SUMMARY

The present report contains a brief exposure of scientific and technical information about MAQUISELECT®/DELPHINOL®, a product from MAQUI NEW LIFE S.A., a bio-science company from Chile.

Our product, MAQUISELECT®/DELPHINOL®, is a standardized extract from Maqui berry, Aristotelia chilensis, a fruit with high antioxidant capacity.

The report includes generalities about Maqui berry, as well as the composition of our product MAQUISELECT®/DELPHINOL®, a dry standardized extract of freeze-dried Maqui berry fruit.

General information on Maqui and in particular on MAQUISELECT®/DELPHINOL® will be shown and discussed.

Also included in the report is scientific evidence of the effect of the different antioxidant components present in MAQUISELECT®/DELPHINOL®, such as anthocyanins and delphinidins, in four main aspects of human health: as an immune system booster, as an inflammatory control, as anti-cancer treatment, and as a glucose balance composition.

Further technical information includes Industrial Property Protection of our product, MAQUISELECT®/DELPHINOL®, and a general brief of patent documents and scientific publications about delphinidins and their uses and effects.
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MAQUISELECT®/ DELPHINOL®

MAQUISELECT®/ DELPHINOL® is a purified, concentrated, standardized dried extract from Maqui berry freezed fruit, Aristotelia chilensis.

MAQUISELECT®/ DELPHINOL® contains fruit solids, is a free flowing deep purple powder, 100% soluble in water, with good taste, typical of Maqui fresh fruit.

The total content of anthocyanins in MAQUISELECT®/ DELPHINOL® is NLT 35%, and the total content of delphinidins is NLT 25%. MAQUISELECT®/ DELPHINOL® also shows less than 5% of humidity (Certificate of Analysis 10/0174/LRF).

### GENERAL ISSUES OF MAQUISELECT®/ DELPHINOL®

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Aristotelia chilensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant part extracted</td>
<td>the fruit</td>
</tr>
<tr>
<td>Indian name</td>
<td>&quot;MAQUI&quot;</td>
</tr>
<tr>
<td>Fruit origin</td>
<td>Patagonia area in southern Chile, South America</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Indena Group</td>
</tr>
<tr>
<td>Extract ratio</td>
<td>25 – 40 : 1</td>
</tr>
</tbody>
</table>

### PHYSICAL PROPERTIES OF MAQUISELECT®/ DELPHINOL®

<table>
<thead>
<tr>
<th>Humidity (w/w %)</th>
<th>≤ 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Free flowing deep purple powder</td>
</tr>
<tr>
<td>Taste</td>
<td>Good taste, typically Maqui fruit fresh</td>
</tr>
<tr>
<td>Water solubility</td>
<td>Good solubility in water</td>
</tr>
</tbody>
</table>

### BIOACTIVE COMPOUNDS OF MAQUISELECT®/ DELPHINOL®

<table>
<thead>
<tr>
<th>Average value of ORAC FN (µmol TE/g of extract)</th>
<th>25,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ANTHOCYANINS %</td>
<td>35 NLT</td>
</tr>
<tr>
<td>Total DELPHINIDINS %</td>
<td>25 NLT</td>
</tr>
</tbody>
</table>

### MICROBIOLOGICAL SPECIFICATIONS OF MAQUISELECT®/ DELPHINOL®

<table>
<thead>
<tr>
<th>TAMC Total Aerobic Microbial Count</th>
<th>≤ 3,000 CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYMC Total Combined Yeast/Moulds Count</td>
<td>≤ 500 CFU/g</td>
</tr>
<tr>
<td>Bile-tolerant gram negative Bacteria</td>
<td>≤ 100 CFU/g</td>
</tr>
<tr>
<td>Escherichia Coli</td>
<td>Absent /g</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Absent /25g</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Absent /g</td>
</tr>
</tbody>
</table>
### MICROBIOLOGICAL SPECIFICATIONS OF MAQUISELECT® / DELPHINOL®

| Staphylococcus Aureus    | Absent /g |

### Another information about MAQUISELECT® / DELPHINOL®

| Pesticides determination (for food products) | Complies |
**BACKGROUND INFORMATION**

**Pharmacognosy**

In southern Chile, a wild fruit grows which boosts the immune system, contains anti-inflammatory properties, is effective to control blood sugar and has higher levels of antioxidants than any other berry fruit: the Maqui berry.

The Maqui berry is just 4 mm in diameter. It has a dry flavor and contains four seeds. It grows on an evergreen bush that reaches a height of about 4 meters, which is also known as Maqui (*Aristotelia chilensis*).

**Traditional use**

Maqui’s therapeutic qualities have been known for centuries to the Mapuches, indigenous people who have traditionally lived in the southern part of Chile. Besides eating the fruit, they also consumed fresh and fermented Maqui juice. They used it to treat stomach ailments, sore throats or wounds, and also as an analgesic and fever reducer, and as a natural colorant.

Mapuches also used dry leaves infusions or directly powdered dry leaves to treat wounds. Fresh leaves infusions were used to alleviate feverish conditions, diarrhea, dysentery, indigestion, to alleviate sore throat symptoms, tonsil inflammation, and mouth ulcers. The juice of fresh leaves was also drank or used as ointment topically.

The research team at Pharmacology and Morphophysiology Institute, of the Faculty of Veterinary Sciences of Universidad Austral de Chile, one of the partners of Maqui New Life developing MAQUISELECT®/DELPHINOL®, is conducting ongoing research that started three years ago. Their findings have proven the exceptional properties of Maqui, revealing their chemical origin and identifying other properties which were not known to the Mapuches.

