Eevia Health Plc.

Research article

A comparative bioactivity profiling of two pine bark extracts - Fenoprolic® and Pycnogenol®: Considering Bioequivalence and Extract equivalence over Herbal equivalence, when validating bioactivity and formulating compendial monographs for dietary supplement ingredients

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Abstract: Eevia Health has extensively studied the bioactivity of its pine bark extract, **Fenoprolic®**, together with a competitor product of equal concentration of oligomeric proanthocyanidins (OPCs), **Pycnogenol®**, using the **BioMAP® Phenotypic** screening and profiling platform by Eurofins. The platform can simulate the biological responses in twelve separate human primary cell tissue model systems by assessing the activity of almost 150 biomarkers in the said systems. Comparing **Fenoprolic®** to **Pycnogenol®** in a neutral, third-party screening platform, the study found near-identical results across almost all the biomarkers studied. The similarity of results demonstrated that neither **Fenoprolic®** nor **Pycnogenol®** is inferior to the other product in terms of up- or down-regulating the panel of biomarkers in the various health systems. The study aimed to select the downstream research focus and input for the design for human clinical investigations of **Fenoprolic®**. However, the results led to the realization that wide-range bioactivity screening could revolutionize the efficacy validation of nutraceuticals, providing a means to validate claims and combat fraud. The study suggests shifting focus from proving herbal equivalence to demonstrating bioactivity and bioequivalence. Comprehensive bioactivity screening could be a powerful tool in the fight against misleading claims and fraud in the industry. The emergence of "candyceuticals" accentuate, and unhinged retail platforms such as Amazon, accepting adulterated products and misleading price comparisons of non-comparable consumer products. Proving bioequivalence through bioactivity in vitro screening platforms and further downstream testing models may allow the industry and authorities to validate efficacies and sort the authentic products from the imposters.

Keywords: Pine Bark Extract; Fenoprolic®, Pycnogenol®, BioMAP Analysis; Adulterated supplements, Bioactivity Profiling; Phytoeqvivalence, Herbal equivalence, Bioactive Potential, Candyceuticals, Immunomodulatory Activities, Anti-inflammatory Compounds, Antiproliferative Effects, Cardiovascular Health, Tissue Remodeling, Hemostasis

1. INTRODUCTION

Providing customers and consumers with accurate scientific evidence about dietary supplements' bioactivity, clinical benefits, and health effects should be a cornerstone for any nutraceutical operator. However, conducting robust scientific trials for these products takes time and effort. Even larger companies struggle to justify the return on investment for such trials [1]. Companies may be tempted to skirt the rigorous standards of scientific research, conducting smaller, simpler, and more inexpensive trials. The methods and results may not coherently or sufficiently match or validate the marketing claims. Academic research, while necessary and valuable, isn't a perfect guarantor for valid knowledge either [2].

As a small ingredient company, Eevia Health faces the challenge of prudently substantiating health effects. In Europe, the route to EFSA-approved health claims is, in practice, blocked for many years to come, as EFSA's application takes years. This block reduces the benefit of clinical studies here. Furthermore, in an increasingly complex and competitive market environment, even well-recognized and renowned brands start bending their consumer product formulas, reducing the amount of active ingredients and, hence, the efficacy strength of their products. In some instances, the dilution leads to products that are merely "candyceuticals" – formulas containing fillers that taste good but have no biological effects. Indeed, you find dietary supplements with mostly sugars and colors making health claims. Additionally, shifts in how nutritional supplements are marketed and sold have compromised the integrity of product claims. One can observe Amazon's growing dominance as a supplement outlet with its acceptance of many adulterated, counterfeit, and misbranded products.

In this jungle, compendial claims are often made by referring to the composition of ingredients measured with methods stated in monographs such as USP (United States Pharmacopeia), NNHPD (Health Canada), or Ph. Eur. (European Pharmacopeia). However, compendial methods for analyzing an ingredient's content or chemical composition, especially for botanical extracts, can sometimes become inadequate. For pine bark extracts, monographs such as the one in the USP prescribe a simple spectrophotometric analysis that provides a quantification of total OPCs found in the product as a measure of procyanidin after acid-butanol reaction. While the method is effective at measuring the concentration of OPCs in the pine bark extract, the results do not tell anything about the more detailed features of the OPCs. The method is not able to for example make a distinction between the smaller, more bioactive short-chain OPCs and the mostly inactive high-molecular weight polymerized OPC fractions, which is a significant flaw.

Therefore, to protect the investments in clinical studies, Eevia wishes to contribute to analytical fingerprinting to reveal adulteration. This can be done through more advanced analytical profiling of the composition of botanical extracts, for which we have initiated another study and method development. However, herbal, compositional, or extract equivalence also has its own pitfalls.

Consequently, our interest has grown in studying the bioactive profile of our products and, in a competitive context, comparing bioequivalence. Thus, in 2023, Eevia Health studied the bioactivity of our organically certified pine bark extract **Fenoprolic®**, utilizing the BioMAP® Phenotypic Profiling system operated by Eurofins. For benchmarking purposes, Eevia added a competitor product with the same concentration of active compounds, **Pycnogenol®**, to the study.

Eurofins BioMAP® system [3] is a screening and profiling platform with a methodological framework that simulates various human disease models to decode the bioactivity of test agents in human primary cells across a spectrum of biological contexts. The comprehensive analysis of **Fenoprolic®** and **Pycnogenol®** revealed significant antiproliferative, anti-inflammatory, and immunomodulatory activities, among others. The desire to build a rational basis for the design of clinical studies for **Fenoprolic®** also motivates these efforts. A natural route is through comprehensive in vitro testing on human primary cells to reveal bioactivity and elucidate possible modes of action.

2. METHODOLOGY

2.1. BioMAP Diversity PLUS

BioMAP Diversity PLUS analysis panel is a primary human cell-based system designed to model different aspects of the human body in an in vitro format [4–10]. Based on twelve (12) systems of various human primary cells modeling vascular biology, systemic immune response, airway inflammation, and connective tissue biology, the panel allows the characterization of nutraceutical ingredients unbiasedly across a broad set of systems modeling various human conditions. Primary cells mean that the cells used in the analysis platform are not laboratory cell lines used in research, but cells extracted from actual human donors.

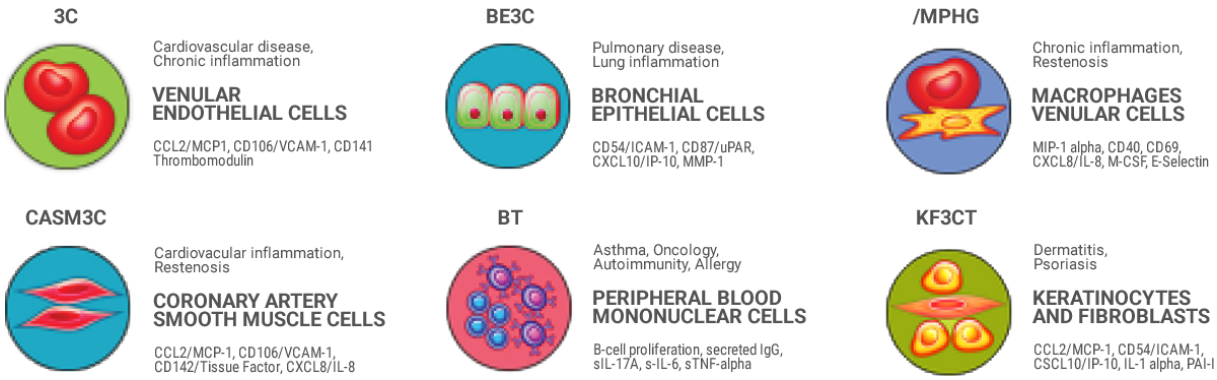


Figure 1. An example of a system found in the BioMAP platform. System names are on top of the illustration. On the right side of each picture, information about the target indication, tissue type, and examples of biomarkers measured can be found.

