Le 3 S dei Botanicals da colture in vitro: Sicurezza, Standardizzazione e Sostenibilità

In-vitality, 20 novembre 2019

Elena Sgaravatti,
Co-Founder & CEO DemBiotech®

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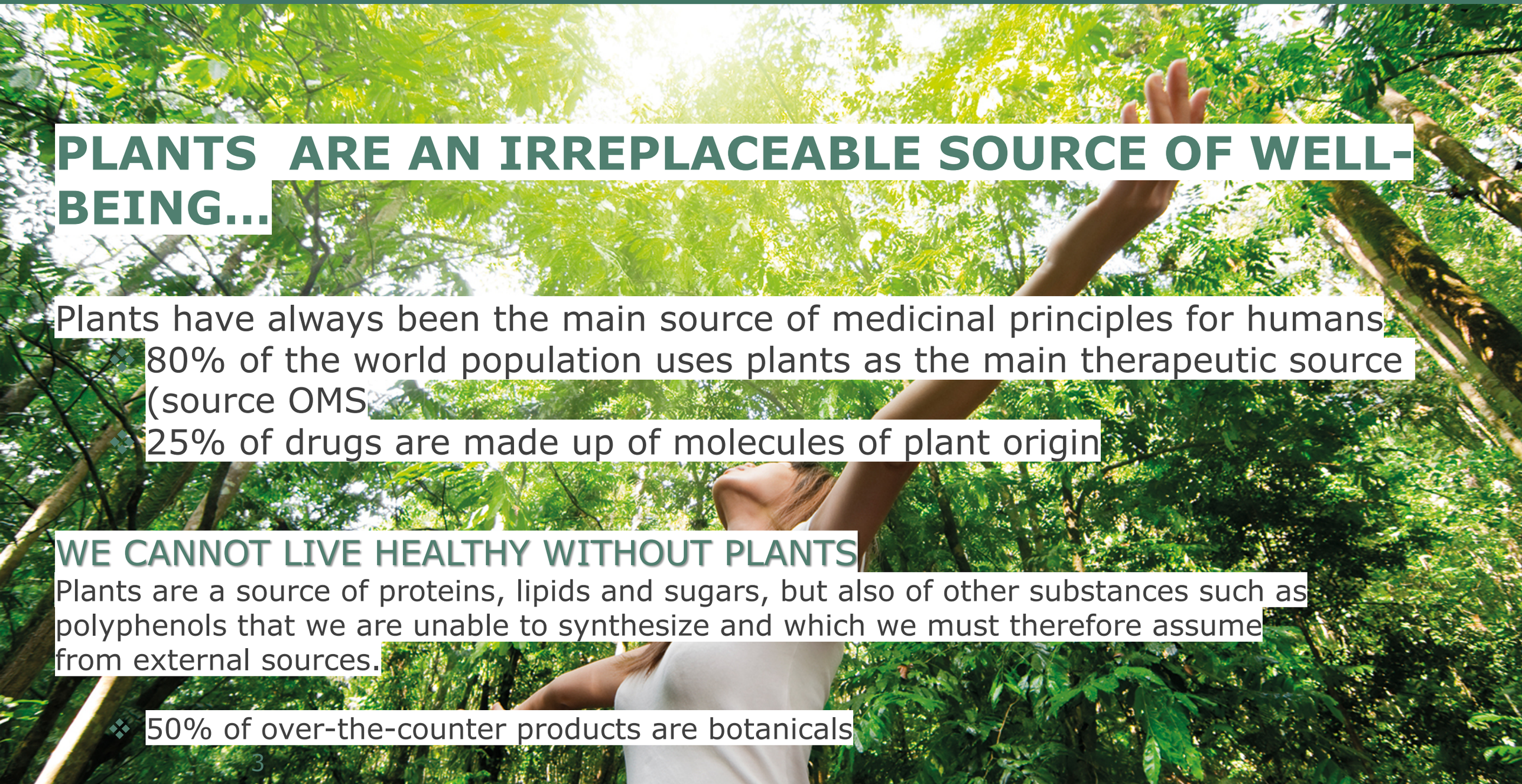
- **DEMETHRA BIOTECH SRL**

- Founded in March 2016 in Vicenza: part of Cereal Docks Groups

- 100% Italian Green Biotech company

- OUR VISION

  For those customers who demand excellent quality standards and environmental sustainability, we want to be a reference point in the world of the accessible supply of botanicals , for the well-being of People, for benefit of Health and the Environment.



PLANTS ARE AN IRREPLACEABLE SOURCE OF WELL-BEING…

Plants have always been the main source of medicinal principles for humans

- 80% of the world population uses plants as the main therapeutic source (source OMS
- 25% of drugs are made up of molecules of plant origin

WE CANNOT LIVE HEALTHY WITHOUT PLANTS

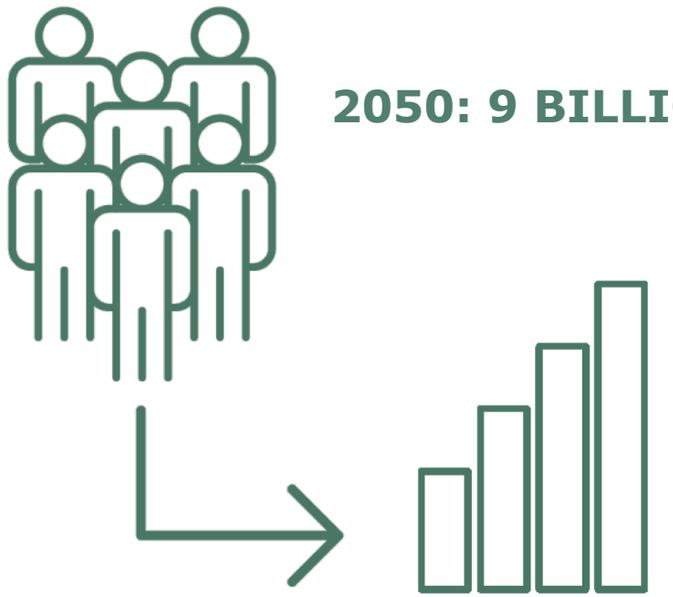
Plants are a source of proteins, lipids and sugars, but also of other substances such as polyphenols that we are unable to synthesize and which we must therefore assume from external sources.

- 50% of over-the-counter products are botanicals

… BUT NOT INEXHAUSTIBLE

The increase in world population requires a 70% increase in agricultural production in the face of:

- shortage of soil, water, environmental resources
- increase in pesticides / environmental contamination
- reduction of biodiversity



2050: 9 BILLIONS

AGRICULTURAL PRODUCTION : + 70%

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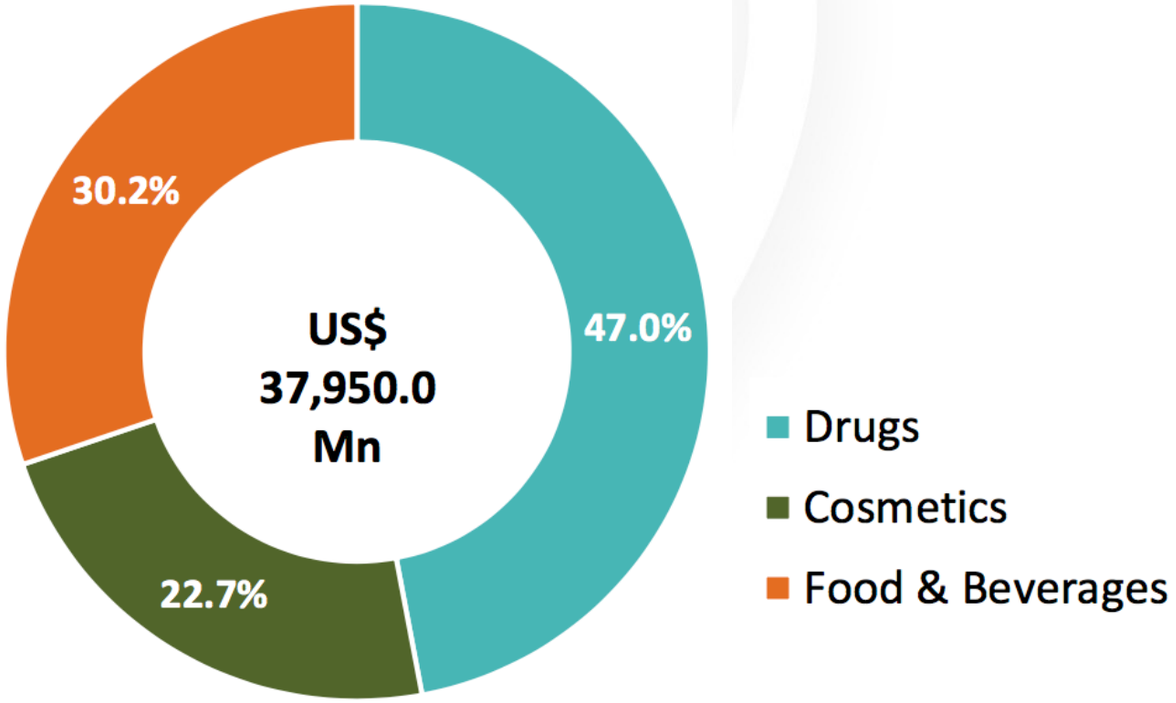
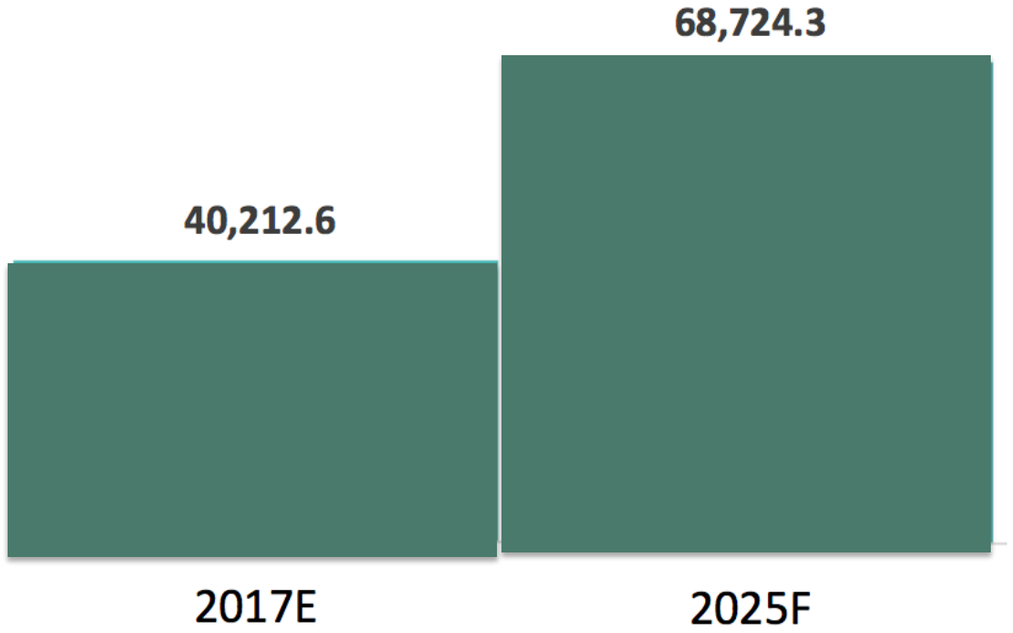
BOTANICALS ARE A SOURCE OF HIGHLY VALUABLE APPLICATIONS



