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PhD DEFENCE

Development of innovative methods to preserve a new line of infant food supplements free from synthetic additives

Sviluppo di metodi innovativi di conservazione nella produzione di una nuova linea di integratori alimentari per la prima infanzia privi di additivi sintetici

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LIST OF ABBREVIATIONS

AAE	Annurca apple extract
ADR	Adverse drug reactions
APC	Adenomatous polyposis coli protein
CK1α	Casein kinase1α
CSC	Cancer stem cells
CVD	Cardiovascular disease
DI-FT-ICR-MS	Direct Infusion Fourier Transform-ion cyclotron resonance mass spectrometry
DMEM	Dulbecco Modified Eagle Medium
Dvl	Dishevelled
EC	European Commission
EFSA	European Food Safety Authority
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FFA	Free fatty acids
FISH	Fluorescence in situ Hybridization
FT-ICR	Fourier transform-ion cyclotron resonance mass spectrometry
FZD	Frizzled receptor
GC/MS	Gas chromatography-mass spectrometry
GMP	Good Manufacturing Practices
GPCR	G-protein-coupled receptors
GSK3β	Glycogen synthase kinase3β
HF	Hair follicle
ILSI	International Institute of Life Sciences
LAE	Limoncella apple extract
LDL	Low-density lipoprotein
LEF	Lymphoid enhancer factor
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MOE	Margin of Exposure
MS	Metabolic syndrome
NFAT	Nuclear Factor of Activated T-cells
OECD	Organization for Economic Co-operation and Development
OXPHOS	Oxidative phosphorylation
PKC	Protein Kinase C
ROS	Reactive oxygen species
TCF	T-cell factor
TG	Test Guidelines
TMAO	Trimethylamine N-oxide
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TTC	Toxicological risk values

ABSTRACT

In last two decades, research studies on nutraceuticals, functional foods and natural products have acquired increasing scientific importance. Unfortunately, this is not only for the interest in this scientific field, but also because the discoveries regarding the healthy effect of these products have the power to further boost enormous profit of the nutraceutical market.

Natural products, such as plants and fruit extracts, provide unlimited opportunities for the development of pharmaceuticals and nutraceuticals. This is due to the amazing chemical diversity and the plethora of physiological activity of their chemical components. The areas of application for nutraceuticals, botanicals, and herbal products are thus becoming more and more varied: from maintenance of well-being to cosmetic applications, from reestablishment of homeostasis to prevention of cardiovascular and dysmetabolic pathologies, from neuro- to chondroprotection, from strengthening the immune system to preserving regular intestinal functions, form weight loss to improvement of muscle performance.

However, being “natural” does not necessarily mean “being safe”. Despite the considerable interest received by nutraceuticals, the proof of their biological activity and of their therapeutic effects is mostly postulated based on the traditional usage but almost never confirmed by scientifically sound approaches.

Nowadays, the European Commission (EC) has proposed harmonized approaches, made of *in vitro* and *in vivo* experimental pipelines, necessary to demonstrate and/or confirm the effectiveness of almost every pharmaceutical preparation before the latters can be sold on the market. The approval procedure is very complex, as it requires “companies' requests” to be collected by the responsible national institutions of the various member countries and sent to EC. Final decisions are based on the "opinions" of an external Authority, the European Food Safety Authority (EFSA), which periodically organizes meetings of experts’ panels, judging data and results presented and expressing a favorable or unfavorable opinion.

Some nutraceuticals complete preclinical and clinical pharmaco-toxicology phases, and meta-analysis of their clinical trials are sometimes available. However, in many cases, specific data on the individual nutraceutical, which can characterize it as an effective aid in medical practice, are absent.

In this scenario, it is therefore necessary to perform preclinical researches on nutraceuticals in order to confirm their activity and their safety in a scientifically sound, valid and reproducible way.

This thesis presents and discusses the development and the application of biological platforms aimed to: 1) validate the health-promoting potential of phytocomplexes and nutraceuticals; 2) investigate their mechanism of action; 3) confirm their biotoxicological safety.

The results are presented in five chapters.

CHAPTER 1, describes the regulatory framework that is relevant for the marketing of nutraceuticals and functional foods in the European Union (EU), and the regulatory authorities who manage the state-of-art worldwide. It also defines how this legislation was developed as well as some practical consequences for manufacturers, marketers and consumers. In this chapter it is explained the research methodologies to be applied and the scientific endpoints to aim during preclinical and clinical phases of the development on nutraceuticals, based on a step-by-step approach.

CHAPTER 2, describes platforms, pipelines, procedures and results obtained by performing the risk assessment of three nutraceuticals and a novel food. Implementing the approaches suggested by OECD guidelines, I present data on the genotoxic and cancerogenic potential of these products confirming their safety. In particular, the aim of this investigation is to provide preliminary information of their pharmacokinetic and toxicological properties through the study of their *in vitro* toxicological profile and *in vivo* bioavailability. In particular, I herein perform and optimize the most commonly used methods to investigate gene point mutations and chromosome aberrations: bacterial reverse mutation assay in *Salmonella typhimurium* and *Escherichia coli* (OECD 471) and *in vitro* micronucleus assay (OECD 487).

CHAPTER 3, describes experimental platform aimed at the identification of plant extracts endowed with antimicrobial potential and discusses their use in a new line of supplements. I confirm that phytochemicals' broad-spectrum antibacterial activity represents a possible source of effective, cheap and safe antimicrobial agents. Specifically, I perform and optimize extraction procedure on five plants

commonly used in herbal products for early childhood: *Melissa officinalis; Matricaria chamomilla; Mentha spicata; Melissa officinalis* and *Mentha sylvestris.* To evaluate antioxidant activity of the herbal extracts a DPPH assay is performed. In order to identify their antibacterial activity, the sensitivity to these compounds of the Gram-positive microorganism *Staphylococcus Aureus* (strain ATCC 25923) and the Gram-negative *Escherichia Coli* (strain ATCC 25922) is evaluated, through the determination of the MIC and MBC.

CHAPTER 4 presents biological platforms aimed at the identification of the molecular mechanism underpinning nutraceuticals' health claims. The use of botanicals is often validated simply by tradition of usage, but there is a lack of modern specific assays to confirm their pharmacological activity. Herein, I present several *in vitro* and *in vivo* experimental approaches useful to identify pro-metabolic properties of phytocomplexes. To reach the purpose, (1) I make use of isotope labeling and high-resolution mass spectrometry approaches, Deuterium labeling gas chromatography-mass spectrometry (GC/MS) and Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR)**,** as potential validated platform to highlight molecular mechanism of nutraceuticals exerting cholesterol and lipid lowering properties. Metabolomic approaches are extremely useful tools for probing any change in metabolism accompanying drug treatments and provide invaluable insights in the mechanism of action of complex mixtures and phytocomplexes. (2) To evaluate Mitochondrial boosting activity of a nutraceutical formulation, I assess a platform on both *in vitro* cultured hepatic cells and *in vivo* on mice fed a High Fat Diet and treated with the nutraceutical by using the mitochondrial selective probe MitoTracker CMXRos. (3) To demonstrate the insulin-like properties of a natural products, I perform a pipeline assessed the ability of nutraceuticals to modulate glucose uptake via glucose transporter (GLUT), by measuring the uptake of 2-NBDG, a fluorescent analogue of deoxy-glucose covalently linked to the fluorescent molecule nitro blue tetrazolium (NBT).

In **CHAPTER 5**, I describe my work on nutraceuticals as supplements during anti-cancer treatments to support chemotherapy therapies. The aim of this thesis chapter is to present three studies in which I set up new approaches to test nutraceuticals and investigate their mechanism of action. In these studies, I perform a metabolite analysis using Direct Infusion Fourier Transform-ion cyclotron resonance mass spectrometry (DI-FT-ICR-MS) to investigate the molecular mechanism underpinning their nutraceutical activity.

CHAPTER 1

Nutraceuticals

1. Definition of nutraceuticals

In a historical period in which traditional pharmaceutical innovations are increasingly rare, especially in the primary care sector, the nutraceuticals have acquired increasing scientific and commercial importance [1].

The term nutraceutical is a neologism coined in 1989 by Dr. Stephen De Felice combining the terms nutrition and pharmaceutical [2]. Its definition was clarified in 1994 as "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary, supplements and diets to genetically engineered 'designer' foods, herbal products, and processed foods such as cereals, soups, and beverages". The term "functional food" is related to nutraceuticals. It was introduced for the first time in Japan in 1984 and defines a food product supplemented with nutraceutical compounds that produces positive effects on the general body condition and decreases the risk of specific diseases. Today, the accepted definition is the following: "Natural or processed foods that contain known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease"[3][4].

Today, research studies on nutraceuticals, functional foods and natural products are hot topics [5]. This is also because the discoveries regarding the health effect of balanced, vegetable-enriched and fat-free diets have boosted the nutraceutical market. It has been indeed increased the consumer awareness of the potential benefits of compounds naturally present in foods and plants for human health promotion and maintenance. The Mediterranean diet, for example, is rich in nutraceutical components which have functional features with positive effects on health and wellness. As example, many studies have highlighted the existence of a strong relationship between Mediterranean diet and human health. Mediterranean diet is mostly characterized by the consumption of cereals, legumes, vegetables, fruits and olive oil; it also includes fish or seafood, white meat and eggs, small amount of poultry and dairy products. Current scientific knowledge supports the hypothesis that Mediterranean diet may modulate several functions of the body and play a key role in the prevention of some diseases [6][7]. The importance of this diet resides in the nutritional values of these constituents, as well as in the high content of pharmacologically active principles [5].

1.1 Nutraceuticals vs Phytochemicals

Natural products, such as vegetable or fruit extract, provide unlimited opportunities for new active principles discoveries due to the chemical diversity of the molecules they contain [8]. The compounds present in natural products are called phytochemicals ("phyto" means "plant") because they are produced by plants, fruits, vegetables, spices and traditional herbal medicinal plants [9]. In the last two decades, many studies have shown that many foodstuffs in our diet contain a high content of phytochemicals, and can provide protection against various diseases.

Isolation and structural characterization of unknown active principles from daily foodstuffs have become very interesting in order to understand how diet can affect human health, and how these pharmacologically active principles can be used as an alternative to the classical and commercial drugs.

Foodstuffs such as fruits, citrus fruits and vegetables are considered functional foods. These diet components were shown to be endowed with protective effects against chronic diseases such as cardiovascular diseases [10]. Antioxidants such as Vitamins C and E are essential for the protection against ROS, even if the majority of the antioxidant activity of foodstuffs such as fruit or vegetable, derives from polyhydroxylated phenolic compounds. Intake of controlled diets rich in fruits and vegetables significantly increased the antioxidant capacity of plasma. As already mentioned, among the constituents of Mediterranean diet, olive oil is considered another essential functional food, because of its constant intake may provide potential health benefits to humans, lowering the incidence of cardiovascular disease [11]. It was suggested that this effect is due primarily to the antioxidant properties of polyhydroxylated aromatic molecules present in the oil as minor compounds.

Antioxidant compounds such as phenolic molecules are therefore the focus of many recent studies [12]. Their antioxidant activity is predominantly determined by their structures, in particular by the electron delocalization over an aromatic nucleus. When these compounds react with a free radical, the delocalization of the electron over the aromatic nucleus allows the formation of a stable radical which prevents the continuation of the free radical chain reaction [13].

Most nutraceuticals are phytochemicals, for example ß-glucans, tocotrienols and phytosterols, sulfur compounds, polyphenols such as anthocyanins, proanthocyanidins, flavonols, stilbenes, coumarins, ellagic acid, isoflavones [14][15]. Their biological activities are numerous and often well documented in literature. For example, the positive correlation between the consumption of flavonoids and the reduction of cancer risk has been highlighted in several epidemiological studies. Much fewer are nutraceuticals of animal origin, even if some of them, such as the well-known polyunsaturated fatty acids of the series omega-3, are among the most used [16][17] (**Figure 1.1**).

The areas of application of these products are becoming more and more varied [18]: from maintaining well-being, to prevention in the cardiovascular and oncological fields, from neuro- to chondroprotection, from strengthening of the immune system to preserving regular intestinal functions, from weight loss to improvement of muscle performance for athletes.

1.2 Current legislation on Nutraceuticals and their Health Claims

In vitro and *in vivo* experiment, followed by clinical trials are necessary to demonstrate the effectiveness of bioactive compounds. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive nutraceuticals and their formulations (extracts) require a careful evaluation [19]. They must be carefully planned to safeguard the health of participants and to evaluate their positive and immediate and long-term side effects. Therefore, research studies are focused on the development of advanced analytical procedures to determine the quality and the safety of food, through the accurate quantification of nutraceutical molecules [20].

Being natural substances, nutraceuticals have the advantage to be ingested regularly and in significant quantities, exerting their health effect over time, without side effects to the doses provided by current legislation.
To ensure a high level of consumer protection and facilitate their choices, products placed on the market must be safe and properly labeled. Current legislation on Health Claims, relating to food products, including nutraceuticals [21] in the European Community, expressly indicates the need for adequate scientific support for health claims.

The approval procedure is very complex, as it requires “companies' requests” to be collected by the responsible national institutions of the various member countries (in Italy the Ministry of Health) and sent to the European Commission (EC)[22]. EC decisions are based on the "opinions" of an external Authority, the European Food Safety Authority (EFSA), which periodically organizes meetings of experts’ panels, expressing a favorable or unfavorable opinion [23][24]. At present, there are opinions available on over 1000 products, which have been sent by EFSA to the European Commission for the relevant provisions. Most of the opinions were not favorable, because the documentation sent was insufficient to support the health claim. Almost all the opinions on probiotics and herbal supplements were negative, as well as over 50% of those expressed on vitamin and mineral supplements.

Currently, the use of food supplements and functional foods is constantly expanding (**Figure 1.2**), and as a consequence of a growing demand, the supply has increased exponentially (**Figure 1.3**).

In Europe, the market for nutraceuticals is quantified in billions of euros, constantly growing, expecially in Italy, over the last decade even in years of great economic crisis. Italian trust does not depend only on marketing phenomena, but also on the high quality of biological products and the degree of innovation and active research on nutraceuticals in our country. It is appropriate to remember that the Founder, as well as Emeritus President of the Italian Nutraceutical Society, Cesare Sirtori, has published a work on nutraceuticals (when it was not yet called that) as early as 1977 [25]. Moreover, some large pharmaceutical companies, as well as to some small- medium-sized Italian companies, have invested in biotechnologies aimed at improving the bioavailability of nutraceuticals and in clinical research, as well as in the construction of professional medical-scientific information networks and in high quality training and divulgation events. Finally, the Italian scientific world has responded by opening the doors of research institutes and national corporate conferences to nutraceuticals in many areas of medicine.
But how to orient oneself, as prescribers, dispensers, or consumers, in front of 20-30 products containing similar active ingredients, variously associated, which recall a similar efficacy, but often with costs very different from each other?

For some nutraceuticals, complete preclinical and clinical pharmaco-toxicology data exist, and are even available meta-analysis of clinical trials. However, in most of the cases, there are factors that limit data availability to few informations on individual components of the phytocomplex.

Even when the drug-toxicological dossiers are quite complete, in some cases there is a lack of data, in particular those of efficacy and safety after prolonged use in humans. The current legislation does not require for nutraceuticals the development process required for drugs (pre-clinical and clinical phases (I- IV); demonstration of the mechanism of action; efficacy and tolerability results). The nutraceutical, therefore, is "effective" because its administration is related to the achievement of an evidence-based health improvement that testifies it without the need for scientific data, controlled studies and publication on medical journals.
The criteria to recognize a serious nutraceutical, whether it is a mono-component nutraceutical or multi-component, should be:

- Literature on mechanism of action, on pharmaco-toxicological data and on quality control;
- Indication of use, consistent with the known pharmacological mechanism;
- Clearly specified dose and duration of treatment;
- Clearly indicated warnings for use;
- Tolerability and safety

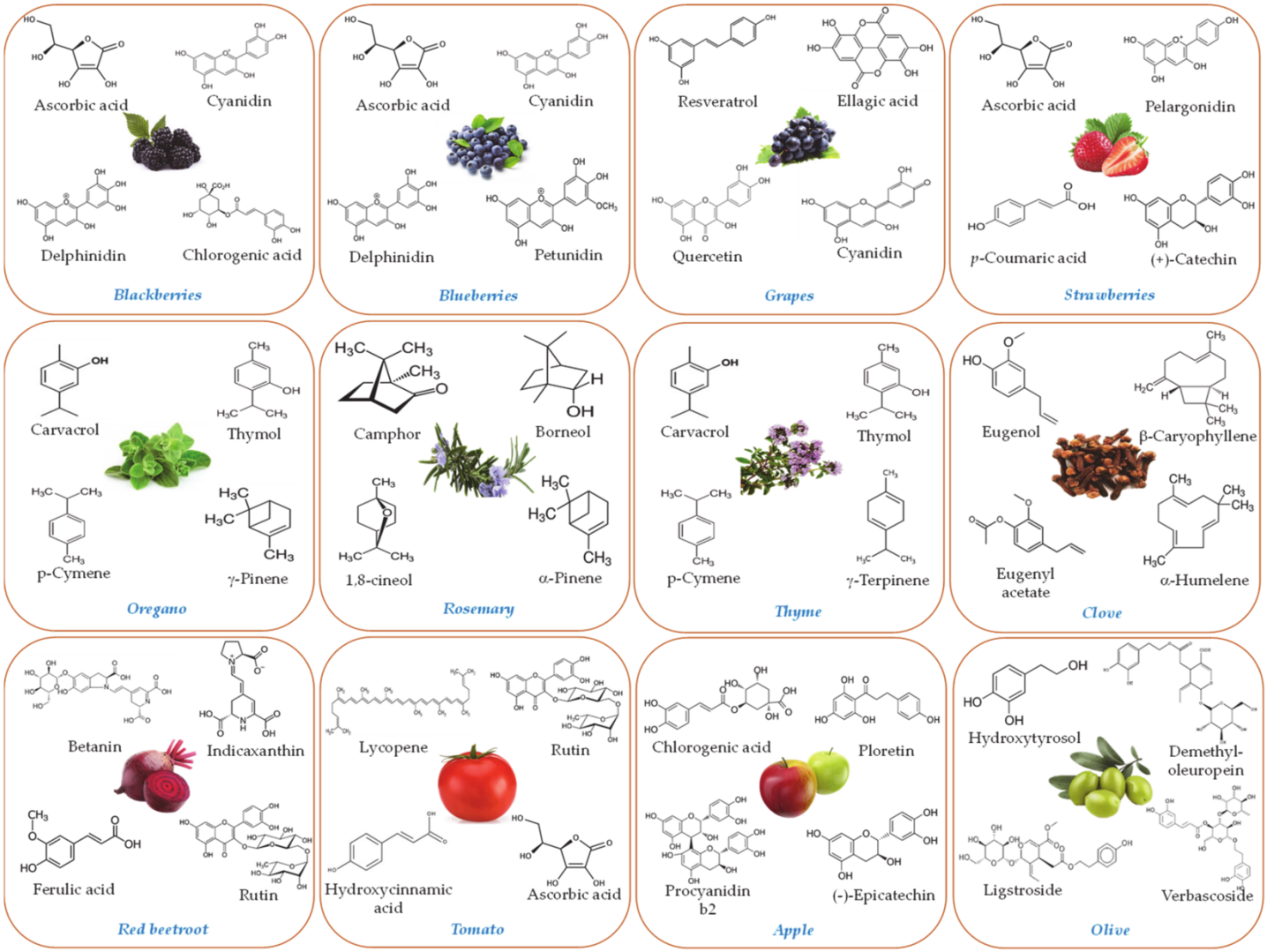

Figure 1.1. Examples of some bioactive agents isolated from natural products

Examples of naturally occurring nutraceuticals

Naturally occurring substances	Select foods containing the substance	Areas with established or emerging evidence of benefit	
Dietary fiber	Fruits, grains, legumes, vegetables	• Lipid control • Arterial hypertension • Glucose control	• Weight control • Intestinal motility
Probiotics (for example, lactobacilli, gram-positive cocci, bifidobacteria)	Many naturally fermented foods (kefir, unpasteurized sauerkraut, soft cheeses, pickled cucumbers)	• Gastrointestinal disorders • Allergies	• Asthma • Cancer • Infections
Prebiotics	Chicory roots, bananas, tomatoes	• Lipid control • Gastrointestinal disorders	• Cancer
Polyunsaturated fats	Fatty fishes	• Cardiovascular disease • Asthma	• Mental health • Diabetes
Antioxidant vitamins (vitamin C, vitamin E, carotenoids)	Citrus fruits, peppers, nuts, seeds, cantaloupe, carrots	• Degenerative disease	
Polyphenols	Tea, dry legumes, berries	• Microbial infection • Neurodegenerative disease	• Diabetes • Cancer • Cardiovascular disease

Figure 1.2. Many naturally substances show evidence of potential health benefits [5]

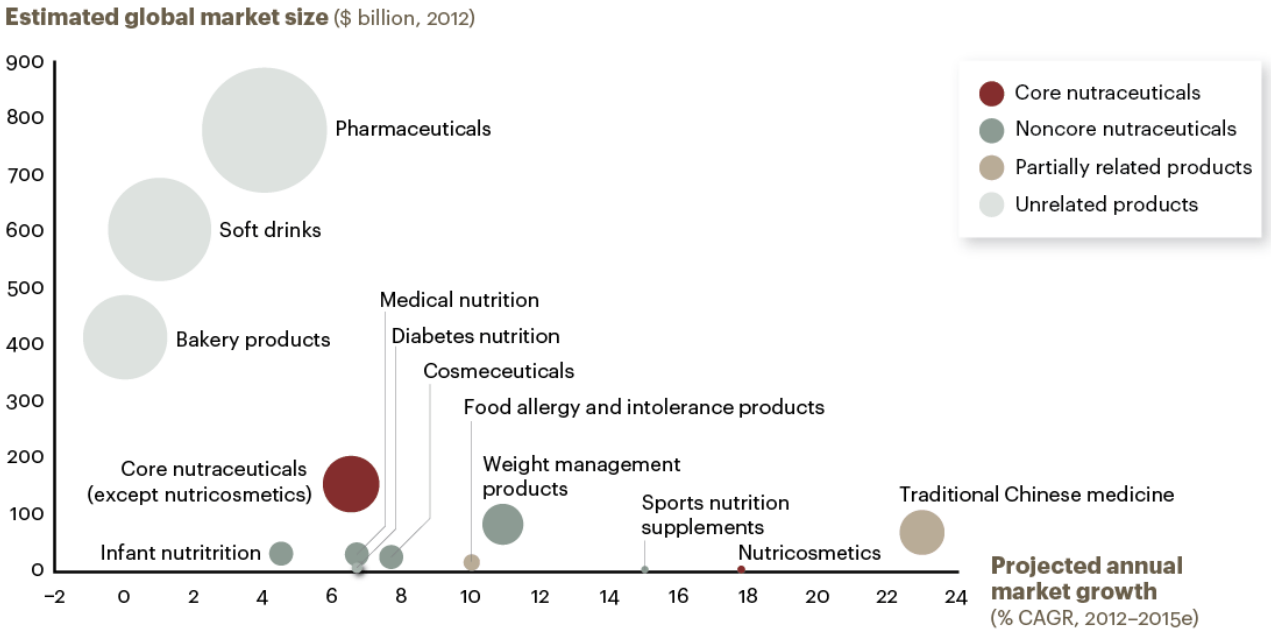

Figure 1.3. Nutraceutical market size and projected growth

1.3 Research methodology applied to nutraceuticals: preclinical pharmacotoxicology

In the United States, nutraceuticals do not require evidence of efficacy for marketing approval by the Food and Drug Administration (FDA). Furthermore, safety is considered, the responsibility of the manufacturer, and his control remains limited to post-marketing surveillance. In Europe, if the product is assigned therapeutic indications, safety and efficacy tests are required, unless it concerns vegetable mixtures with an established history of use; in the latter case, these products are called "traditional herbal medicines" and are subject only to safety and quality requirements, as is the case in the United States.

The Scientific Commission of EFSA has published a document providing guidelines for the safety assessment of preparations containing ingredients of vegetable origin [26].

Despite the recent attention from a regulatory point of view, it cannot be ruled out that some products may pose a health risk. This could result from:
(I) excessive consumption to be attributed to companies that spread misleading claims;
(II) the belief of many consumers to equate the term "natural" with "'safe";
(III) the availability of potentially harmful nutraceuticals from countries in which are not in place Regulations regarding their safety;
(IV) the frequent lack of detailed studies on the safety and quality of nutraceuticals.

A nutraceutical must be a valid and safe product and the guidelines for labeling and Good Manufacturing Practices (GMP) requirements adopted in major markets are helping achieve these goals [27]. It is therefore necessary to identify good practices and harmonized protocols to ensure preclinical research on nutraceuticals to be scientifically sound, valid and reproducible.

1.3.1 Main phases of the preclinical development of safe and effective nutraceuticals

STEP 1. Critical evaluation of the scientific literature relating to botanical or natural products present in the nutraceutical.

The process of developing a new nutraceutical should begin with the consultation of the main scientific search engines, such as PubMed, NAPRALERT, etc., to determine the traditional use of a particular botanical product and the state of art about its safety, efficacy and active components.

STEP 2. Acquire and authenticate the natural product using good agricultural practices and good harvesting practices.

Good agricultural practices for the cultivation of plant products reduce the risks of contamination by pesticides, heavy metals, or biological agents, etc. Part of these practices requires that all plants used for the production of nutraceuticals should be botanically authenticated to avoid misidentification. An incorrect identification of botanical species can not only compromise a subsequent scientific investigation, but can also have tragic results, as was the case with a dietary supplement for slimming use sold in Belgium in 1991[28][29].
For this reason, the authenticity of the botanical product is among the regulatory requirements of the EU and the United States. However, the lack of this evidence is one of the most common violations cited during regulatory inspections.

STEP 3. Determine the mechanism of action and identify active compounds.

Adequate biological tests should subsequently be employed to determine the mechanisms of action and to identify active phytochemicals. Biological tests should include the evaluation of possible synergies between the various constituents of the nutraceutical, as well as the determination of the potential toxic effects of all components.
Identification of active compounds is a necessary process prior to chemical standardization of a complex natural product, as it usually turns out to be a nutraceutical, and the mechanism of action should be determined to then proceed to biological standardization (STEP 4).
Various methods are available for identifying active compounds, such as bio-guided fractionation, or

liquid chromatography interfaced with mass spectrometry, or more recently the use of magnetic beads covered with functionalized surfaces that allow the pre-purification of the sample before analysis in mass spectrometry.

STEP 4. Chemical and biological standardization.

To obtain a reproducible product for subsequent clinical and consumer studies, nutraceuticals should be chemically and biologically standardized through carefully selected tests.
Chemical standardization usually uses analytical methodologies such as chromatography, HPLC-UV or HPLC-MS, to measure the levels of active compounds or any other component that might be useful for the preparation of a reproducible product.
The characterization then proceeds with biological standardization, using specific in vitro tests, such as enzyme assays, receptor binding, gene expression tests on cell cultures and in vivo on laboratory animals, in order to ensure that the nutraceutical will have reproducible biological activities.
These chemical and biological standardization tests help to ensure the reproducibility of the nutraceutical ingredient in other laboratories for research purposes, but above all they allow the production of safe products for consumers.