In total, the analysis panel contains 148 diverse protein biomarker readouts from cell surface receptors, cytokines, chemokines, matrix molecules, and enzymes that can capture biological changes that occur within each system's physiological context. Each test agent generates a signature BioMAP profile from the changes in protein biomarker readouts within individual system environments.

The **CAS3MC** system, for example, is designed to study cardiovascular inflammation together with restenosis. It uses coronary artery smooth muscle cells to measure the activity and response of biomarkers such as **CCL2/MCP1, CD142 Tissue Factor,** and **CD106/VCAM-1.** The supplemental material (REF) provides a complete and comprehensive list of the systems, biomarkers, and their descriptions.

Using data mining tools, a nutraceutical BioMAP profile can be compared against a proprietary reference database of > 4000 bioactive agents of known Mode of Action (biologics, drugs, chemicals, experimental compounds) to classify and identify similar bioactivity profiles. By overlaying the profiles, one can also compare directly against other nutraceutical ingredients measured simultaneously to identify activities with similar or different responses across all systems studied. In addition to the profile comparison, **a Heat Map analysis** can also be used to study the activity profiles.

This robust data platform allows rapid evaluation and interpretation of BioMAP profiles by unbiased mathematical comparison of similar activities. Specific activities have been correlated to in vivo biology, and profiles have been used to distinguish compounds based on Mode of Action and target selectivity. Thus, the profiles can provide a predictive signature for in vivo outcomes across diverse physiological systems.

Specific BioMAP activities have been correlated to in vivo biology [13–15], and BioMAP profiles have been used to distinguish compounds based on Mode of Action and target selectivity [11, 14] in multivariate analyses. The profiles can thus provide a predictive signature for various in vivo toxicological outcomes (e.g., vascular toxicity, developmental toxicity, etc.) across diverse physiological systems [11–12, 16].

2.2. Profile Plot

The main output from the platform is the **Product Profile plot,** which is a graphical overlay of all the measured biomarker activities in several concentrations for a particular test agent. Significant biomarker activities and their readouts are annotated, key activities classified, and listed into biologically relevant categories. Profile plots also identify dose-dependent, cytotoxic, antiproliferative, and potential off-target secondary effects.

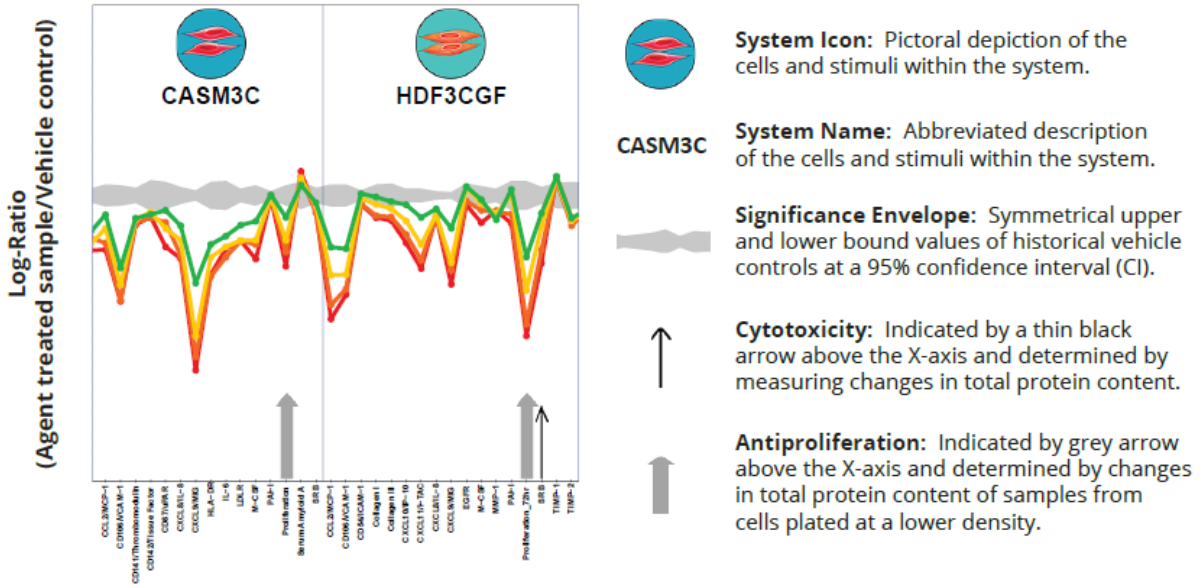


Figure 2. Part of a BioMAP Profile. A graphical representation of the signature biomarker readout changes quantitated within BioMAP systems in response to test agent treatment. **The X-axis represents the Biomarker Readouts,** the therapeutically relevant cell surface, or secreted proteins validated with well-characterized agents or mechanism-of-action compounds. **The y-axis is designated for Log-Ratio Values** of the biomarker activities. The values are divided by the average of vehicle controls to generate a Log_{10} transformed ratio.

The scaling of Biomarker Expression in the Y-axis can be easily understood by understanding the relationship between the Log_{10} scale and the fold change of the normalized biomarker readout expressions.

A value of -0.3 in the Log_{10} scale corresponds to approximately a 50 % decrease (downregulation of the biomarker), while a readout of 0.3 in the Log_{10} scale corresponds to about a two-fold (2x) increase.

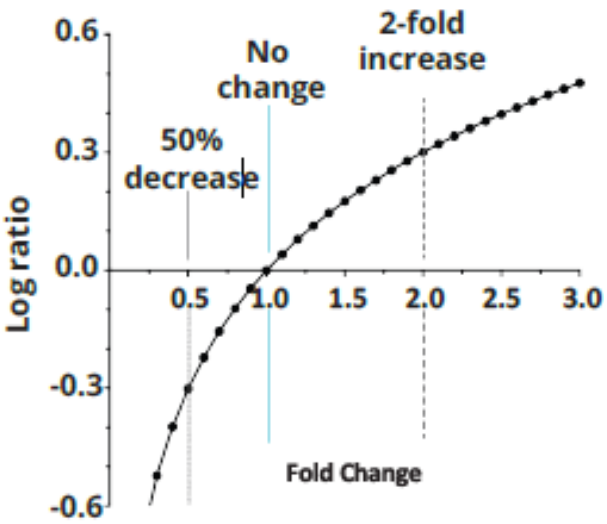


Figure 3. Log_{10} scale vs. Fold change. This is a graphical representation of the relationship between Log_{10} scale values and fold change.

