclude that both water-soluble and non-polar antioxidants are present in the product in forms that are available to provide protection to live cells.

The doses used of Sterol 117™'s PBS extract allowed the % inhibition to reach an IC50. The IC50 is a measure of the effectiveness of a compound in inhibiting (in the case of the CAP-e assay) oxidative damage. If the product is potent enough to show more than 50% inhibition within the dose range tested, then an IC50 can be calculated.

The point on the graph where the red IC50 line intersects the curve reflects the IC50 dose of the test product, i.e. the dose that provided 50% inhibition of oxidative damage. This IC50 dose is compared to the IC50 dose of the known antioxidant Gallic Acid (which is used as a control in the assay), resulting in a CAP-e value reported in Gallic Acid equivalent units.

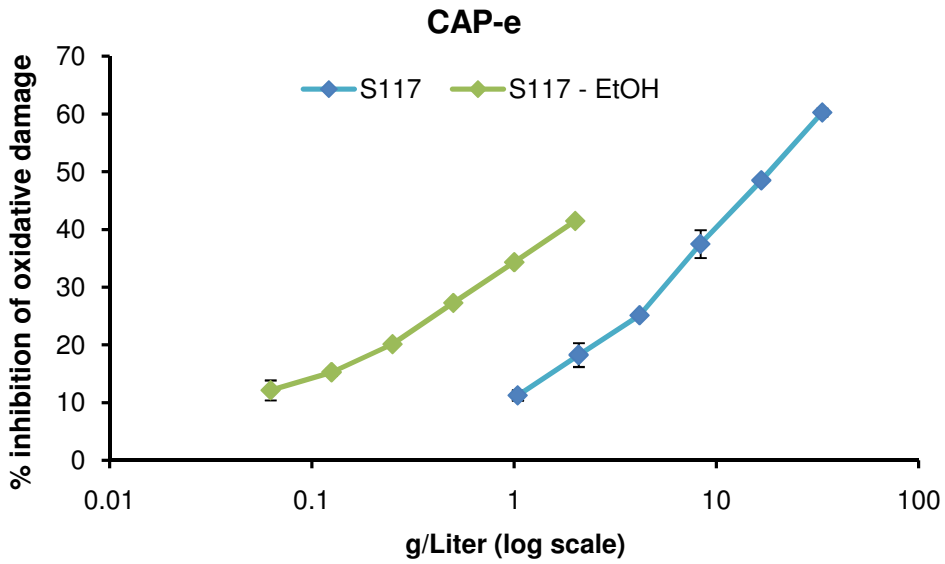
The PBS extract of Sterol 117™ indeed reached an IC50 giving it a CAP-e value of 1.7 CAP-e units per gram.



Even though the diluted EtOH extract did not reach an IC50, it was clearly more efficient – i.e. contained more antioxidants per weight, capable of providing a biological protection.

This is shown below.

The graph below is a comparison of the two extracts from the test product. The ethanol extract shows a higher antioxidant protection since it provided greater inhibition of oxidative damage to the cells at the lower doses than the PBS extract.



For example, this can be seen when comparing the antioxidant protection at 1g/L where the aqueous extract provided 11% protection, in contrast to the 34 % protection provided at the same 1 g/L dose of product in an ethanol extract.