STEP 5. Absorption, metabolism and bioavailability of active compounds.

The active compounds and other natural products found in the nutraceutical should also be evaluated regarding absorption and metabolism. Bioavailability, to which absorption, intestinal metabolism and hepatic first pass metabolism contribute, is often neglected, but important, because it helps determining the effectiveness of nutraceuticals.
Indeed, only those compounds that cross intestinal epithelium are likely to produce systemic effects, and the lack of absorption could explain why many clinical studies with nutraceuticals, or with natural extracts, have not shown any useful biological effects, although in vitro studies suggest otherwise.
For example, in vitro tests have shown hepatoprotective and antioxidant activity for silybin A and B flavolignans present in thistle-based supplements (*Silybum Marianum*).
However, the phase I clinical trial on milk thistle has shown that the bioavailability of these compounds is less than 0.5% "and that their half-life is very short (< 2 hours) due to the rapid Phase II metabolism.
These data suggest that dietary supplements containing milk thistle have limited beneficial effects and a short duration of action. The poor bioavailability of flavolignans present in milk thistle,

resulting from poor absorption and rapid conjugation by phase II enzymes, could have been predicted prior to clinical trials using the Caco-2 cell line [30], or in *in vivo* animal models. Caco-2 cells are a good model of intestinal permeability and transport, and constitute a standard approach for predicting intestinal absorption of drug candidates, as they show a good correlation with human absorption.

The metabolism of products of natural origin usually involves the formation of biologically less active polar metabolites which can be excreted more rapidly in the bile or urine. Rarely metabolism produces more active compounds than their precursors.

As with preclinical drug metabolism studies, rat liver microsomes and human hepatocytes are ideal models for studying phase I and II metabolism.

Furthermore, it is essential to identify the enzymes responsible for the metabolic transformation of the natural products contained in the nutraceutical, as they could be inhibited or induced, with consequences on the metabolism, serum levels, and bioavailability of some agents. Furthermore, it has been postulated that the gut microbiota may also play a role in metabolism, generating more active compounds than their precursors.

The composition of the intestinal microbiota can be modified by the diet and by the consumption of nutraceuticals itself.

The role of the intestinal microflora in the metabolism of natural products contained in nutraceuticals is a relatively new area of investigation.

A major challenge for studying the absorption, metabolism and bioavailability of nutraceuticals is the evaluation of these products as complex blends. Although specific natural products can be isolated and therefore evaluated as individual candidates, this approach does not consider possible interactions, such as the synergy between the various constituents.

Therefore, whenever possible, the assessment of the absorption, metabolism and bioavailability of the compounds present in the nutraceutical should be carried out using the mixtures of components, as they will actually be used by consumers.

STEP 6. Preclinical studies of inhibition and induction of drug-metabolizing enzymes.

It is known that mechanisms of enzymatic induction and/or inhibition by nutraceuticals can be the basis of adverse reactions (ADR-adverse drug reactions), in particular those related to therapeutic failure [31].

Drug-nutraceutical interactions may include induction or inhibition of 1) citP450 enzymes involved in drug metabolism, 2) UDP-glucuronyl-transferase, 3) other phase I and II enzymes, and 4) transport or efflux proteins [32].

A documented example is represented by the interaction between CYP3A4 and *Hypericum perforatum* [33]. Like all inducers of citP450, hypericum reduces the plasma levels of drugs that are usually metabolized by the citP450 system. For example, hypericum may reduce the anticoagulant effect of warfarin. Its use has also been associated with unexpected genital bleeding in women undergoing treatment with oral contraceptives [34].

In Europe, pregnancies were considered to be "probably linked" to this type of drug interaction. To determine whether natural products contained in nutraceuticals inhibit specific CYPs, human hepatocytes, human liver microsomes and recombinant enzymes can be used.

Many drug-nutraceutical interaction studies are based only on data obtained from in vitro studies without considering bioavailability and clinical significance.

Consequently, in vitro results sometimes correlate poorly with drug interaction data in clinical trials. This inconsistency is mainly attributable to the poor bioavailability of many of the natural products contained in nutraceuticals. For example, *in vitro* studies on ginseng (*Panax ginseng*) with recombinant enzymes have shown the ability to inhibit CYP3A4, CYP2C9, CYP2C19, CYP2D674, while clinical studies have shown no interaction [35].

The effects of nutraceuticals on drug metabolism should also include the evaluation of the possible competition between natural products and drugs with plasma proteins, the non-binding of which can transiently increase their serum concentrations, causing toxicity or increasing their clearance.

STEP 7. Preparation of standard GMP formulations.

After the standardization steps described, the production of nutraceuticals in GMP is an essential step to ensure the safety and efficacy of these products, and to prevent contamination of the formulation by microbes, pesticides, heavy metals and other foreign substances. To ensure reproducibility of in vivo research, GMPs should also be used for the preparation of formulations intended for preclinical use, as well as for clinical studies, authenticating the plants used in the formulation, verifying that it contains uniform doses, obtaining a uniform dissolution rate, and ensuring that the material is stable during use.

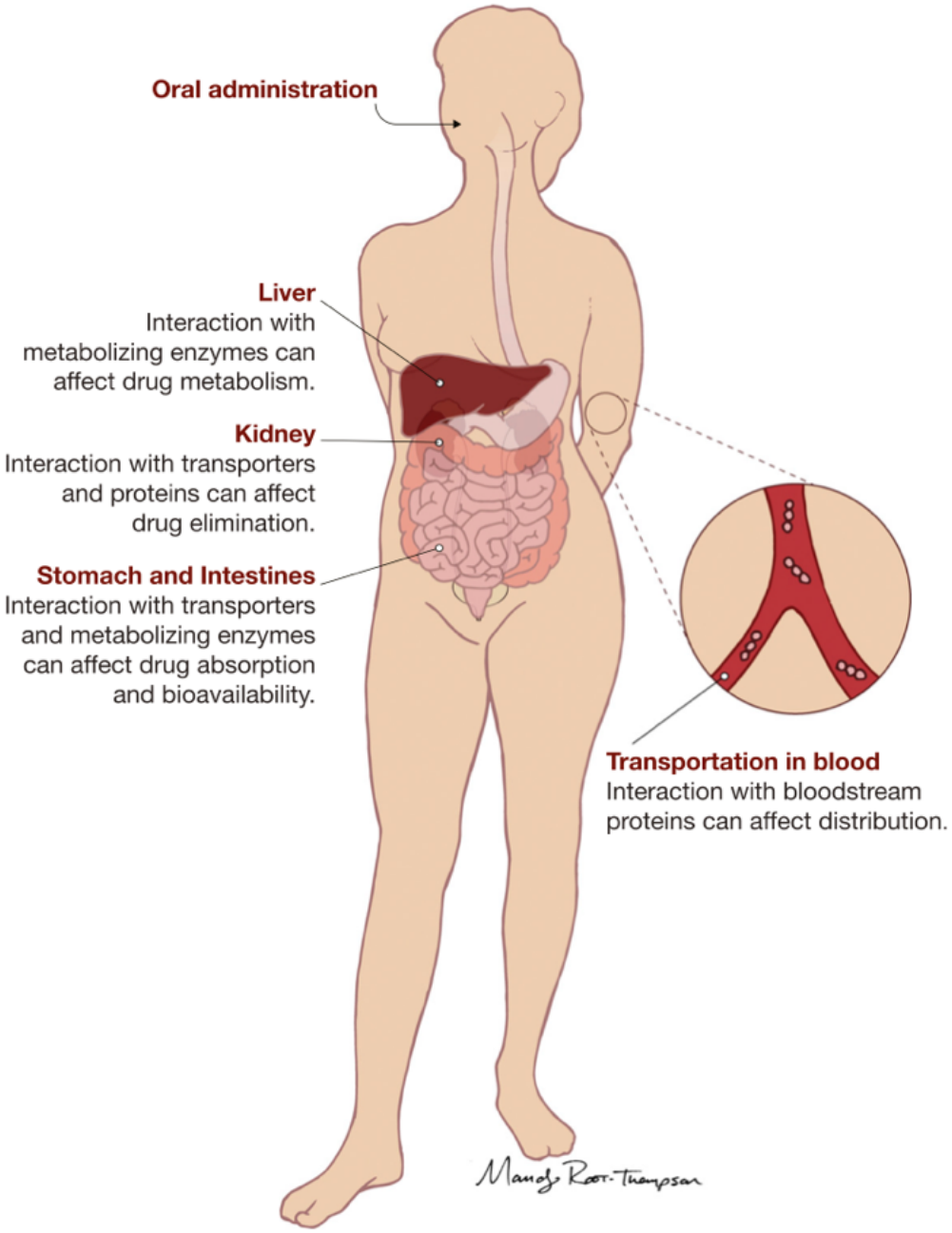

Figure 1.4. Pharmacokinetic drug-botanical interactions. Botanicals can cause pharmacokinetic drug interactions by interfering with drug-metabolizing enzymes in the liver, stomach, and intestines; drug transporters in the kidneys, stomach, and intestines that will alter absorption, bioavailability, and drug elimination; and proteins in the blood that can alter drug distribution [32]

1.3.2 General considerations for the risk and safety assessment of nutraceuticals

At present, an official framework for the safety assessment of nutraceuticals is not available and safety assessments are performed on a national basis or by dedicated bodies. In 2009, the EFSA Scientific Committee published an updated guideline on the scientific data needed to carry out a safety assessment of a botanist or a botanical preparation.

This guideline proposes a scientific approach, depending on the knowledge of the botanical product and the substance(s) it contains. The tiered approach takes into account the nature of the nutraceutical, its uses and intended use.

For preparation having a tradition of food usage no adverse effects on human health should be expected.
On the other hand, for nutraceuticals without a history of consolidated food use, or for those whose intake significantly exceeds traditional intake levels, experimental safety assessment and toxicity data may be required. The EFSA document does not give clear indications on what needs to be assessed when the toxicological profiles of the compounds present in the nutraceutical are not available, but states that the assessment of exposure to the substance in relation to the threshold of toxicological risk values (TTC) may be useful.

1.3.3 Toxicological risk values (TTC)

The TTC method is based on the principle that the likelihood of incurring toxicity is linked to the extent and duration of exposure to a substance.
For many types of toxic effects, it is possible to identify, through experimental studies, a threshold dose below which no adverse effects are observed. Based on published data on the toxicity of numerous chemicals, generic human exposure thresholds (called 'TTC values') have been identified for groups of substances whose chemical structure and probability of toxicity are similar.
The chemical structures have been grouped, with a conservative approach, into three classes of low, medium or high toxicity categories (known, respectively, as Cramer I classes, II and II).
For each of the Cramer classes the TTC values were obtained. Untested substances can be conservatively evaluated by comparing the appropriate TTC value with reliable human exposure data. Therefore, if human exposure to a nutraceutical component is below the toxicological alarm threshold of its structural class, the probability of adverse effects is considered to be very low.

In the event that a component of the nutraceutical contains genotoxic or carcinogenic substances, the assessment of the risk for human health is certainly much more complex.
The presence of genotoxic and carcinogenic substances, even if not desirable, could derive from environmental pollution or depend on production processes. Assuming that a genotoxic and / or carcinogenic compound should not be present in a nutraceutical, if it could not be avoided, the most widely used strategy is based on the concept that the intake of a potentially genotoxic substance should be the lowest reasonably achievable (ALARA principle). However, this approach does not include data on the potency of the carcinogen, nor data on the human exposure. Considering the possible uncertainties and disadvantages associated with the use of the ALARA approach, expert groups from EFSA, FAO / WHO (JECFA) and the International Institute of Life Sciences (ILSI) have recommended the use of the Margin of Exposure (MOE).

The MOE by definition is the relationship between two factors, which for a given population evaluates the dose to which a small but measurable adverse effect is first observed and the level of exposure to the target substance. The MOE is not used to assess the safety of substances subject to regulation deliberately added to the food chain (e.g. food additives or materials in contact with food). However, EFSA's Scientific Committee believes that this could be useful in assessing genotoxic and carcinogenic impurities present at very low levels. In this way, the use of the MOE can help support risk managers in defining any actions necessary to keep exposure to these substances as low as possible.

The EFSA document provides a set of criteria to help prioritize the safety assessment of botanicals in use. The document states that: "Priority should be given to botanical products and preparations: 1) known to have an established history of food use and which have been identified to contain significant levels of potentially hazardous substances; II) that are not allowed / recommended for food use in some European countries, but which are still in use in other EU countries, particularly when expected use levels are high; III) for which some adverse health effects have been reported based on reports of intoxication, epidemiological data or all toxicity data from farmed animals, laboratory animals; IV) for products that are very similar to substances known to have toxic effects; V) for those whose consumption has increased significantly in recent years in the Member States; VI) for those with a limited history of use and limited toxicity data available, and for which expected use levels may be relatively high.

EFSA also disseminated available information on a large number of botanicals that contain substances that may be of concern to human health when used as whole or in specific parts of them or when certain procedures or inadequate procedures are employed in the preparation of the extracts. The compendium, regularly updated, is available on the EFSA website.
Examples of compounds of plant origin with genotoxic and carcinogenic activity are, for example, compounds belonging to the group of pyrrolizidine alkaloids, alkenyl benzenes or aristolochic acid.
It is important underlining that, despite the bans, products containing compounds of toxicological interest may still be present on the market. Another example is the presence of aristolochic acid in herbal preparations used in traditional Chinese medicine, whose metabolites can cause nephropathies and urothelial tumors.

As the use of nutraceuticals around the world continues to increase, the need for a scientific evaluation of their safety and efficacy is becoming an ever more pressing need.
It would be desirable that the same rigor of the preclinical and clinical studies used to discover and develop drugs were applied to the research of nutraceuticals, using a step-by-step approach, as described.
Among the various steps proposed, the requirement of using GMPs in the preparation of these products has significantly contributed to increasing their safety.
However, improving some steps, such as chemical and biological standardization, would help refine the reproducibility of preparations, while further kinetic studies and pharmacological and toxicological investigations on potential drug-nutraceutical interactions could improve our understanding of the safe use of such products. Preclinical studies, with the research of the mechanisms of action and the identification of active compounds, will facilitate the production of more effective and safer nutraceuticals, establishing more and more reliably the advantages for human health and the possible limitations of these products.

Currently, the safety assessment of a nutraceutical is still carried out on a national and often ad hoc basis, following reports of toxicity. Furthermore, even if regulatory measures are in place, nutraceuticals containing toxic compounds can still be offered for sale on unofficial channels, such as websites. It is now clear that "natural" is not the same as "safe" and that nutraceuticals can contain toxic compounds at concentrations higher than those normally found in foods. Furthermore, the tradition of using a compound does not guarantee its safety when used as a supplement. This highlights the need for more widespread and stringent regulation and control for all nutraceuticals, especially considering their ever-expanding market volume.

1.4 Research methodology applied to nutraceuticals: clinical research

For a relatively large number of nutraceuticals preclinical and clinical data, meta-analyses of randomized clinical trials are available. For phytocomplexes, more information would be instead necessary.

However, to market a nutraceutical, it is not necessary to give all the information useful for the characterization of a synthetic drug (from human pharmacokinetics to pharmacodynamics, from efficacy to long-term safety), in some cases certain knowledge should be deepened in order to support the (unofficial) "claims" proposed for the given product. This represents a sure cost, a potential risk (in case the investigations do not lead to positive results) and a slowdown in the marketing process.
For a nutraceutical to be defined as effective and for the end user to recognize it as such, it must have a pharmacological power that exceeds the spontaneous variance of the parameter to be modified.

However, in order not to be equated with the drug it must not have excessive power, so usually no industry has an interest in investing in researching effects on strong outcomes (morbidity, mortality), but tends to support studies on secondary or derivative outcomes. If these outcomes are based on correct parameters, measurable with validated methods, reproducible and predictors or markers of physio-pathological or pathological alterations, then this is acceptable.
Although Italian companies are characterized by an increasing attention to clinical research on their products (even if usually after marketing), a large part of the nutraceuticals marketed in Italy (but also abroad), especially when based on more components or on "innovative" pharmaceutical formulations, it is not clinically studied as such.

The absence of direct clinical studies to support the products presented is not justifiable with the sole selection of high-quality single components supported by literature data or by the investment in techniques, advanced pharmaceuticals to improve bioavailability. Although they are both extremely commendable choices that raise the level of the product, it is not certain the combination of several components to overcome the requirements for correct research methodology.

1.4.1 Clinical pharmacology applied to nutraceuticals

The nutraceutical is usually studied inversely with respect to the drug. That is, starting from epidemiological data or traditional use, a given effect is observed and attributed to a given nutraceuticals. Therefore, a preventive or therapeutic effect is assumed without necessarily knowing the mechanism of action, which will then be studied in subsequent times. However, even when the mechanism of action of a given nutraceutical is well known, it is not certain that it is absorbed and metabolized by the human body and that it does not have interactions with foods or other co-taken drugs.

Clinical pharmacology studies should therefore answer a series of questions:

- Is the nutraceutical absorbed by the human intestine?
- Which pharmaceutical form makes it more absorbable?
- What is peak plasma time, maximum plasma concentration (C_{max}), area under the curve (AUC) and plasma half-life?
- Are these parameters modified by co-administration with food or other active ingredients? In particular with those possibly included in a combined nutraceutical? Does the nutraceutical interact with the absorption or metabolization of any co-administered drugs?

The answers to these questions have been provided for very few nutraceuticals and above all almost never for nutraceuticals co-taken in the same tablet.

In defense of those who do not perform these tests, the important cost of the same, the invasiveness (the number of blood samples to be performed in healthy subjects on the same day can create some perplexity in the ethics committees), and the not easy identification can be considered as elements of the active ingredients and their metabolites in human blood, especially those rapidly metabolized or with low oral bioavailability.

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CHAPTER 2

Setting up harmonized approaches for the risk assessment of genotoxic and carcinogenic substances

2. Experimental pipelines to verify biotoxicological safety of nutraceuticals

Potential risk to human health of carcinogenic and genotoxic substances presented in food is regarded to be the most difficult matters (**Figure 2.1**), especially when these substances cannot be readily eliminated or avoided [1].

These substances may potentially interact with or mutate the DNA, inducing eventually cancer.
Nowadays, several approaches are in use to assess the risk of substances with genotoxic and carcinogenic properties (**Figure 2.2**), within the European Union and at a global scale, but nothing of them can be classify as a gold standard approach [2][3][4].

Increasing of ethical implications about animal experimentation in toxicology testing, most regulatory authorities have recommended their reduction in the use of animals replacing them with appropriate in vitro tests. For this reason, it is essential to focus on the regulatory aspects of development of in vitro methods in order to maximize the reliability, reproducibility, credibility and acceptance of the result obtained from in vitro procedures, [5]. The Organization for Economic Co-operation and Development (OECD) has recommended Test Guidelines (TG) to assess genotoxicity of chemicals and quality control of various products.

In this scenario, it is necessary to perform harmonized approaches for the risk assessment of substances that have both genotoxic and carcinogenic properties in order to investigate toxicity testing, safety assessment, and risk evaluation of compounds [1][6][7].

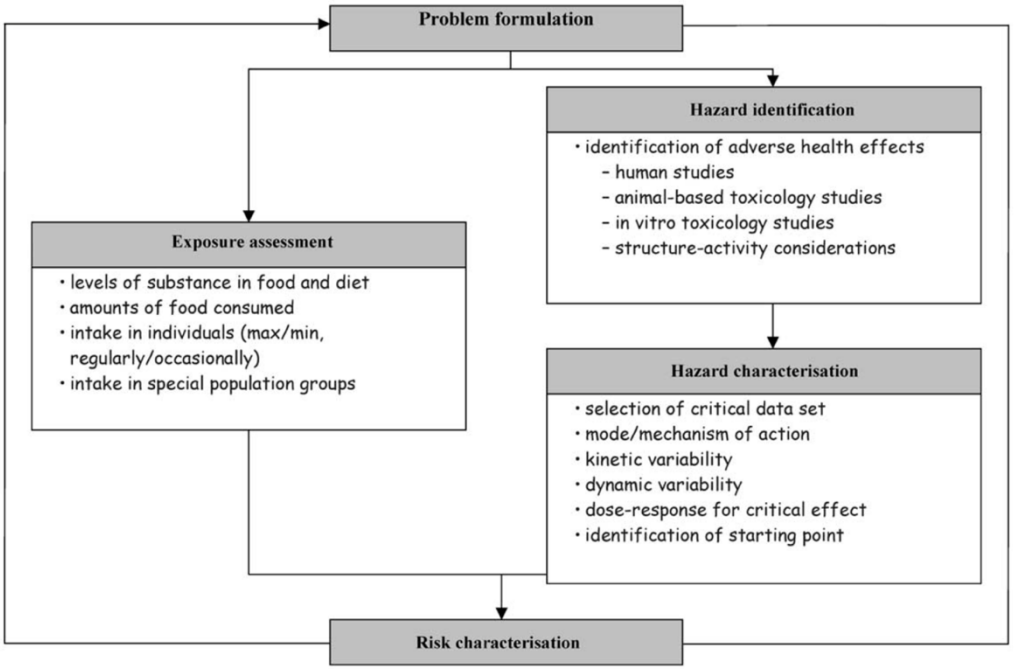

Figure 2.1. The risk assessment paradigm

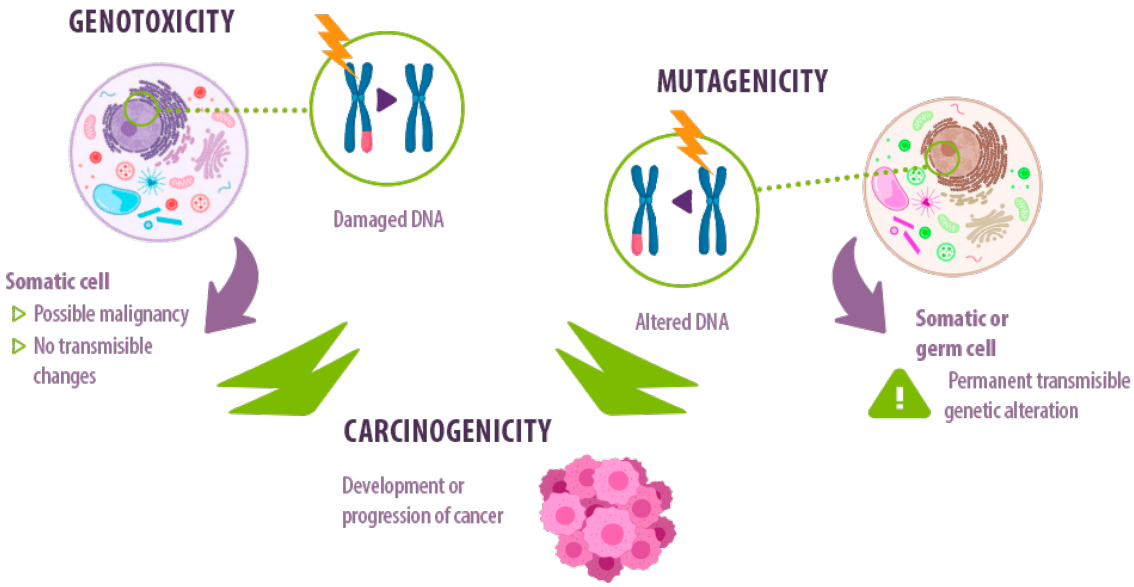

Figure 2.2. Genotoxic and carcinogenic substances carry out different effects

2.1 Regulations on genotoxicity testing strategies applicable to food and feed safety assessment

Organization for Economic Co-operation and Development (OECD) is an international organization, working to establish evidence-based international standards and finding solutions to a plethora of social, economic and environmental challenges.

OECD draws up Guidelines, which are a collection of the most relevant internationally agreed testing methods used by governments, industry and independent laboratories to assess the potential effects of chemicals on human health and the environment. Accepted internationally as standard methods for safety testing, these Guidelines are continuously expanded and updated to ensure they reflect the state-of-the-art science and techniques of member countries regulatory needs (**Figure 2.3**).

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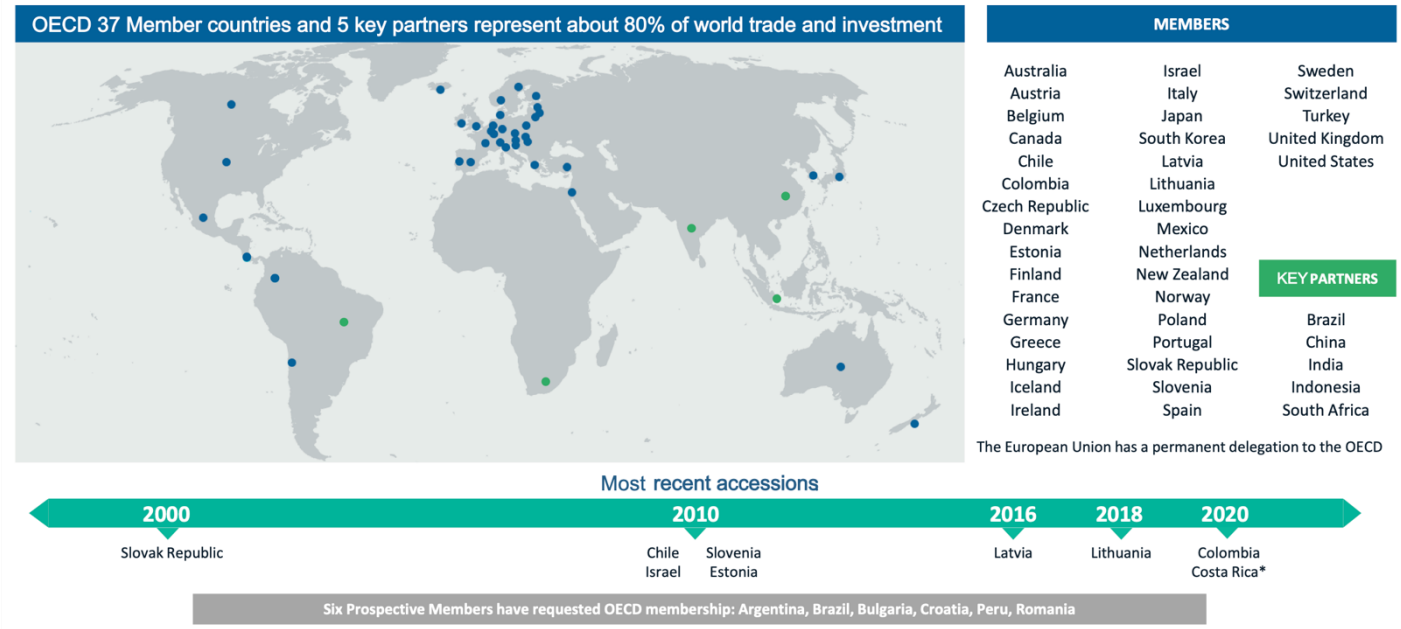

Figure 2.3. OECD Member countries

In 2007, Scientific Committee compiled a report entitled “Overview of the test requirements in the area of food and feed safety”. The document summarizes the state-of-the-science on genotoxicity testing strategies, reminding EFSA’s various scientific panels the need to have appropriate data for risk assessment.