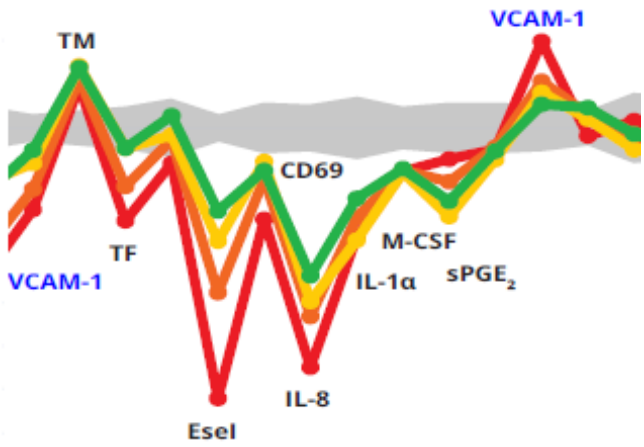


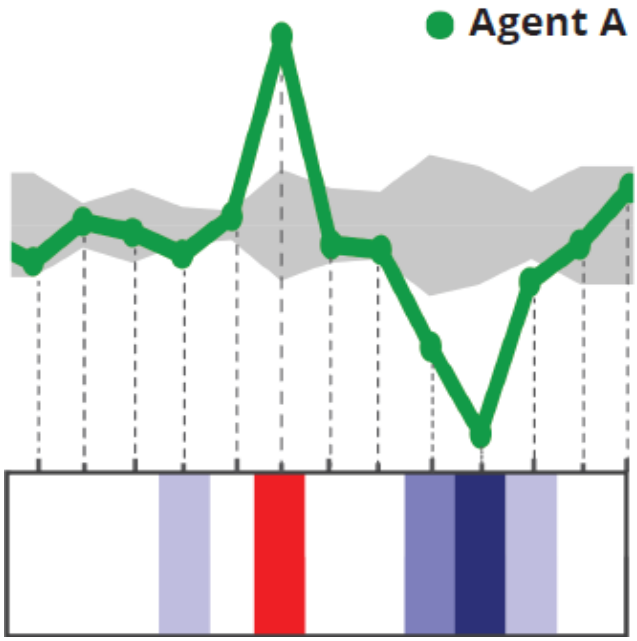
Figure 4. Annotation of Key Activities or Hits. This is a graphical representation of the relationship between Log_{10} scale values and fold change.

Biomarker readouts are designated hits if:

1) Two or more consecutive concentrations are changed in the same direction relative to vehicle controls.
2) Values are outside the significance envelope (grey zone).
3) At least one concentration has an effect size > 20 % vs. vehicle controls.

For a biomarker readout to be classified as *significant*, it must show a substantial change (> 20%) to either direction in at least two different concentrations. The change must be substantial enough to be outside the historical vehicle control area, where even water has been sufficient sometimes to cause a slight change in the biomarker readout. Finally, modulated biomarkers **that are increased in some systems but decreased in others** are annotated in blue color.

2.3. Heat Map Analysis



A heat map analysis can be conducted to observe and compare the biomarker responses a bit deeper. In this data representation type, the activities outside of the significance envelope are depicted with red if the biomarker protein levels are increasing and blue if the levels are decreasing. White corresponds to either unchanged levels or changes within the vehicle control envelope. Darker shades of color represent a more significant change in biomarker activity than control.

Figure 5. Heat Map vs. Profile Plot. Peaks correspond to red bars in the Heat Map, and valleys to blue bars. Peaks within the vehicle control envelope (grey area) remain white in the Heat Map plot.

The benefit of this approach is that it gives equal representation to all biomarker responses **according to their relative scale instead of the absolute scale.** Comparability allows even the more minor but still significant responses to be observed more clearly. Heat Map plots are compelling in comparing two products, comparisons against compounds with known Modes of Action, or consensus mechanisms of specific Modes of Action.

3. THE COMPARISON ANALYSIS

3.1. The test agents and the protocol

Fenoprolic® is an organically certified pine bark oligomeric proanthocyanidin (OPC) extract made from bark peeled from pine trees (Pinus sylvestris) growing in the Arctic and sub-Arctic regions of Finland, at the Northern tip of Europe. Eevia Health Plc, a manufacturer of organic arctic bioactive extracts from Finland, manufactures it. The process includes a water-based extraction combined with an organic certified chromatographic enrichment process and a spray drying step to produce a powder product containing 70 ± 5 % oligomeric proanthocyanidins according to the analytical method described in the United States Pharmacopeia monograph (USP, Maritime Pine Extract).

Pycnogenol® is a conventional pine bark extract made from bark peeled from pine trees (Pinus pinaster) growing in the maritime region of France, in the central part of Europe. Horphag Research, a Swiss company, markets it. Like **Fenoprolic®**, **Pycnogenol®** is purified in a chromatographic process to 70 ± 5 % oligomeric proanthocyanidins (OPC) according to the same USP monograph analytical method. **Pycnogenol®** is regarded as the market leader and a pioneer in pine bark extracts, with a significant body of both in vitro and clinical studies conducted for the product in the past few decades with predominantly positive outcomes.

We procured and analyzed two representative samples of each product, which were determined to be within their respective specifications. The samples were coded (blinded) and shipped to Eurofins for commercial analysis in the BioMAP Discovery Plus services. Before the actual BioMAP analysis, both test agents were evaluated for their toxicity by their ability to inhibit cytokines secreted by the induction of macrophages and monocytes in human peripheral blood mononuclear cells (PBMCs) from a single donor. Eurofins also assessed the test agent's effect on cell viability in parallel with the PBMC alamarBlue® Cytotoxicity Assay. This data was then used to evaluate the final chosen concentration range for the profiling analysis.

Human primary cells used in the BioMAP study systems are used at early passage (passage 4 or earlier) to minimize adaptation to cell culture conditions and preserve physiological signaling responses. All cells were from a pool of multiple donors (N = 2–6), commercially purchased and handled according to the manufacturers' recommendations. Human blood derived CD14+ monocytes were differentiated into macrophages in vitro before being added to the /Mphg system. Systems were derived from either single-cell types or co-culture systems. The researchers cultured Adherent cell types in 96 or 384-well plates until confluence.

Test Agents were prepared in either DMSO or PBS, added at the indicated concentrations one hour before stimulation, and remained in culture for 24–168 hours, depending on the system. Each plate contained positive controls (e.g., legacy control test agent colchicine at 1.1 µM), negative controls (e.g., non-stimulated conditions), and vehicle controls (e.g., 0.1% DMSO) appropriate for each system.

Direct ELISA was used to measure biomarker levels of cell-associated and cell membrane targets. Soluble factors from supernatants were quantified using either **HTRF® detection, bead-based multiplex immunoassay, or capture ELISA.** Overt adverse effects of test agents on cell proliferation and viability (cytotoxicity) were detected by **sulforhodamine B (SRB) staining** for adherent cells and **alamarBlue®** reduction for cells in suspension.

For proliferation assays, Eurofins cultured individual cell types at subconfluence and measured them at time points optimized for each system (48 hours: 3C and CASM3C systems; 72 hours: BT and HDF3CGF systems; 96 hours: SAg system). **Cytotoxicity for adherent cells is measured by SRB and by alamarBlue staining for cells in suspension at the indicated time points.** Additional information can be found in the Supplemental information.

3.2. BioMAP results for Fenoprolic®

At non-cytotoxic concentrations, the BioMAP algorithm found Fenoprolic® to be active and mediate changes in **twenty (20) annotated biomarker readouts.** The following list describes them according to their biological classification.

Table 1. Significant and annotated activities discovered. Some activities are present in multiple systems.