Global Botanical Market Value Forecast (US$ Mn), 2017 and 2025



SOURCE: ©2017 Persistence Market Research

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THE PROBLEMS OF TRADITIONAL CULTIVATION HAVE ALWAYS LIMITED THE USE OF BOTANICALS, LEAVING 3 BASIC NEEDS UNRESOLVED

SAFETY/QUALITY
The presence of pollutants as pesticides, heavy metals and aflatoxins are often detected in many plant actives and extracts. But also adulteration/misidentification

STANDARDIZATION
The variability in the concentration of active substances is a critical limit to the reproducibility of the biological effect

AVAILABILITY
Many problems due to difficult accessibility of plants compromise the availability of Natural Active Substances

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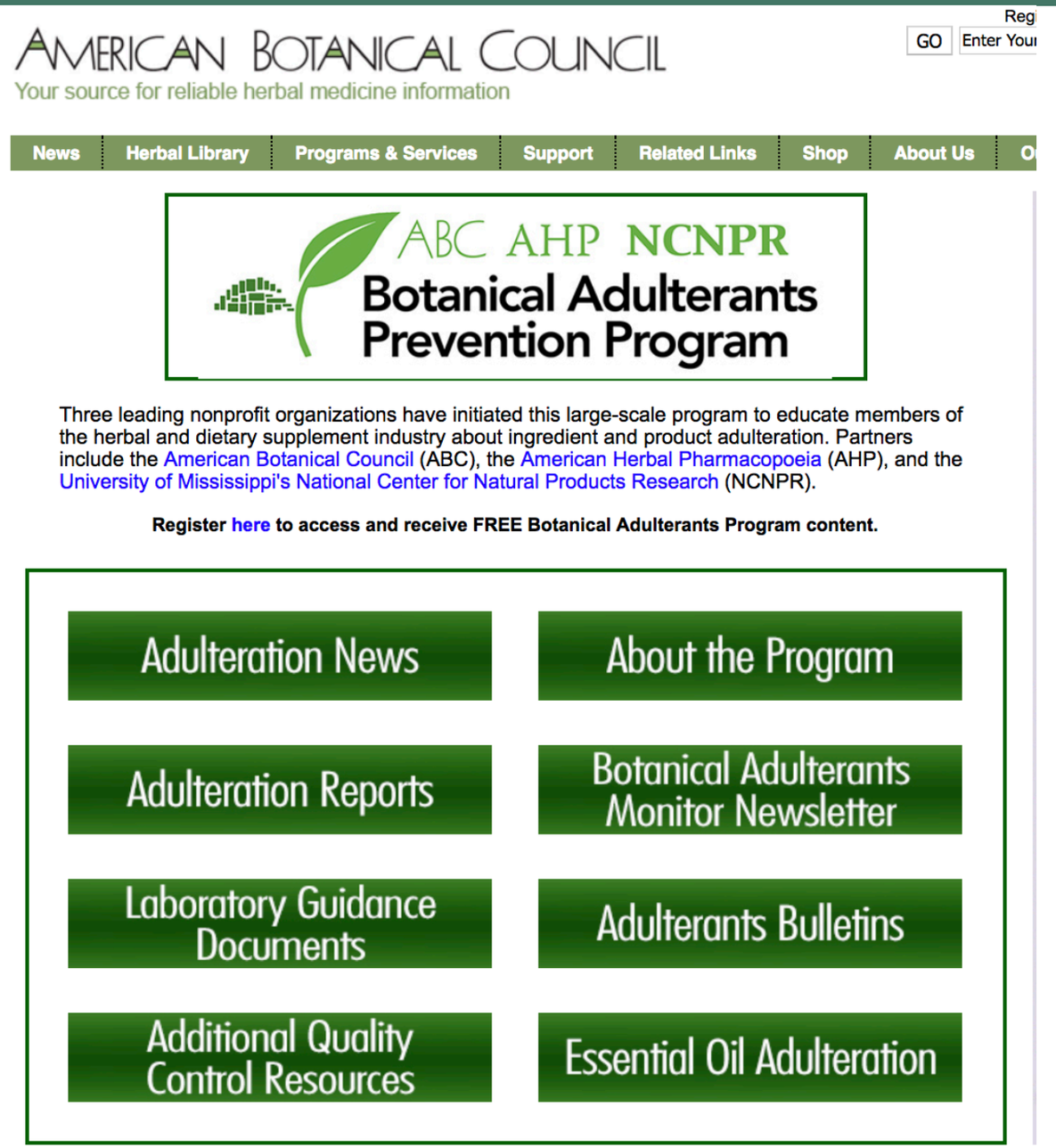
…AND BIGGEST BOTANICAL SOCIETIES ARE TACKLING THIS CRITICAL ISSUE TO TRY TO MITIGATE THE CONSEQUENCES THAT AFFECT THE **PUBLIC SAFETY** AND **SUPPLIER REPUTATION**



7

THE FIRST STEP IN QUALITY CONTROL OF BOTANICAL PREPARATIONS IS ENSURING THE CORRECT IDENTIFICATION OF THE PLANT MATERIAL INTENDED FOR USE

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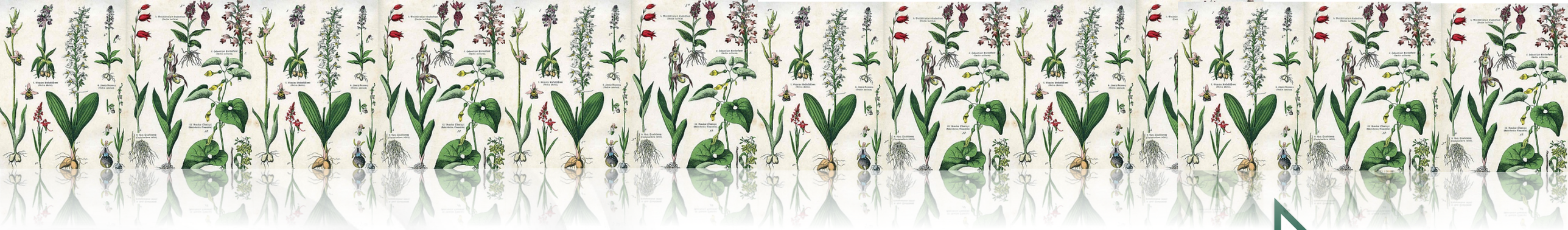

IDENTIFICATION USING **MORPHOLOGY**

The use of morphological characters for herbal identification in a commercial setting remains a viable and important approach.

However, due to the fact that identification of many species requires relatively intact botanical specimens including reproductive structures, the method has limited utility across the broader herbal supply chain.

Ensuring the Specific Identity and Quality of Herbal Products by the Power of DNA by Matthew Cimino HerbalGram. 2010;86:50-57 American Botanical Council

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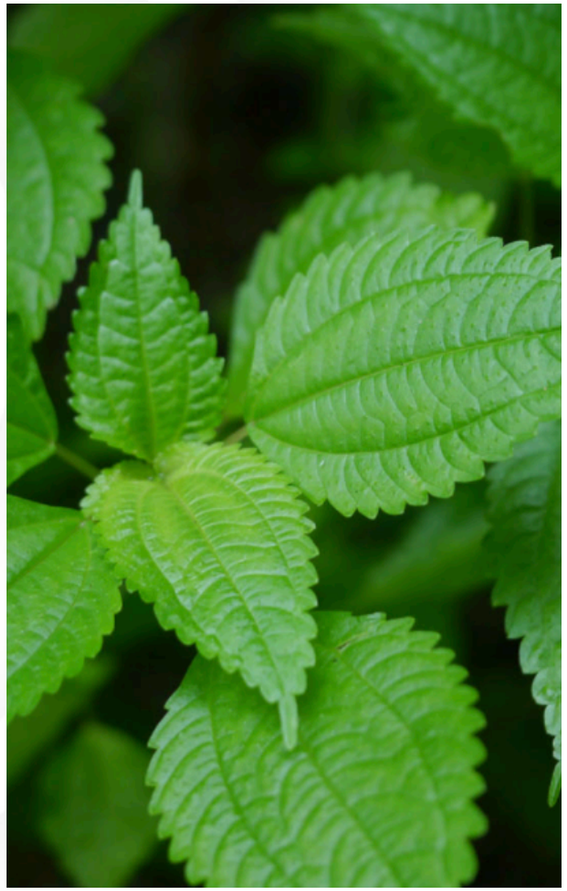
Urtica dioica