In particular, genotoxicity testing’s aims are: (1) to identify dangerous substances; (2) to predict potential genotoxic agents and (3) to understand the mechanism of action of these agents. To pursue these, Scientific Committee recommends a step-wise approach comprising, as first, *in vitro* tests such as a bacterial reverse mutation assay, and an *in vitro* micronucleus test; as second, and only in case of positive results of these tests, review all data on the test substance, and conduct an appropriate *in vivo* study (or studies) to assess whether the genotoxic potential observed *in vitro* is manifested *in vivo* [8].

Even if the bacterial reverse mutation assay covers gene mutations and the *in vitro* micronucleus test covers both structural and numerical chromosome aberrations, Scientific Committee suggests to include, in the first step of testing, an *in vivo* test.
If *in vitro* results are distinctly negative, it is clear the substance has no genotoxic potential. In case of inconclusive, contradictory or equivocal results, it is mandatory perform other tests under different conditions, or performing a different one.

In case of positive results, it may be appropriate to optimize any subsequent *in vivo* testing or to provide additional useful information such as chemical reactivity of the substance (which might predispose to site of contact effects), bioavailability, metabolism, toxicokinetics, and any target organ specificity.

2.2 Aims and rationale of genotoxicity testing

Genetic modifications in somatic and germ cells may cause cancer if mutations occur in proto-oncogenes, tumor suppressor genes and/or DNA damage response genes [9]. These mutations in somatic cells have also been proposed to play a role in degenerative disorders such as accelerated aging, immune dysfunction, cardiovascular diseases [10][11][12]. Alterations in germ cells can lead to spontaneous abortions, infertility or heritable damage to the offspring and possibly to the subsequent generations [13].

The most commonly used methods for assessing the genotoxic potential of substances are classified into studies to investigate gene point mutations (1-2) and studies to investigate chromosome aberrations (3-4):

1. Bacterial reverse mutation assay in *Salmonella typhimurium* and *Escherichia coli* (OECD 471);
2. *in vitro* gene mutation assay in mammalian cells (OECD 476);
3. *in vitro* chromosomal aberration assay (OECD 473);
4. *in vitro* micronucleus assay (OECD 487).

It is very important to do genotoxicity studies so as to avoid the potential damage that can be caused. These genotoxicity tests are useful to identify if a drug or other substance have the potential to cause mutation and genotoxicity.

The aim of these genotoxic tests is to predict the genotoxic potential of the substance in question, to identify its genotoxic carcinogens at an early stage understanding the mechanism of the mutation and genotoxicity thereby paving way to better prevent the frequency of such mutation and genotoxicity [14].

2.2.1 Bacterial reverse mutation test (OECD TG 471)

The most widely used assay to detect gene mutations is the bacterial reverse mutation test [15], also known as Ames test. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. The limitation is that it uses prokaryotic cells which differ from mammalian cells in factors such as uptake, metabolism, chromosome structure and DNA repair processes. There have been developments to use it in high throughput screening [16][17] but the methods have not been developed to a point where they can be routinely used.

The test uses amino-acid requiring strains to detect mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. It has the ability to differentiate between frame-shift and base-pair substitutions with the use of different bacterial strains such as *Salmonella typhimurium* and *Escherichia coli*.

The Ames test's principle is to detect whether a substance is mutagenic by testing its capacity to revert mutations present in the tester strains and restore its ability to synthesize an essential amino acid required for growth. For example, tester mutant bacteria *his- S. typhimurium* carries a mutation that makes them incapable of producing the amino acid histidine, and without it, they cannot grow. If a substance were mutagenic, exposure to the tester mutant bacteria would cause a reverse mutation to *his+ S. typhimurium*. The bacteria would regain its ability to synthesize histidine such that it can grow on a histidine-free medium (**Figure 2.4**).

2.2.2 Example of molecules resulting positive at the Ames Test

If all *in vitro* endpoints are clearly negative in adequately conducted tests, then it can be concluded that the substance has no genotoxic potential. However, as mentioned above, the Scientific Committee notes that a small number of substances that are negative in vitro have positive *in vivo* results, because, for example, the *in vitro* metabolic activation system does not cover the full spectrum of potential genotoxic metabolites generated *in vivo*.

If positive results are obtained in the basic battery of in vitro tests, before embarking on the next step, all relevant data should be reviewed. The next steps may be (a) a conclusion of the assessment without further testing, (b) further *in vitro* testing, and/or (c) *in vivo* testing. One or more positive in vitro tests normally require follow up by in vivo testing. However, on occasion it may be demonstrated that the positive *in vitro* findings are not relevant for the *in vivo* situation, or a decision is taken to complete the assessment for other reasons [18][19][20].

Many flavonoids such as quercetin, rutin [21] have been described to be mutagenic in different strains of *Salmonella typhimurium* in the Ames test as well as in several mammalian cell systems used to assess different toxic end points. In the field of flavonoid mutagenicity research, reports of activity are widely contradictory, probably owing to varying characteristics of chemical structure on inter- and intra-assay variation [22], and it seems that the activity is dependent either on the biotransformation of the compound or on the production of reactive oxygen species [23]. The results suggest that the putative genotoxic metabolites of quercetin fluctuate for unusual genetic mutations considered and that flavonoids' metabolic outcome might partly account for the conflicting data about their genotoxic and carcinogenic activity *in vivo* [24].

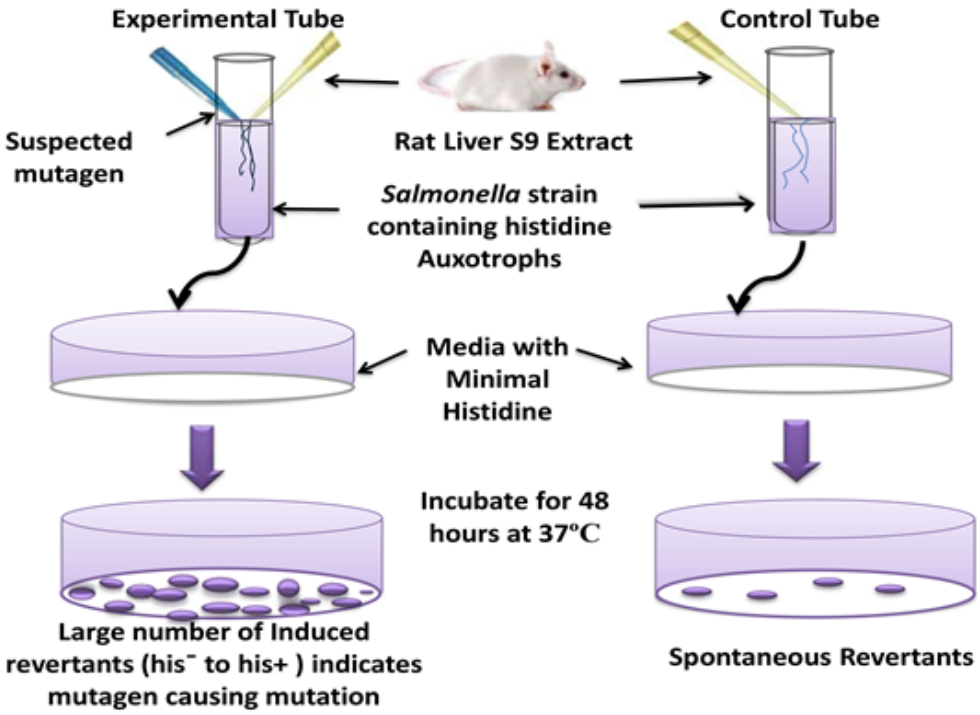

Figure 2.4. Graphical explanation of Ames test

2.2.3 *In vitro* mammalian micronucleus test (OECD TG 487)

The purpose of the *in vitro* micronucleus test (MNvit) [25] is to identify substances that cause structural and numerical chromosomal damage in cells that have undergone cell division during or after the exposure to the test substance. The assay detects micronuclei in the cytoplasm of interphase cells and typically employs human or rodent cells lines or primary cell cultures.

The *in vitro* micronucleus test can be conducted in the presence or in the absence of cytochalasin B (cytoB), which is used to block cell division and generate binucleate cells. The advantage of the using cytoB is that it allows clear identification that treated and control cells have divided in vitro and provide a simple assessment of cell proliferation. The *in vitro* micronucleus test can be combined with FISH (Fluorescence in situ Hybridisation) to provide additional mechanistic information, e.g. on non-disjunction, which is not detected in the standard in vitro micronucleus assay.

The MNvit is rapid and easy to conduct and it is the only in vitro test that can efficiently detect both clastogens and aneugens. Cytotoxicity needs to be controlled to avoid false positive results, as with other in vitro genotoxicity tests conducted in mammalian cells (**Figure 2.5**).

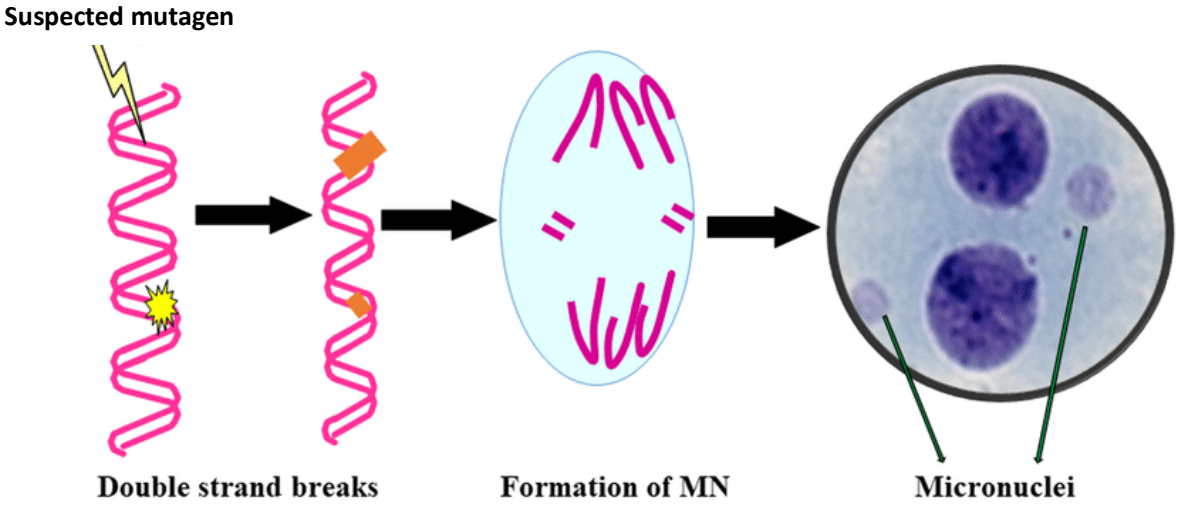

Figure 2.5. Micronucleus formation in the cell exposed to a suspected mutagenic agent [26]

2.3 Risk assessment of natural products as cancerogens

Nutraceuticals containing polyphenols, such as apple or grape pomace extracts, are becoming popular over-the-counter products by virtue of their antioxidant activity and their availability in pharmacies, supermarkets and online specialized shops [27]. They are labelled as "natural", a term that let them be considered as "generally safe". Indeed, the database of botanicals, compiled (and constantly updated) by EFSA, mentions bio-products of apple and grape processing among the natural products that can be included in nutraceutical, food supplements and functional foods in Europe [28].

Nutraceuticals containing grape leaves and/or seeds of the *Vitacea Vitis vinifera L*. can further claim physiological effects on humans. Indeed, their antioxidant activity, as well as their ability to reduce cardiovascular disease (CVD) risk by improving microcirculation and promoting homeostasis of the cardiovascular system, has been scientifically proven. Similarly, nutraceuticals can contain fruits, seeds, buds, cortex *ex radicibus* of the *Rosaceae Malus domestica B*. and *Malus pumila M.,* the consumption of which improves gastrointestinal transit and modulates the intestinal absorption of nutrients.

The list of botanicals allowed in nutraceuticals, however, does not have any legal force, and EFSA itself advices developers and producers to be responsible for the safety of their products, that must comply with the general requirements set out in the general food law. However, as a consequence of possible synergistic or antagonistic effects, the pharmacokinetic parameters, bioavailability, bioaccessibility, bioactivity as well as overall toxicity of the whole product could be different from that of its components [29]. As a consequence, the safety of individual substances cannot be used to draw general conclusions on whole extracts and botanical preparations, whose safety must thus be confirmed.

In 2009, the EFSA published the Guideline entitled "Safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements" [28]. The suggested approach to assess safety divides the evaluation into core areas and includes the assessment of mutagenicity of the food supplement by means of the Ames test (OECD guideline 471)[30].

The aim of this thesis chapter is to present two studies in which I performed the risk assessment of substances based on *in vitro* genotoxicity tests.

2.4 Genotoxicity assessment of three nutraceuticals containing natural antioxidants extracted from agri-food waste biomasses

The reuse of food biomass would increase the volume of recyclable/renewable biomaterial and lower the environmental impact due to the increasing demand for biological products. To this purpose, agri-food waste from particular fruits processing, responsible for the production of a large amount of bio-waste, have become an important source of phytochemicals, and many pharmaceutical industries are using it as starting material to produce dietary supplements, functional foods, and food additives for human consumption.

In virtue of the chemical diversity and complexity of agri-food biowaste, developers and producers of nutraceuticals are advised to assess the safety of their final nutraceutical products, in compliance with EFSA regulation. In this study [31], I use the Ames test to assess the mutagenicity of three nutraceuticals obtained from agri-food waste biomasses.

The first phytocomplex tested is an extract of a grape pomace from *Vitis vinifera* cv 'Aglianico'. This extract is used to prepare 'Taurisolo®', an antioxidant able to reduce serum levels of the cardiovascular risk factor markers oxidized-low-density lipoprotein (LDL) and Trimethylamine N-oxide (TMAO) in humans and in rodents [32][33][34], and improve microcirculation.

The other two nutraceuticals tested contain extracts of two italian apple cultivar, namely *Malus pumila M*. cv 'Annurca' and *Malus domestica B.* cv 'Limoncella'. *Malus pumila Miller* cv. Annurca is a widespread apple and accounts for 5% of Italian apple production. It is listed as a Protected Geographical Indication (PGI) product from the European Council (Commission Regulation (EC) No. 417/2006)). Annurca Apples are used to prepare 'AnnurComplex®', a nutraceutical able to reduce serum cholesterol levels, LDL and lipid uptake; reduce cardiovascular disease (CVD) risk; and promote hair growth in humans [35][36][37][38]. *Malus domestica cv* 'Limoncella' is a juicy and aromatic variety of apple, known since ancient Roman times. Limoncella Apple extract presents high antioxidant activity and it has been shown to reduce colon inflammation and to act as potent inhibitor (*in vitro* and *ex vivo*) [39] of the Wingless-related integration site (WNT)-β catenin pathway, a signaling cascade linked to inflammation, oxidative stress, cell proliferation and cancerogenesis.

By means of the Ames test, we here show that the three nutraceuticals are not mutagenic. Our results support their safety and prove that the use of apple and wine waste product to produce polyphenolic mixtures does not enrich the final nutraceuticals of byproducts and secondary metabolites endowed with mutagenic potential.

2.4.1 Results of Ames Test

Tables A1–A15 present the raw data obtained performing the Ames test on the three nutraceuticals. Each table shows the number of revertants per plate, measured in three replicates, their means, the standard deviation (sd) observed in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and in the *E. coli* strain WP2 trp UvrA upon treatment with different doses of Taurisolo (**Tables A1–A5**), AnnurComplex (**Tables A6–A10**) and Limoncella Apple Extract (**Tables A11–A15**). Treatments were performed in the presence (+S9) and in the absence (−S9) of metabolic activation.

Raw data are summarized in **Table 1, Table 2** and **Table 3**, where the mutagenic index of Taurisolo (**Table 1**), AnnurComplex (**Table 2**) and Limoncella Apple Extract (**Table 3**) observed in the different bacterial strains are compared to known genotoxic substances. Each table shows the number of revertants per plate, measured in three replicates, their means, the standard deviation (sd) observed in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and in the *E. coli* strain WP2 trp UvrA upon treatment with different doses of different nutraceuticals. The mutagenic index is the ratio between the average number of revertants per plate measured upon incubation with the test nutraceutical and the average number of revertants per plate measured upon incubation with the negative (solvent) control. A chemical can be considered mutagenic when a two-fold increase in mutagenic index is observed upon treatment with at least one of the tested concentrations. As shown in **Tables 1,2,3** no mutagenicity was detected for any of the three nutraceuticals obtained from agri-food biowaste, both in the presence (+S9) or in the absence (−S9) of metabolic activation.

Table A1. Number of revertants/plate for *S. typhimurium* TA98 strain treated with Taurisolo.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	23	27	28	26 ± 2.6	38	45	47	43.3 ± 4.7
0.0016	23	23	32	26 ± 5.1	47	51	53	50.3 ± 3
0.005	26	35	28	29.6 ± 4.7	38	43	43	41.3 ± 2.8
0.016	37	28	32	32.3 ± 4.5	39	43	38	40 ± 2.6
0.05	37	24	24	28.3 ± 7.5	47	53	50	50 ± 3
0.16	27	29	31	29 ± 2	42	47	47	45.3 ± 2.8
0.5	32	28	34	31.3 ± 3	41	47	45	44.3 ± 3
1.6	36	32	28	32 ± 4	38	45	50	44.3 ± 6
5	29	38	31	32.6 ± 4.7	40	50	51	47 ± 6
Positive Control	1102	1140	1237	1159.6 ± 69.6	1300	1400	1373	1357.6 ± 51.7

Negative Control: water—100 µL/plate; Positive Control: 2-Nitro Fluorene (2.0 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 22–57 (mean ± s.d. = 29 ± 8). Historical negative in the presence of S9: Range 20–53 (mean ± s.d. = 43 ± 13). Historical positive in the absence of S9: Range 1109–1363 (mean ± s.d. = 1214 ± 46). Historical positive in the presence of S9: Range 236–1348 (mean ± s.d. = 1321 ± 33).

Table A2. Number of revertants/plate for *S. typhimurium* TA100 strain treated with Taurisolo.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	150	170	164	161.3 ± 10.2	203	225	224	217.3 ± 12.4
0.0016	190	187	175	184 ± 7.9	216	225	227	222.6 ± 5.8
0.005	210	222	175	202.3 ± 24.4	214	240	238	230.6 ± 14.4
0.016	175	192	215	194 ± 20	215	238	219	224 ± 12.2
0.05	220	240	233	231 ± 10	220	219	218	219 ± 1
0.16	198	220	183	200.3 ± 18.6	197	215	223	211 ± 13.3
0.5	180	205	183	189.3 ± 13.6	222	210	213	215 ± 6.2
1.6	192	220	170	194 ± 25	215	262	246	241 ± 23.8
5	230	215	206	217 ± 12.1	240	243	215	232.6 ± 15.3
Positive Control	1572	1620	1680	1624 ± 54.1	1750	1742	1763	1751.6 ± 10.5

Negative Control: water—100 µL/plate; Positive Control Sodium Azide (1.25 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 144–240 (mean ± s.d. = 195 ± 15). Historical negative in the presence of S9: Range 176–250 (mean ± s.d. = 211 ± 21). Historical positive in the absence of S9: Range 1428–1620 (mean ± s.d. = 1480 ± 80). Historical positive in the presence of S9: Range 1600–1923 (mean ± s.d. = 1693 ± 72).

Table A3. Number of revertants/plate for *S. typhimurium* TA1535 strain treated with Taurisolo.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	12	18	17	15.6 ± 3.2	22	23	12	19 ± 6
0.0016	12	12	15	13 ± 1.7	24	23	19	22 ± 2.6
0.005	20	16	21	19 ± 2.6	26	27	27	26.6 ± 0.5
0.016	18	21	23	20.6 ± 2.5	28	29	31	29.3 ± 1.5
0.05	21	24	23	22.6 ± 1.5	31	27	23	27 ± 4
0.16	19	17	23	19.6 ± 3	20	20	21	20.3 ± 0.5
0.5	21	21	23	21.6 ± 1.1	21	21	20	20.6 ± 0.5
1.6	24	23	21	19.6 ± 3	23	25	30	26 ± 3.6
5	30	28	25	27.6 ± 2.5	23	23	27	24.3 ± 2.3
Positive Control	206	215	216	212.3 ± 5.5	210	215	213	212.6 ± 2.5

Negative Control: water—100 µL/plate; Positive Control: Sodium Azide (1.25 µg/plate) in the absence of S9 and 2-aminoanthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 12–45 (mean ± s.d. = 26 ± 5). Historical negative in the presence of S9: Range 17–35 (mean ± s.d. = 23 ± 9). Historical positive in the absence of S9: Range 195–240 (mean ± s.d. = 202 ± 36). Historical positive in the presence of S9: Range 222–286 (mean ± s.d. = 232 ± 26).

Table A4. Number of revertants/plate for *S. typhimurium* TA1537 strain treated with Taurisolo.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	21	23	27	23.6 ± 3	21	19	17	19 ± 2
0.0016	27	21	20	22.6 ± 3.7	17	17	21	18.3 ± 2.3
0.005	23	23	23	23	23	26	26	25 ± 1.7
0.016	26	28	21	25 ± 3.6	30	29	21	26.6 ± 4.9
0.05	29	27	25	27 ± 2	28	23	23	24.6 ± 2.8
0.16	32	30	32	31.3 ± 1.1	26	22	23	23.6 ± 2
0.5	24	29	36	29.6 ± 6	34	31	30	31.6 ± 2
1.6	36	35	40	37 ± 2.6	28	29	36	31 ± 4.3
5	30	30	31	30.3 ± 0.5	24	24	26	24.6 ± 1.1
Positive Control	198	215	201	204.6 ± 9	218	242	243	234.3 ± 14.1

Negative Control: water—100 µL/plate; Positive Control: 9-aminoacridine HCl (50.0 µg/plate) in the absence of S9 and 2-amino anthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 19–40 (mean ± s.d. = 32 ± 10). Historical negative in the presence of S9: Range 18–43 (mean ± s.d. = 31 ± 6). Historical positive in the absence of S9: Range 187–250 (mean ± s.d. = 210 ± 42). Historical positive in the presence of S9: Range 223–270 (mean ± s.d. = 216 ± 34).

Table A5. Number of revertants/plate for *E. coli WP2 Uvr A* strain treated with Taurisolo.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	31	42	34	35.6 ± 5.6	39	41	37	39 ± 2
0.0016	31	35	35	33.6 ± 2.3	45	47	42	44.6 ± 2.5
0.005	30	32	34	32 ± 2	48	48	42	46 ± 3.4
0.016	35	42	34	37 ± 4.3	48	49	55	50.6 ± 3.7
0.05	37	41	46	41.3 ± 4.5	56	55	51	54 ± 2.6
0.16	37	42	42	40.3 ± 2.8	54	54	52	53.3 ± 1.1
0.5	40	40	37	39 ± 1.7	50	50	56	52 ± 3.4
1.6	39	39	43	40.3 ± 2.3	57	48	58	54.3 ± 5.5
5	42	52	40	44.6 ± 6.4	50	52	54	52 ± 2
Positive Control	180	182	186	182.6 ± 3	242	248	226	238.6 ± 11.3

Negative Control: water—100 µL/plate; Positive Control: 4-nitroquinoline-N-oxide (1.0 µg/plate) in the absence of S9 and 2 amino- anthracene (20 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 37–58 (mean ± s.d. = 45 ± 11). Historical negative in the presence of S9: Range 32–70 (mean ± s.d. = 44 ± 6). Historical positive in the absence of S9: Range 109–195 (mean ± s.d. = 177 ± 29). Historical positive in the presence of S9: Range 193–263 (mean ± s.d. = 210 ± 34).

Table A6. Number of revertants/plate for *S. typhimurium* TA98 strain treated with AnnurComplex.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	24	27	33	28 ± 4.5	31	37	42	36.6 ± 5.5
0.0016	21	27	27	25 ± 3.4	34	34	37	35 ± 1.7
0.005	32	30	23	28.3 ± 4.7	37	39	43	39.6 ± 3
0.016	30	31	34	31.6 ± 2	38	40	40	39.3 ± 1.1
0.05	25	27	27	26.3 ± 1.1	42	46	42	43.3 ± 2.3
0.16	32	33	33	32.6 ± 0.5	38	48	48	44.6 ± 5.7
0.5	35	38	36	36.3 ± 1.5	40	41	41	40.6 ± 0.5
1.6	27	34	21	27.3 ± 6.5	42	44	38	41.3 ± 3
5	34	38	38	36.6 ± 2.3	38	32	34	34.6 ± 3
Positive Control	1180	1250	1228	1219.3 ± 35.7	1280	1340	1330	1316.6 ± 32.1

Negative Control: water—100 µL/plate; Positive Control: 2-Nitro Fluorene (2.0 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 22–57 (mean ± s.d. = 29 ± 8). Historical negative in the presence of S9: Range 20–53 (mean ± s.d. = 43 ± 13). Historical positive in the absence of S9: Range 1109–1363 (mean ± s.d. = 1214 ± 46). Historical positive in the presence of S9: Range 236–1348 (mean ± s.d. = 1321 ± 33).

Table A7. Number of revertants/plate for *S. typhimurium* TA100 strain treated with AnnurComplex.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	167	183	182	177.3 ± 8.9	182	203	212	199 ± 15.3
0.0016	184	185	163	177.3 ± 12.4	207	209	217	211 ± 5.2
0.005	212	214	197	207.6 ± 9.2	230	214	206	216.6 ± 12.2
0.016	194	210	185	196.3 ± 12.6	219	223	204	215.3 ± 10
0.05	221	232	207	220 ± 12.5	215	215	217	215.6 ± 1.1
0.16	202	224	268	231.3 ± 33.6	197	203	234	211.3 ± 19.8
0.5	235	210	222	222.3 ± 12.5	222	214	232	222.6 ± 9
1.6	196	210	177	194.3 ± 16.5	214	239	227	226.6 ± 12.5
5	223	221	207	217 ± 8.7	229	229	230	229.3 ± 0.5
Positive Control	1631	1677	1642	1650 ± 24	1756	1738	1742	1745.3 ± 9.4

Negative Control: water—100 µL/plate; Positive Control Sodium Azide (1.25 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 144–240 (mean ± s.d. = 195 ± 15). Historical negative in the presence of S9: Range 176–250 (mean ± s.d. = 211 ± 21). Historical positive in the absence of S9: Range 1428–1620 (mean ± s.d. = 1480 ± 80). Historical positive in the presence of S9: Range 1600–1923 (mean ± s.d. = 1693 ± 72).

Table A8. Number of revertants/plate for *S. typhimurium* TA1535 strain treated with AnnurComplex.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	12	12	15	13 ± 1.7	18	17	19	18 ± 1
0.0016	17	17	22	18.3 ± 2.8	21	23	19	21 ± 2
0.005	16	12	22	16.6 ± 5	24	23	27	24.6 ± 2
0.016	17	15	22	18 ± 3.6	25	28	35	29.3 ± 5.1
0.05	19	19	22	20 ± 1.7	27	28	19	24.6 ± 4.9
0.16	27	16	24	22.3 ± 5.6	17	28	22	22.3 ± 5.5
0.5	19	17	22	19.3 ± 2.5	21	21	21	21
1.6	24	24	23	23.6 ± 0.5	24	21	23	22.6 ± 1.5
5	26	26	24	25.3 ± 1.1	25	28	31	28 ± 3
Positive Control	215	220	207	214 ± 6.5	214	227	206	215.6 ± 10.5

Negative Control: water—100 µL/plate; Positive Control: Sodium Azide (1.25 µg/plate) in the absence of S9 and 2-aminoanthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 12–45 (mean ± s.d. = 26 ± 5). Historical negative in the presence of S9: Range 17–35 (mean ± s.d. = 23 ± 9). Historical positive in the absence of S9: Range 195–240 (mean ± s.d. = 202 ± 36). Historical positive in the presence of S9: Range 222–286 (mean ± s.d. = 232 ± 26).