MAQUISELECT®/DELPHINOL® extract is obtained from the wild Maqui fruit, gathered in southern Chile. It has a standardized content of anthocyanins (35% NLT) and an astonishing level of delphinidins (25% NLT), the highest among all food ingredients which are currently available.
Current situation

The oxidation processes in our cells produce free radicals, which are atoms or molecules with an unpaired electron. These free radicals are highly reactive with other substances and therefore damaging to the human organism.

Antioxidants are molecules which can neutralize free radicals, stopping the stress caused by oxidants and slowing down the cell aging process.

Figure 1: ORAC capacity of different berry fruits.

The ORAC (Oxygen Radical Absorbance Capacity) index measures the antioxidant activity and free radical neutralization properties in food. Also, a high ORAC value for a food is often associated with protection against various chronic inflammatory diseases or metabolic disorders.

Maqui fresh fruit has the highest ORAC score among berries known so far (Figure 1). Ronald L. Prior and Guohua (Howard) Cao from the Jean Mayer USDA
Human Nutrition Research Center on Aging at Tufts University in Boston suggest a daily intake of 3,000 to 5,000 ORAC units. However, based on average fruit and vegetable consumption and their ORAC content, most people consume only about 1,200 ORAC units. Recently, Chilean Government, through the Health Ministry included Maqui in a list of “Traditional Herbal Medicines”, recognizing its anti-inflammatory, antispasmodic, astringent and analgesic properties.

In the United States of America, the use of Maqui berries in food is approved as safe by the FDA and the import directives established by the USDA, as established in the list of “Approved Fruits and Vegetables” version 04/2010.

In Europe, Maqui berries have been imported before 1997, and thus would not fall in the category of novel food, according to regulation 258/97/EC.

Use as food ingredient

The idea to commercialize Maqui was introduced by our team to the US market. Given the high content of antioxidants in the Maqui fruit, its use as an ingredient in different food products has increased in the last years.

Alone, or in combination with other plants or fruit extracts, Maqui is present in drinks, capsules, makeup powders, dietary supplements, nutraceuticals, with different marketing focus, and in different configurations, from lyophilized powder, concentrated juice extracts, fruit pulp, etc.

More than 60 products containing Maqui are available, such as Monavie's Pulse, Monavie's EMV, which are marketed as energizing and anti-oxidant beverages; Lancome's Maqui Loose powder, commercialized as a makeup powder; Tahiti Trader's Maqui 100 and Organic Maqui 100, sold as beverages with high antioxidant content; Swanson's Full Spectrum Maqui Berry capsules; Live Superfoods' Maqui Berry powder; “Genuine Whole Food Now” Maqui Super Antioxidant Juice; S4Labs' MaquiMaxx; Health Spark Limited's Maqui Extreme; MäritzMayers Laboratories' Super Maqui–1200; Health Essentials' Maqui Berry Active; Biovea's Maqui Berry capsules; Dr. Scurr's Zinopin capsules; Honest Tea's Kombucha beverage; sold as dietary supplements, among many others. Nevertheless, none of the available products contains a Maqui concentrated extract standardized in its delphinidin content as MAQUISELECT®/DELPHINOL® does.
Antioxidant content of berries

The intake of super berries has a positive impact on human health, well being and the prevention of certain diseases, including cardiac, neurodegeneration, aging, obesity, and also certain types of cancer, such as esophagus and digestive system. The biological properties are due to the content of polyphenols. Polyphenols include flavonoids (anthocyanins, flavonoles, and flavonoids), condensed tannins (proanthocyanidins) and hydrolyzed tannins (ellagittannins and galotannins), estilbenoids and phenolic acids [61].

Among polyphenols, anthocyanin (pigments that give the attractive blue violets, red colors), have demonstrated antioxidant, anti cancer, and anti-inflammatory effects [62–65]. The concentrations of polyphenols, either condensed proanthocyanidines or ellagittannins, vary considerably among berries [65]. For example, blueberries contain principally proanthocyanidines, whereas blackberries, black raspberries, raspberries and strawberries contain principally ellagittannins. Therefore, the type and specific chemical structure of the compounds present in a berry can contribute significantly to the biological properties. For example, the antibacterial anti-adhesive properties of red cranberry are explained by the oligomeric proanthocyanidins, that possess a type A structural link [66]. Similarly, the biological effects of blueberries (rich in proanthocyanidine) compared to strawberries (rich in elagittannins) on neuronal and cognitive deficit in adult rats can be due to the effect of these compounds in different regions of the brain [67,68]. A diet rich in pterostilben (present in berries and grapes) is able to revert the negative effects of cognitive involution and behavior performance [69].

The phenolic compounds from berries are better known for their antioxidant capacities [70]. In fact, they regulate the activity of the enzymes that metabolize and modulate the nuclear receptors, genetic expression, signalization paths, and DNA oxidative damage repair [70, 71]. Animal and human studies have shown that polyphenols are poorly absorbed due to the low levels found in blood. However, the polyphenols from berries are also metabolized and converted by the micro flora of the colon in other related compounds. These compounds can persist in vivo, accumulating in the tissues and contributing to the biological effects. Polyphenols from berries have a role in the prevention and treatment of different chronic diseases [72].
**Delphinidins**

Delphinidins are a class of anthocyanidines of which Maqui fruit, *A. chilensis* has the highest content of all known fruits, and our product, MAQUISELECT®/DELPHINOL® contains up to 28.6%.