Laportea canadensis

Pilea pumila

Boehmeria cylindrica

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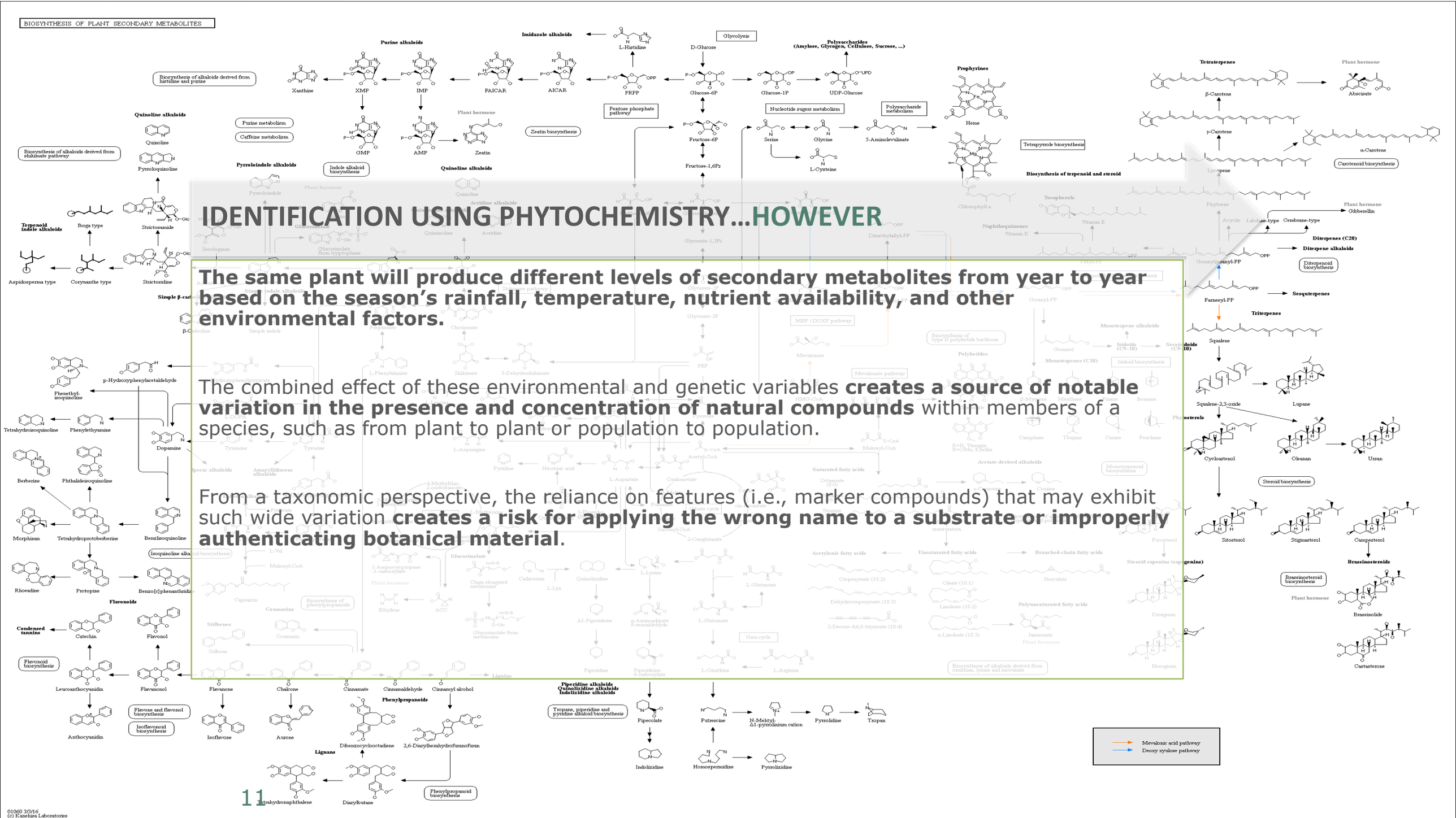

BIOSYNTHESIS OF PLANT SECONDARY METABOLITES
IDENTIFICATION USING PHYTOCHEMISTRY…HOWEVER
The same plant will produce different levels of secondary metabolites from year to year based on the season's rainfall, temperature, nutrient availability, and other environmental factors.
The combined effect of these environmental and genetic variables creates a source of notable variation in the presence and concentration of natural compounds within members of a species, such as from plant to plant or population to population.
From a taxonomic perspective, the reliance on features (i.e., marker compounds) that may exhibit such wide variation creates a risk for applying the wrong name to a substrate or improperly authenticating botanical material.
Mevalonic acid pathway
Deoxy xylose pathway
01060 3/3/16
(c) Kanehisa Laboratories

FACTORS AFFECTING ECHINACEA QUALITY

W. Letchamo, L.V. Polydeonny, N.O. Gladisheva, T.J. Arnason, J. Livesey, and D.V.C. Awang

→ E. purpurea infected by a mycoplasma - like organism in commercial fields.

← View of the damage caused by flower borer flies

Table 4. Effects of plant selection and flower developmental stages on chemical content of *E. purpurea* and *E. angustifolia* clones under commercial cultivation in the US.

Flower developmental stages	Content (% dry matter)					
	Cichoric acid		Echinacoside		Isobutylamides	
	Before selection	After selection	Before selection	After selection	Before selection	After selection
	E. purpurea 'Sorgogo'					
1 (early)	2.56	3.97	0.002	0.007	0.008	0.011
2 (medium)	1.89	2.35	0.023	0.011	0.004	0.012
3 (mature)	0.39	0.76	0.034	0.081	ND	0.016
4 (overblown)	0.06	0.43	0.048	0.072	ND	0.015
Mean	1.23	1.88	0.027	0.043	0.006	0.014

↑ A slow but sure death of E. purpurea due to a leaf spot or shoot fungus (Cercospora sp.) infection is common in commercial cultivations.

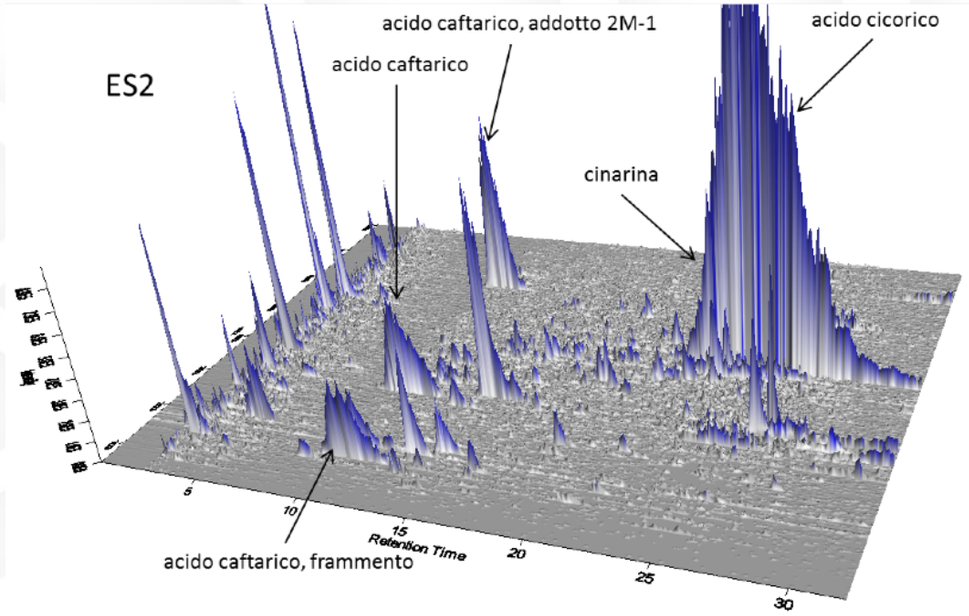
FINAL RECOMMENDATION IS TO USE THE MOST GUARANTEED SOURCE

IS YOUR ECHINACEA REALLY ECHINACEA? ADULTERATION OF YOUR HERBAL SUPPLEMENTS

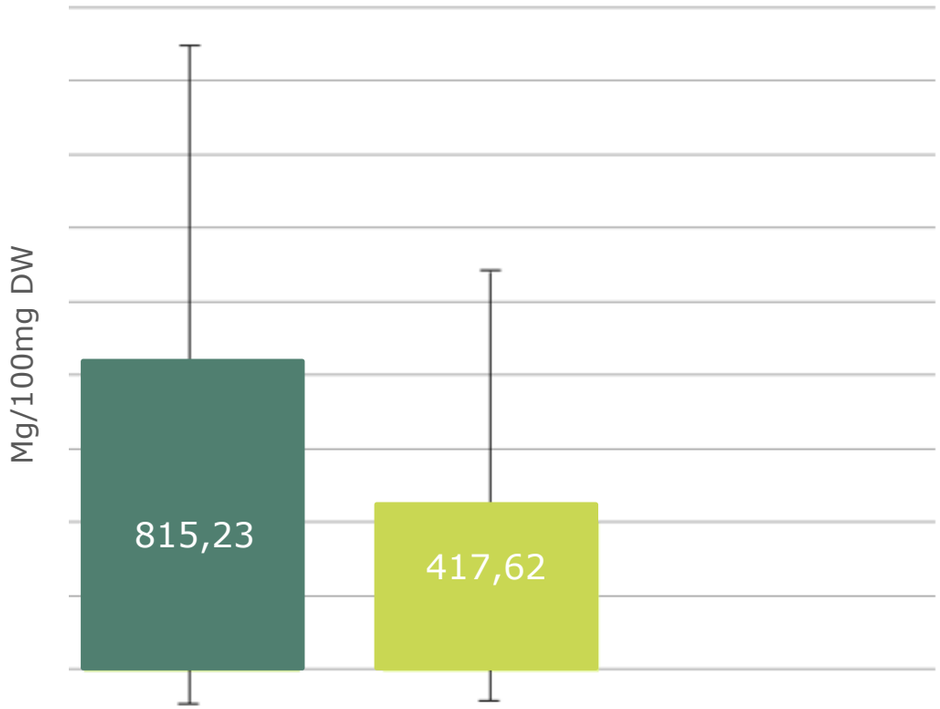
A research group headed by Steven Newmaster used a technique called DNA barcoding to conduct a blinded test of the authenticity for 44 herbal products representing 12 companies.

- ➔ Less than half (**48%**) of the products contained some of the claimed ingredients. One-third of these also contained contaminants and or fillers not listed on the label.
- ➔ **Product substitution** occurred in **30/44** of the products tested. Only 2 of 12 companies had products free of substitution, contamination or fillers.
- ➔ Some of the **contaminants** posed serious health risks to consumers due to potential organ toxicity, side effects or food or skin allergy issues.
- ➔ The conclusions of the study were that the products tested were of **poor quality**, included considerable **product substitution**, contamination and use of fillers which posed **health hazards**.

Source: DNA barcoding detects contamination and substitution in North American herbal products Steven G *BMC Medicine* 2013 **11**:222



HPLC-MS profiles
Commercial sample of Echinacea p. extracts



Average and standard deviation of total polyphenols and total chicoric acid (in HPLC-DAD) six commercial samples

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THE ADOPTION AND INTEGRATION OF DNA-BASED TESTING CAN PROVIDE AN INDEPENDENT, SENSITIVE, AND SPECIFIC MEANS TO ASCERTAIN THE SPECIFIC IDENTITY OF BOTANICAL MATERIALS

DNA fingerprint

- The opportunity to remove the ambiguity associated with botanical ingredient identity will allow to more prominently recognize 2 distinct attributes of herbal ingredients: **identity as well as quality.**

- **The combination of DNA-based identification (or, where possible, morphology-based authentication) complemented by measurements of biochemical compound concentration contained in a particular lot of raw material would be especially beneficial.**

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CHANGE OF PARADIGM, WE VALUE THE POTENTIAL OF PLANTS

Since 1994 FAO endorsed plant tissue culture technology as a reliable alternative for the production of food substances and metabolites

"Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented.
These problems include: **environmental factors** (drought, floods, etc.), **disease, political and labor instabilities** in the producing countries (often Third World countries), **uncontrollable variations in the crop quality**, inability of authorities to prevent **crop adulteration**, **losses in storage and handling**."

Dr. Masanaru Misawa, PLANT TISSUE CULTURE: AN ALTERNATIVE FOR PRODUCTION OF USEFUL METABOLITE, Bio International Inc. Toronto, Canada, (**FAO AGRICULTURAL SERVICES BULLETIN No. 108**, Food and Agriculture Organization of the United Nations Rome 1994)

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BOTANICAL DRUGS ARE, BY NATURE, PLANT-DERIVATIVE MATERIALS AND THEIR COMPLEXES

This makes them unfit for conventional “single-target/single-drug” development processes and thus have been largely disregarded in the field of medicine.