Table A9. Number of revertants/plate for *S. typhimurium* TA1537 strain treated with AnnurComplex.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	18	20	18	18.6 ± 1.1	20	18	19	19 ± 1
0.0016	22	26	20	22.6 ± 3	23	21	23	22.3 ± 1.1
0.005	24	24	28	25.3 ± 2.3	25	27	24	25.3 ± 1.5
0.016	28	28	21	25.6 ± 4	32	28	23	27.6 ± 4.5
0.05	30	30	34	31.3 ± 2.3	26	21	21	22.6 ± 2.8
0.16	26	36	34	32 ± 5.3	21	25	25	23.6 ± 2.3
0.5	30	29	31	30 ± 1	31	34	27	30.6 ± 3.5
1.6	28	27	31	28.6 ± 2	30	32	28	30 ± 2
5	32	36	34	34 ± 2	26	32	28	28.6 ± 3
Positive Control	191	197	213	200.3 ± 11.3	240	229	226	231.6 ± 7.3

Negative Control: water—100 µL/plate; Positive Control: 9-aminoacridine HCl (50.0 µg/plate) in the absence of S9 and 2-amino anthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 19–40 (mean ± s.d. = 32 ± 10). Historical negative in the presence of S9: Range 18–43 (mean ± s.d. = 31 ± 6). Historical positive in the absence of S9: Range 187–250 (mean ± s.d. = 210 ± 42). Historical positive in the presence of S9: Range 223–270 (mean ± s.d. = 216 ± 34).

Table A10. Number of revertants/plate for *E. coli WP2 Uvr A* strain treated with AnnurComplex.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	34	38	34	35.3 ± 2.3	38	40	40	39.3 ± 1.1
0.0016	31	34	38	34.3 ± 3.5	45	47	47	46.3 ± 1.1
0.005	29	31	34	31.3 ± 2.5	49	43	47	46.3 ± 3
0.016	28	47	34	36.3 ± 9.7	52	52	50	51.3 ± 1.1
0.05	37	41	41	39.6 ± 2.3	50	51	51	50.6 ± 0.5
0.16	39	35	35	36.3 ± 2.3	47	50	41	46 ± 4.5
0.5	38	28	34	33.3 ± 5	49	47	51	49 ± 2
1.6	37	29	32	32.6 ± 4	53	51	50	51.3 ± 1.5
5	41	29	43	37.6 ± 7.5	41	43	48	44 ± 3.6
Positive Control	161	181	154	165.3 ± 14	228	262	223	237.6 ± 21.2

Negative Control: water—100 µL/plate; Positive Control: 4-nitroquinoline-N-oxide (1.0 µg/plate) in the absence of S9 and 2 amino- anthracene (20 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 37–58 (mean ± s.d. = 45 ± 11). Historical negative in the presence of S9: Range 32–70 (mean ± s.d. = 44 ± 6). Historical positive in the absence of S9: Range 109–195 (mean ± s.d. = 177 ± 29). Historical positive in the presence of S9: Range 193–263 (mean ± s.d. = 210 ± 34).

Table A11. Number of revertants/plate for *S. typhimurium* TA98 treated with Limoncella Extract.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	26	27	24	25.6 ± 1.5	43	42	44	43 ± 1
0.0016	28	33	32	31 ± 2.6	43	44	47	44.6 ± 2
0.005	29	26	27	27.3 ± 1.5	38	37	38	37.6 ± 0.5
0.016	24	27	27	26 ± 1.7	39	41	44	41.3 ± 2.5
0.05	24	29	31	28 ± 3.6	43	47	49	46.3 ± 3
0.16	26	29	32	29 ± 3	43	40	45	42.6 ± 2.5
0.5	27	25	26	26 ± 1	34	36	39	36.3 ± 2.5
1.6	34	37	31	34 ± 3	41	37	38	38.6 ± 2
5	30	36	37	34.3 ± 3.7	39	33	39	37 ± 3.4
Positive Control	1121	1237	1222	1193.3 ± 63	1290	1310	1360	1320 ± 36

Negative Control: water—100 µL/plate; Positive Control: 2-Nitro Fluorene (2.0 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 22–57 (mean ± s.d. = 29 ± 8). Historical negative in the presence of S9: Range 20–53 (mean ± s.d. = 43 ± 13). Historical positive in the absence of S9: Range 1109–1363 (mean ± s.d. = 1214 ± 46) Historical positive in the presence of S9: Range 236–1348 (mean ± s.d. = 1321 ± 33).

Table A12. Number of revertants/plate for *S. typhimurium* TA100 treated with Limoncella Extract.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	162	168	176	168.6 ± 7	184	184	203	190.3 ± 10.9
0.0016	178	179	176	177.6 ± 1.5	205	209	217	210.3 ± 6.1
0.005	195	215	199	203 ± 10.5	231	227	234	230.6 ± 3.5
0.016	189	207	189	195 ± 10.3	226	239	229	231.3 ± 6.8
0.05	217	219	203	213 ± 8.7	219	227	234	226.6 ± 7.5
0.16	187	217	189	197.6 ± 16.7	206	212	232	216.6 ± 13.6
0.5	197	210	180	195.6 ± 15	225	209	242	225.3 ± 16.5
1.6	174	180	174	176 ± 3.4	217	239	227	227.6 ± 11
5	195	200	209	201.3 ± 7	230	228	234	230.6 ± 3
Positive Control	1537	1499	1573	1536.3 ± 37	1635	1750	1749	1711.3 ± 66.1

Negative Control: water—100 µL/plate; Positive Control Sodium Azide (1.25 µg/plate) in the absence of S9 and Benzo(a)pyrene (6 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 144–240 (mean ± s.d. = 195 ± 15); Historical negative in the presence of S9: Range 176–250 (mean ± s.d. = 211 ± 21). Historical positive in the absence of S9: Range 1428–1620 (mean ± s.d. = 1480 ± 80). Historical positive in the presence of S9: Range 1600–1923 (mean ± s.d. = 1693 ± 72).

Table A13. Number of revertants/plate for *S. typhimurium* TA1535 treated with Limoncella Extract.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	10	20	18	16 ± 5.2	18	22	27	22.3 ± 4.5
0.0016	20	17	22	19.6 ± 2.5	23	27	25	25 ± 2
0.005	16	22	21	19.6 ± 3.2	20	26	32	26 ± 6
0.016	20	20	22	20.6 ± 1.1	26	25	23	24.6 ± 1.5
0.05	20	24	32	25.3 ± 6.1	24	28	23	25 ± 2.6
0.16	20	18	23	20.3 ± 2.5	21	19	26	22 ± 3.6
0.5	19	21	23	21 ± 2	22	20	32	24.6 ± 6.4
1.6	26	21	24	23.6 ± 2.5	27	27	26	26.6 ± 0.5
5	20	32	24	25.3 ± 6.1	24	22	22	22.6 ± 1.1
Positive Control	190	191	232	204.3 ± 23.9	210	210	234	218 ± 13.8

Negative Control: water—100 µL/plate; Positive Control: Sodium Azide (1.25 µg/plate) in the absence of S9 and 2-aminoanthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 12–45 (mean ± s.d. = 26 ± 5). Historical negative in the presence of S9: Range 17–35 (mean ± s.d. = 23 ± 9). Historical positive in the absence of S9: Range 195–240 (mean ± s.d. = 202 ± 36). Historical positive in the presence of S9: Range 222–286 (mean ± s.d. = 232 ± 26).

Table A14. Number of revertants/plate for *S. typhimurium* TA1537 treated with Limoncella Extract.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	18	18	26	20.6 ± 4.6	20	28	24	24 ± 4
0.0016	26	26	27	26.3 ± 0.5	25	25	27	25.6 ± 1.1
0.005	24	27	29	26.6 ± 2.5	26	26	24	25.3 ± 1.1
0.016	30	30	28	29.3 ± 1.1	30	29	29	29.3 ± 0.5
0.05	31	34	30	31.6 ± 2	25	27	31	27.6 ± 3
0.16	29	30	33	30.6 ± 2	24	23	26	24.3 ± 1.5
0.5	31	29	36	32 ± 3.6	29	31	32	30.6 ± 1.5
1.6	30	35	33	32.6 ± 2.5	27	31	31	29.6 ± 2.3
5	35	38	33	35.3 ± 2.5	26	30	26	27.3 ± 2.3
Positive Control	210	217	196	207.6 ± 10.6	210	215	240	221.6 ± 16

Negative Control: water—100 µL/plate; Positive Control: 9-aminoacridine HCl (50.0 µg/plate) in the absence of S9 and 2-amino anthracene (2 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 19–40 (mean ± s.d. = 32 ± 10). Historical negative in the presence of S9: Range 18–43 (mean ± s.d. = 31 ± 6). Historical positive in the absence of S9: Range 187–250 (mean ± s.d. = 210 ± 42). Historical positive in the presence of S9: Range 223–270 (mean ± s.d. = 216 ± 34).

Table A15. Number of revertants/plate for *E. coli WP2 Uvr A* strain treated with Limoncella Extract.

	−S9				+S9			
mg/plate	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**	**Replicate1**	**Replicate2**	**Replicate3**	**Mean ± sd**
Negative control	36	38	38	37.3 ± 1.1	38	40	44	40.6 ± 3
0.0016	37	35	35	35.6 ± 1.1	43	47	43	44.3 ± 2.3
0.005	29	34	40	34.3 ± 5.5	51	41	43	45 ± 5.2
0.016	40	40	30	36.6 ± 5.7	48	52	50	50 ± 2
0.05	40	43	38	40.3 ± 2.5	53	48	51	50.6 ± 2.5
0.16	37	37	40	38 ± 1.7	47	51	43	47 ± 4
0.5	39	39	40	39.3 ± 0.5	47	47	47	47
1.6	35	39	50	41.3 ± 7.7	48	53	61	54 ± 6.5
5	42	43	43	42.6 ± 0.5	44	45	49	46 ± 2.6
Positive Control	180	179	204	187.6 ± 14.1	230	237	249	238.6 ± 9.6

Negative Control: water—100 µL/plate; Positive Control: 4-nitroquinoline-N-oxide (1.0 µg/plate) in the absence of S9 and 2 amino- anthracene (20 µg/plate) in the presence of S9. Historical negative in the absence of S9: Range 37–58 (mean ± s.d. = 45 ± 11). Historical negative in the presence of S9: Range 32–70 (mean ± s.d. = 44 ± 6). Historical positive in the absence of S9: Range 109–195 (mean ± s.d. = 177 ± 29). Historical positive in the presence of S9: Range 193–263 (mean ± s.d. = 210 ± 34).

Table 1. Mutagenicity of Taurisolo.

Mean Revertants										
		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	**E.coli WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	26 ± 2.6	161.3 ± 10.2	15.6 ± 3.2	23.6 ± 3	35.6 ± 5.6	43.3 ± 4.7	217.3 ± 12.4	19 ± 6	19 ± 2	39 ± 2
0.0016	26 ± 5.1	184 ± 7.9	13 ± 1.7	22.6 ± 3.7	33.6 ± 2.3	50.3 ± 3	222.6 ± 5.8	22 ± 2.6	18.3 ± 2.3	44.6 ± 2.5
0.005	29.6 ± 4.7	202.3 ± 24.4	19 ± 2.6	23	32 ± 2	41.3 ± 2.8	230.6 ± 14.4	26.6 ± 0.5	25 ± 1.7	46 ± 3.4
0.016	32.3 ± 4.5	194 ± 20	20.6 ± 2.5	25 ± 3.6	37 ± 4.3	40 ± 2.6	224 ± 12.2	29.3 ± 1.5	26.6 ± 4.9	50.6 ± 3.7
0.05	28.3 ± 7.5	231 ± 10	22.6 ± 1.5	27 ± 2	41.3 ± 4.5	50 ± 3	219 ± 1	27 ± 4	24.6 ± 2.8	54 ± 2.6
0.16	29 ± 2	200.3 ± 18.6	19.6 ± 3	31.3 ± 1.1	40.3 ± 2.8	45.3 ± 2.8	211 ± 13.3	20.3 ± 0.5	23.6 ± 2	53.3 ± 1.1
0.5	31.3 ± 3	189.3 ± 13.6	21.6 ± 1.1	29.6 ± 6	39 ± 1.7	44.3 ± 3	215 ± 6.2	20.6 ± 0.5	31.6 ± 2	52 ± 3.4
1.6	32 ± 4	194 ± 25	19.6 ± 3	37 ± 2.6	40.3 ± 2.3	44.3 ± 6	241 ± 23.8	26 ± 3.6	31 ± 4.3	54.3 ± 5.5
5	32.6 ± 4.7	217 ± 12.1	27.6 ± 2.5	30.3 ± 0.5	44.6 ± 6.4	47 ± 6	232.6 ± 15.3	24.3 ± 2.3	24.6 ± 1.1	52 ± 2
Positive Control	1159.6 ± 69.6	1624 ± 54.1	212.3 ± 5.5	204.6 ± 9	182.6 ± 3	1357.6 ± 51.7	1751.6 ± 10.5	212.6 ± 2.5	234.3 ± 14.1	238.6 ± 11.3
Mutagenic Index		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	**E.coli WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.0
0.0016	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.2 ± 0.2	1.0 ± 0.1	1.1 ± 0.1
0.005	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.3	1.3 ± 0.1	1.2 ± 0.1
0.016	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.5 ± 0.3	1.4 ± 0.2	1.3 ± 0.1
0.05	1.1 ± 0.2	1.4 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.4 ± 0.3	1.3 ± 0.1	1.4 ± 0.1
0.16	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.2	1.2 ± 0.1	1.4 ± 0.0
0.5	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.2	1.7 ± 0.1	1.3 ± 0.1
1.6	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	1.6 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.3	1.6 ± 0.2	1.4 ± 0.1
5	1.3 ± 0.1	1.3 ± 0.1	1.8 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.0
Positive Control	44.6 ± 3.0	10.1 ± 0.4	13.6 ± 1.6	8.6 ± 0.7	5.1 ± 0.5	31.3 ± 2.1	8.1 ± 0.3	11.2 ± 2.1	12.3 ± 0.9	6.1 ± 0.2

Mean revertants per plate and mutagenic index measured in bacterial strains TA98, TA100 and TA1535 and 1537 and WP2 treated with Taurisolo at various doses, with (+S9) or without (−S9) metabolic activation. Negative controls consisted of 100 µL water. Positive controls consisted: for *S. typhimurium* TA100 NaN3 (−S9) and BAP (+S9); for *S. typhimurium* TA98 2NF (−S9) and BAP (+S9); for *S. typhimurium* TA1535 NaN3 (−S9) and 2AA (+S9); for *S. typhimurium* TA1537 9AC (−S9) and 2AA (+S9); for *E. coli* WP2 trp UvrA NQO (−S9) and 2AA (+S9).

Table 2. Mutagenicity of AnnurComplex.

Mean Revertants										
		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	28 ± 4.5	177.3 ± 8.9	13 ± 1.7	18.6 ± 1.1	35.3 ± 2.3	36.6 ± 5.5	199 ± 15.3	18 ± 1	19 ± 1	39.3 ± 1.1
0.0016	25 ± 3.4	177.3 ± 12.4	18.3 ± 2.8	22.6 ± 3	34.3 ± 3.5	35 ± 1.7	211 ± 5.2	21 ± 2	22.3 ± 1.1	46.3 ± 1.1
0.005	28.3 ± 4.7	207.6 ± 9.2	16.6 ± 5	25.3 ± 2.3	31.3 ± 2.5	39.6 ± 3	216.6 ± 12.2	24.6 ± 2	25.3 ± 1.5	46.3 ± 3
0.016	31.6 ± 2	196.3 ± 12.6	18 ± 3.6	25.6 ± 4	36.3 ± 9.7	39.3 ± 1.1	215.3 ± 10	29.3 ± 5.1	27.6 ± 4.5	51.3 ± 1.1
0.05	26.3 ± 1.1	220 ± 12.5	20 ± 1.7	31.3 ± 2.3	39.6 ± 2.3	43.3 ± 2.3	215.6 ± 1.1	24.6 ± 4.9	22.6 ± 2.8	50.6 ± 0.5
0.16	32.6 ± 0.5	231.3 ± 33.6	22.3 ± 5.6	32 ± 5.3	36.3 ± 2.3	44.6 ± 5.7	211.3 ± 19.8	22.3 ± 5.5	23.6 ± 2.3	46 ± 4.5
0.5	36.3 ± 1.5	222.3 ± 12.5	19.3 ± 2.5	30 ± 1	33.3 ± 5	40.6 ± 0.5	222.6 ± 9	21	30.6 ± 3.5	49 ± 2
1.6	27.3 ± 6.5	194.3 ± 16.5	23.6 ± 0.5	28.6 ± 2	32.6 ± 4	41.3 ± 3	226.6 ± 12.5	22.6 ± 1.5	30 ± 2	51.3 ± 1.5
5	36.6 ± 2.3	217 ± 8.7	25.3 ± 1.1	34 ± 2	37.6 ± 7.5	34.6 ± 3	229.3 ± 0.5	28 ± 3	28.6 ± 3	44 ± 3.6
Positive Control	1219.3 ± 35.7	1650 ± 24	214 ± 6.5	200.3 ± 11.3	165.3 ± 14	1316.6 ± 32.1	1745.3 ± 9.4	215.6 ± 10.5	231.6 ± 7.3	237.6 ± 21.2
Mutagenic Index		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
0.0016	0.9 ± 0.1	1.0 ± 0.0	1.4 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.0
0.005	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.0
0.016	1.1 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.3 ± 0.0
0.05	0.9 ± 0.1	1.2 ± 0.0	1.5 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	1.4 ± 0.2	1.2 ± 0.1	1.3 ± 0.0
0.16	1.2 ± 0.1	1.3 ± 0.1	1.7 ± 0.3	1.7 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
0.5	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.6 ± 0.1	1.2 ± 0.0
1.6	1.0 ± 0.1	1.1 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	1.3 ± 0.0
5	1.3 ± 0.1	1.2 ± 0.1	1.9 ± 0.2	1.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.0	1.2 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.1 ± 0.1
Positive Control	43.5 ± 3.2	9.3 ± 0.4	16.5 ± 1.3	10.7 ± 0.5	4.7 ± 0.3	35.9 ± 0.3	8.8 ± 0.4	12.0 ± 0.5	12.2 ± 0.4	6.0 ± 0.3

Mean revertants per plate and mutagenic index measured in bacterial strains TA98, TA100 and TA1535 and 1537 and WP2 treated with AnnurComplex at various doses, with (+S9) or without (−S9) metabolic activation. Negative controls consisted of 100 µL water. Positive controls consisted: for *S. typhimurium* TA100 NaN3 (−S9) and BAP (+S9); for *S. typhimurium* TA98 2NF (−S9) and BAP (+S9); for *S. typhimurium* TA1535 NaN3 (−S9) and 2AA (+S9); for *S. typhimurium* TA1537 9AC (−S9) and 2AA (+S9); for *E. coli* WP2 trp UvrA NQO (−S9) and 2AA (+S9).

Table 3. Mutagenicity of Limoncella Apple Extract.

Mean Revertants										
		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	25.6 ± 1.5	168.6 ± 7	16 ± 5.2	20.6 ± 4.6	37.3 ± 1.1	43 ± 1	190.3 ± 10.9	22.3 ± 4.5	24 ± 4	40.6 ± 3
0.0016	31 ± 2.6	177.6 ± 1.5	19.6 ± 2.5	26.3 ± 0.5	35.6 ± 1.1	44.6 ± 2	210.3 ± 6.1	25 ± 2	25.6 ± 1.1	44.3 ± 2.3
0.005	27.3 ± 1.5	203 ± 10.5	19.6 ± 3.2	26.6 ± 2.5	34.3 ± 5.5	37.6 ± 0.5	230.6 ± 3.5	26 ± 6	25.3 ± 1.1	45 ± 5.2
0.016	26 ± 1.7	195 ± 10.3	20.6 ± 1.1	29.3 ± 1.1	36.6 ± 5.7	41.3 ± 2.5	231.3 ± 6.8	24.6 ± 1.5	29.3 ± 0.5	50 ± 2
0.05	28 ± 3.6	213 ± 8.7	25.3 ± 6.1	31.6 ± 2	40.3 ± 2.5	46.3 ± 3	226.6 ± 7.5	25 ± 2.6	27.6 ± 3	50.6 ± 2.5
0.16	29 ± 3	197.6 ± 16.7	20.3 ± 2.5	30.6 ± 2	38 ± 1.7	42.6 ± 2.5	216.6 ± 13.6	22 ± 3.6	24.3 ± 1.5	47 ± 4
0.5	26 ± 1	195.6 ± 15	21 ± 2	32 ± 3.6	39.3 ± 0.5	36.3 ± 2.5	225.3 ± 16.5	24.6 ± 6.4	30.6 ± 1.5	47
1.6	34 ± 3	176 ± 3.4	23.6 ± 2.5	32.6 ± 2.5	41.3 ± 7.7	38.6 ± 2	227.6 ± 11	26.6 ± 0.5	29.6 ± 2.3	54 ± 6.5
5	34.3 ± 3.7	201.3 ± 7	25.3 ± 6.1	35.3 ± 2.5	42.6 ± 0.5	37 ± 3.4	230.6 ± 3	22.6 ± 1.1	27.3 ± 2.3	46 ± 2.6
Positive Control	1193.3 ± 63	1536.3 ± 37	204.3 ± 23.9	207.6 ± 10.6	187.6 ± 14.1	1320 ± 36	1711.3 ± 66.1	218 ± 13.8	221.6 ± 16	238.6 ± 9.6
Mutagenic Index		−S9					+S9			
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	***E.coli* WP2 Uvr A**
Negative control	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
0.0016	1. 2 ± 0.1	1.1 ± 0.0	1.2 ± 0.3	1.3 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
0.005	1.1 ± 0.1	1.2 ± 0.0	1.2 ± 0.3	1.3 ± 0.2	0.9 ± 0.1	0.9 ± 0.0	1.2 ± 0.0	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
0.016	1.0 ± 0.1	1.2 ± 0.0	1.3 ± 0.3	1.4 ± 0.2	1.0 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
0.05	1.1 ± 0.1	1.3 ± 0.0	1.6 ± 0.4	1.5 ± 0.2	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
0.16	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.3	1.5 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
0.5	1.0 ± 0.0	1.2 ± 0.1	1.3 ± 0.3	1.5 ± 0.2	1.1 ± 0.0	0.8 ± 0.0	1.2 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	1.2 ± 0.1
1.6	1.3 ± 0.1	1.0 ± 0.0	1.5 ± 0.3	1.6 ± 0.2	1.1 ± 0.1	0.9 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
5	1.3 ± 0.1	1.2 ± 0.0	1.6 ± 0.4	1.7 ± 0.1	1.1 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Positive Control	46.5 ± 2.1	9.1 ± 0.3	12.8 ± 2.6	10.0 ± 1.3	5.0 ± 0.2	30.7 ± 0.6	9.0 ± 0.4	9.8 ± 1.2	9.2 ± 1.0	5.9 ± 0.3

Mean revertants per plate and mutagenic index measured in bacterial strains TA98, TA100 and TA1535 and 1537 and WP2 treated with Limoncella Apple Extract at various doses, with (+S9) or without (−S9) metabolic activation. Negative controls consisted of 100 µL water. Positive controls consisted: for *S. typhimurium* TA100 NaN3 (−S9) and BAP (+S9); for *S. typhimurium* TA98 2NF (−S9) and BAP (+S9); for *S. typhimurium* TA1535 NaN3 (−S9) and 2AA (+S9); for *S. typhimurium* TA1537 9AC (−S9) and 2AA (+S9); for *E. coli* WP2 trp UvrA NQO (−S9) and 2AA (+S9).

2.4.2 Ames Test experimental procedure

Ames test was performed following the guidelines of OECD 471

Chemicals and solvents. Mitomycin C (Mit C. CAS Number 50-07-7. code 3514. LOT. NO F1318) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ampicillin (code 26-810), Tetracycline (code 26-811), Crystal Violet (code 26-813), Benzo(a)pyrene (BAP. CAS 50-32-8. code 60-114.6. LOT. NO 8197BP), Sodium azide (NaN3, code 60-103.1), 2-aminoanthracene (2AA, code 60-107.21), 2-nitrofluorene (2-NF, code 60-111), 4-nitroquinoline-N-oxide (4-NQO, code 60-121.3), 9-aminoacridine (9AA, code 60-147.5) were all purchased from Trinova Biochem GmbH (Geissen. Germany) as well as MutazymeTM, 10%, Lyophilized Rat Liver S9 Mix (20 mL/vial, code 11-402L). When indicated chemicals were dissolved in sterile dimethyl sulfoxide (DMSO. CAS Number 67-68-5. JT Baker).

Bacteriological Media. Minimal Glucose Agar Plates: Agar (15 g/L), Vogel-Bonner salts ($MgSO_4$ × $7H_2O$ (200 mg/L), Citric Acid × H_2O 2 g/L, KH_2PO_4 10 g/L, (NH4) $NaHPO_4$ × $4H_20$ (3.5 g/L)), D-Glucose (4.0 g/L), pH 7.0.
Oxoid Agar Plates: Agar (15 g/L), Vogel-Bonner salts ($MgSO_4$ × $7H_2O$ (200 mg/L), Citric Acid × H_2O 2 g/L, KH_2PO4 10 g/L. (NH_4) $NaHPO_4$ × $4H_2O$ (3.5 g/L)), D-Glucose (2.0 g/L), Oxoid No.2 Broth (25 g/L), pH 7.0.
Top Agar: Agar (7 g/L), NaCl 5 g/L, L-Histidine HCl (10.4 mg/L), L-Tryptophan HCl (10.1 mg/L), D-Biotin (12.2 mg/L), pH 7.0. Media were steam sterilized at 15 lbs/sq for 20 min at 121 °C

Bacterial Growth and storage. Bacterial cultures were grown at the temperature of 37 °C in Pyrex flasks with 9 volume of air per volume of broth. Flasks were allocated in an incubator for prokaryotes on an orbital shaker shaking at the speed of 230 rpm. Cultures were grown up to late exponential phase (approximately 10^9 cells per mL. Optical Density at λ = 600 nm of 1.0 ± 0.1). Titers were determined by plating a dilution 1:250,000 of the culture on Oxoid Agar Plates to then count the number of viable cells after 24 h of incubation. Long term storage of the bacterial strain was performed by keeping bacterial stocks at −80 °C in Oxoid No. 2 Broth supplemented with 20% of sterile Glycerol.

Bacterial Strain. The five bacterial strains *S. typhimurium* TA1535 (LOT. NO 5294D), *S. typhimurium* TA1537 (LOT NO. 5295D), *S. typhimurium* TA98 (LOT NO. 5293D), *S. typhimurium* TA100 (LOT NO. 5325D) and *E. coli WP2 trp UvrA* were purchased at Trinova Biochem (Giessen, Germany).