<table>
<thead>
<tr>
<th>Berry</th>
<th>mg of anthocyanin per 100mg fruit</th>
<th>Main kind of anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberry</td>
<td>25-495</td>
<td>delphinidin-3-galactoside(13,5%), malvidin-3-galactoside(12,9%), malvidin-3-glucoside(11,9%), malvidin-3-arabinoside(11,9%), delphinidin-3-arabinoside</td>
</tr>
<tr>
<td>Cranberry</td>
<td>45-100</td>
<td>peonidin-3-galactoside, peonidin-3-arabinoside, cyanidin-3-galactoside, cyanidin-3-arabinoside</td>
</tr>
<tr>
<td>Raspberry</td>
<td>20-60</td>
<td>cyanidin-3-sophoroside, cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-glucosylrutinoside</td>
</tr>
<tr>
<td>Blackberry</td>
<td>83-326</td>
<td>cyanidin-3-glucoside(57%), cyanidin-3-rutinoside(30%)</td>
</tr>
<tr>
<td>Maqui</td>
<td>1400</td>
<td>delphinidin-3-sophoroside-5-glucoside(33,4%), delphinidin-3-5-diglucoside(16,2%), cyanidin-3-sophoroside-5-glucoside(14,8%), delphinidin-3-sophoroside(9%), delphinidin-3-glucoside(12,4%), cyanidin-3-sophoroside(4%), cyanidin-3-glucoside(4,4%)</td>
</tr>
</tbody>
</table>

**Maqui berry, Aristotelia chilensis**

*A. chilensis* is an endemic plant of the Elaeocarpaceae family present geographically in central and southern Chile, up to 2,500 m altitude, also in the archipelago of Juan Fernández and south-western of Argentina. It grows in soil rich in organic matter. Frequently *A. chilensis* forms pure communities that receive the name of “macales”. The fruit has more pulp than other berries and its flavor is described as “astringent but fresh”. The leaves and the fruits of *A. chilensis*, have been used for sore throat, ulcer, fever, hemorrhoids, inflammation, diarrhea, lesions, migraines and scars [74–76].

Maqui has become a top super berry in the food and beverage industry in USA. This fruit contains one of the highest contents of anthocyanins, polyphenols and has a great capacity to trap free radicals (ORAC), with values between 4 to 30 times higher than other berries (mangosten, acai, etc.) [77].

The anthocyanins present in the fruit of Maqui constitute a 0.2% and are associated to a great anti-oxidant capacity. In the fruit 8 pigments are found:
1. Delphinidin-3-O-samb-5-O-gluc;
2. Delphinidin-3,5-O-diglucoside;
3. Cyanidin-3-O-samb-5-O-gluc;
4. Cyanidin-3,5-O-diglucois;
5. Delphinidin-3-O-sambubioside;
6. Delphinidin-3-O-glucoside;
7. Cyanidin-3-O-sambubioside;
8. Cyanidine-3-O-glucoside

Where the principal anthocyanin is delphinidin-3-sambubioside,5-glucoside, that constitute 34% of total anthocyanins. In the fresh fruit, the mean total anthocyanins is 137.6 ± 0.4mg/100g; and in dried fruit 211.9 ± 0.6 mg/100g. [78]. Moreover, the fruits contain a high content of minerals, 100gr of fruit, provide 27% of the daily recommended dose of calcium, 28% of potassium and 70% of iron, demonstrating a potential as a dietary supplement and functional food [82].

The juice of Maqui can inhibit the oxidation of lipoproteins of low density (LDL) and protect the endothelial cells against intracellular oxidative stress, as anti-atherogenic [73]. Methanolic extract of the fruits in in vivo studies have demonstrated a preventive protective effect in reperfusion/ ischemia in the heart of rats, probably due to a reduction in the lipidic oxidation and oxidative stress[79].

MAQUISELECT®/DELPHINOL® has a high concentration of delphinidins (28.6%).

It is known that anthocyanins have a positive effect in many diseases. Recently, anti-inflammatory [68], antiviral [69], and antitumoral [67] effects were demonstrated for anthocyanins. It is known that anthocyanins, activate PPARγ modulating the inflammatory responses and inhibiting NfκB, a nuclear transcription factor that controls the expression of several proinflammatory proteins. When NFκB is inhibited, the pro inflammatory cytokines (Interferon gamma, Interleukin 2) and pro inflammatory agents (COX–2, iNOS) are reduced.

MAQUISELECT®/DELPHINOL® has demonstrated the capacity to inhibit COX2 and to reduce inflammation induced by carrageen using the rat paw model. It is experimentally proven that MAQUISELECT®/DELPHINOL® activates PPARγ and inhibits NFκB without toxicity and collateral effects. NFκB is activated in the inflammation of upper respiratory tract as asthma and allergies and in autoimmune diseases.
Pharmacodynamics and pharmacological effects of anthocyanins

In the murine model Apc\textsuperscript{min}, which is highly susceptible to spontaneous intestinal adenoma formation, the effect of anthocyanins obtained from red grape pomace extract (oenocyanin) were tested for its cancer chemopreventive properties. Mice received oenocyanin (0.3%) in their diet until week 16, when adenoma number and burden were recorded. In mice which had consumed oenocyanin, overall adenoma burden was halved and adenoma number was marginally reduced when compared with mice on control diet. Oenocyanin anthocyanins and glucuronide metabolites were found in the urine and intestine but not in plasma [132].

A further study [131] analysed the effect of mirtocyan, an anthocyanin extracted from bilberries, in colorectal cancer human patients, considering the pharmacodynamics. The study found that anthocyanin concentrations in plasma and urine were roughly dose–dependent, reaching approximately 179 ng/gram in tumor tissue at the highest dose. In tumor tissue from all patients on mirtocyan, proliferation was decreased by 7% compared with preintervention values. The low dose caused a small but nonsignificant reduction in circulating IGF–I concentrations. In conclusion, repeated administration of bilberry anthocyanins exerts pharmacodynamic effects and generates concentrations of anthocyanins in humans.