However, it is widely understood in synthetic medicine that the single-drug "magic bullet" strategy is not adequate for treating chronic illnesses (e.g. immune disorders, mental illnesses, cardiovascular diseases, lifestyle diseases) due to their complex pathogenetic mechanisms, and that a "multi-target/multi-component" approach involving control over a number of target sites is more effective

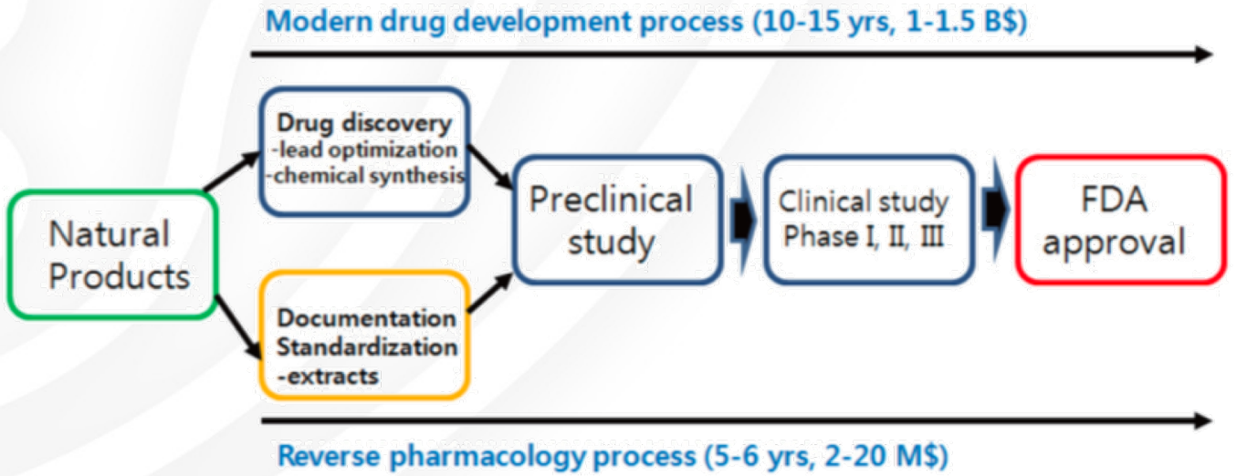
The worldwide trend of using botanical drugs and strategies for developing global drugs
BMB Rep. 2017; 50(3): 111-116

TRADITIONAL HERBAL MEDICINE HAVE THE LONGEST HYSTORY OF TREATMENT OF CHRONIC ILLNESSES WITH PROVEN SAFETY AND CLINICAL EFFICACY

Traditional herbal medicine, itself being a mixture of various components, corresponds to the “multi-target/multi-component” approach,

with therapeutic effects that are clinically confirmed-- albeit with no analytically defined mechanisms—

through experience and knowledge accrued over a long history of treatment of chronic illnesses.



THE STRATEGY FOR DEVELOPING NOVEL BOTANICAL DRUGS BY REVERSE-ENGINEERING OF TRADITIONAL HERBAL MEDICINE IS CALLED **REVERSE PHARMACOLOGY**

The worldwide trend of using botanical drugs and strategies for developing global drugs
BMB Rep. 2017; 50(3): 111-116

3 FUNDAMENTAL ABILITIES OF PLANTS

Totipotency

- ✓ The potential or inherent capacity of a plant cell to develop into an entire plant if suitably stimulated. It implies that all the information necessary for growth and reproduction of the organism is contained in the cell.

Dediferentiation

- ✓ Capacity of mature cells to return to meristematic condition and development of a new growing point, followed by redifferentiation which is the ability to reorganize into new organ

Competency

- ✓ The endogenous potential of a given cells or tissue to develop in a particular way

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THE PROCESS

1. From Mother Plant to Mother Culture

- Plant selection
- DNA fingerprint analysis
- Tissue sanitization
- Growths plantlets

2. Explants and callogenesis induction

- Preparation of explants and transfer in different growth culture medium
- Selection of the callus tissue

3. Stabilization in solid medium and metabolic analysis

- Selection of the most friable calli
- Increase of plant biomass
- Optimization and growth stabilization on solid medium
- Analysis of metabolic profile by UPLC-MS
- Set up of quantification methods of markers metabolites by UPLC-DAD
- Mother culture selection

4. Stabilization in liquid medium

- Transfer of selected mother culture in liquid medium
- Optimization and growth stabilization on liquid medium

5. Phytocomplex profile

- Monitoring of markers metabolites content by UPLC-DAD
- Yield optimization of secondary and primary metabolites
- Characterization of marker metabolites

6. Scale up

- Set up of cellular growth parameter in liquid medium in bioreactor
- Optimization of fermentation parameters in bioreactor
- Quantification of markers metabolites content

7. Biological activities

In vitro efficacy

8. Product Specification

- Validation of Production method
- Definition of Product Specifications

PECULIARITY OF IN VITRO CULTURE

MERISTEMATIC CELLS ARE MAINLY PRESENT IN THE BUDS AND IN THE APICAL PART OF THE ROOTH:

WHERE THE GROTWH IS NEEDED BUT FRAGILE CONDITIONS ARE PRESENT …

SO THAT, IT IS A SORT OF HYPER GEMMOTHERAPY

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INNOVATIVE
BOTANICAL
TRADITION®

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INNOVATIVE BOTANICAL TRADITION®

Deep innovation to benefit from the traditional use of plants

- Profound innovation able to overcome by process the limits inherent to traditional collection that open field production exposes: Safety limits, Standardization, limits of availability and environmental and social Sustainability.

- Limits the weakness of the results obtained from clinical trials should be ascribed, mostly affected by the use of non-standardized extracts that compromise the reproducibility of the results obtained.

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CROP®

Controlled Release of Optimized Plants

DemBiotech®
Science-based natural ingredients

TRADITIONAL CULTIVATION VS. CROP ® PLATFORM

Traditional cultivation

No significant changes in the composition or structure of the products (nutritional value, metabolism or level of undesirable substances)

BUT EVEN ALSO RISK OF

Pesticides, aflatoxins, heavy metals, solvent residues, alkaloids, pyrrolizidine derivatives..

In Vitro Propagation technique

No significant changes in the composition or structure of the products (nutritional value, metabolism or level of undesirable substances)

CROP®
Controlled Release of Optimized Plants

Phytocomplex: **100 %** of primary and secondary metabolites useful for the growth and defense of plants, beneficial to humans

> QUALITY AND SAFETY

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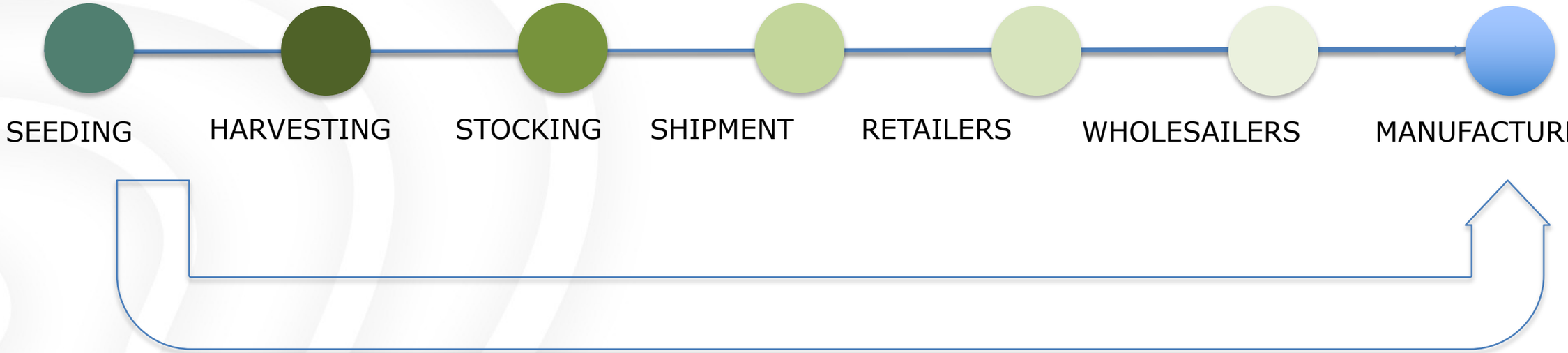
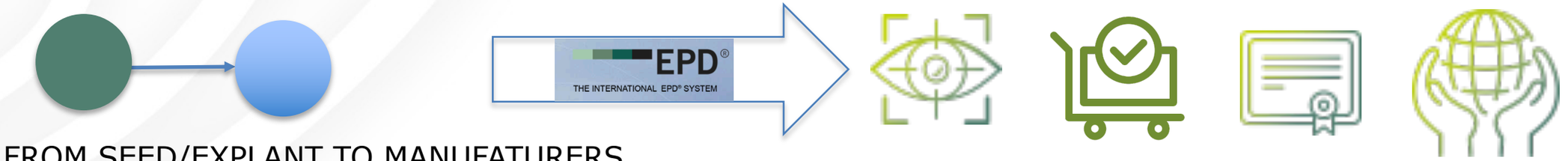
THE SHORTEST SUPPLY CHAIN





FROM SEED/EXPLANT TO MANUFATURERS

DemBiotech®
Science-based natural ingredients

KEY ADVANTAGES by CROP® TECHNOLOGY

The whole phytocomplex with primary as well as secondary metabolites, with less impact on odor and taste, assuring:

STANDARDIZATION

Batch-to-batch highest reproducibility (composition & efficacy); less impact of taste and odor

SAFETY

No environmental contaminants or crop adulteration since cells grow in a sterile medium, by process: no pesticides; no solvents; no pyrrolizidine derivatives; no aflatoxin, no heavy metals, no GMO

ECO-SUSTAINABILITY

Totally eco-friendly approach and truly eco-sustainable with dramatic saving of natural resources; possibility to exploit rare or endangered plants without touching flora
and biodiversity.
Full access to natural substances included rare and difficult to synthetize substances; independence from seasonal-climatic and geographical limits

DemBiotech®
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Water Saving **1:1000**
Soil **1:1000**
Total Abolishment of
Pesticides 0
Fertilizer 0
Solvents 0

producing in 1 months what in field is done in years

DemBiotech®
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EchiPure-PC® from CROP® Technology

Echinacea purpurea

Naturali difese dell'organismo. Funzionalità delle vie urinarie. Funzionalità delle prime vie respiratorie

Echinacea purpurea is a North Americas plant, a member of the daisy family (Compositae) or Asteraceae, present to some extent in the wild in much of the eastern, southeastern and midwestern United States as well as in Ontario. The generic name comes from the Greek word for hedgehog (echinos) inspired by the spiky projections in the centre of its flower head in the seed stage. The specific epithet purpurea refers to the purple colour of the flowers.
Traditional use: indigenous medicine of the native American Indians, the plant was used externally for wounds, burns, and insect bites, chewing of roots for toothache and throat infections; internal application was used for pain, cough, stomach cramps, and snake bites