BIOLOGICAL RELEVANCE CATEGORY	DECREASED ACTIVITY	INCREASED ACTIVITY
Inflammation-related activities	VCAM-1, ICAM-1, IP-10, ITAC, MCP-1, MIG	IL-8 (3 systems)
Immunomodulatory activities	M-CSF, sIL-17F	-
Tissue remodeling activities	PAI-I, Col-I, Col-III, Col-IV	MMP1
Hemostasis-related activities	-	TF

Fenoprolic® was demonstrated to impact **inflammation-related activities** (decreased MCP-1, VCAM-1, I-TAC, ICAM-1, MIG, IP-10; increased IL-8), **immunomodulatory activities** (decreased M-CSF, sIL-17F), **tissue remodeling activities** (decreased Collagen I, Collagen III, Collagen IV, PAI-1; increased MMP-1), and **hemostasis-related activities** (increased TF).

Increased activities (3) were found to be considerably lower compared to the total of twelve downregulated activities. The most affected systems were **3C (venular endothelial cells), HDF3CGF (dermal fibroblasts), and MyoF (lung fibroblasts).**

The BioMAP Profile Plot across the twelve systems studied for the two concentrations used in the statistical analysis of significant biomarker activities for **Fenoprolic®** is illustrated in the following picture:

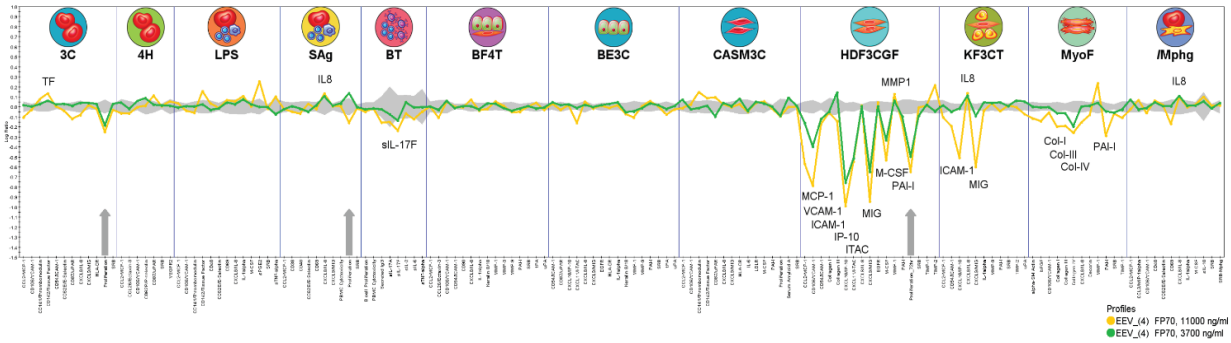


Figure 6. BioMAP Profile Plot of Fenoprolic® in two concentrations together with annotations.

The X-axis lists the biomarker readouts measured in each system. At the same time, the **Y-axis** represents a Log-transformed ratio of the biomarker readouts for the treated sample over vehicle controls. The grey region around the Y-axis represents the 95 % significance envelope generated from historical vehicle controls.

Biomarker activities are annotated when two (2) or more consecutive concentrations change in the same direction relative to vehicle controls, are outside of the significance envelope, and have at least one concentration with an effect size > 20 % (|log10 ratio| > 0.1). Biomarker key activities are described as modulated if these activities increase in some systems but decrease in others. Cytotoxicity is indicated on the profile plot by a thin black arrow above the X-axis, and a thick grey arrow indicates antiproliferative effects.

Fenoprolic® had detectable cytotoxicity in more than three of the systems studied at concentrations of 33 µg/mL and 100 µg/mL, so the Profile Plot data acquired from those concentrations has been omitted from the analysis. Data reduction somewhat limits the detection of significant activities, as the criteria always require activity detected in at least two concentrations.

3.3. BioMAP results for Pycnogenol®

At non-cytotoxic concentrations, the BioMAP algorithm found Pycnogenol® to be active and mediate changes in **sixteen (16) annotated biomarker readouts.** The following list describes them according to their biological classification:

Table 2. Significant and annotated activities discovered. Some activities are present in multiple systems.

BIOLOGICAL RELEVANCE CATEGORY	DECREASED ACTIVITY	INCREASED ACTIVITY
Inflammation-related activities	VCAM-1, ICAM-1, IP-10, ITAC, MCP-1, MIG	IL-8 (1 system)
Immunomodulatory activities	M-CSF, sIL-17F	-
Tissue remodeling activities	Col-I, Col-IV	MMP1
Hemostasis-related activities	-	TF

Pycnogenol® was demonstrated to impact **inflammation-related activities** (decreased MCP-1, VCAM-1, I-TAC, ICAM-1, MIG, IP-10; increased IL-8), **immunomodulatory activities** (decreased M-CSF, sIL-17F), **tissue remodeling activities** (decreased Collagen I, Collagen IV, modulated MMP-1), and **hemostasis-related activities** (increased TF).

Increased activities (3) were found to be considerably lower compared to the total of twelve downregulated activities. The most affected systems were also **3C (venular endothelial cells), HDF3CGF (dermal fibroblasts), and MyoF (lung fibroblasts).**

The BioMAP Profile Plot across the twelve systems studied for the two concentrations used in the statistical analysis of significant biomarker activities for **Pycnogenol®** is illustrated in the following picture:

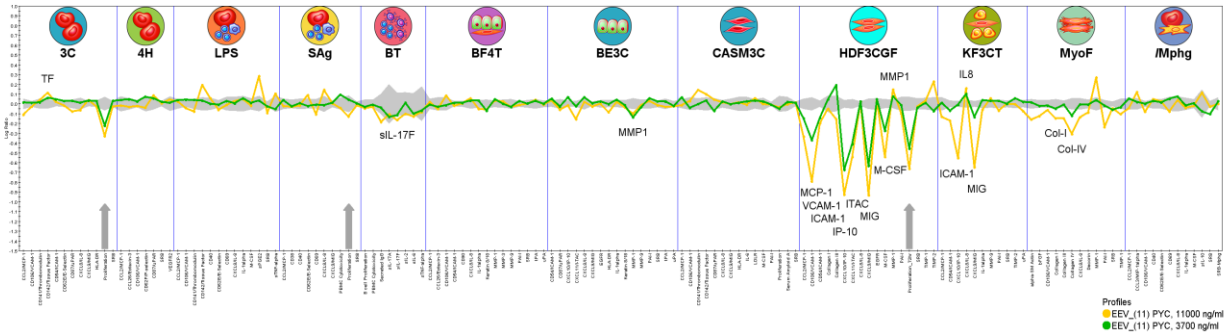


Figure 7. BioMAP Profile Plot of Pycnogenol® in two concentrations together with annotations.

The X-axis lists the biomarker readouts measured in each system. At the same time, the Y-axis represents a log-transformed ratio of the biomarker readouts for the treated sample over vehicle controls. The grey region around the Y-axis represents the 95 % significance envelope generated from historical vehicle controls.

Biomarker activities are annotated when two (2) or more consecutive concentrations change in the same direction relative to vehicle controls, are outside of the significance envelope, and have at least one concentration with an effect size > 20 % (|log10 ratio| > 0.1). Biomarker key activities are described as modulated if these activities increase in some systems but decrease in others. Cytotoxicity is indicated on the profile plot by a thin black arrow above the X-axis, and a thick grey arrow indicates antiproliferative effects.

Like **Fenoprolic®**, **Pycnogenol®** had detectable cytotoxicity in more than three of the systems studied at concentrations of 33 µg/mL and 100 µg/mL, so the Profile Plot data acquired from those concentrations have been omitted from the analysis.