The pharmacological effect of delphinidins has been widely researched, and scientific evidence has been found, either \textit{in vitro} or \textit{in vivo}, relating the application of delphinidins with antioxidant effects [1, 2, 3, 5, 6, 7, 11, 12, 13, 14, 19, 20, 21, 24, 25, 26, 28, 29, 30, 31, 32, 35, 36, 37, 38, 39, 41, 43, 44, 47, 52, 54, 56, 57 and 59]; anti–inflammatory effects [5, 24, 34, 50, 51, 53 and 55], prevention in tumor generation, cancer suppressor, and apoptosis induction in cancerous cells [5, 8, 10, 15, 17, 18, 22, 24, 32, 33, 40, 42, 44, 45, 46, 49, 50, 51, 55, 58 and 60]; angiogenesis inhibition in cancer [9, 23, 25, 27 and 56]; inhibition of nitric oxide production [4, 16 and 25]; and effect on metabolic syndrome [48].

Our team identified four main application fields for anthocyanins, more particularly delphinidins, in human health. These fields are application of delphinidins as an immune system booster, application of delphinidins with an anti–inflammatory effect, application of delphinidins in cancer treatment, application of delphinidins to maintain glucose balance in metabolic syndrome associated conditions.

In the following sections, further detail will be given to the use of delphinidins in the fields of application identified by our team, as well as results from our own research, demonstrating the effects of our product, \textit{MAQUISELECT®/DELPHINOL®}, in these situations.
Application 1: Immune System Booster

As previously discussed in this report, our product, MAQUISELECT®/DELPHINOL®, comprises up to 28.6% of delphinidins, thus, analysing the function or effect of delphinidins is of major importance.

Current research on the effect of delphinidins in the immune system is non-existent. Nevertheless, Maqui New Life is analysing the effect of delphinidins in the immune system, firstly in an in vitro set of experiments.

Our Research

Our research group has performed several studies to determine the effect of the main components of our product, MAQUISELECT®/DELPHINOL®, on the immune system.

Calcium, apart from being important as a major bone constituent, is also relevant as a second messenger in many cell types. In particular, lymphocytes.

In cell signaling, calcium is maintained at a low intracellular concentration, and in the case of lymphocytes, the activation of antigen receptors induces an influx of calcium from the extracellular space. One of the main routes is the Store-Operated Calcium (SOC) channels.

Therefore, the research of our team has been focused in studying the effects of the main components of MAQUISELECT®/DELPHINOL® in calcium mobilization in lymphocytes, more particularly, T cells. This includes analysing the effect of delphinidin in Jurkat cells, which are an immortalized line of T lymphocyte cells frequently used for in vitro studies of immune system, as well as similar studies using human lymphocytes obtained from peripheral blood.

In a first approach, our research determined that delphinidin induces calcium mobilization in a dose-dependent manner in Jurkat E6-1 cells. As can be seen in Figure 2, with increasing concentration of delphinidin, from 10 micromolar to 100 micromolar, the calcium fluxes are also increased.
Figure 2: Calcium fluxes in Jurkat cells. A dose-dependent effect of delphinidin is shown.

As mentioned earlier, the mobilization of calcium in lymphocytes is primarily through Store-Operated Calcium Entry (SOCE) system. In order to prove that delphinidins induce calcium flux at SOCE level, Jurkat cells were incubated with gadolinium, BTP-2, and 2–APB, known as SOCE inhibitors.

Figures 3 and 4 show the effect of known SOCE inhibitors, 2–APB and gadolinium, in delphinidin–induced calcium fluxes in Jurkat cells.
Figure 3: Jurkat cells incubated with delphinidin and 2-APB SOCE inhibitor. A) Calcium flux in response to delphinidin in the presence of variable concentrations of 2-APB, B) Calcium flux response in Jurkat cells to an increase in extracellular calcium.

Figure 4: A) Jurkat cells incubated with delphinidin and gadolinium, a SOCE inhibitor.
Apart from SOCE, there are also other types of calcium mobilization which are modulated by the application of delphinidins. Intracellular calcium as well as PLC are essential in delphinidin–induced calcium mobilization. This is clear when Jurkat cells are incubated with variable concentrations of U–73122, a known PLC inhibitor, and delphinidins as shown in Figure 5.

![Figure 5](image)

**Figure 5:** Calcium release in response to delphinidin, challenged with PLC inhibitor U–73122.

Further research was focused in following the effects of calcium signaling through SOCE system in the production of cytokines involved in immune response, IL–2 and Interferon γ.

Intracellular calcium in resting cells is approximately 0.1 micromolar, while in an activated cell the concentration of intracellular calcium increases to 1 micromolar. This increase in turn induces the expression of different cytokines. As can be seen in the plot below, IL–2 production is significantly increased with 50 micromolar delphinidin (Figure 6).
It is postulated that delphinidin-induced IL-2 production is through calcium mobilization through SOCE, since addition of known SOCE inhibitor BTP-2 (1, 5, 10 µM), greatly affects IL-2 production, as can be seen in Figure 7.

All previous results are experiments using Jurkat cells. Nevertheless, a similar effect is observed using T-cells. We observed an increase in IL-2 and INF-γ induced by delphinidin in human lymphocytes obtained from peripheral blood, as shown in Figure 8.