For *S. typhimurium* TA98 strain, phenotype confirmation was performed by growing an overnight culture in Oxoid No. 2 broth to then challenge 1–2 × 10^8 of bacteria as follows: (a) the strain did not grow on agar minimal plate in the absence of L-His confirming the his- phenotype; (b) the strain manifested zonal growth inhibition on agar minimal plate in the presence of L-His and of a 10 μg Crystal Violet disc. confirming the rfa phenotype; (c) the strain did grow on agar minimal plate containing L-His and a 2 μg Ampicillin disc confirming the presence of the R-factor plasmid; (d) the strain did not grow on agar minimal plate containing L-His, 2 μg Ampicillin and 1 μg Tetracycline disc confirm the absence of the pAQ1 plasmid; (e) the strain did not grow on agar minimal plate containing L-His and 0.2 μg Mitomycin disc confirming the uvrA/B phenotype and thus the absence of an active excision repair. The *S. typhimurium* TA98 strain yielded spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and within the range reported in the literature. Mean revertant per plates: (water: number of colonies 26), Daunomycin (6 μg; number of colonies 954), ICR191 (1 μg; number of colonies 38), Mitomycin C (0.5 μg; number of colonies 10), NaN_3 (1.5 μg; number of colonies 21 colonies).

For *S. typhimurium* TA100 strain, phenotype confirmation was performed by growing an overnight culture in Oxoid No. 2 broth to then challenge 1–2 × 10^8 of bacteria as follows: (a) the strain did not grow on agar minimal plate in the absence of L-His confirming the his- phenotype; (b) the strain manifested zonal growth inhibition on agar minimal plate in the presence of L-His and of a 10 μg Crystal Violet disc confirming the rfa phenotype; (c) the strain did grow on agar minimal plate containing L-His and 2 μg Ampicillin confirming the presence of the R-factor plasmid; (d) the strain did not grow on agar minimal plate containing L-His and of a 2 μg Ampicillin and 1 μg Tetracycline disc confirming the absence of the pAQ1 plasmid; (e) the strain did not grow on agar minimal plate containing L-His and a 0.2 μg Mitomycin disc confirming the uvrA/B phenotype and thus the absence of an active excision repair. The *S. typhimurium* TA100 strain yielded spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and within the range reported in the literature. Mean revertant per plates: (water: number of colonies 92), Daunomycin (6 μg; number of colonies 229), ICR191 (1 μg; number of colonies 117). Mitomycin C (0.5 μg; number of colonies 50), NaN3 (1.5 μg; number of colonies 558).

For *S. typhimurium* TA1535 strain, phenotype confirmation was performed by growing an overnight culture in Oxoid No. 2 broth to then challenge 1–2 × 10^8 of bacteria as follows: (a) the strain did not

grow on agar minimal plate in the absence of L-His confirming the his- phenotype; (b) the strain manifested zonal growth inhibition on agar minimal plate in the presence of L-His and 10 μg Crystal Violet disc confirming the rfa phenotype; (c) the strain did not grow on agar minimal plate containing L-His and a 2 μg Ampicillin disc confirming the absence of the R-factor plasmid; (d) the strain did not grow on agar minimal plate containing L-His and a 2 μg Ampicillin and a 1 μg Tetracycline disc confirming the absence of the pAQ1 plasmid; (e) the strain did grow on agar minimal plate containing L-His and 0.2 μg Mitomycin disc confirming the uvrA/B phenotype and thus the absence of an active excision repair. The S. typhimurium TA1535 strain yielded spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and within the range reported in the literature. Mean revertant per plates: (water, number of colonies 6), Daunomycin (6 μg, number of colonies 5), ICR191 (1 μg, number of colonies 7), Mitomycin C (0.5 μg, number of colonies 0 colonies). NaN_3 (1.5 μg, number of colonies 329 colonies).

For *S. typhimurium* TA1537 strain, phenotype confirmation was performed by growing an overnight culture in Oxoid No. 2 broth to then challenge 1–2 × 10^8 of bacteria as follows: (a) the strain did not grow on agar minimal plate in the absence of L-His confirming the his- phenotype; (b) the strain manifested zonal growth inhibition on agar minimal plate in the presence of L-His and a 10 μg Crystal Violet disc confirming the rfa phenotype; (c) the strain did not grow on agar minimal plate containing L-His and a 2 μg Ampicillin disc confirming the absence of the R-factor plasmid; (d) the strain did not grow on agar minimal plate containing L-HisAnd a 2 μg Ampicillin and 1 μg Tetracycline disc confirming the absence of the pAQ1 plasmid; (e) the strain did grow on agar minimal plate containing L-His and 0.2 μg Mitomycin disc confirming the uvrA/B phenotype and thus the absence of an active excision repair. The *S. typhimurium* TA1537 strain yielded spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and within the range reported in the literature. Mean revertant per plates: water (number of colonies: 6), Daunomycin (6 μg, number of colonies: 10), ICR191 (1 μg, number of colonies: 60), Mitomycin C (0.5 μg, number of colonies: 1), NaN_3 (1.5 μg, number of colonies: 10).

For *E. coli WP2 trp UvrA* strain, phenotype confirmation was performed by growing an overnight culture in Oxoid No. 2 broth to then challenge 1–2 × 10^8 of bacteria as follows: (a) the strain did not grow on agar minimal plate in the absence of L-Trp confirming the trp- phenotype; (b) the strain did not grow on agar minimal plate containing L-Trp and a 2 μg Ampicillin disc confirming the absence of the R-factor plasmid pKM101; (c) the strain did not grow on agar minimal plate containing L-Trp and a 0.2 μg Mitomycin disc confirming the UvrA/B phenotype and thus the absence of an active excision repair. The *E. coli WP2 trp UvrA* strain yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and within the

range reported in the literature. Mean revertant per plates: water (number of colonies: 47), Methyl methanesulfonate (MMS) (2.5 μL, number of colonies: 528).

Metabolic activation. Metabolic activation of nutraceuticals was achieved by exogenous metabolization using S9 post-mitochondrial fraction. S9 (code 11-402L. LOT NO. 4026) prepared from livers of Sprague Dawley male rats treated with Aroclor 1254 (500 mg/Kg i.p.). Lyophilized S9 was purchased from Trinova Biochem already supplemented with glucose-6-phosphatedehydrogenase (180 mg/mL) Nicotinamide adenine dinucleotide phosphate (25 mg/mL), Potassium chloride (150 mM) mixed in the ratio 2:1:1:1. S9 was reconstituted in deionized water and stored at −80 °C. The protein concentration of S9, assayed with the Lowry Method, was 3.5 mg/mL. To prove S9 able to activate pro-mutagens, we measured the number of revertant colonies of TA98 and TA1535 strains growing in the presence of S9 and of ethidium bromide and cyclophosphamide. TA98 strain yielded 52 colonies in the presence of ethidium bromide and TA1535 yielded 430 colonies in the presence of cyclophosphamide, respectively. Dilution of S9, ranging from 0.6 to 10% were tested for their ability to activate benzo (a) pyrene and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. The final concentration of S-9 fraction in the test system was 7% v/v. Cultures treated in the absence of S9 received an equivalent volume of 0.1 M phosphate Buffer pH 7.4 in place of S9 mix.

Nutraceutical test conditions. Taurisolo, AnnurComplex and Limoncella Apple Extract are highly soluble in water that was thus used as vehicle for all the experiments. Mother stocks of Nutraceuticals 50 mg/mL were freshly prepared in water. The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate. None of the nutraceutical gave precipitation on the surface of the agar plate. However, the grape pomace stained the agar plate in a dark purple color.

Test dilutions were obtained by diluting mother stocks in water. We tested eight dilutions for each of the nutraceuticals (namely, 0.0016, 0.005, 0.016, 0.05, 0.16, 0.5, 1.6. and 5 mg/10 cm plate) (volume 100 μL). Up to 5 mg/plate and on Oxoid Agar Plates, none of the nutraceutical-induced growth inhibition of the bacterial strains here tested, confirming that in the range of dilution here assayed, all the tested nutraceuticals were not cytotoxic for the bacteria strains.
Negative controls consisted of 100 μL water. Positive controls consisted: for *S. typhimurium* TA100, NaN_3 1.25 μg/10 cm plate in the absence of S9 and BAP 6.0 μg/10 cm plate in the presence of S9; for *S. typhimurium* TA98, 2NF 2.0 μg/10 cm plate in the absence of S9 and BAP 6.0 μg/10 cm plate in the presence of S9; for *S. typhimurium* TA1535, NaN_3 1.25 μg/10 cm plate in the absence of S9

and 2AA 2.0 μg/10 cm plate in the presence of S9; for *S. typhimurium* TA1537, 9AC 50.0 μg/10 cm plate in the absence of S9 and 2AA 2 μg/10 cm plate in the presence of S9; for *E. coli WP2 trp UvrA*, NQO 1.0 μg/10 cm plate in the absence of S9 and 2AA 20.0 μg/10 cm plate in the presence of S9.

Experimental Procedure. We used the plate incorporation method. Briefly, 0.1 mL of test solutions (the appropriated amount of nutraceutical dissolved in 0.1 mL of water), 0.1 mL of fresh bacterial culture containing 108 viable cells and either 0.5 mL of 0.1 M Phosphate buffer (pH 7.4) or 0.5 mL of S9 were mixed with 2.0 mL of Top Agar. For the assay with metabolic activation, 0.5 mL of metabolic activation mixture contained 7% post-mitochondrial fraction. The contents of each tube were mixed and poured over the surface of a minimal agar plate. The overlay agar was allowed to solidify before incubation. Experiments were performed in triplicates for each condition. Plates were incubated at 37 °C for 72 h. After the incubation period, the number of revertant colonies per plate was counted.

Acceptance of the test. Acceptance of the test was based on the following criteria: (a) all experimental conditions requested by OECD 471 were tested; (b) the results obtained for the negative control were consistent with the laboratory's historical negative control database; (c) concurrent positive controls induced responses that were compatible with those generated in the laboratory's historical positive control database and produced a statistically significant increase compared with the concurrent negative control.

2.5 *In vivo* bioavailability and *in vitro* toxicological evaluation of the new butyric acid releaser N-(1-carbamoyl-2-phenyl-ethyl) butyramide

Novel food is defined as food that had not been consumed to a significant degree by humans in the EU before 15 May 1997, when the first Regulation on novel food came into force.
'Novel food' can be newly developed, innovative food, food produced using new technologies and production processes, as well as food which is or has been traditionally eaten outside of the EU.
The underlying principles underpinning Novel Food in the European Union are that Novel Foods must be:
- Safe for consumers;
- Properly labelled, so as not to mislead consumers;
- If novel food is intended to replace another food, it must not differ in a way that the consumption of the Novel Food would be nutritionally disadvantageous for the consumer.

FBA (N-(1-carbamoyl-2-phenyl-ethyl) butyramide, **Figure 2.6**), as a nutrient with an intentionally modified molecular structure, is classed as a novel food in Europe in accordance with European law (Regulation (EU) 2283/2015), and therefore cannot be placed on the market or used in foods for human consumption until it is included in “the Union list” of novel foods authorized to be placed on the market within the European Union.
Thus, the aim of this investigation is to provide preliminary information of its toxicological properties through the study of its *in vitro* toxicological profile by means of the Ames Test and Micronucleus Test.
In conclusion, FBA is a new butyric acid releaser that can overcome the disadvantages of butyric acid while maintaining the same pharmacokinetic properties and safety profile, as shown by the results of the preliminary *in vitro* toxicological studies performed in this investigation.

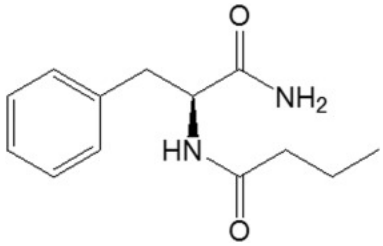

Figure 2.6. FBA (N-(1-carbamoyl-2-phenyl-ethyl) butyramide chemical structure

2.5.1 Results of Ames Test and Micronucleus Test

The results reveal that FBA does not show any in vitro genotoxicity as it is non mutagenic in the Ames Test (**Table 3**) and results to be unable to induce chromosome breaks in the Micronucleus Test (**Table 4-5**) [40].

	-S9					+S9				
mg/plate	**TA98**	**TA100**	**TA1535**	**TA1537**	**E.Coli WP2 Uvr A**	**TA98**	**TA100**	**TA1535**	**TA1537**	**E.Coli WP2 Uvr A**
Negative control	27 ± 2	173 ± 87	17 ± 2	23 ± 3	36 ± 4	43.3±4.7	217.3±12.4	19±6	19±2	39±2
0.0016	34 ± 3	184 ± 16	18 ± 3	22 ± 4	35 ± 2	50.3±3	222.6±5.8	22±2.6	18.3±2.3	44.6±2.5
0.005	32 ± 3	200 ± 12	18 ± 2	26 ± 1	33 ± 6	41.3±2.8	230.6±14.4	26.6±0.5	25±1.7	46±3.4
0.016	22 ± 1	190 ± 15	19 ± 2	27 ± 1	38 ± 7	40±2.6	224±12.2	29.3±1.5	26.6±4.9	50.6±3.7
0.05	31 ± 4	210 ± 17	28 ± 1	31 ± 3	43 ± 4	50±3	219±1	27±4	24.6±2.8	54±2.6
0.16	29 ± 2	196 ± 18	19 ± 2	31 ± 4	39 ± 4	45.3±2.8	211±13.3	20.3±0.5	23.6±2	53.3±1.1
0.5	27 ± 2	195 ± 12	22 ± 1	32 ± 4	39 ± 2	44.3±3	215±6.2	20.6±0.5	31.6±2	52±3.4
1.6	36 ± 2	184 ± 16	29 ± 4	33 ± 4	41 ± 7	44.3±6	241±23.8	26±3.6	31±4.3	54.3±5.5
5	37 ± 3	215 ± 14	33 ± 2	36 ± 3	46 ± 7	47±6	232.6±15.3	24.3±2.3	24.6±1.1	52±2
Positive Control	**1214 ± 12**	**1542 ± 33**	**213 ± 11**	**200 ± 7**	**169 ± 19**	**1357.6±51.7**	**1751.6±10.5**	**212.6±2.5**	**234.3±14.1**	**238.6±11.3**

Table 3. Number of revertants⁄plate for each strain in the presence or absence of metabolic activation (S9 mix) treated with FBA at different concentration, DMSO and positive controls (mean±sd)

Table 4
6 h Treatments with the indicated chemicals and 28 h incubation in the presence of CytB, in the presence and absence of S9-mix (*statistically increase $p < 0.05$).

Dose	Citotoxicity					Genotoxicity		% Cytostasis	
FBA (mM)	S9-mix	Mononucleated cells	Binucleated cells	Polinucleated cells	% Citotoxicity	Binucleated cells with micronuclei	% of Binucleated cells with micronuclei	Replication index (RI)	CBPI
0	–	9650	2621	350	0	9	0.3	100.0	1.26
0	+	9755	2874	550	1.1	10	0.4	114.6	1.30
0.01	–	9574	2736	370	−0.8	4	0.15	104.2	1.27
0.01	+	9928	2321	540	2.9	2	0.09	101.1	1.27
0.03	–	9836	2968	350	1.9	6	0.2	106.0	1.28
0.03	+	9547	3128	400	−1.1	3	0.1	114.2	1.30
0.1	–	9328	2523	260	−3.3	2	0.08	95.5	1.25
0.1	+	9725	2626	530	0.8	8	0.3	108.8	1.29
0.3	–	9758	2558	330	1.1	3	0.12	96.7	1.25
0.3	+	9826	2380	270	1.8	3	0.13	88.9	1.23
1	–	9521	2723	600	−1.3	5	0.18	116.1	1.31
1	+	9723	2428	500	0.8	4	0.16	103.0	1.27
3	–	10,236	2761	430	6.1	6	0.22	102.5	1.27
3	+	10,257	2923	500	6.3	5	0.17	109.0	1.29
MitC									
0.15 μg/mL	–	12,345	2521	128	27.9	32	1.3*	70.4	1.19
BAP									
3 μg/mL	+	11,074	2210	110	22.3	42	1.9*	68.9	1.18

Historical negative (n > 20 experiments): % micronucleated binucleated cells upon treatment with vehicle of 0.30 ± 0.2 and of 0.4 ± 0.3. in the presence of Cyt B and in the absence of Cyt B, respectively.
Historical positive values (n > 20) = % micronucleated binucleated cells upon treatment with 1.2 ± 0.3% upon treatment with MitC in the absence of Cyt B and of 1.7 ± 0.3% for BAP in the presence of Cyt B [range 1.5–3].

Table 5
34 h Treatment with FBA at different concentrations of the indicated chemicals in the presence of CytB and in the absence of S9-mix (*statistically increase $p < 0.05$).

Dose	Citotoxicity					Genotoxicity		% Cytostasis	
FBA (mM)	S9-mix	Mononucleated cells	Binucleated cells	Polinucleated cells	% Citotoxicity	Binucleated cells with micronuclei	% of Binucleated cells with micronuclei	Replication index (RI)	CBPI
0	–	9487	2430	295	0	8	0.33	100.0	1.25
0.01	–	9318	2621	302	2.3	7	0.27	106.5	1.26
0.03	–	9532	2432	287	1.7	2	0.08	99.2	1.25
0.1	–	9278	2050	280	3	6	0.29	90.9	1.22
0.3	–	9001	2460	288	2.5	7	0.28	104.5	1.26
1	–	9120	2555	327	3	5	0.20	108.1	1.27
3	–	9870	1860	300	12	7	0.38	82.7	1.20
ColC									
0.1 μg/mL	–	11,428	2727	87	23.3	60	2.2*	77.4	1.20

Historical negative (n > 20 experiments): % micronucleated binucleated cells upon treatment with vehicle of in the absence of Cyt B = 0.29 ± 0.20.
Historical positive values (n > 20): % micronucleated binucleated cells upon treatment with for ColC in the absence of Cyt B = 2.0 ± 0.4%.

Table 4- 5. Results of Micronuclei Assay Test

2.5.2 Ames Test and Micronucleus Test experimental procedure

***In vitro* toxicity tests**

Toxicological studies were performed in compliance with OECD principles of good laboratory practice (GLP), guidelines for testing of chemicals (OECD guideline 471 and 487) and in accordance with the Standard Operating Procedures (SOPs) of the laboratories of Nutraceuticals.

Ames Test

Briefly, four cultures of *S. typhimurium* (strains TA98, TA100, TA1535, TA1537) and a culture of *E. coli* (strain ECWP2UvrA), were prepared from their main strain plates and used in their late exponential growth phase. Using the incorporation method, a negative control plate (with DMSO), positive control plates (with different chemicals depending on the bacterial strain), and FBA plates containing FBA solubilized into DMSO (used as solvent), were prepared at eight growing concentrations ranging from 0.0016 to 5 mg/plate (0.0016, 0.005, 0.016, 0.05, 0.16, 0.5, 1.6, and 5 mg/plate). In each tube, 0.1 mL of FBA solutions were added to 0.1 mL of fresh bacterial culture (containing approximately 10^8 viable cells), 0.5 sterile buffer, and 2.0 mL of overlay agar. The experiments were performed both in the presence and in the absence of metabolic activation (S9-mix), at a post-mitochondrial fraction concentration of 7% v/v. The contents of each tube were mixed and poured over the surface of a minimal agar plate. After solidification, the plates were incubated at 37 ◦C for 72 h. After the incubation period, the number of revertant colonies per plate was counted and their frequency was compared with that for the negative control group. Negative and positive control plates and FBA plates were tested in triplicate, the results expressed as number of revertant colonies per plate, and mean ± SD.

Acceptance of the test. Acceptance of the test was based on the following criteria: a) all experimental conditions requested by OECD guideline 471 were tested; b) considering FBA solubility, the criteria for the selection of FBA top test concentration (5 mg/plate) was consistent with those described in OECD guideline 471; c) none of the test concentrations exhibited a statistically significant increase in terms of number of revertants per plate compared with the concurrent negative controls; d) no concentration-related increase, nor any other trend, could be identified, e) all results were below the historical range of negative control data, f) all concurrent positive controls gave a statistically significant increases in terms of number of revertants compared with the concurrent negative controls.

In vitro mammalian cell Micronucleus Tests

A Micronucleus Tests was performed according to OECD guideline 487.

Cells. Chinese Hamster Ovary K1 (CHO-K1) cells were chosen for micronucleus testing of FBA, in virtue of their extensive and validated use in this specific test [41]. Cells were grown in Ham's F-12 Medium, containing 2 mM L-alanyl-L-glutamine, 1500 mg/L sodium bicarbonate and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin. Mother stocks were maintained in 75 cm^2 flasks (Corning, Tewksbury, MA, USA) in a cell culture incubator at 37 ◦C and with 5% CO_2 and 95% humidity. Duplication time of the CHO-K1 clone used in this set of experiments was of about 14 h, according to published cell characteristics [41]. The exponential growth phase was achieved by trypsinization using 0.25% (w/v) trypsin and 0.53 mM EDTA and by sub-cultivating at a 1:4 /1:8 ratio, with medium renewal once between cell splitting. CHO-K1 with passage numbers above 30 were not used for the assay. The absence of Mycoplasma contamination was confirmed before the beginning of each experiment by immunofluorescence identifying Mycoplasma DNA by DAPI staining.

FBA preparation and test condition. Since FBA is insoluble in water at room temperature, mother stocks (50 mg/mL) of FBA were prepared just prior to treatment by dissolving the solid test chemical in sterile DMSO, which is a well-established solvent for the Micronucleus Tests [41]. Test concentrations were obtained by diluting mother stock in complete cell culture medium. In a preliminary set of experiments, the insolubility of FBA at the test concentration of 3 mM was verified, with massive turbidity of the cell culture medium solution. This insolubility could not be rescued by increasing the DMSO content. As OECD guideline 487 includes the lowest concentrated turbid solution among test concentrations, a FBA 3 mM solution was therefore included. Six FBA concentrations were tested (3, 1, 0.3, 0.1, 0.03, and 0.01 mM) with the highest allowed DMSO concentration of 1% v/v. The pH of the FBA test solutions was checked and found to be 7.4. As expected in virtue of the chemical nature of FBA and the vehicle used to dissolve it, the osmolality of test dilutions was found to be 286 ± 22 mOSM/kg, and did not change compared to negative controls. FBA solutions with concentrations lower than 3 mM did not show signs of serum protein precipitation or other deleterious interaction between FBA and cell culture medium. Negative controls consisted of 1% v/v DMSO dissolved in cell culture medium. Positive controls for clastogenicity and aneugenicity consisted of MitC, (1.5 µg/mL, 4.5 mM), BaP (3 µg/mL, 12 mM) and

Metabolic activation. With regards to the metabolic activation of FBA and BaP, this was achieved by exogenous metabolization using lyophilized S9 (S9-mix) previously supplemented with glucose-6- phosphate dehydrogenase, nicotinamide adenine dinucleotide and potassium chloride. S9-mix was reconstituted in deionized water and stored at − 80 ◦C. The activity of the S9-mix was tested by measuring its ability to activate BaP in the Ames Test. When indicated, S9-mix was used at a concentration of 2% (v/v) in the final test dilution.

Treatment schedules. The length of exposure to FBA was chosen to enable cell growth, chromosome damage and formation of micro- nuclei. The polyclone of CHO-K1 cells used showed a cell cycle length of ~14 h. Thus, the following treatment schedules were chosen:
– short treatment in the presence of metabolic activation: CHO-K1 were exposed to FBA and S9-mix for 6 h, then cultivated in the absence of FBA and in the presence of CytB for a further 28 h (2.0 normal cell cycle lengths after the beginning of treatment);
– short treatment in the absence of metabolic activation: CHO-K1 were exposed to FBA for 6 h to be then cultivated in the absence of FBA in the presence of CytB for a further 28 h;
– long treatment in the absence of metabolic activation: CHO-K1 were continuously exposed to FBA for 34 h in the presence of 6 μg/mL CytB.
Since any sign of cell detachment could be detected during the washing-out of FBA, it was not necessary to recover cells from conditioned media.

Micronucleus Test procedure. For evaluation of cytotoxicity, CHO-K1 cells were propagated from stock cultures and seeded into 25 cm^2 culture flasks with 5 mL of a (~ 0.6–0.7) × 10^5 cells/mL stock and incubated for 16 h. This cell density value was chosen so that monolayers would continue to grow exponentially until harvest time, and would not reach confluence. Before adding FBA solutions, a cell count was taken from two specified flasks. Medium was then replaced with FBA solutions according to the treating schedules. Negative controls (cells treated with vehicle) and positive controls (cells treated with MitC, BaP and ColC) were also processed in the same way as FBA treated cultures. Upon the 28h time point, cells were harvested by trypsinization and counted by using the automatic Cell counter Luna (Logos Bio- system, South Korea). Measurement of cell proliferation was performed to assure that sufficient treated cells underwent mitosis during the test.
For micronucleus induction, parallel cultures of CHO-K1 cells were seeded in 6-well slide chambers with 3 mL of cell suspension, resulting in a plating density of ~50,000 cells/cm^2, and incubated for 16 h. Medium was then replaced with FBA solutions according to the treating schedules. Negative controls consisted of solvent diluted in the culture medium. Positive controls were processed in the

same way as the FBA treated cultures. Upon 28 h of incubation, the medium was removed and cells were rinsed once with 1 mL of PBS and fixed in paraformaldehyde 3.7% for 30 min. The wells were then washed twice in PBS 1X, permeabilized in 0.1% Triton X100 diluted in PBS (10 min) and stained with DAPI (30 μM). DAPI fluorescence was measured using following parameters: λ excitation 351 nm, λ emission 450 nm, using an IRIS fluorescent microscope (Logos Biosystem, South Korea).

Cells were analyzed manually for the presence of micronuclei using the criteria developed by Eastmond and Tucker [42]. Binucleate cells with irregular shapes or where the two nuclei differed greatly in size were excluded. Poorly spread multi-nucleate cells were excluded. Cells containing more than two main nuclei were not analyzed for micronuclei.

The cytokinesis-block proliferation index (CBPI) and the Replication Index (RI) were measured to estimate the cytostatic activity of each treatment by comparing values in the treated and control cultures.

CBPI = (N◦ mononucleate cells + 2 x N◦ binucleate cells + 3 x N◦ multinucleate cells) / (Total number of cells).

RI = ((N◦ binucleated cells + 2x N◦ multinucleate cells)/total number of cells in treated cultures)/((N◦ binucleated cells + 2x N◦ multi- nucleate cells)/total number of cells in control cultures) X 100.

Cell counting verified duplication of cells in culture during or following treatment with FBA as well as cytotoxicity of the test compounds.

% Citotoxicity = 100–100*(((Total number of cells at the end of the assay-Total number of cells plated at time 0) treated)/((Total number of cells at the end of the assay-Total number of cells plated at time 0) untreated)).

The historical negative values were obtained by averaging more than 20 experiments and resulted in a percentage of micronuclei in binucleated cells upon treatment with vehicles of 0.30 ± 0.2 (range 0.2–1; 95% CI [0.25–0.36]) and of 0.4 ± 0.3 (range 0.2–1.1; 95% CI [0.30–0.50]), in the presence of Cyt B and in the absence of Cyt B, respectively.