3.4. Discussion of results and comparison between Fenoprolic® and Pycnogenol®

One of the essential features of the BioMAP platform **is the option to compare the test agents in selected concentrations against selected reference benchmark molecules or against each other.** In this comparison, common or differentiating biomarker activities are annotated and listed by the system, along with a description of the Reference Benchmark agent. Because the Reference Benchmark is another nutraceutical product analyzed in the platform simultaneously on the same human primary cells, it allows a direct comparison of the profile, magnitude, and characterization of biological activity of the two products.

For **Fenoprolic®** and **Pycnogenol®**, the overlaid comparison Profile Plot appears like this (**Fenoprolic®** in Red, **Pycnogenol®** in Black):

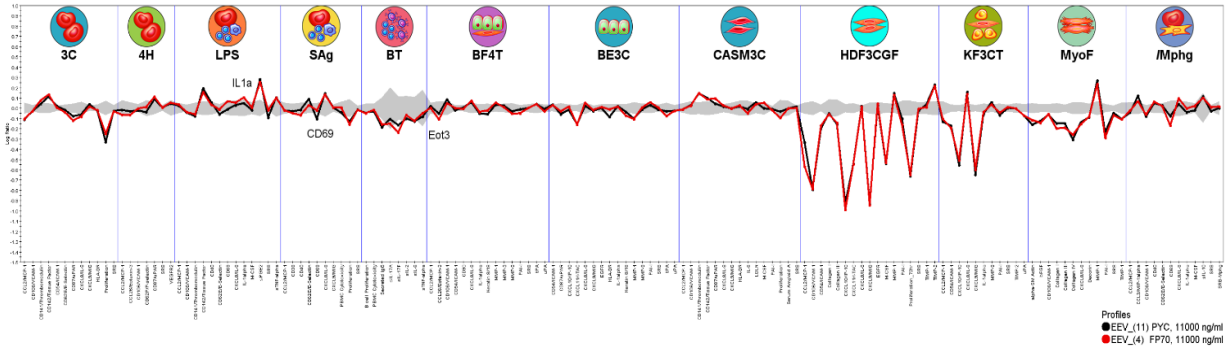


Figure 8. BioMAP Profile Plot Comparison of Fenoprolic® and Pycnogenol®.

As can be seen from the chart, **the two profiles show remarkable similarities.** The overlaid profiles are almost identical, sharing **significant similarities in measured biomarker activities in forty-one (41) common biomarkers (MCP-1, TF, sTNF-alpha, IL-8, etc.)** within nine (9) systems out of the whole twelve, with only three biomarkers (IL-1 alpha, CD69 and Eotaxin 3) showing activation not seen in the other sample studied that can be considered statistically significant. We observed two in **Fenoprolic®** alone, and one from **Pycnogenol®** alone. It must be noted that the differences measured are very minor on an absolute scale. Thus, there are insufficient findings to make meaningful conclusions from this limited data set.

The table of all biomarker activities measured, both common and different, and their respective systems are summarized in Table 2 below:

Table 3. Significant common and different biomarker activities of Fenoprolic® and Pycnogenol®.

SYSTEM	COMMON ACTIVITIES	DIFFERENT ACTIVITIES	ACTIVE IN
3C	MCP-1, TF, Proliferation	-	
4H	-	-	
LPS	TF, sPGE2, sTNF-alpha	IL-1 alpha	Fenoprolic®
Sag	IL-8, Proliferation	CD69	Pycnogenol®
BT	SigG, sIL-17F, sIL-6	-	
BF4T	-	Eotaxin 3	Fenoprolic®
BE3C	I-TAC, MMP1	-	
CASM3C	Thrombomodulin, (TF)	-	
HDF3CGF	MCP-1, VCAM-1, ICAM-1, Collagen III, IP-10, I-TAC, MIG, M-CSF, MMP-1, PAI-1, Proliferation, TIMP-2	-	
KF3CT	MCP-1, ICAM-1, IP-10, IL-8, MIG	-	
MyoF	Alpha-SM Actin, bFGF, Collagen I, Collagen III, Collagen IV, IL-8, MMP-1, PAI-1, TIMP-1	-	
Mphg	-	-	

Suppose the discovered ***differences*** are taken into consideration first. In that case, the profile plot shows that we are dealing more with statistical differences than clearly quantifiable differences in absolute scale. The Profile Plots move across the Y-axis almost in unison, making all the differences reported relatively small.

Collagen III and **PAI-1** biomarker activities in the MyoF system and **IL-8** in the Sag system were the same in **Fenoprolic®** and **Pycnogenol®** at the concentration used in this comparison. Still, neither was found to change significantly in the stand-alone analysis of **Pycnogenol®**. In basic terms, these activities were found in **Pycnogenol®** at the concentration used to compare the two products but were not found to respond significantly in the other concentrations tested.

Collagen III is an extracellular matrix protein and fibrillar collagen found in various connective tissues, especially in the **vascular circulatory system,** as a structural element of blood veins. Collagen III is centrally involved in cell adhesion, cell migration, and tissue remodeling. **PAI-I** is a Plasminogen activator inhibitor involved in tissue remodeling and fibrinolysis. Elevated levels of PAI-I are also considered a risk factor in the prognosis of various cardiac conditions, such as thrombosis and atherosclerosis [16].

IL-8, in the context of the SAg system of venular endothelial cells, **measures the activity related to neutrophil recruitment into acute inflammatory sites,** categorized as an inflammation-related activity modeling T-cell-driven Th1 vascular inflammation. In the KF3CT system of keratinocytes and dermal fibroblasts, where IL-8 was also reported as a joint activity between the two products, IL-8 activity is used for modeling Th1-type cutaneous inflammation.

Otherwise, we found all the other **Pycnogenol®** significant biomarker activities identical to the ones observed for **Fenoprolic®** (see Table 1). Given that the dataset in this comparison had only two non-cytotoxic concentrations available, and since these activities were found in **Pycnogenol®** in the higher concentrations tested, we conclude that these biomarkers should be considered significantly active in **Pycnogenol®** as well.

The activities classified as responding differently, **IL-1 alpha, CD69,** and **Eotaxin 3** are all biomarkers involved in the recruitment and proliferation of various types of white blood cells during either Th1 or Th2 inflammation reactions.

While it is worthwhile to note these differences detected, **none were found to be significantly responding for either product in their respective individual analyses,** so it is not valuable to formulate any meaningful conclusions from these differences. Overall, the biomarker responses across all systems are exceptionally similar.

In the considerably more extended list of forty-one (41) commonly reported activities, there are multiple exciting findings, even with the limitations of the available dataset. Both products are influencing key biomarkers in several systems (3C, LPS, CASM3C) like Thrombomodulin and Tissue Factor (TF) for hemostasis-related activities. Similarly, when looking at biomarkers of **cardiac system inflammation,** activities of the same direction and magnitude can be observed in several biomarkers (IL-8, VCAM-1, sTNF-alpha) in almost all of the venular endothelial cell systems (3C, LPS, Sag).

In **connective tissue-related activities,** both products appear to have almost identical activities across nearly all of the biomarkers included in those systems (HDF3CGF, KF3CT, MyoF), suggesting extensive shared efficacy in tissue matrix remodeling, fibrotic events, and control of chronic inflammation in fibroblast-influenced tissues. Finally, some interesting **shared immunomodulatory activities** can be seen, especially in the downregulation of sIL-17F in mononuclear white blood cells (BT system) and VCAM-1 and CD69 in the macrophage system (/Mphg).

In the following pages, we present the activities in Heat Map illustrations. Then, critical common activities are illustrated and explored in more detail and scope in different picture variations.