![Figure 6: Delphinidin-induced IL-2 production.](image)

![Figure 7: Delphinidin induced IL-2 is inhibited with BTP-2.](image)
Figure 8: Delphinidin-induced IL-2 and INF-γ production in human peripheral blood lymphocytes.

Figure 9: Delphinidin induced IL-2 and INF-gamma production is mediated by SOCE.

In order to prove that human peripheral blood lymphocyte IL-2 and IFN-γ production induced by delphinidins is also mediated by SOCE system, human
Peripheral blood lymphocytes were incubated with a SOCE inhibitor, BTP–2. The results, shown in Figure 9 prove this hypothesis.

Further to our research on the effects of delphinidin on the immune system, our group found that delphinidin–induced production of cytokines (IL–2 and INF–γ) is through the NFALT pathway, as was measured by dephosphorylation of NFAT and the activation of pNFAT/pRL.

Our research has demonstrated that delphinidins elevate the release of intracellular calcium in Jurkat cells, which may activate the production of cytokines such as IL–2 and INF–γ in this cell line and in human T lymphocytes. Since cytokine production in T lymphocytes is activated through the NFAT transcription factor, and production of IL–2, induced by the delphinidins, is significantly reduced by the cyclosporin A (CsA) calcineurin inhibitor, it is evident that delphinidins have the ability to activate NFAT. All of these effects result in strengthening the cells of the immune system.
Application 2: Anti-Inflammatory Effect

General Research

Inflammation processes are biological responses of tissues to harmful stimuli. These processes are mediated by many different enzymes, the most common pathways in inflammation including cyclooxygenases 1 and 2 (COX-1, COX-2) and 5-lipoxygenase (5-LOX). Thus, the inhibition of these enzymes using different drugs has been proved to alleviate the symptoms of inflammation.

There are several reports describing specific effects of delphinidins in inhibiting the above mentioned enzymes COX and LOX, thus, an anti-inflammatory effect is expected.

A research reports the inhibition of up to 12% of COX-1 and COX-2 using concentrations of delphinidins of 40 micromolar, compared to common use anti-inflammatory drugs, such as ibuprofen (10 micromolar) with an inhibition of nearly 40% [5]. A similar research, this time using 100 micromolar delphinidins reported an inhibition of COX-1 and COX-2 of up to 49% [24].

Other study reports that delphinidins inhibit TNF–alpha induced COX–2 expression [51], while other study shows that delphinidins block the activation of NF–κ–B by UVB, and thus blocked the NF–κ–B induced COX–2 expression.

Also, delphinidins have been found to act as lipoxygenase inhibitors [53] suggesting that delphinidins could be used for anti-inflammatory therapy.

Our Research

We have conducted preliminary tests to find the effect of MAQUISELECT®/DELPHINOL® in an in vivo model of inflammation.

The anti-inflammatory effect of A. chilensis using the acute subplantar model of inflammation in rats was studied. Carrageenan 1% was injected subplantarly. The degree of inflammation was evaluated with the measurements of the width of the mice leg using a digital caliper, from time 0 (before injection) till 24 hrs. The maximum inflammatory effect was observed at 3 hours post injection. A. chilensis, was injected i.p. at 100 mg/kg. A. chilensis had a significant anti-inflammatory effect at 4 hours after injection. This effect was similar to the one observed with diclofenac between 4 and 24 hours (Figure 10).
Figure 10: Evolution of edema size in time for SS saline solution, *A. chilensis* 100mg/kg, *A. chilensis* 0.025mg/kg and diclofenac.

Figure 11: Histological comparison of rat paw tissue between experimental groups at 6 hours after treatment with carrageenan. (a) control subject; (b) treated with *A. chilensis* 100mg/kg at time 0; (c) treated with *A. chilensis* 0.025mg/kg at time 0; (d) treated with diclofenac 2mg/kg. All pictures with 10X magnification.
Application 3: Cancer Treatment

General Research

Inflammation has been identified as a critical component in tumor progression, where tumor microenvironments are frequently invaded by inflammatory cells. Also, quantitative aspects of wound repair or inflammatory gene expression often correlate negatively with cancer stage and prognosis. Furthermore, NF-κB signaling pathway has been described in inflammatory processes as well as cancer.

Delphinidins, apart from research focused in inflammatory processes, have been widely studied to determine their effect in cancer. There are reports suggesting that delphinidins have a tumor suppressor function, can induce apoptosis, or can have a potential role inhibiting angiogenesis in cancer.

For example, a study with 3-O-beta-galactopyranoside, a delphinidine, shows inhibition of COX-1 and COX-2, suggesting a protecting role in tumor formation and a role as anti-inflammatory compound [5].

Other reports show the effect of delphinidines present in berries inducing apoptosis in leukemia cells HL60 [8, 10, 22], humane uterine carcinoma, colon adenocarcinoma [15], hepatoma [18] and colon cancer cells HCT116 [8, 51].

A very important discovery, by Dr. Beliveau team, showed that delphinidins inhibit VEGF receptor 2 in neovascularization processes of tumor genesis [130].

Further studies show the effect of delphinidins inhibiting the proliferation of mammary cancer cells [17], human umbilical cord endothelial cells [25], colon cancer, lung, stomach, and mammary [24], adenocarcinoma and HT–29 cells [45] and murine hepatoma cells [33].

Thus, the effect of delphinidins in cancer is at many different levels, acting as a tumor suppressor, inhibiting proliferation of cells, inducing apoptosis, and inhibiting angiogenesis among others.