The historical negative values for 36 h incubation with Cyt B in the absence of S9-mix was 0.29 ± 0.20 (range 0.2–1.1; 95% CI [0.2–0.35]). The historical positive values were obtained by averaging more than 20 experiments and resulted in a percentage of micronuclei in binucleated cells of 1.2 ± 0.3% (range 1.2–1.7; 95% CI [1.1–1.3]) upon treatment with MitC in the absence of Cyt B, 2.0 ± 0.4% (range 2–3.1; 95% CI [1.8–2.2]) for ColC in the absence of Cyt B, and of 1.7 ± 0.3% (range 1.5–3; 95% CI [1.6–1.8]) for BaP in the presence of Cyt B.

Acceptance of the test. Acceptance of the test was based on the following criteria: a) all experimental conditions requested by OECD guideline 487 were tested; b) more than 10000 cells were scored and six FBA concentrations were tested; c) considering FBA solubility, the criteria for the selection of FBA top test dilution (FBA 3 mM) was consistent with those described in OECD guideline 487; d) the results obtained from the negative control were consistent with the laboratory's historical negative control database; e) concurrent positive controls induced responses that are compatible with those generated in the lab- oratory's historical positive control database and produced a statistically significant increase compared with the concurrent negative control; f) as shown by the reported CBPI and RI values, cell proliferation criteria in the solvent control, in the positive controls and in the test dilutions of FBA were all fulfilled.

2.6 Conclusions

Results of the *in vitro* toxicological tests can be considered to be a promising starting point for other *in vivo* toxicological studies. In fact, although interesting, the Ames Test presents the limitation that it is carried out on prokaryotic cells, which differ from mammalian cells, and therefore cannot entirely mimic mammalian *in vivo* conditions. In addition, there are many substances found to be positive in this test which exhibit mutagenic activity in other tests, other substances, found negative in this test, do exhibit mutagenic activity in other tests, and finally, other substances again for which the test overestimates mutagenic activity.

Ames assay uses *Salmonella typhimurium* strains and so it is not a perfect model for human. Mice liver S9 hepatic fraction is used as mimicry of mammalian metabolic activations by the hepatic system so that the mutagenicity of metabolites can be assessed. There are several differences between human and mice metabolism which can affect the mutagenicity of testing substances. Major disadvantages of fluctuation test are slower and slightly more laborious than Ames protocol. The test is primarily used for testing aqueous samples containing low levels of mutagen and therefore, this test is well adapted for evaluating the mutagenicity of wastewater samples.

Thus, to show the safety of these substances, other *in vivo* toxicological investigation must be performed following the tiered approach.

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CHAPTER 3

Setting-up of a biological platform to study the antimicrobial potential of herbal extracts commonly used for infants

3. Experimental pipelines to identify nutraceuticals as antimicrobial agents

An antibiotic is a substance able to inhibit or kill microorganisms [1]. It has biological, semisynthetic or synthetic source and it is dispensed orally, topically, or by injection.
Based on its mechanism of action, an antibiotic can be classified in two ways [2]:

- a bactericidal works by killing bacteria; usually interfering with either the formation of the bacterial cell wall or its cell contents.
- a bacteriostatic works by preventing bacterial duplication.

Antibiotics are considered as a cornerstone of modern medicine and are prescribed to solve several infectious diseases [3]. However, their excessive use worldwide has caused the huge health issue of the multi-drug bacterial resistance, manifesting with antibiotics inefficiency. Finding novel therapeutic approaches based on natural compounds with antibacterial effects could be considered a promising option.

Several studies have demonstrated the antimicrobial and antioxidant properties of secondary metabolites of many plants, which have been found to slow or completely inhibit the growth of bacteria, yeasts and molds. Phytochemicals have broad-spectrum antibacterial activity representing a possible source of effective, cheap and safe antimicrobial agents [4][5].
In virtue of this property, some natural products, such as essential oils and herbal extracts, could be used as an alternative to additives chemicals in food preservation, especially if the latter are intended for children [6].

In this scenario, EFSA's evaluation on food additives is opportune and its opinion will be relevant to future uses of herbal extracts within the food industry.
Overall, it would be wise setting harmonized approaches to identify nutraceuticals as antimicrobial agents providing information about their activity.

3.1 Food preservation and additives

Nowadays there are different preservation methods which allow food conservation and can be divided as follow [7]:

- inhibiting chemical weakening;
- inactivating microorganisms;
- avoiding recontamination.

Various synthetic antimicrobials, including several organic acids and salts (sodium benzoates and propionates, potassium sorbates, sorbic acid, sulfites, chlorides, nitrites, triclosan, nisin, natamycin, potassium lactate, ascorbic acid, citric acid, tartaric acid, etc. [8]) have been approved by regulation and are used as food additives. The use of some of these, however, represents nutritional or health threats for the consumer.

For example, sulphites and sulphiting agents are well known food additives endowed with antimicrobial properties that constitute an important and versatile category within the food additives. They are widely used in food industry and they have some applications in other industries [9]. Despite all their uses, sulphites are associated with some adverse reactions such as asthmatic and anaphylactic episodes in some sensitive individuals. They have also been related with some anti-nutritional consequences such as degradation of thiamine (vitamin B1) [10].

New antimicrobial could be indeed identified in food additives. These can be subdivided into different classes such as additives of synthetic (such as butyl hydroxy toluene or butyl hydroxyanisole), animal (such as lysozyme) and vegetable origin (ascorbic acid or tocopherols) [11].

Moreover, there is an increasing rejection among consumers on the use of synthetic additives, as well as a demand for a better food quality, free of artificial preservatives, but maintaining its long shelf life. For all these reasons, research focus has been on finding natural alternatives to traditional solutions.

A good natural antimicrobial has to fulfil some requirements such as: (a) to be active in low concentrations in its natural form, (b) to be low-cost, (c) not to alter sensorial properties of the product, (b) to inhibit a wide range of spoilage and pathogenic microorganisms, and (e) not to be toxic [12][13].

Many food preservation strategies can be used for the control of microbial spoilage and oxidation; however, these quality problems are not yet controlled adequately. Although synthetic antimicrobial agents are accepted in many countries, there is an increased request of food consumers and producers to use natural, safe and effective preservatives [14]. Many plant occurring bioactive compounds can be considered as good alternatives to synthetic antimicrobial and antioxidant food additives [15][16]. These compounds are mostly derived from plants and their antimicrobial and antioxidant in vitro testing have resulted in many publications in the last decade [17][18][19]. However, the selection of the plant sources to extract these compounds must be guided for the safe use of food additives. A possible alternative could be medicinal plants that have been used for thousands of years to treat health disorders and prevent diseases.

Medicinal plant parts such as roots, leaves, branches/stems, flowers, and fruits are commonly rich in terpenes (carvacrol, citral, linalool, and geraniol) and phenolics (flavonoids and phenolic acids), and these compounds have been effective as food additives [20].

For example, lemongrass is a medicinal plant utilized as stomachic, antispasmodic, carminative, and antihypertensive agent [21]. Other medicinal plants that could be used to sustain the idea of generating extracts with potential as food additives are: *Chenopodium ambrosioides* rich in terpenes (used to control menses disorders, fibroids, uterine hemorrhage, and parasitic diseases); *Euphorbia stenoclada* rich in phenolics (used to control skin diseases, gonorrhoea, migraine, intestinal parasites and wart cures); *Geranium mexicanum* rich in terpenes and phenolics (used as remedy against tonsillitis, cough, whooping cough, urticaria, dysentery and diarrhea); *Gnaphalium oxyphyllum* rich in phenolics (used to treat gripe, fever, asthma, bronchitis, and cough); *Helianthemum glomeratum* rich in flavonoids (used to treat bloody and mucoid diarrheas and for the relief of abdominal pain); *Larrea tridentata* rich in phenolic compounds (used to treat respiratory infections as tuberculosis); *Marrubium vulgare* rich in terpenes and phenolics (used mainly as an expectorant); *Peumus boldus* rich in phenolics and alkaloids (regulator of the hepatic function, antispasmodic, digestive stimulant, and nervous sedative); *Eysenhardtia polystachya* rich in flavonoids (used to treat kidney and bladder infections, diuretic, antispasmodic and febrifuge).

3.2 Nutraceuticals as antimicrobial agents in food safety

Food safety deals with the production, the handling, the storage and the preparation of food in a manner that prevents infections or diseases and retains enough nutrients for a healthy diet. Nowadays, food safety is regarded to be the key public health issue that is compromised by the presence of pathogenic bacteria [8][22]. Food poisoning is associated with organisms such as *Staphylococcus Aureus, Salmonella Spp., Bacillus Cereus, Clostridium Botulinum, Clostridium Perfringens, Listeria Monocytogenes, Campylobacter Jejuni, E. Coli O157:H7, and Toxoplasma Gondii* [23].

Increasing food-borne diseases and enteric diseases [24] prompted the researches to set up innovative methods to eliminate food-borne pathogens in addition to the consumers' demand of "green" or natural substances to be used as food additives [25][26]. Foodborne diseases (FBD) are a global health issue that has key impact on human health. Many bacterial pathogens including *Salmonella, Campylobacter, Enterohaemorrhagic Escherichia Coli (EHEC), Listeria Monocytogenes* as well as norovirus and typhoid are responsible for large foodborne illnesses [27][28][29].

In recent years, consumers, increasingly aware of the quality of food, have shown on the one hand the need of food products without synthetic additives, preferring natural ones, and on the other to have long shelf-life food products, especially for the target groups of population belonging to early childhood. In children the exposure to synthetic additives could exert negative effects such as neuro developmental behavioral disorders (i.e. hyperactive behaviors and poor attention) that could persist into adulthood. Actually, in children, the exposure to synthetic additives could exert negative effects such that could persist into adulthood. Even now in the regulation of No EU 1333/2008 [30], food additives shall not be used in foods for infants.

The study of the possible use of natural additives as preservatives in infant food products, among which food supplements, is of crucial importance, especially in view of the promulgation of new European law that might limit the use of synthetic food additives in infant food supplements.

3.3 Development of innovative methods to preserve a new line of infant food supplements free from synthetic additives

Some medicinal plants may be good source of antimicrobial and antioxidant agents for the food industries. The research around this topic reflects that several studies have explored the extraction, antioxidant, and antimicrobial efficacy of medicinal plant tissues; however, the practical use of these sources of bioactive compounds as food additives has been overlooked.

Future research is suggested for exploring different medicinal plants traditionally used as antimicrobial and/or antioxidant treatment. Different extraction procedure could be performed to characterize the composition of the medicinal plants and special attention should be paid to this process considering that new molecules could be identified, even when the focus should be in those structures indicating a safe use in food systems. Different technologies, such as nanoemulsions, edible films or coatings, controlled release from encapsulation systems, among others, can be used to apply medicinal plant extracts in food matrices. Moreover, the impact of the treatments on the sensorial characteristic of the treated products should be contemplated. Finally, the conception of functional foods could be considered through the addition of the medicinal plants extracts; however, several experimental approaches could be considered to prove this statement. This proposal looks forward to promoting the use of natural extracts and fulfilling consumer demands for healthier foods.

The study of the possible use of natural additives as preservatives in infant food products, among which food supplements, is of crucial importance, especially in view of the promulgation of new European law that might limit the use of synthetic food additives in infant food supplements.

In this scenario, the aim of this study was to develop a new conservation method of food supplements for infants to be used in the production phase. This aim was achieved through the selection of safe plants with a history of use in infants, the development and the optimization of extraction methods to obtain plant extracts rich in antioxidant or antimicrobial agents and the evaluation of their use in a new line of free from food supplements. The final purpose is to replace the current synthetic additives with our vegetal extracts to provide a safer and healthier food supplements.

3.3.1 Experimental procedure

To reach our purpose I have first identified 12 different safe plants commonly used in early childhood using databases like Scopus or the NCBI. Then I selected, using literature data's, few of this plant and prepared their extracts. To select the single components of every chosen plant we consulted the annex 1 and the annex 1 bis of the Belfrit's list [34], where are reported all the plants and their components allowed in supplement foods.

The preparation and the standardization of the extraction procedure was thus performed. To verify the quality and the proprieties of the extracts, we proceeded, as first, with the evaluation of the polyphenols concentration and with the evaluation of the antioxidant activity. After these preliminary tests the most important phase consisted in the analysis of MIC and MBC. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism.
The MBC is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 h under a standardized set of conditions. The bactericidal endpoint (MBC) has been subjectively defined as the lowest concentration, at which 99.9% of the final inoculum is killed.

The experimental design pipeline was thus:

- First of all, we identified 12 different safe plants commonly used in early childhood, then we selected 5 of them and we performed and optimized the extraction method (to verify the quality and the properties of each extracts evaluating antioxidant activity via the DPPH assay).
- Determination of MIC and MBC for the common pathogens involved in contamination process, in particular we tested our extracts on both gram-positive and gram-negative bacteria. We focused our attention on two different bacteria strains: *Staphilococcus Aureus* and *Escherichia Coli*. These strains were chosen because they demonstrated an acceptable range of sensitivity to a wide range of antimicrobials and antibiotics.
- Further investigations to determine cell viability to evaluate cytotoxic effect of the extracts.

3.3.1.1 Preparation of herbal extracts

The natural compounds, for which antimicrobial activity has been evaluated, all boast a traditional use in products intended for children. They were extracted from the following matrices:

a. Melissa officinalis, powder*;*
b. Matricaria chamomilla;
c. Mentha spicata;
d. Melissa officinalis, raw plant*;*
e. Mentha sylvestris.

The extraction method deals with the maceration of starting materials into prechilled hydroalcoholic solution, taking in account that different parameters like pH, temperature, amount of starting material, particle size, % of organic solvent and time of extraction can influence the features of the final product. A direct aqueous extraction is preferable to the organic ones, especially because the company's food supplement, that will contain the extract, is aimed to infants.
The powder or the raw plant of each product (3 g) was extracted with 60 ml of 50% ethanolic solution. The samples pH was adjusted to 2.5. Samples were subjected to magnetic stirring for 3h keeping on ice and protecting from light, to avoid oxidation during preparation, followed by centrifugation at 6000 rpm for 10 min. The precipitate was separated from the supernatant. The same procedure was repeated three times and the supernatants of each sample were collected and filtered through 0,45 and 0,22 μM Whatman cellulose filters. The filtrate was concentrated in a rotary evaporator at a temperature lower of 30 °C and submitted to freeze drying. The dry extracts were kept at −20 °C for subsequent investigations or stored at 4 C° protected from light for immediately use. They were resuspended in bacteria media, Mueller Hinton, in order to determine their antimicrobial activity on *S.Aureus* and *E.Coli* at different concentrations.

The drugs used were extracted according to the procedures described above, which confirmed the efficiency and yield of the methodology.

3.3.1.2 MIC and MBC determination

In order to identify their antibacterial activity, the sensitivity to these compounds of the Gram-positive microorganism *Staphylococcus Aureus* (strain ATCC 25923) and the Gram-negative *Escherichia Coli* (strain ATCC 25922) was evaluated, through the determination of the MIC (minimum concentration of compound inhibiting the growth of the microorganism) and MBC (minimum bactericidal concentration).

The bacterial medium used in setting up the MIC was Mueller-Hinton (MH) broth for both bacterial strains. The broth is prepared as indicated by the manufacturer and the pH must be between 7.2 and 7.4 at room temperature (+25 °C). Subsequently, the broth is autoclaved and brought to +4 °C overnight or kept in an ice bath if used the same day.

Each extract, in the form of powder, was weighed and dissolved in MH so that it had a concentration equal to 60 mg/mL. Subsequently the mother solutions were filtered using cellulose acetate filters with pore diameter of 0.22 μm (Millipore, Italy) to ensure their microbiological sterility and stored at –80 °C until their use.

The bacterial strains, previously stored at –80 °C, were taken from their respective stabs and resuspended in 5 ml of Tryptic Soy Broth (TSB), the ideal culture medium for both strains of interest. The inoculates were placed overnight at +37 °C, in order to reach a density of 0.5 Mc Farland ($\simeq$ $1X10^8$ CFU, colony forming units per ml). Each suspension was also further diluted 1:100 in a sterile Mueller Hinton Broth to reach the appropriate inoculum concentration ($\simeq 10^6$ CFU/ml).
The preparation of the MIC was carried out using the broth microdilution method. Multiwell-96 (Falcon) plates were used. 100 μl of MH broth was added to each well of the plate. A volume of 100 μl of stock solution was added to one of the wells, with a final concentration of 15 mg/ml. Starting from this well, base two serial dilutions were performed, obtaining wells having a concentration range between 15 mg/ml and 0.45 mg/ml. Finally, wells were prepared in which no extract was added (negative control) and others in which the antibiotic Gentamicin was added (positive control); 100 μl of the bacterial suspension was added to each well. The plates thus prepared were incubated at 37 ° C for 18-20 hours in aerobic conditions.

The multiwells' measurement was performed using the EnVision 2105 MultiPlate Reader. MIC is considered to be the lowest concentration of a substance capable of inhibiting the visible growth of the microorganism. The graphs shown below show the inhibition of bacterial growth as the concentration of the extract of interest increases. The inhibition of the microorganism is represented by the absorbance value (in the absence of extracts and antibiotic the turbidity and the absorbance value of the well increase, highlighting the growth of the microorganism; vice versa, a non-increase in OD indicates an inhibition of bacterial growth or in some cases the bactericidal action of the extract).

The determination of the MIC does not allow to distinguish between the bacteriostatic and bactericidal action of a substance. For this purpose, at the end of each experiment 10 µl of bacterial culture were taken from each of the wells and transferred to Petri dishes without extract and containing Tryptic Soy Agar (TSA), an optimal solid culture medium for the growth of *S. Aureus* and by *E.coli.* Plates are incubated at 37 °C overnight. The next day, the possible absence of bacterial colonies establishes the bactericidal nature of the extract or confirms its purely bacteriostatic nature.

3.3.2 Results

I. Results of the antimicrobial potential of vegetable extracts on *S.Aureus*

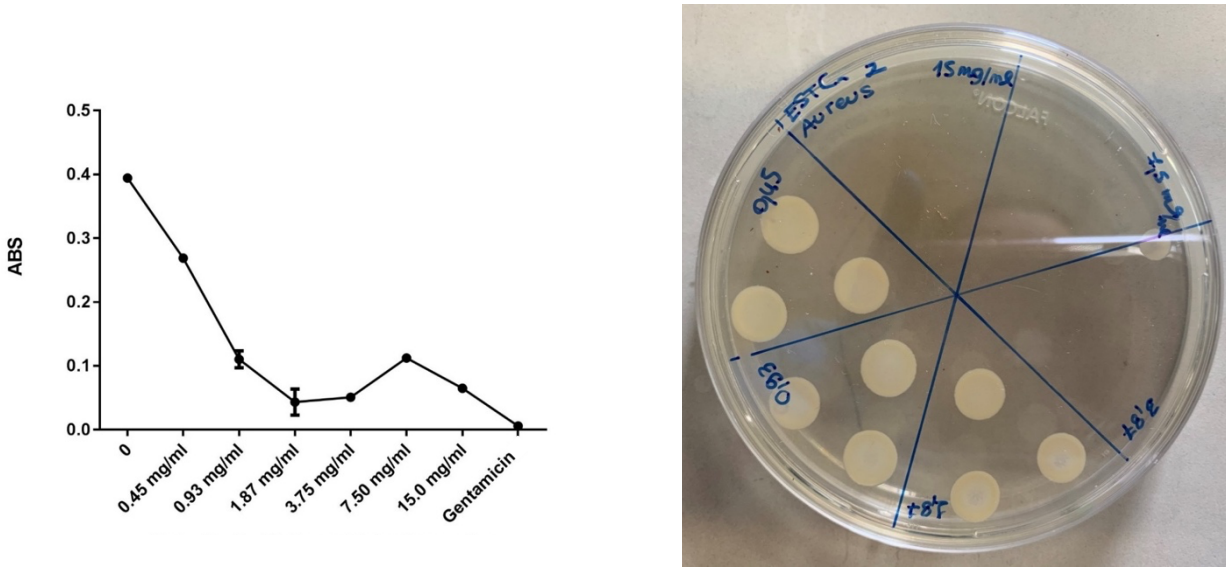


Figure 3.1. *Melissa officinalis* - powder shows a MIC of 1.87 mg/ml and at this concentration it is bacteriostatic. In fact, the bacterium resumes growth once it is plated in the absence of the extract. The trend is similar to that found when treating bacteria with the antibiotic.

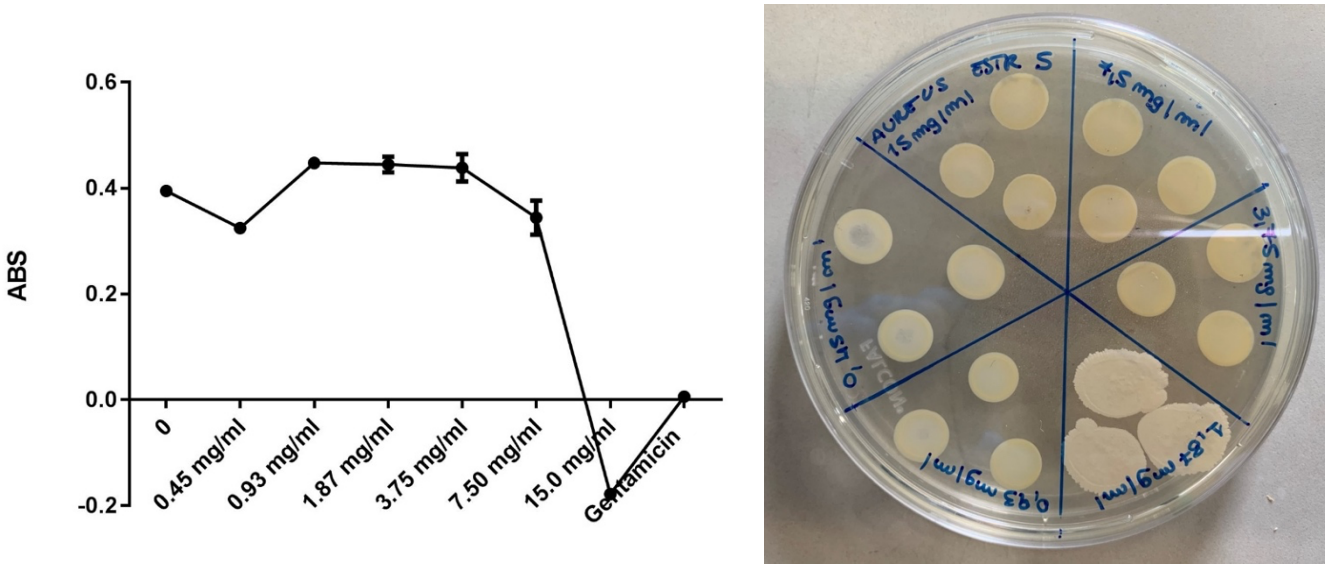


Figure 3.2. *Matricaria chamomilla* has a MIC of 15 mg/ml, this value is due to a bacteriostatic action of the extract.

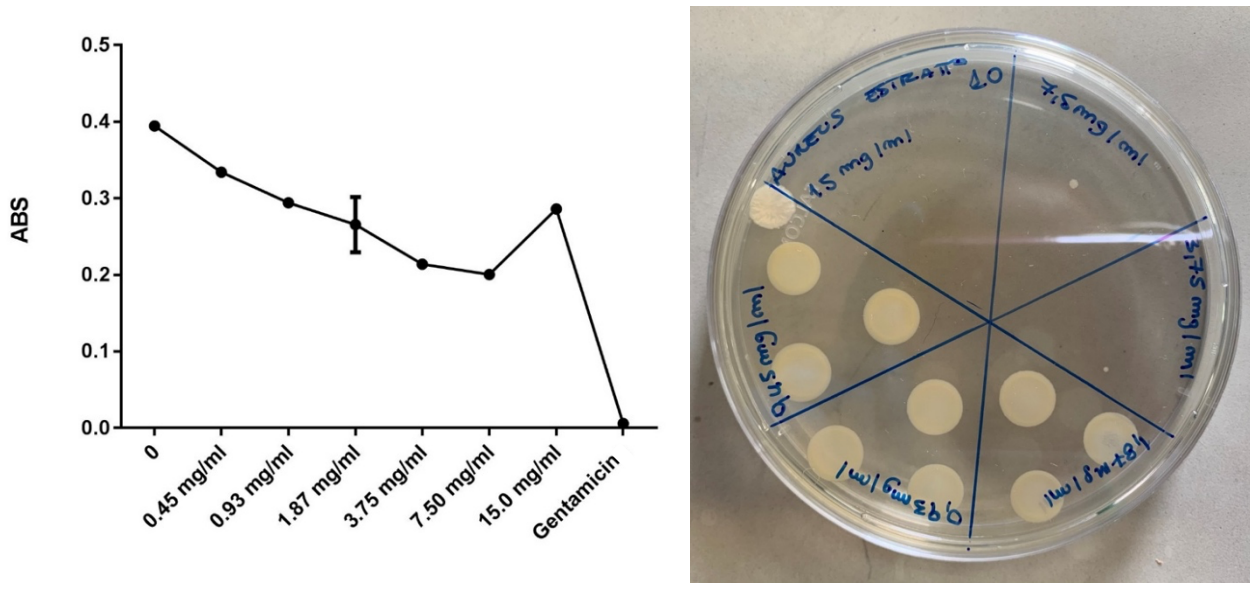


Figure 3.3. *Mentha spicata* shows a MIC of 7.5 mg/ml and is bacteriostatic at this concentration.

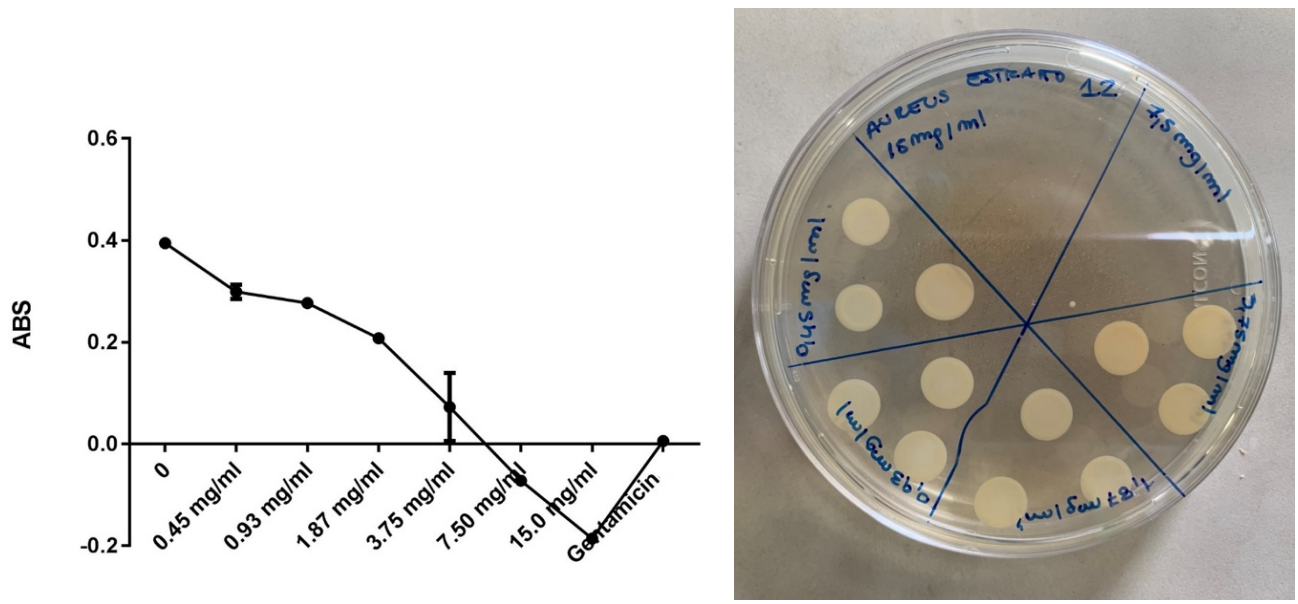


Figure 3.4. *Melissa officinalis* - raw plant shows a MIC of 3.75 mg/ml and is bacteriostatic at this concentration.