EchiPure-PC®

Valori Nutrizionali

Analisi	Estratto secco da colture in vitro EchiPureP-PC(g/100g)
Proteine	14,8
Lipidi	0,8
Carboidrati	64,1
Ceneri	13,8
Umidità	6,5
Valore Energetico (kJ)	1349,30
Valore Energetico (kcal)	322,8

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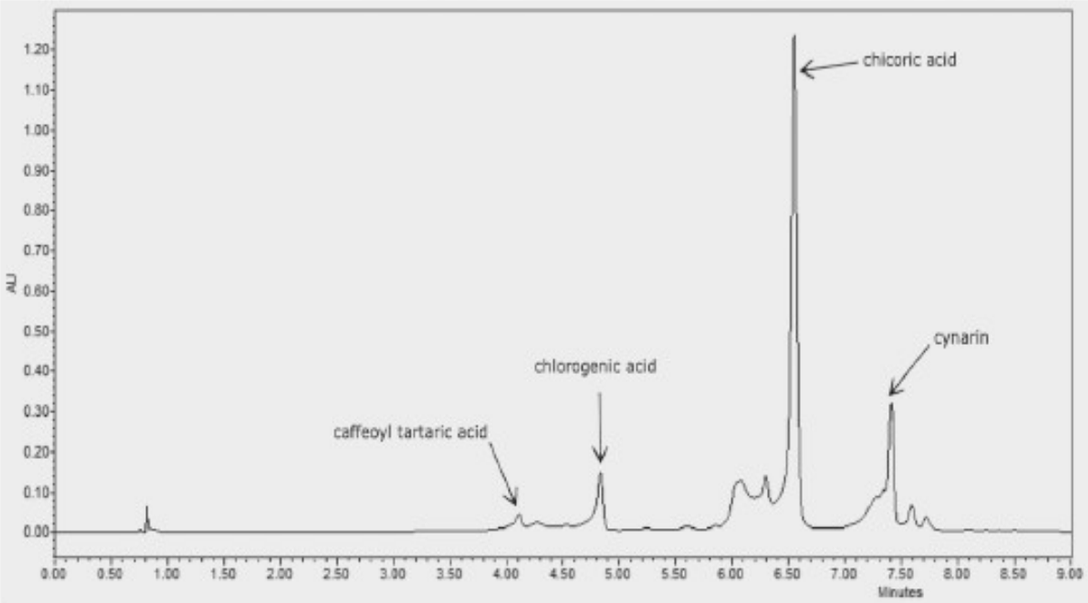
EchiPure®-PC

is titrated in total polyphenols (chlorogenic acid, caftaric acid, cichoric acid and cynarine) ≥0,8 % P/P by HPLC-DAD
content of caftaric acid+cichoric acid ≥0,5% P/P by UPLC-DAD

ACTIVE COMPOUNDS POLYPHENOLS



UPLC profile at 330 nm.

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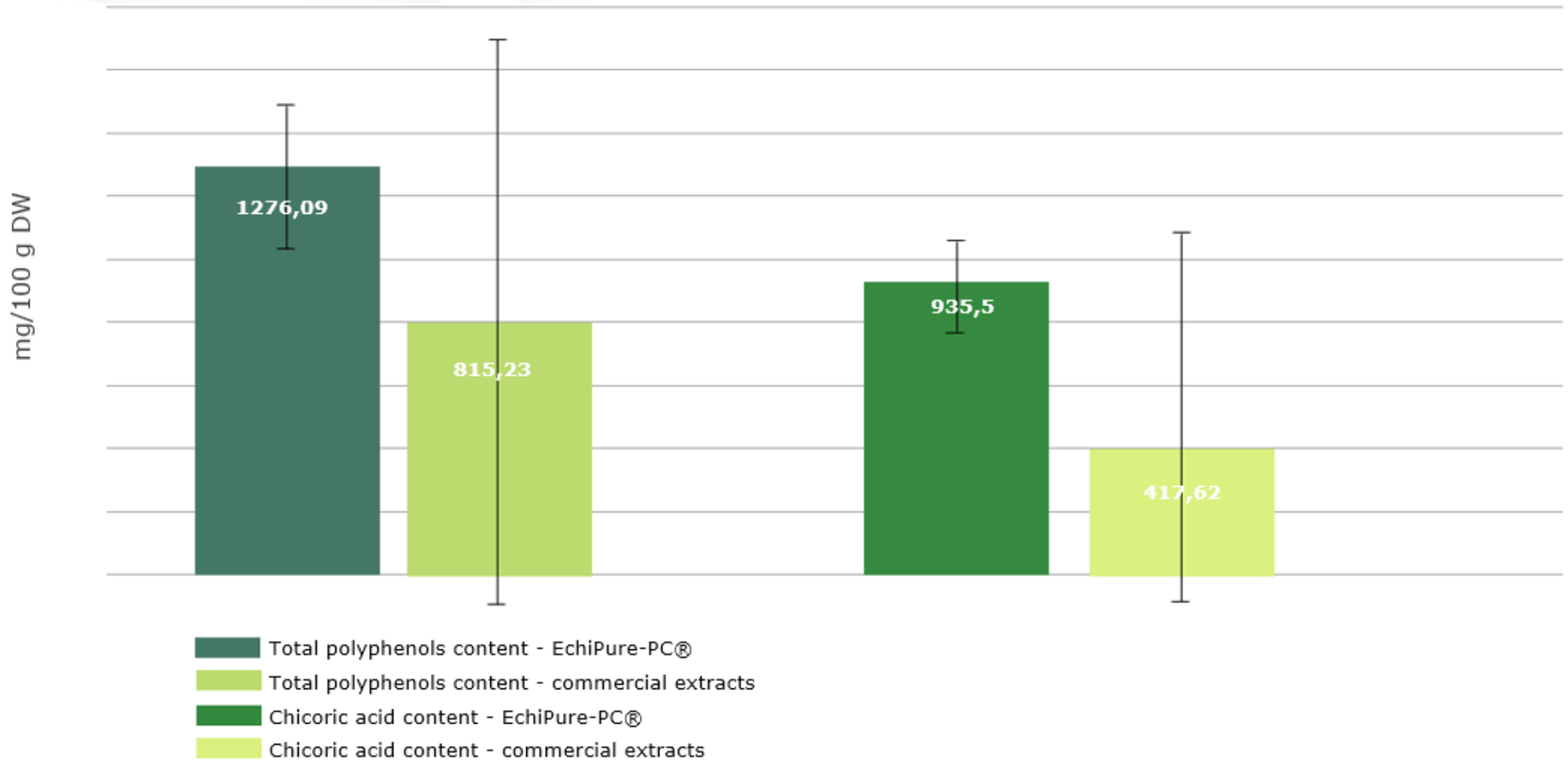

Average and standard deviation of total polyphenols and total chicoric acid (in HPLC-DAD) in five EchiPure-PC® samples and six commercial samples

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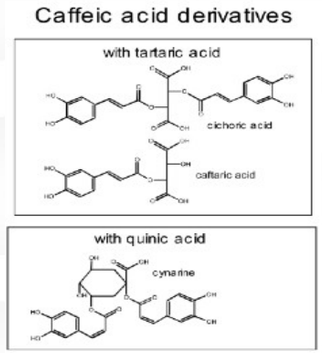
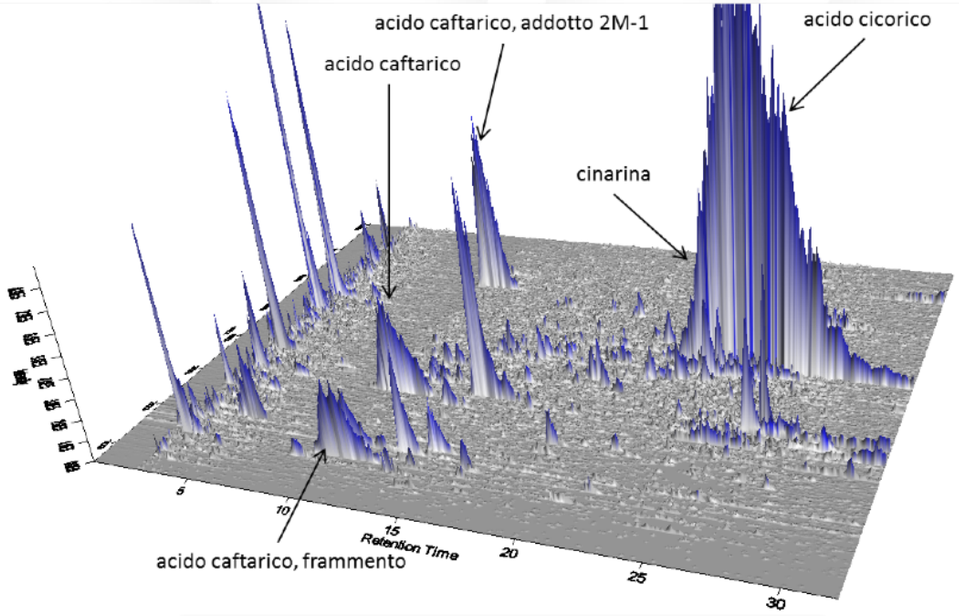
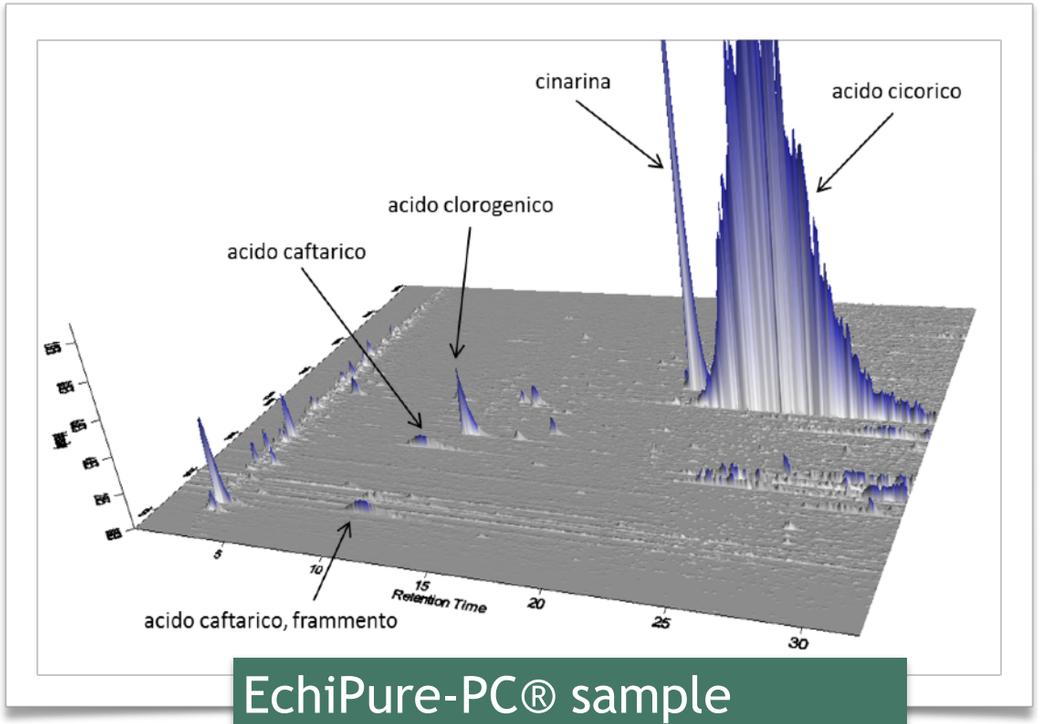
HPLC-MS profiles