Our Research

In the study, conducted by our research group, we evaluated the effects of a juice berry A. chilensis on COX–2 expression, intracellular signalling pathways, and cell viability in colon cancer cells. Caco–2 cells were incubated with 5 μg/ml A. chilensis and the COX–2 expression was analyzed by immunoblot and real time PCR. The effect of A. chilensis on NF–κB pathway activation was studied by immunoblot of
IκBα, p65 NF-κB localization by immunocytochemistry and NF-κB activity by luciferase reporter plasmid. Also, the effect of A. chilensis on ERK1/2 and Akt phosphorylation, and c-fos expression was analyzed by immunoblot. The cell viability and apoptosis in Caco-2 and HT-29 cells was assessed by MTT assay, flow cytometry of annexin V–propidium iodide and immunoblot of apoptotic proteins. It was observed that A. chilensis reduced expression of protein and mRNA COX–2 and inhibited the TNF–α–induced activation of NF–κB. A. chilensis transiently reduced the levels of IκBα in cytoplasm at 2 and 4 h of incubation and mildly increased the nuclear localization of p65 NF–κB at 4 h. The treatment with A. chilensis transiently increased the ERK1/2 and Akt phosphorylation, at 4 h and 30 min, respectively, and an increase in the c–fos expression after 4 h of treatment was observed. The MTT and annexin V–PI analysis showed that A. chilensis did not affect Caco–2 cell viability, at concentrations that reduced COX–2 expression. On the contrary, high concentrations of A. chilensis induced an increase in apoptosis and necrosis of Caco–2 and HT–29 cells. In conclusion, we demonstrated that A. chilensis reduces COX–2 expression and TNF–α–induced activation NF–κB in Caco–2 cells suggesting a putative anti–carcinogenic and anti–inflammatory effect of A. chilensis.

Figure 12. Effect of A. chilensis on COX–2 expression. Caco–2 cells were incubated with 5 μg/ml A. chilensis (A.ch.) for 0, 24, 48 or 72 h and total proteins or RNA was isolated. The COX–2 protein level (A) was analyzed by immunoblotting using an antibody against COX–2, and the blot was stripped and re-probed with anti–β–actin antibody. (B) The mRNA expression of COX–2 at 24 h of treatment with A. chilensis was assessed by real time PCR. The graphs show the mean ± S.E. from three independent experiments. * p < 0.05 compared to the control.
Figure 13. Effect of *A. chilensis* on NF-κB activation. Caco-2 cells were transiently transfected with the pGL3-NF-κB and pRL-TK plasmids for 24 h, treated with *A. chilensis* or vehicle for 30 min and TNF-α or solvent was added for 16 h. Luciferase activity was measured in a luminometer. Each bar represents mean ± S.E., n=3, * p<0.05 compared with TNF-α.
Figure 14. Effect of A. chilensis on cell viability. Caco-2 cells were incubated with 5 µg/ml A. chilensis for 24 h and then stained with AnnexinV-FITC and PI, or analyzed by MTT assay. The AnnexinV–FITC and PI signal was detected by flow cytometry (A–C) and the formazan crystal produced from MTT was registered in a microplate lector (D). Each bar represents the mean ± S.E., n=8. ** p<0.01 vs control.
Application 4: Glucose Balance

General Research

Hyperglycemia is the central characteristic of all metabolic imbalances of sugar, lipids and ketones and amino acids [83]. This pathologic condition is the most common and known as diabetes type 2 (about 90% of all types of diabetes), it is considered an epidemic disease, especially in occidental countries with an incidence of 2 to 6% [84].

Diabetes type 2 is preceded during years by an abnormal condition known as glucose intolerance, that is characterized by higher levels of glucose than normal, with a concentration (between 140 mg / dL and 199 mg / dL) 2 hours after a standard charge of glucose, but not as high as in diabetes (> 200 mg / dL) [25]. There are characteristics that are associated to a mayor risk in the progression of glucose intolerance to diabetes type 2. Among these, alteration in the secretion of insulin, insulin resistance, obesity and aging occur [86–88 y 127]. Physical exercise and some diets can be of help in the control of pre-pathologic hyperglycemia [89]. Many diets and natural product can be of help in the control and prevention of hyperglycemia [90]. In particular, there are many medicinal plants with hypoglycemic effects [91–92]. In this context, our product MAQUISELECT®/DELPHINOL® could be significant for the control of hyperglycemia [93–94].

Diabetes mellitus is a chronic disease caused by an inherited deficiency and/or acquired in the production of insulin, or a lack of response to insulin. This is caused by an increase in the concentration of glucose in the blood that affects various tissues and organs. This is a global epidemic affecting approximately 143 million people [89,95,96]. In Chile the prevalence of diabetes estimated by the OMS is 6–8%, similar to countries as USA, Canada, Argentina and Uruguay [97].

The effect of A. chilensis in diabetes or renal pathologies is unknown. However, it is known that the fruit and vegetable intake rich in polyphenols diminishes the incidence of diabetes type 2. It is possible that the anthocyanins exert an antioxidant/anti-inflammatory effect at the kidney level since the concentrations of these substances is 3 times higher than the ones reported in the plasma [98]. It is known that antioxidants from diet, including anthocyanins, protect the pancreatic beta cells from oxidative stress induced by the increase in glucose. Studies have described that anthocyanins are able to induce the release of insulin and therefore reduce hyperglycemia. Polyphenols from the skin of grapes or whole grapes are able to increase the secretion of insulin and inhibit COX–2. Anthocyanins can reduce the pathologies associated to elevated glycemia such as cataracts. In this sense, combination of semi synthetic anthocyanins and natural can treat cataracts. In
diabetes there is microcirculation problems (thickening of capillary layers) that are involved in the pathogenesis of micro vascular diseases associated to diabetes. Delphinidin induces an increase in the microvascular permeability and a reduction in the leukocyte adhesion in the venules in diabetic hamsters [99]. In diabetes and other diseases, vasodilatation of the endothelium induced by agonists is reduced [40], due to a decrease in the release of nitric oxide [100]. Wine extract, or products from grapes and plants with high polyphenolic content (among them anthocyanins), increase the vasorelaxation probably due to an increase in nitric oxide via liberation of NO or increase of its biological effects [101–104]. Anthocyanins from V. myrtillus has vasodilatating [105], and ophthalmologial [106] activities. Furthermore, 10 mg/L delphinidin increase the expression of eNOS in adherent BAEC, augmenting the nitric oxide production, exerting vasorelaxation and preventing cardiovascular diseases [129].