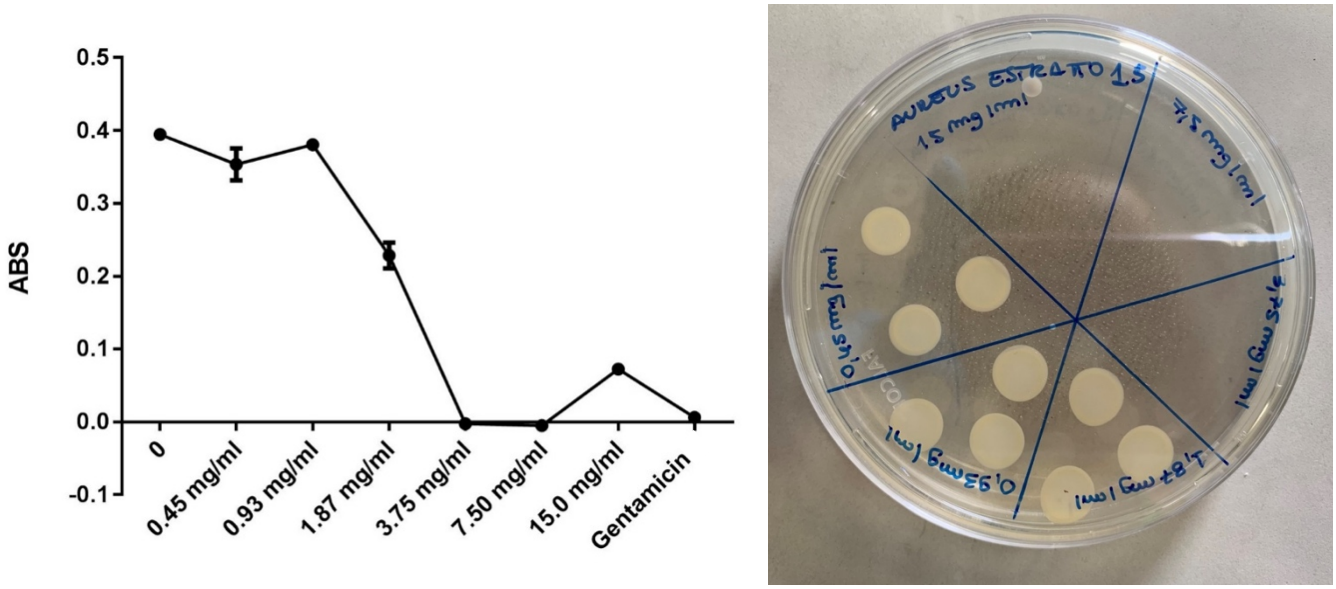
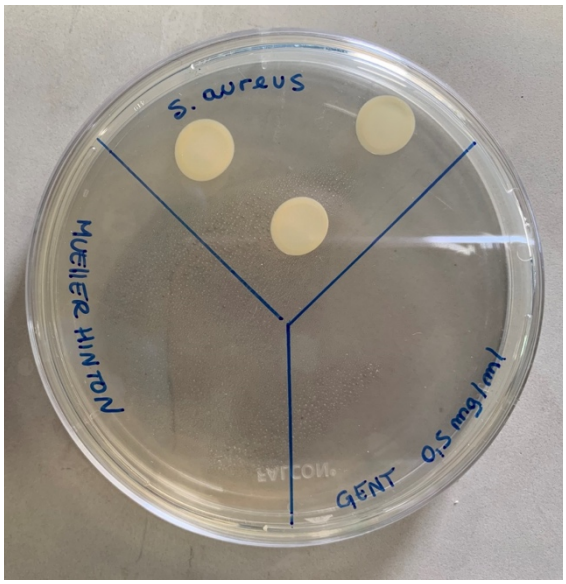


Figure 3.5. The only extract that shows bactericidal action is *Mentha sylvestris* to which a MIC of 3.75 mg/ml corresponds to a bactericidal action.



The results obtained are summarized in **Table 3.1.**

HERBAL EXTRACT	MIC (mg/ml)	MBC (mg/ml)
MELISSA OFFICINALIS POWDER	1.87	> 3.75
MATRICARIA CHAMOMILLA	15	> 15
MENTHA SPICATA	7.5	> 7.5
MELISSA OFFICINALIS RAW PLANT	3.75	> 3.75
MENTHA SYLVESTRIS	3.75	> 1.87

Table 3.1. The table shows the extracts used, the MIC and MBC expressed in mg/ml determined for each.

II. Results of the antimicrobial potential of vegetable extracts on *E.coli*

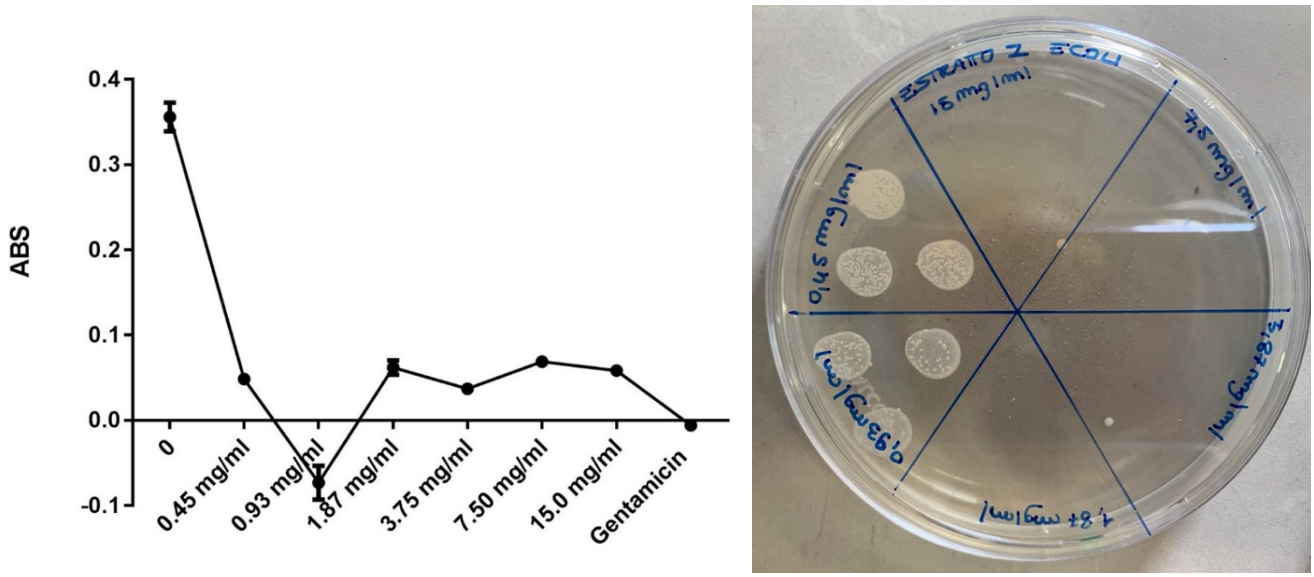


Figure 3.6. *Melissa officinalis* - powder shows a MIC of 0.93 mg/ml and is bacteriostatic at this concentration

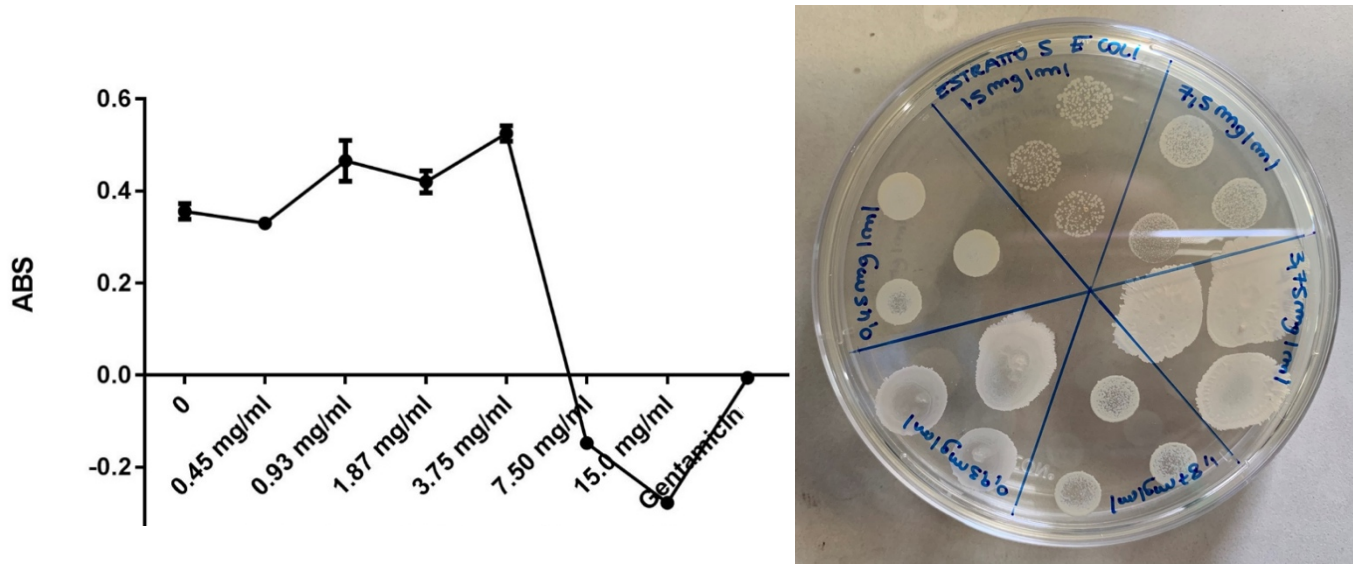


Figure 3.7. *Matricaria chamomilla* has a MIC of 7.5 mg/ml and is bacteriostatic at this concentration

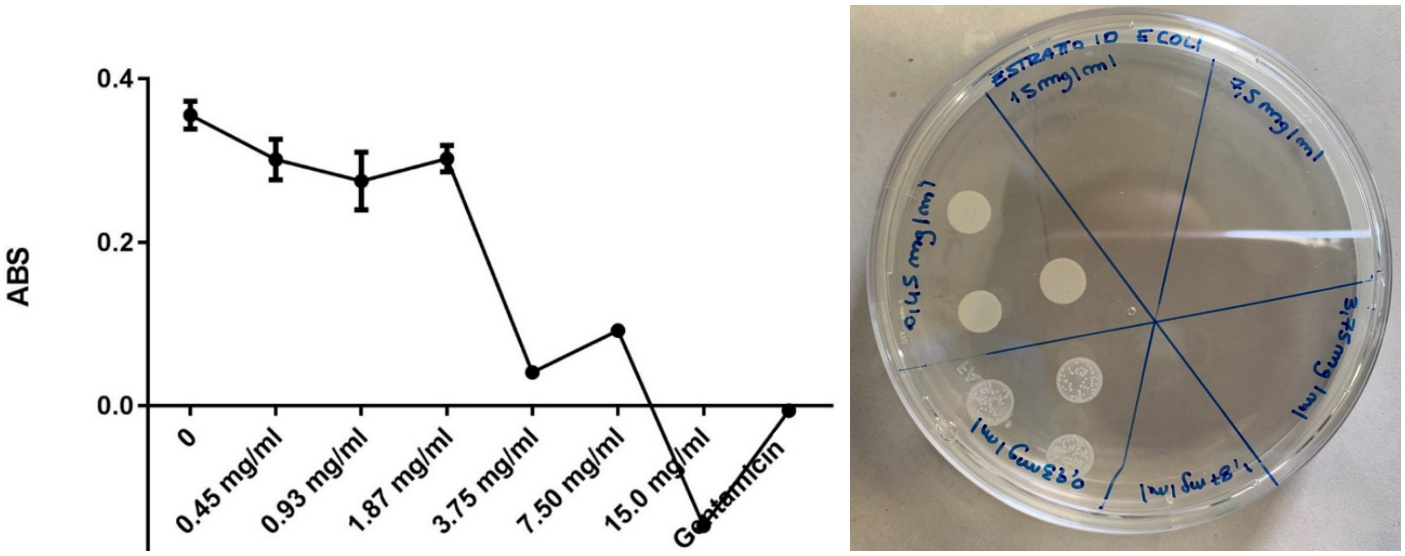


Figure 3.8. *Mentha spicata* shows a MIC of 0.93 mg/ml and at this concentration it is bacteriostatic.

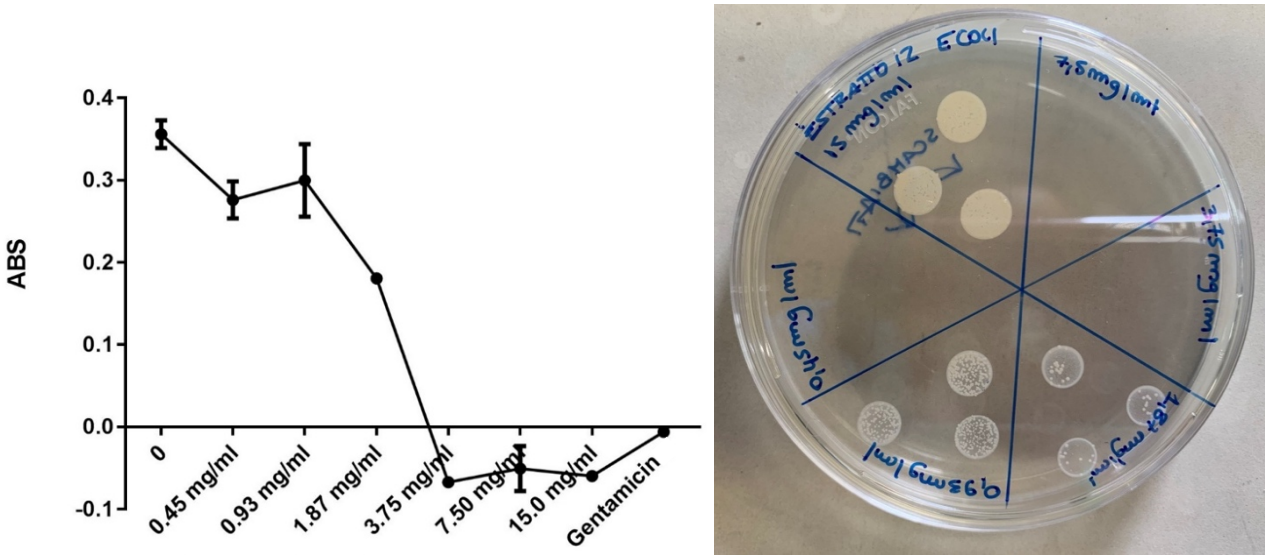


Figure 3.9. *Melissa officinalis* shows a MIC of 1.87 mg/ml and is bacteriostatic at this concentration.

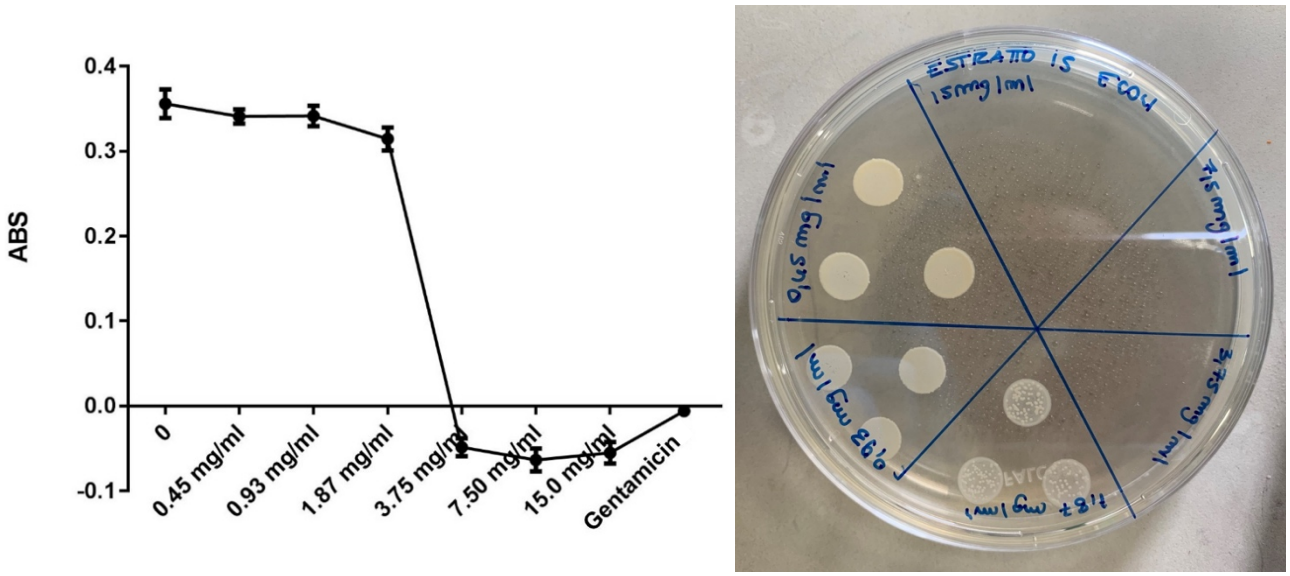
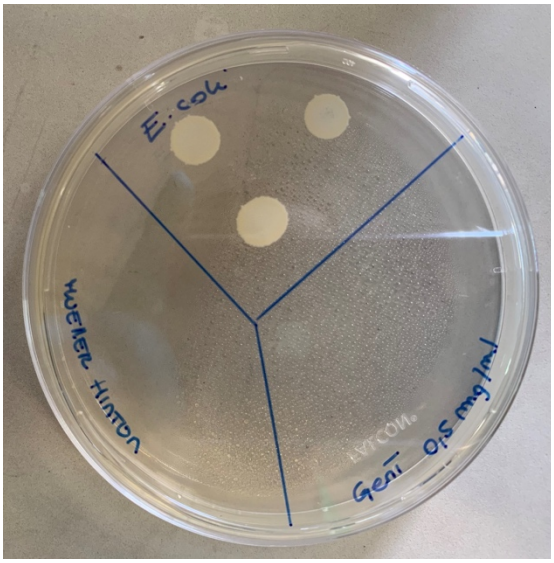


Figure 3.10. *Mentha sylvestris* shows a MIC of 3.75 mg/ml and at this concentration it is bactericidal.



The results obtained are summarized in **Table 3.2**.

HERBAL EXTRACT	MIC (mg/ml)	MBC (mg/ml)
MELISSA OFFICINALIS POWDER	0.93	> 0.93
MATRICARIA CHAMOMILLA	7.5	> 15
MENTHA SPICATA	0.93	> 0.93
MELISSA OFFICINALIS RAW PLANT	1.87	> 3.75
MENTHA SYLVESTRIS	3.75	> 1.87

Table 3.2. The table shows the extracts used, the MIC and MBC expressed in mg/ml determined for each extracts.

III. Results of DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) assay

The antimicrobial activity of the natural extracts tested turns out to be different. Reasonably, this difference is due to the different composition and chemical nature of the extracts. However, to rule out if the difference in antimicrobial activity was reflecting a mere difference in extraction yield of bioactive molecules, we decided to perform a DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) test to correlate the antioxidant potentials of the five extracts. These potentials will allow for a better comparison of their antimicrobial activities.

The procedure is very simple, efficient, inexpensive, and quick and require a UV–Vis spectrophotometer. DPPH method was developed by Blois and modified by Brand Williams et al. to produce the current widely used form. DPPH is a stable free radical which possesses a deep purple color and a strong absorption around 517 nm. The antioxidant compounds present in the medium convert DPPH radical to a more stable DPPH molecular product by donating an electron or a hydrogen atom. The color change from purple of DPPH radical to pale yellow of reduced form of DPPH allows the spectrophotometric determination of the antioxidant activity. The results are either expressed as SC50 (otherwise called the IC50 value), the concentration of the antioxidant causing 50% DPPH scavenging or as % scavenging of DPPH at a fixed antioxidant concentration for all the samples. Precisely, the inhibition ratio (%) was obtained from the following equation:

$$\text{Inhibition ratio (\%)} = (A1 - A2) \times 100/A1$$

where A1 is the absorbance of the addition of ethanol instead of testing sample and A2 is the absorbance of testing sample solution.

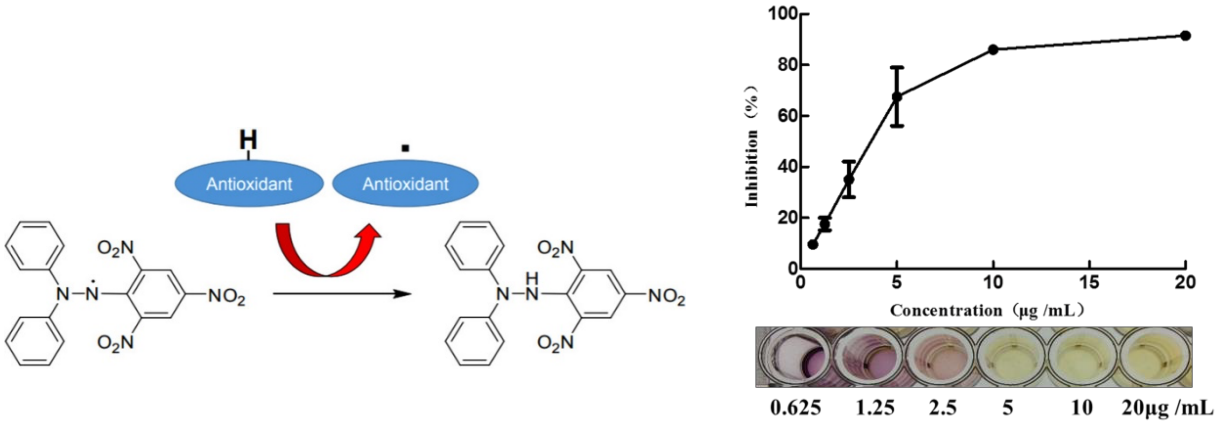


Figure 3.11. Reaction between DPPH and antioxidant to form DPPH. The color change from the purple of DPPH radicals to the pale yellow of its reduced form allows the spectrophotometric determination of antioxidant activity.

DRUGS	% ARA	DEV.ST
MELISSA OFFICINALIS POWDER	74.58	0.85
MATRICARIA CHAMOMILLA	12.06	3.62
MENTHA SPICATA	52.58	2.73
MELISSA OFFICINALIS RAW PLANT	67.23	1.76
MENTHA SYLVESTRIS	40.08	3.71

Table 3.3. The table shows the extracts used, ARA (Antiradical activity) % ± SD determined for each extracts.

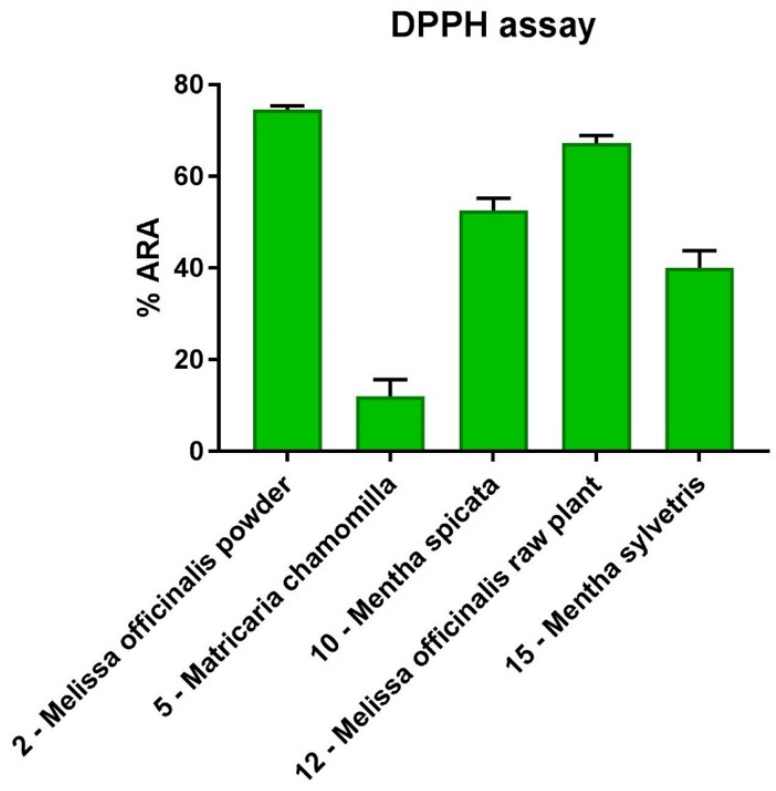


Figure 3.12. DPPH assay of herbal extracts. Bar graph shows comparison of antiradical activity (%) between *Melissa officinalis* powder, *Matricaria chamomilla*, *Mentha spicata*, *Melissa officinalis* raw plant, *Mentha sylvestris*.

3.4 Conclusions

The antimicrobial activity of an extract can be attributed to a single molecule or to a set of them. One of the possible mechanisms of action of those we tested could consist of their interference with bacterial membranes. The extracted biomolecules, by virtue of their hydrophobicity, could penetrate and interfere with the bacterium's membrane, altering its structure and functionality.

This mechanism of action would explain the greater inhibitory activity that the extracts showed towards the Gram-negative microorganism (*E.Coli*) compared to the Gram-positive (*S. Aureus*). The greater thickness of the peptidoglycan, in the latter, slows the entry of hydrophobic substances into the cell itself, protecting the bacterium.

The tested extracts, in addition to altering the cell wall structure, could interfere with the functionality of the cell wall proteins, inhibiting the transport processes of essential compounds for the cell. Although the data in this regard suggest various hypotheses on the mechanisms of action of natural extracts, we do not yet have data available on their mechanism of action.

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CHAPTER 4

Setting-up of biological platforms to identify the pro-metabolic properties of phytocomplexes

4. Experimental pipelines to identify nutraceuticals endowed with pro-metabolic effects

In recent years, epidemiological studies have shown that food has a very powerful mean for maintaining a well-being status and for health prevention [1]. These studies have consistently shown that dietary habit is one of the most important determinants for chronic diseases such as cardiovascular diseases, type-II diabetes, neurodegenerative diseases, and several types of cancer (e.g., gastrointestinal cancer). Food and dietary habits have thus a direct impact on health and diseases.

Nutrients or bioactive compounds can precisely target and control numerous aspects of the mechanism underlying the pathology itself. Phytocomplexes are groups of molecules contained in plants that often have a positive biological value that justifies their use as ingredients in food supplements or drug formulations. Their potential lies in the fact that these organic compounds bind to the same target as drugs.