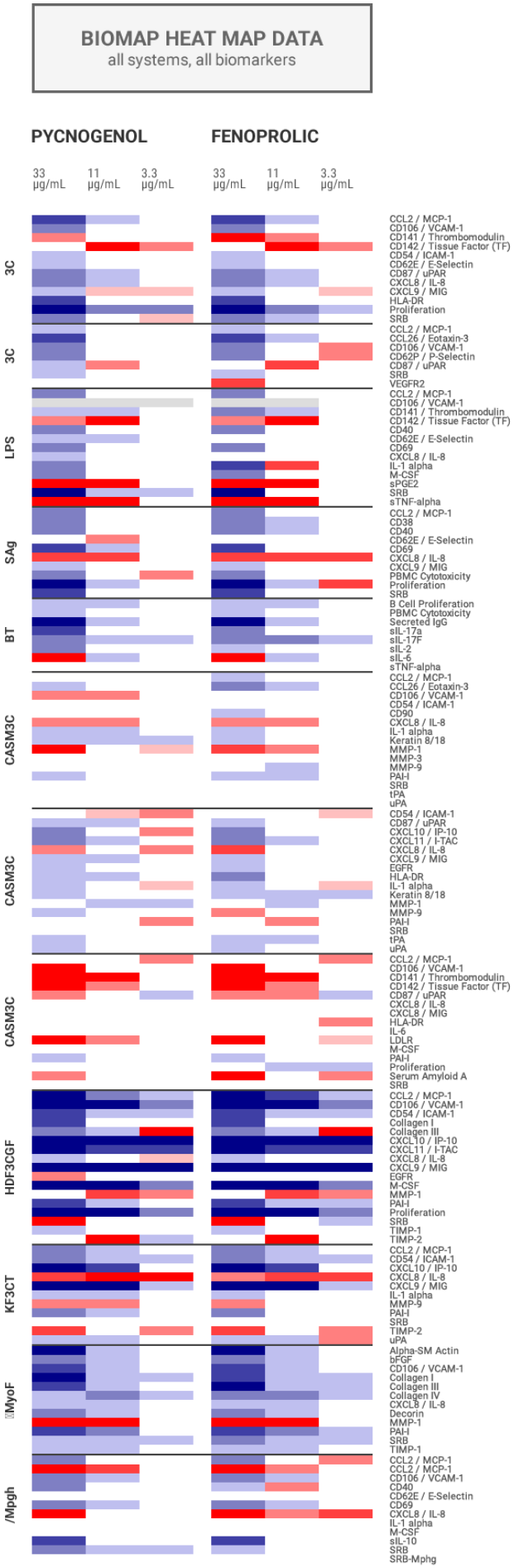


Figure 9. Heat Map comparison plot in hemostasis-related activities for **Pycnogenol®** and **Fenoprolic®.**

All activities measured for the two products are represented in a **Heat Map plot,** where three different concentrations tested for each product are depicted in columns in descending order according to the concentration used. **Pycnogenol®** activities occupy the first three columns, and the **Fenoprolic®** activities the next three.

The columns under the product concentrations are filled with colored bars that correspond to either **upregulated (red color) or downregulated (blue color) activities of biomarkers.** The codes of the biomarkers, both the genes and the proteins, are annotated with their codes on the right side of the Heat Map. White spaces (blanks) and even empty rows mean a biomarker with no detected activity or activity within the vehicle control space.

Lastly, the Heat Map is divided with black horizontal lines into sections according to the systems, which are annotated on the left side of the figure.

As can be easily seen, **most of the biomarkers were significantly activated either by up- or downregulation** by the two compounds, especially as the product concentration increased. Only a few biomarkers remained completely static during the product addition to the cell growth medium.

Figure 10. Heat Map comparison plot for hemostasis-related activities for **Pycnogenol®** and **Fenoprolic®**. All hemostasis-related or non-responding activities have been greyed out, and the hemostasis-related activities have been emphasized with an accent color. Colored bars under the product concentrations correspond to either up- (red color) or down-regulated (blue color) activities in their respective systems. Biomarker names are annotated with their codes on the right side of the Heat Map.

For both products tested, **a significant hemostasis-related activity can be seen** in two venular endothelial cell systems (3C, LPS) and the coronary artery smooth muscle cell system (CASM3C).

CD141 / Thrombomodulin is a cell surface receptor for complement factor 3b with anticoagulant, anti-inflammatory, and cytoprotective activities during fibrinolysis, coagulation, and thrombosis. Thrombomodulin is categorized as a hemostasis-related activity in all the influenced systems modeling Th1 vascular inflammation in endothelial and smooth muscle cells.

CD142 Tissue Factor (TF) is a cell surface receptor for coagulation factor VII that promotes the formation of thrombin during the process of vascular thrombosis and coagulation. TF is categorized as a hemostasis-related activity in the 3C system modeling Th1 vascular inflammation.

Thrombomodulin appears to be upregulated with the Tissue Factor (TF) for both 3C and CASM3C systems. Still, it has an opposite trend in the LPS system, showing downregulation, while TF upregulation remains the same.

Hemostasis and angiogenesis are central mechanisms responsible for **regulating vascular endothelial functionality,** such as tissue healing, tissue repair, and endothelial barrier stability [17]. When combined with the anti-inflammatory effects through **modulation of activated C-protein** [18], the response of these biomarkers by both products could support the past clinical findings and the reputed efficacy of OPC extracts in maintaining vascular system health.

Figure 11. Heat Map comparison plot for vascular inflammation-related activities for **Pycnogenol®** and **Fenoprolic®**. All cardiac inflammation-related or non-responding activities have been greyed out, and the cardiac inflammation-related activities have been emphasized with an accent color. Colored bars under the product concentrations correspond to either up- (red color) or down-regulated (blue color) activity in their respective systems, which are annotated with their code on the right side of the bars.

Based on the data, **almost all the systems involving venular endothelial cells appear to have some activity for biomarkers involved in vascular inflammation.** On the upregulation side of things, a practically symmetrical response emerges between the two products in activati**ng sPGE2, sTNF-α,** and **Interleukin IL-8.**

Secreted Prostaglandin E2 (sPGE2) is an immunomodulatory lipid mediator involved in muscle contractility, inflammatory pain, and kidney function. At the same time, **Secreted Tumor Necrosis Factor-alpha (sTNFa)** is a secreted proinflammatory cytokine involved in Th1 vascular inflammation. Both sPGE2 and sTNFa are categorized as inflammation-related activities in the LPS system modeling monocyte-driven Th1 vascular inflammation. **Interleukin-8 (IL-8/CXCL8)** is a chemokine that mediates neutrophil recruitment into acute inflammatory sites. IL-8 shares the categorization of sPGE2 and sTNFa as inflammation-related activities and as a model activity for T cell-driven Th1 vascular inflammation.

Interestingly, even though IL-8 was significantly upregulated and activated in the SAg and /Mphg systems, in the 3C system, the same IL-8 biomarker **was found to be activated in the opposite direction and downregulated instead of upregulated.** While the upregulation in the SAg and /Mphg system was found to be almost constant and independent of the actual concentration used, the downregulation in the 3C system showed clear signs of dose-dependent downregulation.

Understanding the timing of IL-8's activity in various systems is important, especially in the research of **atherosclerosis,** where IL-8 is an important mediator of angiogenesis that may contribute to plaque formation via its angiogenic properties [19].

Figure 12. Heat Map comparison plot for connective tissue-related activities for **Pycnogenol®** and **Fenoprolic®**.