Antioxidant molecules diminishes the oxidative stress and hypertension associated to the resistance to insulin in rats fed with fructose [102]. Oranges complex supplements (anthocyanins, flavanones and hidroxinamic acid) improved significantly the activity of glutathione in the serum reducing the levels of free radicals [103]. Pomegranate juice consumption in diabetic patients showed antioxidant effects in the serum, and in the oxidative state of monocytes and macrophages [104], probably due to the presence of anthocyanins [105].

The sugar fraction of pomegranate juice, in difference to the juice of White grapes, diminishes the oxidative state in macrophages in normal and diabetic conditions suggesting an anti tatarogenic effects. Anthocyanins and procyanidins, lower triglycerides and increases the HDL cholesterol in rats. In acute studies in animals, the glycosides of anthocyanins (derived from leucopelargonine) from F. bengalensis showed hypocholesterolemic and antioxidant properties [116–118]. Adipocytes are the primary place for energy storage and due to the excess of nutritional triglycerides. In recent years, the adipocyte dysfunction plays a key role in the development of obesity and insulin resistance. Adipocites sensitize and secret biologically active molecules known as adipokines [119]. Adiponectin is one of the most important and highly expressed in adipocitokines in adipocytes. The plasmatic concentration of adiponectin and the level of expression of ARNm are inferior in obese and insulin resistant individuals [120, 121].

To the best of our knowledge, we have found only one study [48] directly linking the effects of delphinidin, among other compounds, in the prevention of metabolic syndrome. The study considered administration of 2.1mg/kg of delphinidin to a rat model of metabolic syndrome, where said administration prevented insulin resistance.
Our Research

Our research group conducted a study where a diabetic rat model was used. After 4 months of treatment with 20 mg/kg, a decrease from 380 mg/dl to 90 mg/dl in glycemia was found (Figure 16), a 76% reduction. Triglycerids also showed a decrease of a 52.5% (Figure 15).

Figure 15: Triglycerids measured as mg/dl in a rat diabetic model after 4 months of treatment with 20 mg/kg of MAQUISELECT®/DELPHINOL®.

Figure 16: Glycemia measured as mg/dl in rat diabetic model after 4 months of treatment with MAQUISELECT®/DELPHINOL®.
Clinical Study

The results obtained in the laboratory using a rat diabetic model provided sufficient evidence to initiate a human clinical trial.

Our research group, in association with a local University, Universidad Austral, started a clinical trial for a dried standardized extract of 300mg of MAQUISELECT®/DELPHINOL®, for oral administration previous dissolution in fresh water.

All studies and clinical trials are performed in accordance with the approval of the Scientific Ethical Committee of the Occidental Metropolitan Health Service, Ministry of Health.

Phase I Trial is designed to evaluate the efficacy of the oral administration of MAQUISELECT®/DELPHINOL® on the post-prandial levels of glucose and insulin and tolerability in individuals with glucose intolerance.

The design of the study is a double blind randomized crossover, and the event under study is the change in the post-prandial plasmatic blood levels of glycemia and insulin. The principal parameter to evaluate is the measurement of post-prandial plasmatic blood levels of glucose and insulin, and the second parameter is the determination of adverse effects and/or events.

Individuals selected for the study comprise individuals with high levels of glycemia between 110 y 125 mg/dL, age 18 and 55 years, with a total of 10 patients. Preliminary results for glycemia and insulinemia are shown in Figure 17.

Preliminary results show a first effect of MAQUISELECT®/DELPHINOL® increasing AMPK activity. In fact, MAQUISELECT®/DELPHINOL® increases phosphorilation of AMPK which acts like obesity control (Figure 18). AMPK is a key target in the effect of well known drugs such as metformin and thiazolidinediones.
Figure 17: Preliminary results of insulinemia and glycemia of patients receiving 300 mg of MAQUISELECT®/DELPHINOL®.

Figure 18: Phosphorylation of AMPK is shown as a consequence of treatment with insulin and delphinidin. The control shows no phosphorylation of AMPK.
Safety in relation to traditional and well established use

Several preparations with Maqui are used in Chile. Furthermore, chilean government, through the Health Ministry, recognized the use of Maqui as a traditional herbal medicine, promoting its use for treatment of diarrhea, dysentery, indigestion, to alleviate sore throat symptoms, tonsil inflammation, and mouth ulcers. The way of administration is internal or topical.

For internal preparations, the recommended dosage is adding 1 tea spoon of dried or fresh ground leaves, or 1 spoon of Maqui berries prepared to 1 liter of boiling water, and drink 3 to 4 cups per day.

For External preparations, the same infusion prepared for drinking is used to wash wounds, or for gargling. Crushed fresh leaves are also used as cataplasm for back pain.