Commercial sample



EchiPure-PC® sample

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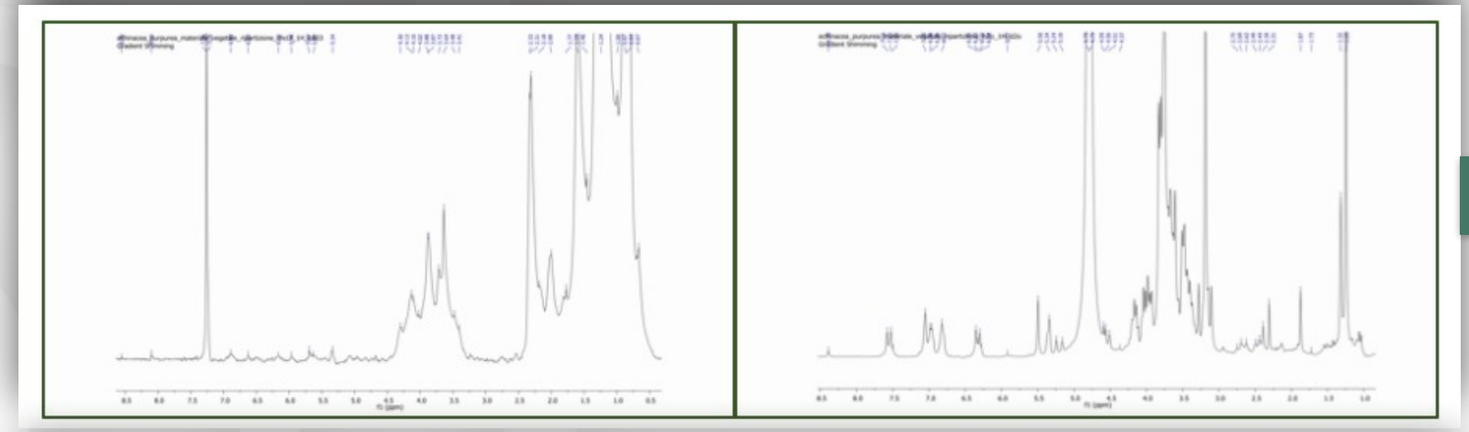
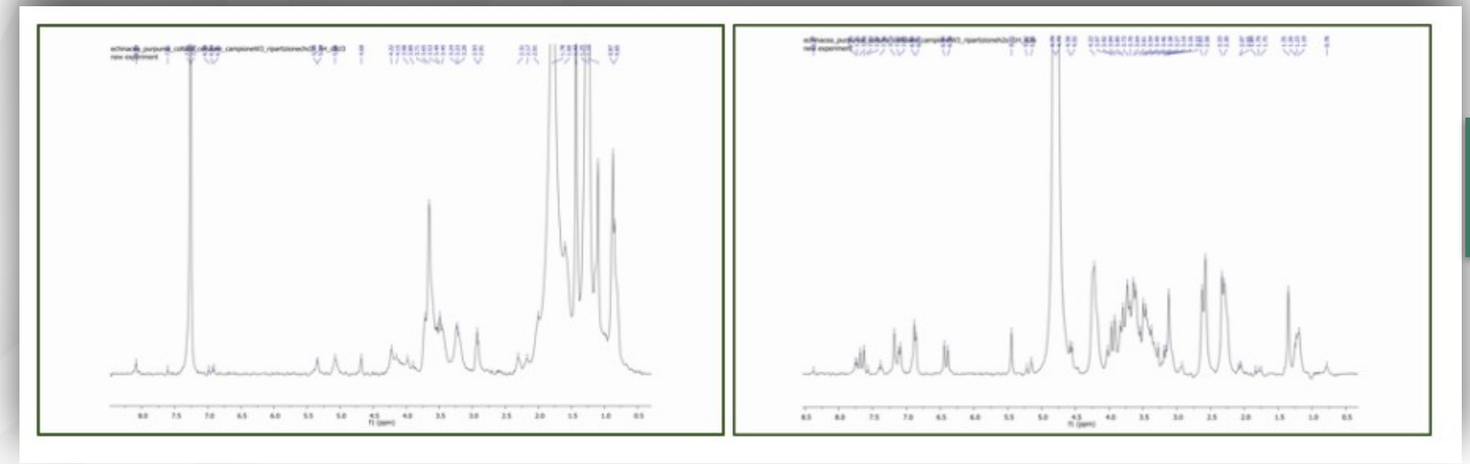


Commercial sample



EchiPure-PC® sample

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EchiPure-PC® activities

EchiPure-PC® has **anti-inflammatory** effects through:

- Modulation of inflammatory pathway (IkB-α), pro-inflammatory enzymes (iNOS and COX-2) and cytokines (TNFα and IL-β)

EchiPure-PC® has **antioxidant** properties reducing:

- The release of nitrites
- Lipid peroxidation

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MeliPure-PC™ from CROP® Technology

Melissa officinalis

Funzione digestiva. Regolare motilità gastrointestinale ed eliminazione dei gas. Rilassamento e benessere mentale. Normale tono dell'umore. Antiossidante.

Melissa officinalis, also known as lemon balm, common balm or sweet balm, is a well-known perennial medicinal plant native to the Mediterrean region belonging to the Lamiaceae.
Modern pharmacological studies demonstrate that *Melissa officinalis* has several biological activities including antioxidant, hypoglycemic, hypolipidemic, antimicrobial, anticancer, antidepressant, anxiolytic, anti-inflammatory and spasmolytic properties. All of these actions of Melissa officinalis extract are advantageous in nutrition efficacy to prevent the damage caused by ROS.

MeliPure-PC™
Valori Nutrizioinali

Analisi	MeliPure-PC™ (g/100g)
Proteine	15,8
Lipidi	15,2
Carboidrati	50,3
Ceneri	14,0
Umidità	4,7
Valore Energetico (kcal)	401,2
Valore Energetico (kJ)	1677,01

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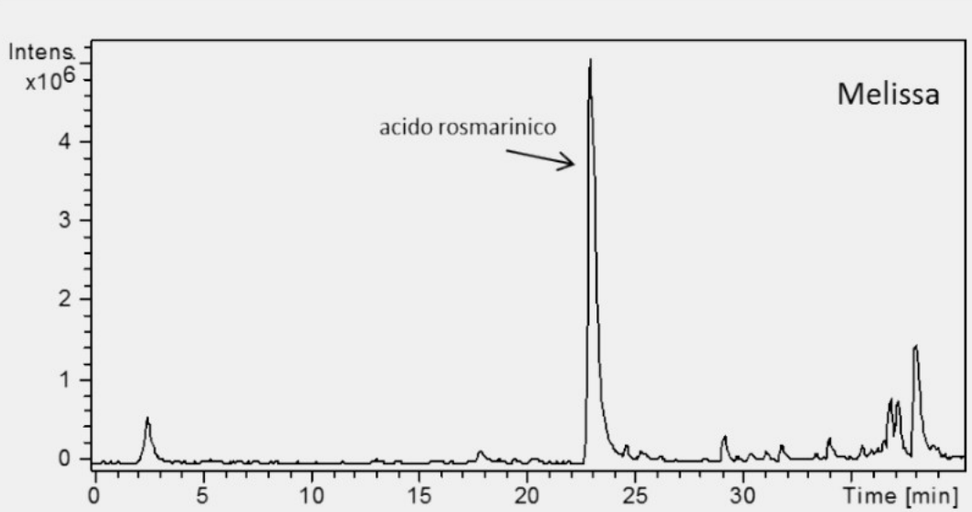
MeliPure-PC™

is titrated in rosmarinic acid ≥1%P/P by UPLC-DAD

ACTIVE COMPOUNDS
POLYPHENOLS



UPLC profile of polyphenol content at 330 nm.