2. Effect on activation of NK cells

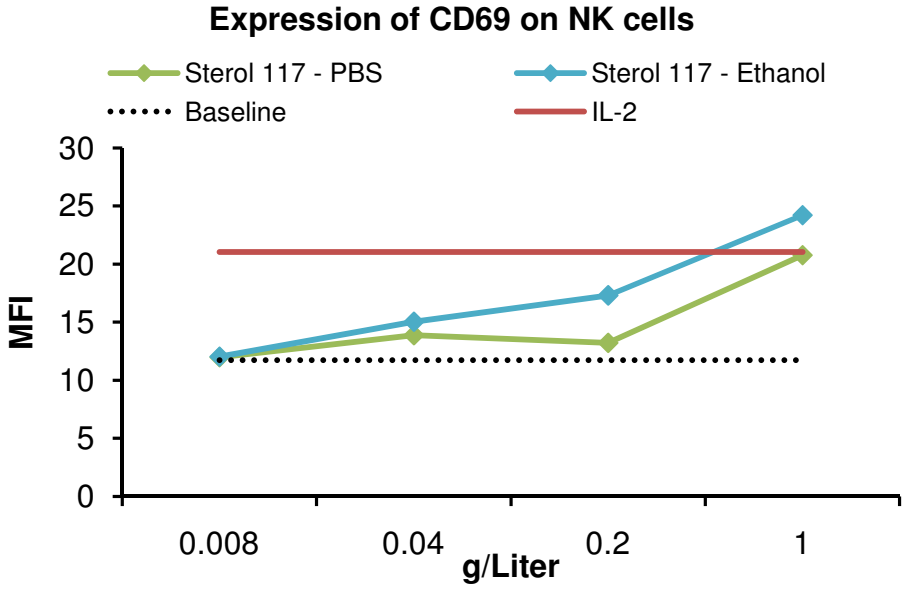
Our body's primary defense mechanisms towards cancers and viral diseases involve a group of cells called Natural Killer (NK) cells. These cells travel in our blood stream in a state of rest, but can be immediately recruited into tissues by chemical signals and activated through various mechanisms to a) kill cancer cells, b) divide and make more NK cells, and c) secrete substances that attract other cells into the site.

We performed one pilot test to evaluate whether there would be data to support further work on immune modulating effects of the product. The test is normally performed three times on primary human cells from three different blood donors. In preparation for this test, normally an overnight cell viability test is performed to establish the optimal working dose for a given product for further in vitro testing in biological assays. Based on budget limitations, only a single NK test was performed. Test conditions were tested in singlet, in contrast to the duplicates we normally do. This, the data serve to guide further work, but repeats are necessary before the data will be considered publishable.

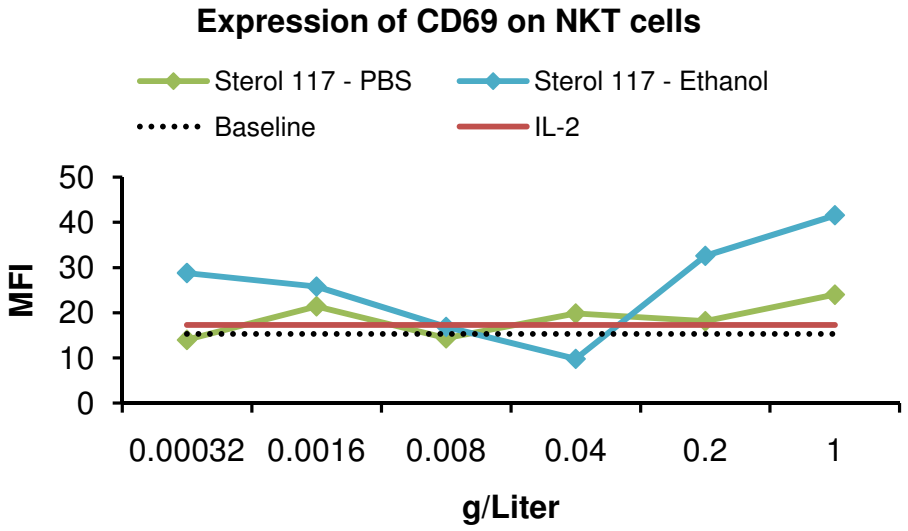
Freshly purified human peripheral blood mononuclear cells were used for this assay. The cells were plated in micro-well plates. Negative control wells in triplicate are left untreated. Positive controls are treated with IL-2 at a dose of 100 international units per mL (IU/mL). Serial dilutions of the test product in *singlet* were applied to the cells and the cells were cultured overnight at 37°C with 5% CO_2.