Both products **exhibit the most pronounced effects when measured quantitatively on an absolute scale in connective tissue-related systems.** Almost all of the biomarkers included in every system (HDF3CGF, KF3CT, MyoF) are activated (predominantly downregulated), suggesting product efficacy in tissue matrix remodeling, fibrotic events, and control of chronic inflammation in fibroblast-influenced tissues.

The almost universal activation of **the HDF3CGF system** is exciting due to its capacity to model wound healing and matrix/tissue remodeling in the context of Th1-type inflammation. The HDF3CGF system is biologically relevant for various diseases, including fibrosis, psoriasis, stromal biology in tumors, and rheumatoid arthritis, where the system has been demonstrated to have predictive power for *in vivo* biology.

One fascinating observation is that the most downregulated activity in the whole panel is **IP-10 (Interferon-gamma-inducible protein 1),** a central chemokine mediating T cell, monocyte, and dendritic cell chemotaxis. IP-10 is classified as a model of Th1 inflammation in the HDF3CGF system.

The significance of this downregulating comes from the published science of a widely reported correlation between its **presence and overexpression in the pathogenesis of several chronic autoinflammatory conditions,** such as rheumatoid arthritis and osteoarthritis [20], something which has also been the subject of successful clinical research for **Pycnogenol®** in the past in studies of joint health benefits.

In addition to the IP-10, also **MIG (Monokine induced by gamma interferon (MIG/CXCL9)**, a chemokine mediating T cell recruitment with a similar standing as a central core inflammatory biomarkers in osteoarthritis and Chron's disease [21], was found to be heavily downregulated by both products.

Figure 13. Heat Map comparison plot for immunomodulating activities for **Pycnogenol®** and **Fenoprolic®**. All non-immunomodulating or non-responding activities have been greyed out, and cardiac inflammation-related activities are emphasized with an accent color. Colored bars under the product concentrations correspond to either up- (red color) or downregulated (blue color) activity in their respective systems, which are annotated with their code on the right side of the bars.

The key finding in this space involves a modes downregulation of **lnterleukin-17F {ll-17F), a proinflammatory cytokine produced by T cells** that induce cytokine, chemokine, and adhesion molecule production and mediates neutrophil recruitment to sites of inflammation.

IL-17F is a proinflammatory cytokine with a central role associated with the pathogenesis of many diseases, such as autoimmune diseases. It has an important role, for example, in asthma, where it has been well characterized both *in vitro* and *in vivo* to have a proinflammatory role in the development of the condition [22]. The expression level of IL-17F correlates with the severity of the disease.

It is also heavily involved in intestinal inflammation. Expression of IL-17F in the colon is associated with Inflammatory Bowel Disease (IBD), Crohn's disease, and Ulcerative colitis [23–24], **suggesting a role in chronic, uncontrolled low-grade inflammation.**

Vascular Cell Adhesion Molecule 1 (VCAM-1/CD106) reacting in the /Mphg system is a cell adhesion molecule that mediates the adhesion of monocytes and T cells to endothelial cells.

Active in the same system, **CD69 is a cell surface activation antigen**-induced early during immune activation and involved in macrophage activation. CD69, like VCMA-1, is categorized as an immunomodulatory-related activity in the /Mphg system modeling macrophage-driven Th1 vascular inflammation.

Lastly, we looked at the database hits found in the **BioMAP reference compound database.**

An unsupervised search was conducted against the BioMAP Reference Database of > 4,500 agents for each non-cytotoxic concentration of the test agents. The similarity between agents was determined using a combinatorial approach that accounts for the characteristics of BioMAP profiles by filtering (Tanimoto metric) and ranking (BioMAP Z-Standard) the Pearson's correlation coefficient between the two profiles. Profiles are identified as having mechanistically relevant similarity if Pearson's correlation coefficient is ≥ 0.7. Additional information can be found in the Supplemental material.

Table 4. Top BioMAP Reference Database matches for both **Fenoprolic®** and **Pycnogenol®**.

TEST AGENT	DATABASE MATCH	Z-SCORE	PEARSON SCORE	COMMON READOUTS	MECHANISM CLASS
Fenoprolic® 11 µg/mL	Chlorquinaldol	10.469	0.701	148	Antimicrobial Agent
	BAY 11-7085	8.429	0.670	111	NFκB Inhibitor
	Sertaconazole Nitrate	8.200	0.592	148	Antifungal Agent
Fenoprolic® 3.7 µg/mL	Chlorquinaldol	11.196	0.730	148	Antimicrobial Agent
	BAY 11-7085	8.979	0.698	111	NFκB Inhibitor
	Sertaconazole Nitrate	8.937	0.630	148	Antifungal Agent
Pycnogenol® 11 µg/mL	Chlorquinaldol	9.929	0.678	148	Antimicrobial Agent
	BAY 11-7085	8.340	0.665	111	NFκB Inhibitor
	DIDS	7.728	0.644	105	Anion Exchanger 1 inhibitor
Pycnogenol® 3.7 µg/mL	Chlorquinaldol	11.295	0.734	148	Antimicrobial Agent
	GSKj4	9.050	0.636	148	Histone Demethyl. Inhibitor
	BAY 11-7085	8.643	0.681	111	NFκB Inhibitor

In an unsupervised search for mathematically similar compound profiles from the BioMAP Reference Database, in the lowest concentration tested, both **Fenoprolic®** and **Pycnogenol®** were found to be most similar to Chlorquinaldol (Pearson's correlation coefficients r = 0.730 and 0.734, respectively). **The Pearson's correlation coefficients between the two test agents and Chlorquinaldol are above the determined threshold (r ≥ 0.7), indicating these compounds share a mechanistically relevant similarity.** Chlorquinaldol is an antimicrobial and antiseptic agent formerly used for topical treatment of skin conditions and vaginal infections.

In the other concentration tested, only **Fenoprolic®** was similar to Chlorquinaldol (Pearson's correlation coefficients r = 0.701). The Pearson's correlation coefficient between these two profiles is above the determined threshold (r ≥ 0.7), indicating these compounds share a mechanistically relevant similarity. Chlorquinaldol is an antimicrobial agent formerly used for topical treatment of skin conditions and vaginal infections.

There were 17 common activities for the reference match for Chlorquinaldol within the following systems: 3C (Proliferation), LPS (sTNFα), SAg (Proliferation), BT (sIL-17F, sIL-6), HDF3CGF (MCP-1, VCAM-1, ICAM-1, Collagen III, IP-10, I-TAC, MIG, M-CSF, PAI-1, Prolif 72), and MyoF (αSMA, MMP-1).

Another close match appearing from the database search just under the significance threshold (Pearson's correlation coefficients between 0.65–0.70) for both products was for compound **BAY 11-7085, a well-researched NFκB Inhibitor,** and irreversible inhibitor of IκBα phosphorylation capable of preventing activation of NF-κB by cytokines and lipopolysaccharides. BAY 11-7085 is an interesting anti-inflammatory compound that has been researched in hundreds of publications for its antitumor properties and capacity to induce apoptosis [25–26].

4. DISCUSSION AND FUTURE EFFORTS

This research article aims to report the observed findings of and between the two products tested on a qualitative scale and in terms of profile and magnitude across a wide range of in vitro biomarkers. The purpose of this article is not to start a deep discussion of the observed biomarker activities, their clinical significance, or their precise relationship to the existing published data and science. The authors acknowledge a need for more data and insight, which are still required to establish clear connections. However, we can conclude that **the study provided a near-identical profile of activities** between **Fenoprolic®** and **Pycnogenol®** across the 148 biomarkers studied in the BioMAP panel.