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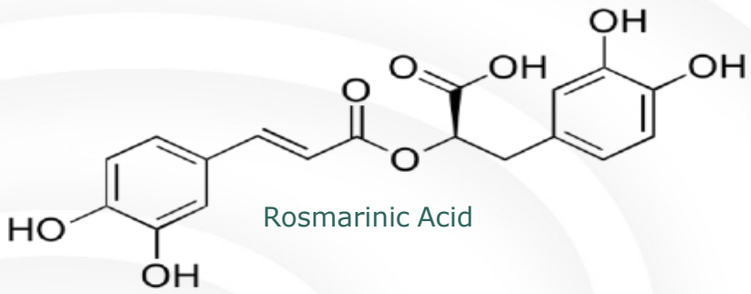
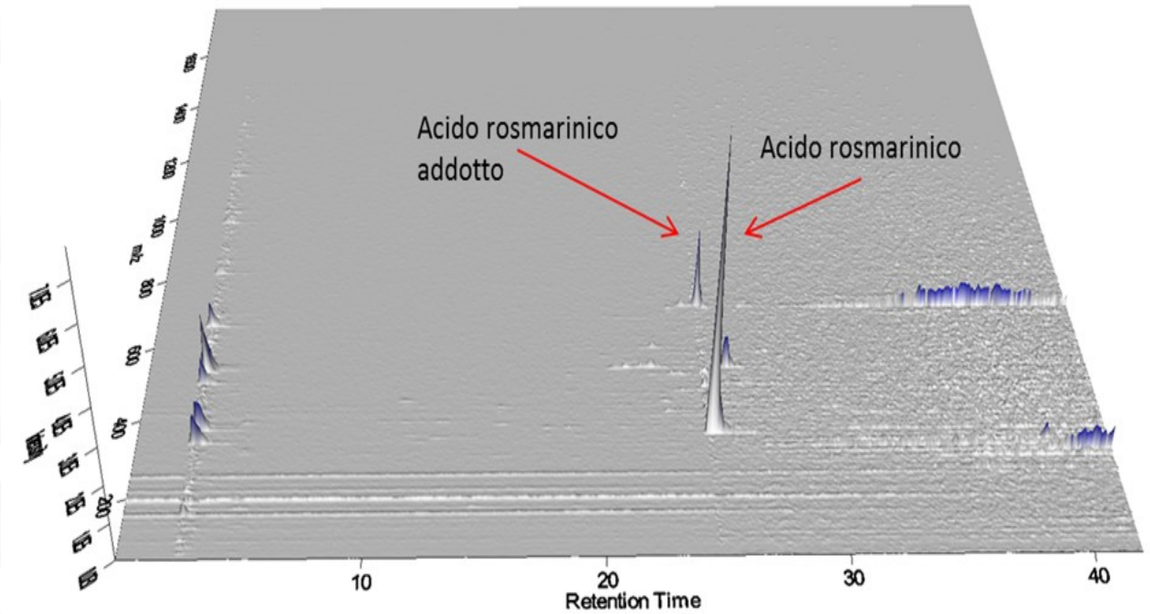
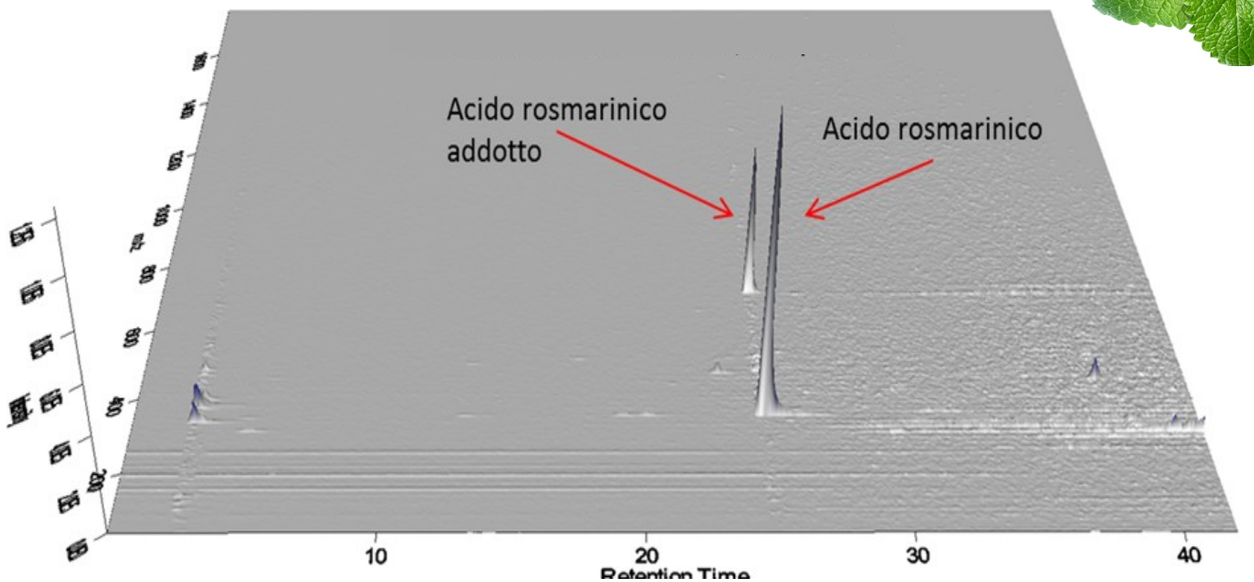


Rosmarinic Acid

HPLC-MS profiles



Commercial sample



MeliPure-PC ™ sample

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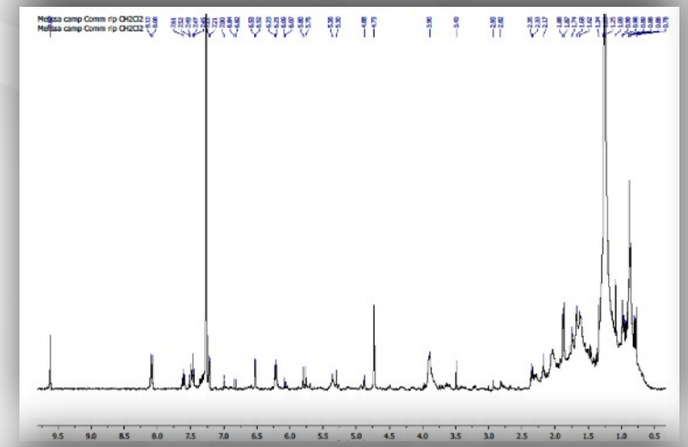
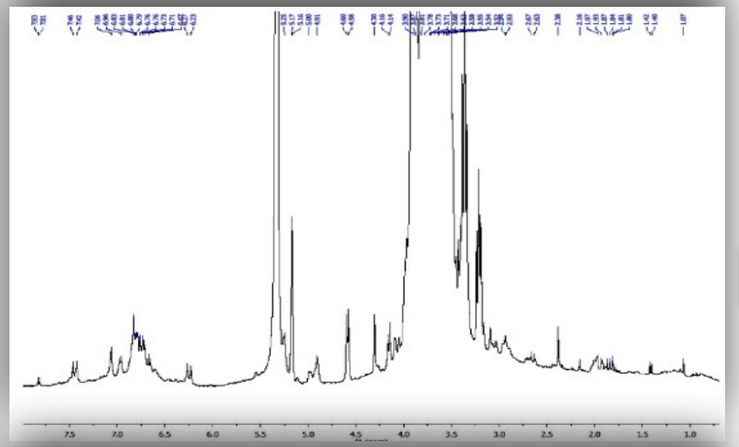
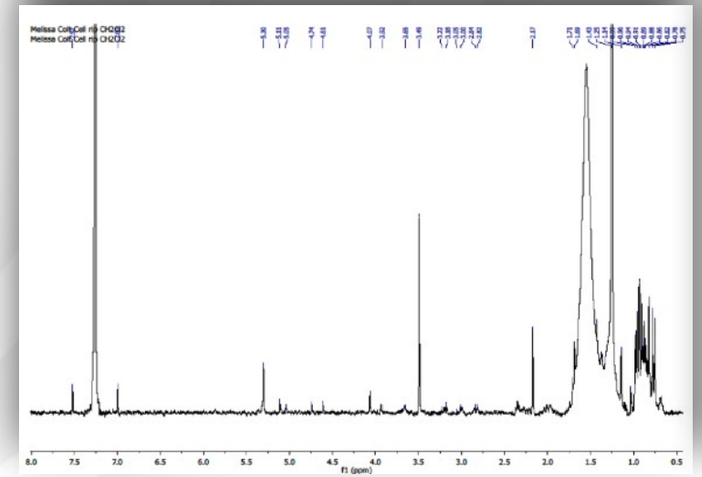
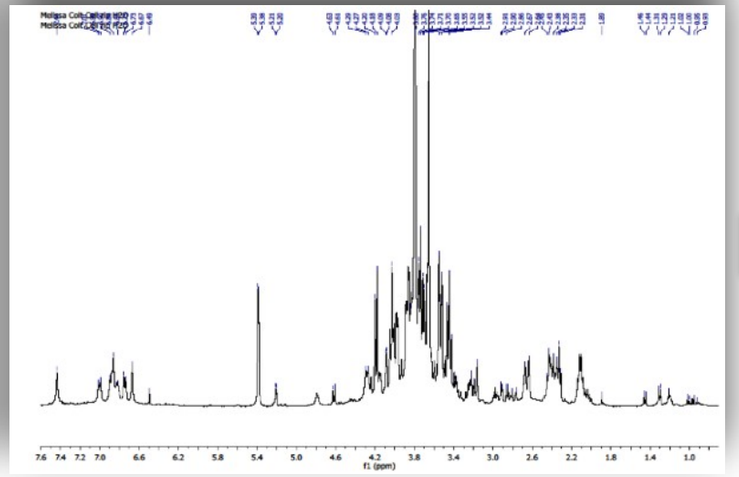




Commercial sample





MeliPure-PC ™ sample

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Melissa officinalis extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomised, placebo controlled trial

*S Akhondzadeh, M Noroozian, M Mohammadi, S Ohadinia, A H Jamshidi, M Khani

Objective: To assess the efficacy and safety of *Melissa officinalis* extract using a fixed dose (60 drops/day) in patients with mild to moderate Alzheimer's disease.

Design: A four month, parallel group, placebo controlled trial undertaken in three centres in Tehran, Iran.

Methods: Patients with mild to moderate Alzheimer's disease aged between 65 and 80 years (n = 42; 18 women, 24 men) with a score of ⩾ 12 on the cognitive subscale of Alzheimer's disease assessment scale (ADAS-cog) and ⩽ 2 on the clinical dementia rating (CDR) were randomised to placebo or fixed dose of *Melissa officinalis* extract. The main efficacy measures were the change in the ADAS-cog and CDR-SB scores compared with baseline. Side effects were systematically recorded.

Results: At four months, *Melissa officinalis* extract produced a significantly better outcome on cognitive function than placebo (ADAS-cog: df = 1, F = 6.93, p = 0.01; CDR: df = 1, F = 16.87, p < 0.0001). There were no significant differences in the two groups in terms of observed side effects except agitation, which was more common in the placebo group (p = 0.03).

Conclusions: *Melissa officinalis* extract is of value in the management of mild to moderate Alzheimer's disease and has a positive effect on agitation in such patients.

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EFFECT OF MELIPURE-PC ON A PARKINSON'S DISEASE CELL MODEL

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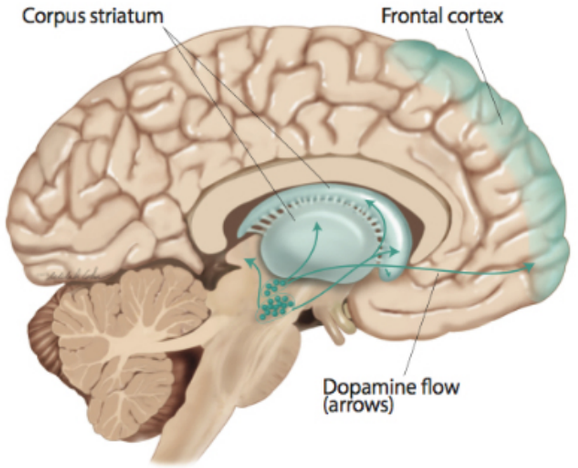
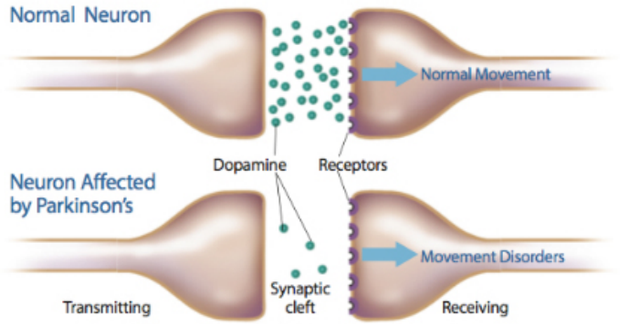
Parkinson's disease is a neurodegenerative disease that affects 1% of the population over the age of 60 and involves the dopaminergic neurons of the substantia nigra of the midbrain. Neuronal death leads to a rigid hypokinetic syndrome or agitating paralysis. Its causes are unknown and there is no cure for now.