After 18 hours of culture, cells are stained for the activation molecule CD69 on the surface of CD3-negative, CD56-positive NK cells. The analysis allows us to detect if compounds in a test product directly activate NK cells in vitro by flow cytometry.

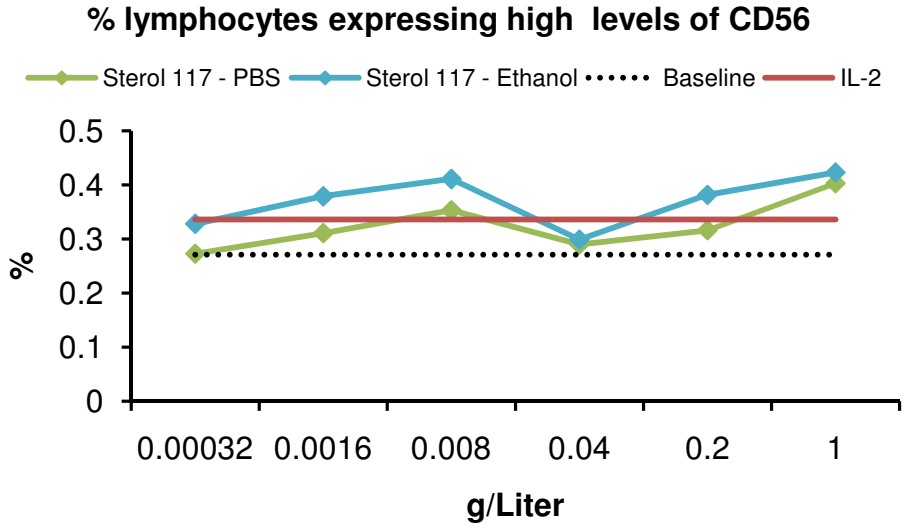
The graph below shows the data from the pilot test performed on Sterol 117™ in an extract of PBS (water extract) and in a 95% ethanol extract. The data show that the two extracts are comparable in their effect on NK activation as seen by the expression of the marker CD69 on NK cells. The ethanol extract was slightly better than the PBS extract and the IL-2 control at the highest dose.



The graph below shows more promising effects of Sterol 117™ extracts in PBS and EtOH on the expression of CD69 on NKT cells. This small subset of cells plays an important role in regulating the immune system. Again, the ethanol extract of Sterol 117™ demonstrates an effect on NKT cells at the highest dose.



The molecule CD56 (or NCAM) has been implicated as having a role in NK cell function. Here we have analyzed the data for the number of NK cells that express high levels of this marker. We examined if the product Sterol 117™ was able to affect the cells in culture to express more CD56. Although the number of cells examined was only a small subset of the total population, we do believe the effect of Sterol 117™ on the expression of CD56 further supports that further work on the immune modulating properties of Sterol 117™ is justified.



Conclusions

- Contained both water-soluble and water-insoluble antioxidants capable of providing a biologically meaningful antioxidant protection of living cells.

- Contained both water-soluble and water-insoluble compounds that appears capable of activating various functions of human Natural Killer (NK) and NKT cells in vitro. Further work is needed before this is conclusive.

Suggested further work

The following work is suggested as a progression from the data presented here:

Further documenting antioxidant properties:

1. CAP-a: Protection of cell viability under conditions of oxidative stress;
2. CAP-m: Protection of cellular energy production (i.e. mitochondrial function) under oxidative stress;

Further documenting effects on the innate (immediate) immune defense mechanisms:

3. NK activation (anti-viral defense)
4. Phagocytosis (anti-bacterial defense)

Further work involving anti-inflammatory properties of the product:

5. Effect on ROS formation by polymorphonuclear (PMN) cells.

Clinical pilot testing of

6. Antioxidant bioavailability in humans;
7. Immune modulating activities;
8. Anti-inflammatory properties.