Repeated Dose toxicity, Short term Toxicity testing

Repeated-dose administration of Maqui to albino rats or rabbits at 1g/kg PO once daily for 7 days did not significantly change the body weight, blood chemistry, hepatic and renal functions and histology of important organs. Similar results were obtained with the injection administered to rats at 84 mg/kg i.p. for 10 days also produced no toxic effects.

Subchronic toxicological study in pigs of MAQUISELECT®/ DELPHINOL®

The aim of the study was to evaluate the sub-chronic peroral toxicity of MAQUISELECT®/ DELPHINOL®. This study was carried out in Landrace piglets for 2 months using MAQUISELECT®/ DELPHINOL®. The product was stored in a dark room, in sealed plastic containers, under controlled conditions of humidity (18%) and temperature (21±5°C).

Test samples of MAQUISELECT®/ DELPHINOL® were prepared mixing the product with the food in 3 final different doses, 0.171 g/kg, 0.857 g/kg and 1.379 g/kg using powdered industrial food as vehicle. Group size was 6 piglets. Group 1 control, group 2: 0171g/kg group 3: 0857 g/kg and group 4: 1.371 g/kg. The dosage scheme was based on the “group size” for all calculations of dosages. Each week MAQUISELECT®/ DELPHINOL® was mixed in a known quantity of food and given during the whole week. The animal used in the test were landrace weaned
piglets, three per sex, per dose, with an initial body weight of 15 ± 3 kg, supplied by Lorenzini Piglet Farm, Molina, Chile.

All animals were individually identified by ear tattoo. Upon arrival all animals were caged in groups according to the dose of MAQUISELECT®/DELPHINOL® and control. A quarantine period of 7 days of acclimatisation was used before the test started. The animals received a prefabricated food mix of high quality. Water was given ad libitum. Environmental conditions were as follows: temperature (ambient), humidity (relative) 40% RH and natural light.

Ventilation: The level of ammonia and other volatile products from excrement’s were kept at a low level due to the design of the building and strict cleansing activities twice a day.

Sampling and testing

Sampling of blood from each animal was performed at day 0, 15, 30 and 60. The blood was drawn from the vena cava cranialis with the animal resting on its back.

Haematological tests:

EDTA was utilized for the hemogram in solutions of 1%, 1:9 and the blood samples were analysed by standard laboratory procedures.

Red Blood Cells, White Blood cells, Vol. Packed RBC. Haemoglobin, differential count of leukocytes were controlled in haematological tests.

Blood chemistry:

The samples were centrifuged in order to obtain serum for analysis of the following parameters.

Urea–N, Glucose, Protein, Alkaline phosphatases AP, Aspartate aminotransferase (ASAT), Alanine aminotransferase (ALAT) were controlled in blood chemistry tests.

All haematological and biochemical data were evaluated to the following statistical methods: basic statistics including mean (M), standard error of the mean (SEM), standard deviation (SD), number (N), etc. Where appropriate, Bartlett test for homogeneity or variance, ANOVA, one way and multiple comparison test of Tukey were used. A level of p<0.05 was used for significance. A two way ANOVA (factorial 2x4) was utilised if the variables (effects of treatment) were significant a Scheffe’s test was also used. The non-parametric test, Kruskal–Wallis was utilised for all variables. Sequentially a box–design graphical statistical was employed. For the evaluation of body weight the same descriptive statistics as above were used.
Results

Body weight

In all three doses of MAQUISELECT®/ DELPHINOL®, no significant changes in the weight of the piglets were observed. The treatment lasted 42 days.

Food intake

Initially due to the bitterness of the herb extract, a decrease in the intake of food was observed, but this was recovered after two to three days. The intake of food was restricted for the animals in all groups.

Behaviour and appearance

All piglets looked healthy, playful with no changes in behaviour in all groups during treatment.

Mortality

Not observed.

Haematological tests

Apart from some minor changes observed in white blood cells (dose: 0.171g/kg, day 15; and 0.857g/kg, day 30) and differential leukocyte counting, these are not due to the effect of MAQUISELECT®/ DELPHINOL®, as they are within the normal ranges for the piglet species.

Blood chemistry

With all doses of MAQUISELECT®/ DELPHINOL® there was a significant decrease in proteins at day 0 which recovered afterwards. At day 30, a mild decrease with the dose of 0.171g/kg was observed.

At day 15 all doses of MAQUISELECT®/ DELPHINOL® induced lower levels of ALT enzyme as compared to the control, a fact already described in the literature. Alkaline phosphatase showed a mild decrease at a dose of 1.370g/kg at day 15.

Histopathological examination

No changes were observed in the histopathological examination
Chronic toxicity testing

In chronic toxicity test carried out in dogs, MAQUISELECT®/ DELPHINOL® at doses 10–15 times those used clinically exhibited no toxicity. The LD$_{50}$ of a water extract (1:1) samples varied from 5.4 to 6.8 g kg$^{-1}$ (Wagner).
Patent Applications

An international patent application, PCT/IB2010/002698 has been filed on October, 21st, 2010. The matter protected by this application comprises a composition of a plurality of anthocyanins and/or anthocyanidins, wherein (a) at least or about 35% of the composition, by weight, is an anthocyanin or anthocyanidin, (b) at least or about 15% of the anthocyanins and/or anthocyanidins, by weight, are sugar-free or sugar-containing delphinidins, and (c) the composition is non-toxic and non-naturally occurring.

The patent application also claims protection over an oral formulation, either tablet, capsule, or food product, method of treatment or use as an immune system booster, cancer or acquired immunodeficiency conditions, improving inflammation in a subject, and for improvement on the condition of a patient suffering of metabolic syndrome.

A further composition also covered in the patent application, is the initial composition plus andrographolides, and another plus Vaccinium myrthilus
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