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Culture of LUHMES cells differentiated in mesencephalic dopaminegic neurons.
Parkinson disease is simulated with the addition of rotenone in the presence or absence of MeliPure-PC

Measurements:

- Cytotoxicity (LDH)
- Autophagy (expression of LC3)
- Mitochondrial morphology

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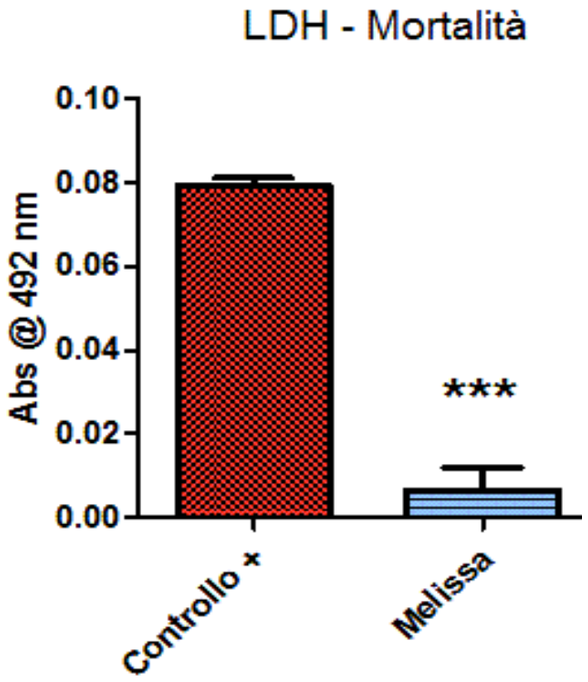
CYTOTOXICITY - LDH

Cells in neurotoxic environment with 5 nM rotenone, pH = 6 for 2 hours with or without MeliPure-PC.

MeliPure-PC protects cells from rotenone-induced damage.



Cell mortality measured by LDH released in the culture medium

Melissa is effective in reducing cell death.
** p<0.01; *** p<0.001 with ANOVA and test post-hoc of Dunnett.

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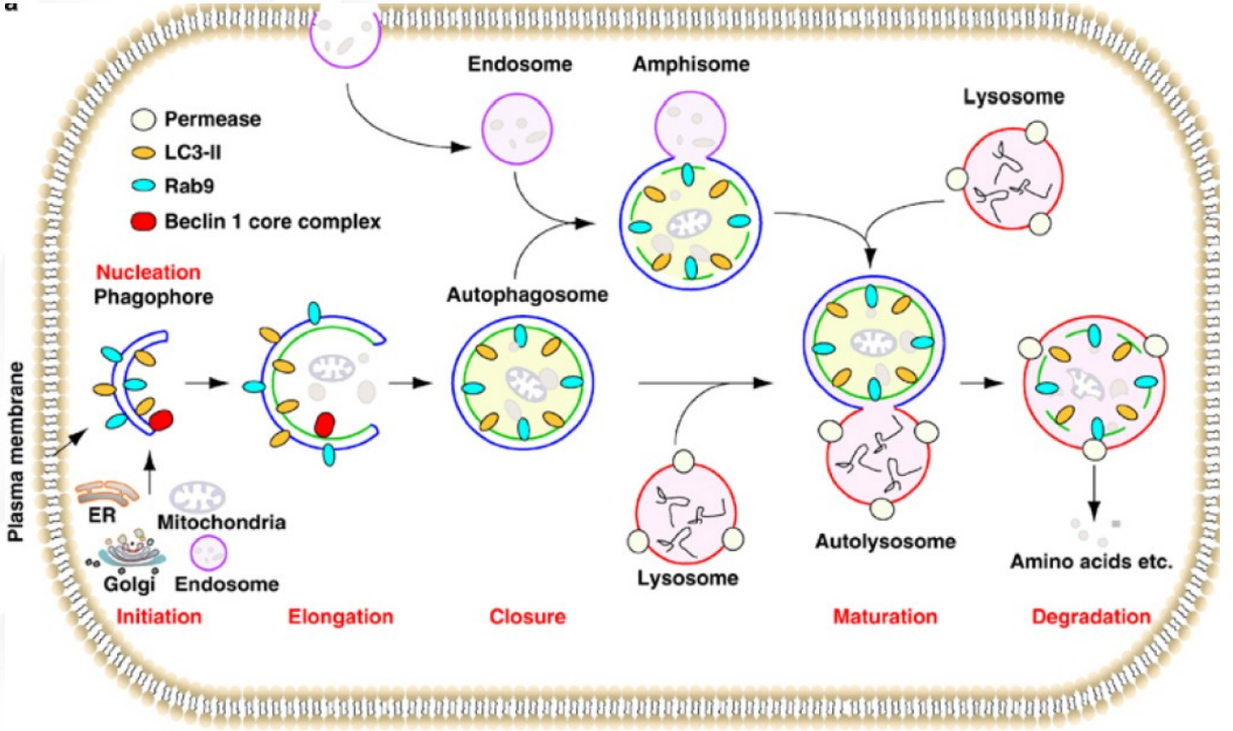
AUTOPHAGY

Autophagy is the key process by which cellular waste is removed or recycled. In general terms, autophagy is the skin's «housekeeping» process. Autophagy helps to detoxify/purify the skin (Elysèe, Scientific Cosmetics, 2015)



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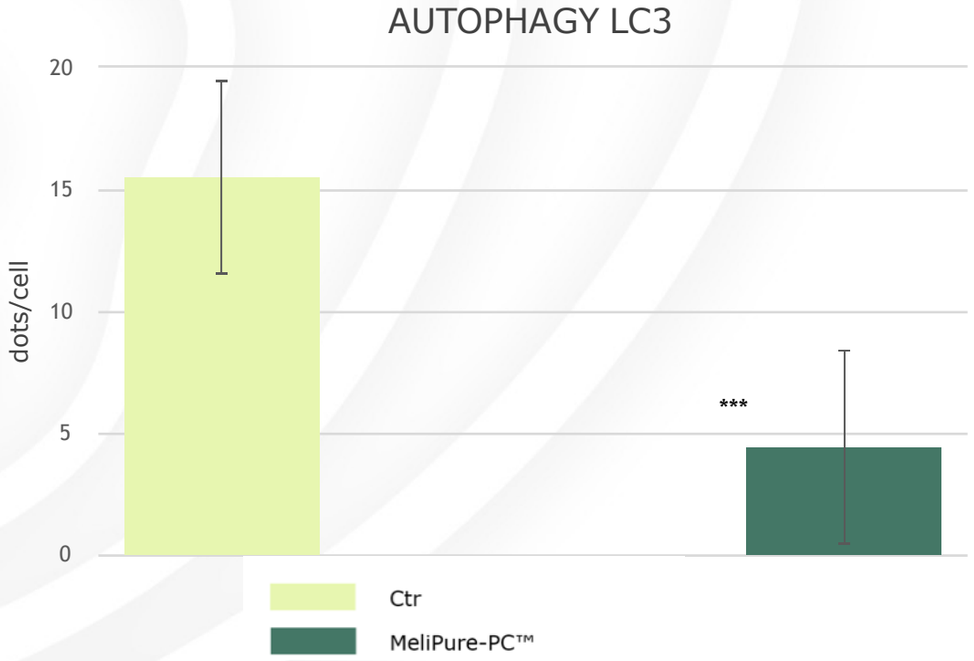
LC3 is a central protein in the autophagy pathway where it functions in authophagy substrate selection and autophagosome biogenesis.
Evaluation of LC3 expression was performed by using LC3 antibody on macrophage cells after treatment with LPS.



LC3 staining after 18h of stimulation with MeliPure-PC™
***p<0,001 with ANOVA

MeliPure-PC™ significantly decreases the expression of LC3 giving cellular protection against oxidative stress induced by LPS.

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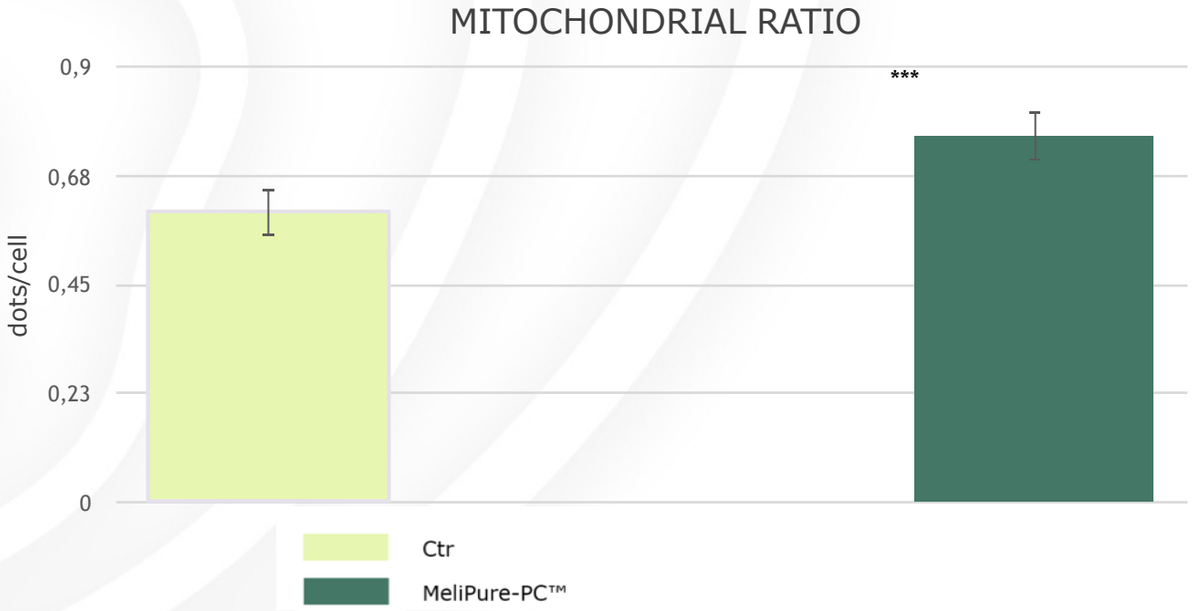
Mitochondria play a key role in the aging process and the health of the mitochondria is directly proportional to their aspect ratio.
Ratio of major axis to minor axis of an approximate mitochondria to an ellipse.



MeliPure-PC™ significantly increases the mitochondrial aspect ratio compared to control.

Mitochondrial morphology after 18h of stimulation with MeliPure-PC ***p<0,001 with ANOVA

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- The application of MeliPure-PC on a Parkinson's disease model (dopaminergic neurons damaged with rotenone) protects cells from damage induced by rotenone itself

- The protection mechanism probably lies in the ability of the extracts to inhibit the damage induced by oxygen radicals (antioxidant activity) that occurs starting from the blockage of the respiratory chain by the rotenone.

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INNOVATIVE BOTANICALS TRADITION

The best of what Nature has to offer preserving biodiversity and overcoming the limits of plant supplies.

Aperitivo DemBiotech: scopri il “rosso” che fa bene.

STAND 417

Thank you for your attention

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