In the non-cytotoxic concentration tested, **both products demonstrated significant activity in the systems and biomarkers associated with modulation of cardiovascular inflammation and hemostasis,** one of the established vital health benefit associations for pine bark oligomeric proanthocyanidins and their metabolites.

Similar activity was evident in the systems associated with connective tissues measuring fibroblast biomarker responses, where almost universal activity could be seen across the biomarkers when exposed to both products**. Inhibition of NF-κB, Matrix Metalloproteinases (MMPs), and inhibition of IP-10** are all hallmark activities in the homeostasis of skin, connective tissue, and better joint health.

In the future, more specific *in vitro* data would be helpful to proceed further in the comparison, for example, investigating these products' possible influence on vascular health and inflammation by **looking at Endothelial nitric oxide synthase (eNOS) activity in more detail.** eNOS produces NO in physiological circumstances, which regulates vascular tone and reduces atherosclerotic lesions. Inhibitors of eNOS may increase peripheral vascular resistance, platelet aggregation, cerebral and cardiac ischemia, decrease renal function, and impair wound healing. Another good choice could be to run an *in vitro* activity panel on **Angiotensin-Converting Enzyme (ACE).** Angiotensin is involved in malignant hypertension or hypertension resulting from stenosis of the renal artery, as well as plasma renin activity. Consequently, ACE inhibitors are useful as antihypertensive agents.

Another reasonable and prudent action would be to repeat the BioMAP study with the same compounds to see if the results can be repeated and to retune the range of concentrations to get more data at the non-cytotoxic range, allowing a more robust evaluation of the responses and their significances.

Eevia is planning further actions also in the chemical characterization of the products. One of the following steps already in motion is a **study of the product compositions in more detail.** In this study, the oligomeric proanthocyanidin fraction of both products will be studied, for example, for their respective **Average Polymerization Rate (APR), cross-linkage features of the monomers, and functional group substitutions.** Working with a world-leading laboratory with expertise in advanced polyphenol analytical methods allows quantification of the main polyphenol fractions, including those outside the OPC group of compounds. Such quantification allows the comparison to continue with the addition of composition-level data, to start evaluating the similarities and differences of the active compound content in the two products, and to determine if and how that reflects the biomarker responses seen so far.

The compositional research could also include method development. The industry still sometimes relies heavily on methods that are becoming obsolete for analyzing ingredients' content or chemical composition, especially for botanical extracts. For pine bark extracts, the compendial method described in the United States Pharmacopeia monograph of *Maritime Pine Extract,* is for a simple spectrophotometric analysis that provides a quantification of OPCs as expression of procyanidins. However, the said method is only able to quantify the amount of OPCs in the product through the acid-butanol reaction product of procyanidins, but the method is not able to say anything about the composition prior to the reaction. This allows ways to

manipulate the results, for example with addition of high molecular weight condensed tannins and other compounds that will show up in the analysis but are unlikely to result into a desirable bioactivity due to compromised bioavailability. And this is all so far just about the active fraction quantification, and not yet about the actual identification of the product. Our aim is to figure out if a more adequate methods for measuring polyphenols and oligomeric proanthocyanidins from pine bark extract could be developed, which could enable the distinction between the various product types that still meet the monograph criteria.

In the literature regarding herbal medicine and botanical ingredients for dietary supplements, the terms “not significantly different”, or “essentially the same” are used for compositional comparison, for example in the "Guidance on the equivalence of herbal extracts in Complementary Medicine" documentation published by TGA (Therapeutic Goods Administration, Department of Health and Ageing, Australian Government). One conclusion of this article and the study within is that perhaps bioactivity comparison from in vitro screening could be another relevant approach to establish equivalence. Instead of herbal equivalence, the compositional equivalence on key bioactive constituents could be validated by a comprehensive screening of in vitro bioactivity.

In an article by *“Understanding plant to extract ratios in botanical extracts,”* the authors Monagas et al. discuss the concept of standardized extracts, extract plant ratios, and the possible use of bioassays. Standardized extracts are categorized by European Medicines Agency (EMA) as those where *the identified constituents are understood to fully account for an extract's proven therapeutic activity,* but relationship of identified constituents to an extract's biological activity may not always be clear. Identity of constituents responsible for the biological activities of a plant extract is rarely clearly established, even with bioassays and clinical studies, and numerous constituents may be active to different degrees and in various respects [27]. The same discussion was also on topic of an earlier paper on the topic, where standardization to either active or marker constituents and bioassays that reflect the underlying mechanisms of action was proposed as one definition choice [28].

While bioassays are acknowledged as a kind of measure of therapeutic activity, they are rarely used for standardization, but some examples do exist. One is the bioactivity measurement to ensure reproducible pharmacological activity of the dragon’s blood (*Croton lechleri*) latex botanical drug [29].

In a discussion of herbal equivalence, although **Fenoprolic®** and **Pycnogenol®** are made from the same genus of trees, the coniferous pines from the genus Pinus in the Pinaceae family are not from the same species. **Fenoprolic®** is from Pinus sylvestris and **Pycnogenol®** from Pinus pinaster. Hence, according to various regulatory agencies, such as the FDA, TGA, and EMA, they are not herbal equivalents. Subject to further information on the process, they may be extract equivalent and subject to current USP and other monographs; both extracts meet the compendial requirements. The information from the ongoing compositional study may elucidate similarities or differences between the main polyphenolic chemical classes.

However, as the reported study shows, we suggest that the industry utilizes bioactivity profiling to identify bioequivalence. At the same time, it may enable the rejection of biologically inactive ingredients and products that claim herbal or compositional compendial equivalence. Characterizing the in vitro features on neutral, repeatable biomarker panels could be used to support plant extract standardization.

5. APPENDIX

Supplementary Materials

For additional information on Eevia Health's BioMAP report, please get in touch with the authors.

For more details on BioMAP, see the references.

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Conflicts of Interest

Petri Lackman and Stein Ulve are employees of Eevia Health Plc. Finland.

Abbreviations

BioMAP	Biological Matrices Analysis Platform
MCP-1	Monocyte Chemoattractant Protein-1
VCAM-1	Vascular Cell Adhesion Molecule-1
I-TAC	Interferon-Inducible T-cell Alpha Chemoattractant
MIG	Monokine Induced by Gamma Interferon
IP-10	Interferon Gamma-Induced Protein 10
IL-8	Interleukin-8
M-CSF	Macrophage Colony-Stimulating Factor
sIL-17F	Soluble Interleukin-17F
PAI-1	Plasminogen Activator Inhibitor-1
MMP-1	Matrix Metalloproteinase-1
TF	Tissue Factor
sPGE2	Prostaglandin E2
sTNF-alpha	Tumor Necrosis Factor Alpha
SIgG	Secreted lgG
sIL-6	Soluble Interleukin-6
TM	Thrombomodulin
ICAM1	lntercellular Adhesion Molecule 1
TIMP-1	Tissue Inhibitor Matrix Metalloproteinase 7
TIMP-2	Tissue Inhibitor Matrix Metalloproteinase
Alpha-SM Actin	Alpha-Smooth Muscle Actin
bFGF	Basic Fibroblast Growth Factor
IL-1 alpha	lnterleukin-1 alpha
CD69	CD69 cell surface activation antigen
BAY 11-7085	NFκB Inhibitor
DIDS	Anion Exchanger 1 inhibitor
GSKj4	Histone Demethylase Inhibitor

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