Identifying the molecular mechanism of nutraceuticals is notoriously difficult. They are characterized by a mixture of hundreds of different metabolites. The attempt to identify the molecular mechanism of their components individually can be misleading, since these components, used as pure molecules, usually show a reduced or even opposite activity compared to the entire phytocomplex [2][3].Their activity seems indeed the result of a synergism between its components [4][5].

In this scenario, it would be advisable setting new and harmonized approaches to probe any change in metabolism accompanying drug treatments and provide invaluable insights in the mechanism of action of complex mixtures and phytocomplexes.

4.1 Dysmetabolic diseases

Metabolic syndrome (MS) is defined by a collection of interconnected physiological, clinical, and metabolic factors that directly rises the risk of non-communicable diseases such as cardiovascular disease and type 2 diabetes mellitus, and indirectly mortality, as a consequence of complex interplay between genetic and environmental factors [6].

Insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypercoagulable state, and chronic stress are the several factors which constitute the syndrome. This collection of unhealthy body measurements and abnormal laboratory parameters include atherogenic dyslipidemia, hypertension, glucose intolerance, proinflammatory state, and a prothrombotic state. Higher socioeconomic status, sedentary lifestyle, and high body mass index (BMI) significantly associate with MS. Insulin resistance seems to act a critical role in the etiology of the metabolic syndrome (**Figure 4.1**) [7][8].
The prevalence of MS varies around the world and, recently, due to the exponential increase in obesity worldwide, has assumed significant importance. MS affects a fifth or more of the population of the USA and about a quarter of the population of Europe. South-east Asia has a lower prevalence of MS but is rapidly moving towards rates similar to the western world [9].

The management of MS is complex and negatively affects prognosis, survival and quality of life of patients. Despite the undoubted progress that has occurred over the last few years both in diagnostic and therapeutic terms, the morbidity and mortality associated with this clinical condition remain high worldwide. Pharmaceutical therapies are aimed at treating the individual components of MS and usually include antihypertensives, statins, and metformin, but they are limited by various factors. Firstly, there is a considerable number of patients who do not respond (or who do not adhere to) the prescribed drug therapy. Secondly, the chronic nature of the components of MS warrants prolonged and often indefinite use of various medications such as statins, leading to an increased burden of drug-related adverse effects and patient noncompliance.

In particular, in MS, insulin resistance appears to be a major abnormality that may be only partially handled by drug treatments. Insulin resistance may of course translate into altered body structure, elevated blood pressure and other metabolic and non-metabolic changes [10].

Insulin resistance-mediated increase in circulating free fatty acids (FFAs) is believed to play a pivotal role in the pathogenesis of MS. Insulin increases glucose uptake in muscle and liver, inhibiting lipolysis and hepatic gluconeogenesis and leading to an increase in circulating FFAs that further reduces the antilipolytic effect of insulin. FFAs inhibit protein kinase activation in the muscle leading to reduced glucose uptake. They increase protein kinase activation in the liver that promotes gluconeogenesis and lipogenesis. The outcome is a hyperinsulinemic state to maintain euglycemia. Eventually, the compensation fails and insulin secretion decreases. FFAs are also lipotoxic to beta cells of the pancreas causing decreased insulin secretion [11].

Insulin resistance contributes to the development of hypertension due to loss of the vasodilator effect of insulin and vasoconstriction caused by FFAs [12] increasing serum viscosity and releases of pro-inflammatory cytokines from the adipose tissue that contribute to increased risk of CVD [13]. Additional mechanisms include increased sympathetic activation and sodium reabsorption in the kidneys.

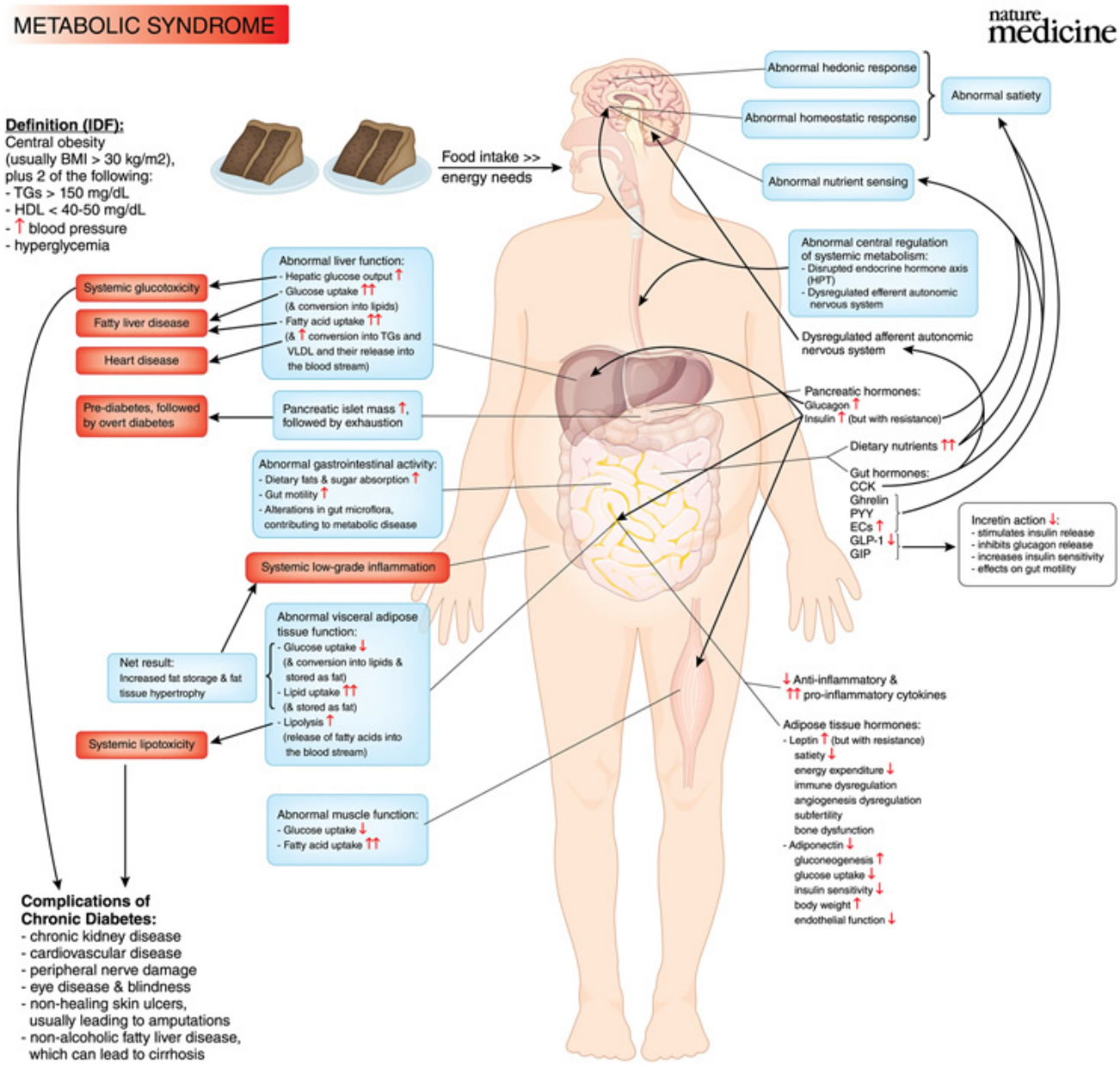


Figure 4.1. Metabolic syndrome (MS) is a multifactorial chronic disease that includes hypertension, central obesity, insulin resistance, and atherogenic dyslipidemia, and is strongly associated with an increased risk of developing non-communicable diseases such as diabetes, atherosclerotic and nonatherosclerotic cardiovascular disease (CVD)

4.2 Use of nutraceuticals in dysmetabolic diseases

The very high number of carriers of MS makes it mandatory to achieve treatment by more widely available nutritional approaches [14]. Some natural compounds and dietary elements have been shown to have some benefit in the treatment of MS [8].
The use of nutraceuticals and functional foods endowed with sound scientific evidence of efficacy would help reducing the use of medicines and concomitantly decreasing their side effects. In this respect, the metabolic syndrome offers nutrition and pharma to merge and have mutual therapeutic goals.

In this context, various natural compounds derived from plant extracts, spices, herbs, and essential oils have demonstrable benefit in the management of patients with MS [15][16][17].
Even if, the benefits of these nutraceuticals are still under investigation, they are readily available and with minimal side effects may represent an area of promise in the development of novel therapies[18][19][20].

Some of these nutraceuticals include [21] (**Figure 4.2**):

- **Curcumin**: the active ingredient, diferuloylmethane, has anti- inflammatory and anti-oxidant properties. Curcumin also inhibits the Wnt/ß-catenin pathway, which is involved in the r development of obesity and activate peroxisome activator receptor gamma in hepatic stellate cells which improves insulin resistance. Curcumin is also known to interrupt leptin signaling, thus increasing adiponectin expressions. Adequate adiponectin levels improve insulin resistance, thus improving glucose intolerance. It also has anti- inflammatory and antithrombogenic properties. The anti-inflammatory, anti-thrombogenic, insulin sensitizing and the negative effect on obesity will impact positively on all components of MS [22].

- **Cinnamon**: Cinnamon extracts and polyphenol have anti-thrombotic, insulin sensitizing, lipid lowering, anti-inflammatory and anti-oxidants properties which are beneficial in the management of MS. Cinnamon polyphenols have insulin-like activity and several studies have reported improvement in glycemic control and lipid levels [23]. All the mechanistic pathways for this benefit in MS is yet to be elucidated, however, some studies in mouse models indicate that cinnamon extracts can regulate adipocyte gene expression to improve glucose transporter (GLUT 4) and insulin signaling [17].

- **Berberine**: it acts through up-regulation of genes involved in energy utilization and down regulation of genes involved in lipogenesis [24]. It has insulin sensitivity action similar to metformin and thiazolidinediones, mediated by adenosine monophosphate associated protein kinase activation in adipocytes.

- **Resveratrol**: it is a polyphenol present in plants such as grapes, nuts, and derivatives such as wine. It is a regulator of the Sirtuin pathway, which regulates several cellular functions related to metabolism, oxidation and aging. It has favorable effects on cellular energy homeostasis, such as decreasing adipogenesis and increasing lipolysis through multiple mechanisms. It also inhibits cyclo-oxygenase with resultant anti- oxidant effects. The use of Resveratrol in MS has been studied in animal models as well as humans. Clinical studies in patients with insulin resistance and non-alcoholic fatty liver disease has shown promising results. Its use has been shown to improve insulin sensitivity, glucose tolerance and overall weight and body mass index in patients with MS [25][26].

- **Quercetin**: a plant derived from flavonoid found in vegetable and fruits such as onions, berries, and teas have been reported to have anti-oxidant and anti- inflammatory metabolic effects. Quercetin acts via the mitochondria pathways and is involved in adipokinesis and lipolysis that affect the development of obesity [20][27].

- **Omega-3-fatty acids**: have been shown to inhibit lipogenesis and induce fatty acid oxidation in liver and adipose tissue via regulation of key transcriptional factors such as peroxisome activated receptors and sterol regulatory element binding protein.

The use of these nutraceuticals is validated by tradition but there is a lack of modern specific assays that precede clinical trials. It is therefore necessary developing biological platforms to identify the molecular mechanism underpinning their applications. Herein, I present several experimental approaches extremely useful to identify the pro-metabolic properties of phytocomplexes, using both in vitro and in vivo strategies, aiming at the selection of molecules active either in reducing cholesterol and fatty acid biosynthesis and/or in improving glucose homeostasis and mitochondrial activity.

Nutraceuticals in metabolic syndrome.

Source	Active ingredient	Actions
Turmeric (*Curcuma longa*)	Curcumin	Anti-inflammatory, antioxidant ↑ Insulin sensitivity ↓ Obesity ↓ Leptin and ↑ adiponectin
Garlic (*Allium sativum*)	Allicin	Anti-inflammatory Antioxidant ↑ Insulin sensitivity ↓ Total cholesterol and triglycerides ↑ Adiponectin levels
Cinnamon (*Cinnamomum verum*)	Polyphenols	Antithrombotic Anti-inflammatory ↑ Insulin sensitivity Improves fasting blood glucose, blood pressure, and body composition
Rhizoma coptidis	Berberine	↑ Insulin sensitivity ↓ Body weight ↓ Triglycerides ↓ Systolic blood pressure
Neem (*Azadirachta indica*)	Neem oil	↑ Insulin secretion ↓ Postprandial hyperglycemia
Bergamot orange (*Citrus bergamia*)	Bergamot essential oil	Anti-inflammatory and antioxidant effects ↓ Lectin-like oxidized low-density lipoprotein receptor-1 expression ↓ Reactive oxygen species formation
Cumin (*Cuminum cyaminum*)	Cuminaldehyde	↓ Lipid levels ↓ Blood glucose levels

Figure 4.2. Nutraceuticals used in the treatment of metabolic syndrome

4.3 Development of an experimental platform to identify cholesterol-lowering nutraceuticals

Reduction in cholesterol blood levels represents one of the therapeutic goals to achieve in order to reduce the occurrence of cardiovascular diseases. Commonly, this goal is attempted by promoting healthy lifestyle behaviors and low-fat diets. Recently, several nutraceuticals have been shown to possess cholesterol-lowering properties and are becoming common over-the-counter products.

Despite this activity, the analysis of the molecular mechanism behind its cholesterol-lowering effect is unclear. Herein, I make use of isotope labeling and high-resolution mass spectrometry approaches as potential validated platform to highlight molecular mechanism of nutraceutical formulation exerting cholesterol-lowering properties.

4.3.1 Deuterium labeling gas chromatography-mass spectrometry (GC/MS)

Several studies have already demonstrated that polyphenolic extracts reduce total cholesterol levels both in vitro, in hepatic cultured cells, as well as in vivo, in humans affected by mild hypercholesterolemia [28][29]. However, despite the many meta-analyses describing their positive effects, data concerning their molecular mechanism of action are incomplete. Cholesterol lowering activity of polyphenols have been explained (i) by a statin-like mechanism, via inhibition of either HMG-CoA reductase, or squalene synthase (another key enzyme in cholesterol biosynthesis) or (ii) by a β-cyclodextrin-like mechanism via sequestering of lipids and avoidance of their intestinal uptake.

Liver is main provider of endogenous Cholesterol production. Cholesterol is synthesized via the combination of two acetyl-CoAs to form acetoacetyl-CoA, releasing CoA-SH in the presence of thiolase. Acetyl-CoA also condenses to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is catalyzed by HMG-CoA synthase. HMG-CoA is reduced by HMG-CoA reductase using NADPH to mevalonate, via mevalonate pathway. The rate limiting reduction of HMG-CoA to mevalonate is an important regulatory step in cholesterol synthesis that is commonly used as therapeutic target in hyperlipidemic disorders utilizing statin drugs.
Mevalonate is phosphorylated by three kinases sequentially utilizing three ATPs and is then decarboxylated to form isopentenyl diphosphate (5C). Three isopentenyl diphosphate molecule joins to form the 15C compound, farnesyl diphosphate. Two 15C molecules fuse to form 30C squalene. Squalene is then cyclized to form lanosterol. Finally, lanosterol is converted to cholesterol in 19 more reactions.

In my studies [30][31], I applied isotope labelling strategy to evaluate cholesterol-lowering properties of nutraceuticals. Deuterated water (D_2O) has been here used tracer how nutraceuticals can affect cholesterol metabolism in liver cells. During biosynthesis, (deuterated) water can be used as a substrate for enzymatic reactions in multiple pathways, leading to the formation of stable carbon-deuterium bonds that are not exchangeable with hydrogen.

At the end of the tracing experiments, chemical species endowed with molecular masses heavier (mainly $\Delta m/z$ of 2–4 Da) than those naturally occurring were found co-eluting with cholesterol and cholesterol fragments. The presence of these heavier species proves the incorporation of Deuterium in newly synthesized cholesterol and thus the possibility to follow cholesterogenesis in liver cells.

Malus Pumila Miller cv Annurca polyphenolic extract (AAE) (**I**) and Hanphyllin and Salvigenin (**II**), from *Achillea Wilhelmsii C. Koch*, were the two nutraceuticals tested.

(**I**) AAE: tested in clinical trials, the oral consumption of AAE exerted a cholesterol-lowering effect similar to the statins Atorvastatin and Simvastatin. The latters are a class of cholesterol-lowering drugs also known as HMG-CoA reductase inhibitors. Statins act by inhibiting HMG-CoA reductase, the rate-limiting enzyme of the cholesterol pathway. Due to their structure similar to HMG-CoA on a molecular level, they will fit into the enzyme's active site and compete with the native substrate. On the contrary, AAE mechanism of action is not yet clear.

In order to monitor cholesterogenesis in AAE treated cells we performed D_2O labeling of *in vitro* cultured hepatoma cells, HuH7 [32]. Cells were grown in a Dulbecco Modified Eagle Medium (DMEM) (41965-039, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (10270, GIBCO), glutamine (35050-061, GIBCO), penicillin and streptomycin (15070-063, GIBCO) and sodium selenite (10 μM) in a cell culture incubator at 37 ◦C and with 5% CO_2. For D_2O labeling, 2×10^6 HuH7 cells were cultivated in a medium supplemented with 5% D_2O (Sigma Aldrich (St. Louis, MO, USA). When indicated, AAE 400 mg/L, Atorvastatin 10 μM or Simvastatin 10 μM (the two statins were here used as positive controls) were added to the cultures for 72 h, a time sufficient to allow incorporation of Deuterium atoms into de-novo synthesized sterols and fatty acids (FAs).

After labeling, lipids were extracted and derivatized with TMS to be easily visualized and quantitated by GC-MS. In particular, For GC-MS analyses, 2×10^6 HuH7 cells were scraped in ice-cold water and centrifuged at 10,000× g for 5 min at 4 ◦C. Membrane pellets were dried and dissolved in 1 mL of ice-cold dichloromethane. Insoluble material was removed by centrifugation at high speed for 10 min at 4◦ C. The supernatants were dried and resuspended in acetonitrile. Sample was solubilized in pyridine (50µL) and derivatized with 25 µL of N,O-Bis(trimethylsilyl(TMS)trifluoroacetamide (BSTFA) with a reaction time of 90 min. One µL was injected, split ratio 1:10. GC-MS analyses were carried out on a Shimadzu GCMS 2010 plus (Kyoto, Japan) with the following parameters. Injection temperature 280 ◦C, Ramp 0–1.00 min 100 ◦C, 1.00–6.00 min 100–320 ◦C, hold for 2.33 min. Column flow 1.10 mL/min Linear velocity 39 cm/s. Helium gas was used. Ion source Temp. 200 ◦C, Interface 320 ◦C, Solvent cut 5.9 min, Scan 35–600 m/z. Detector voltage 0.1 kV. Separation was performed on an Agilent (Santa Clara, CA, U.S.A.) SIL-8, 30 m × 0.25 mm, 0.25 µm.

When HuH7 cells were cultured in the presence 10 µM Simvastatin or 10 µM Atorvastatin, total intracellular cholesterol levels were reduced (Atorvastatin 0.55 ± 0.09 fold induction compared to vehicle mean ± SEM (standard error of mean), $p < 0.001$; Simvastatin 0.52 ± 0.06, $p < 0.001$) (**Figure 4.3C**). Moreover, the intensity of peaks corresponding to deuterated cholesterol molecules were all decreased, confirming, as expected, their molecular mechanism of action resulting in inhibition of de-novo cholesterol synthesis (**Figure 4.3A, B**). AAE treatment (400 mg/L) resulted in a reduction of total cholesterol levels (0.48 ± 0.05 fold, $p < 0.001$) with a potency similar to that exerted by the two statins (**Figure 4.3C**). AAE reduced as well cholesterol deuterated peaks, proving that its cholesterol lowering activity involves inhibition of de novo cholesterogenesis (**Figure 4.3A, B**).

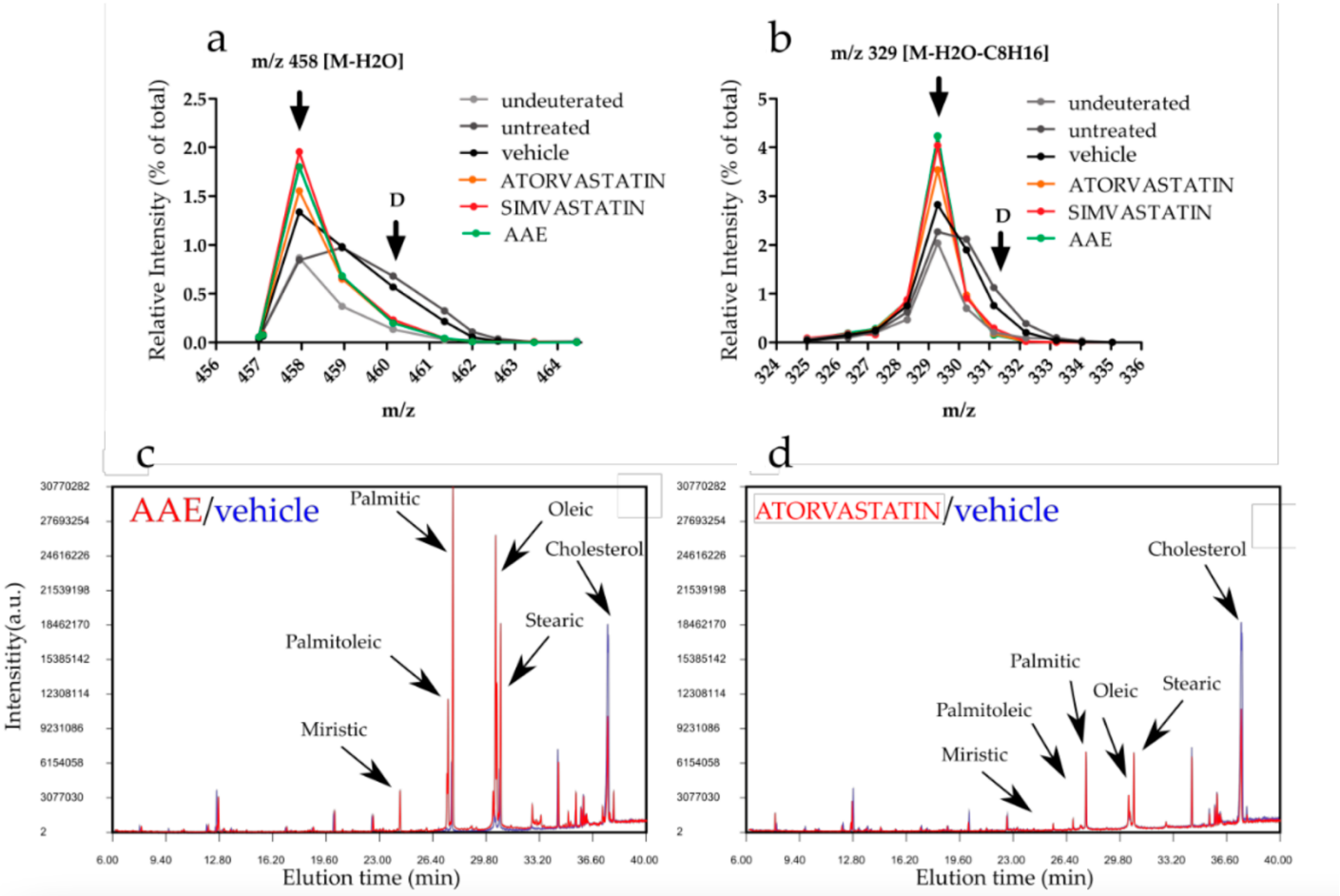


Figure 4.3. AAE inhibits cholesterogenesis in HuH7 cells. HuH7 cells were grown in the presence of D_2O and treated for 72 h in the presence of Atorvastatin (Orange dots and lines), Simvastatin (Red), Annurca polyphenolic extract (AAE (green) or the corresponding volume of vehicle (DMSO, black) or water (untreated, dark gray). HuH7 were as well grown in the absence of D_2O (undeuterated, light gray) or left untreated for comparison. (a,b) MS analysis of GC peaks eluting at 37.5 min and containing undeuterated TMS derivatized cholesterol (m/z 458 M-H20), a cholesterol fragment (m/z 329 M-H20-C8H16) and their corresponding deuterated forms (arrows labeled with letter D). (c,d) Comparison of gas chromatography-mass spectrometry (GC/MS) spectra of samples extracted from HuH7 cells treated with AAE (c, red profile), vehicle (c,d, blue profile) or Atorvastatin (d, red profile). Arrows indicated cholesterol and fatty acid over-represented in AAE treated samples.

(**II**) *Achillea Wilhelmsii* metabolites were the second group of nutraceuticals tested. To identify *A. Wilhelmsii* secondary metabolites responsible for the effects on metabolic syndrome ascribed to the plant extract, I used the same screening pipelines, aiming at the selection of molecules active either in reducing cholesterol and fatty acid biosynthesis. Briefly, *in vitro* growing hepatic HuH7 cells were labelled with D_2O and cultured in the presence of *Achillea Wilhelmsii* compounds for 72 h (**Figure 4.4**). In virtue of its activity against cholesterogenesis, simvastatin (30 μM) was used as positive control molecule. Upon extraction in the organic solvent, cellular lipids were derivatized with TMS and analyzed by GC/MS (**Figure 4.5**). Among the tested *Achillea Wilhelmsii* metabolites and similarly to simvastatin, cells treated with hanphyllin and santoflavone showed lower amount of total cholesterol compared to untreated HuH7 cells (**Figure 4.5A, B**). To highlight any effect of hanphyllin on cholesterogenesis, I measured the amount of Deuterium incorporated in newly synthesized cholesterol molecules. Chemical species endowed with molecular masses heavier than those naturally occurring (mainly $\Delta m/z$ =1–2 Da) were found co-eluting with molecular ions and their fragments. The presence of these heavier species in untreated cells indicates that cholesterogenesis was occurring in HuH7 cells (**Figure 4.5C**). As expected, when HuH7 cells were cultured in the presence of simvastatin (**Figure 4.5D**), m/z peaks corresponding to deuterated cholesterol molecules were all decreased. Treatment with hanphyllin resulted in a reduction of cholesterol deuterated peaks, proving that, such as simvastatin, its cholesterol lowering activity involves inhibition of de novo cholesterogenesis. A similar reduction in cholesterol biogenesis was obtained upon incubation with santoflavone.

Next, we used GC/MS to measure the intracellular levels of palmitic acid, one of the most abundant lipids of biological membranes, (**Figure 4.6**). In virtue of its lipogenesis inhibitory activity, apple polyphenolic extract (APE, 400 mg/L) was used as positive control. Compared to cells left untreated or treated with vehicle, incubation with hanphyllin did not exert any effect on the total amount of fatty acid present in the cell. However, we did measure a reduction of palmitic acid (**Figure 4.6A, B**) in cells treated with santoflavone and salvigenin, suggesting their effect on fatty acid homeostasis. The rate of deuterium incorporation in newly synthesized palmitic acid molecules confirmed that the palmitate lowering activity of the two flavone derivatives involves inhibition of de novo lipogenesis. Indeed, compared to cells left untreated (**Figure 4.6C**) or treated with vehicle (**Figure 4.6D**), HuH7 treated with compound salvigenin presented a reduction in the intensity of palmitic acid deuterated m/z peaks (**Figure 4.6E**).

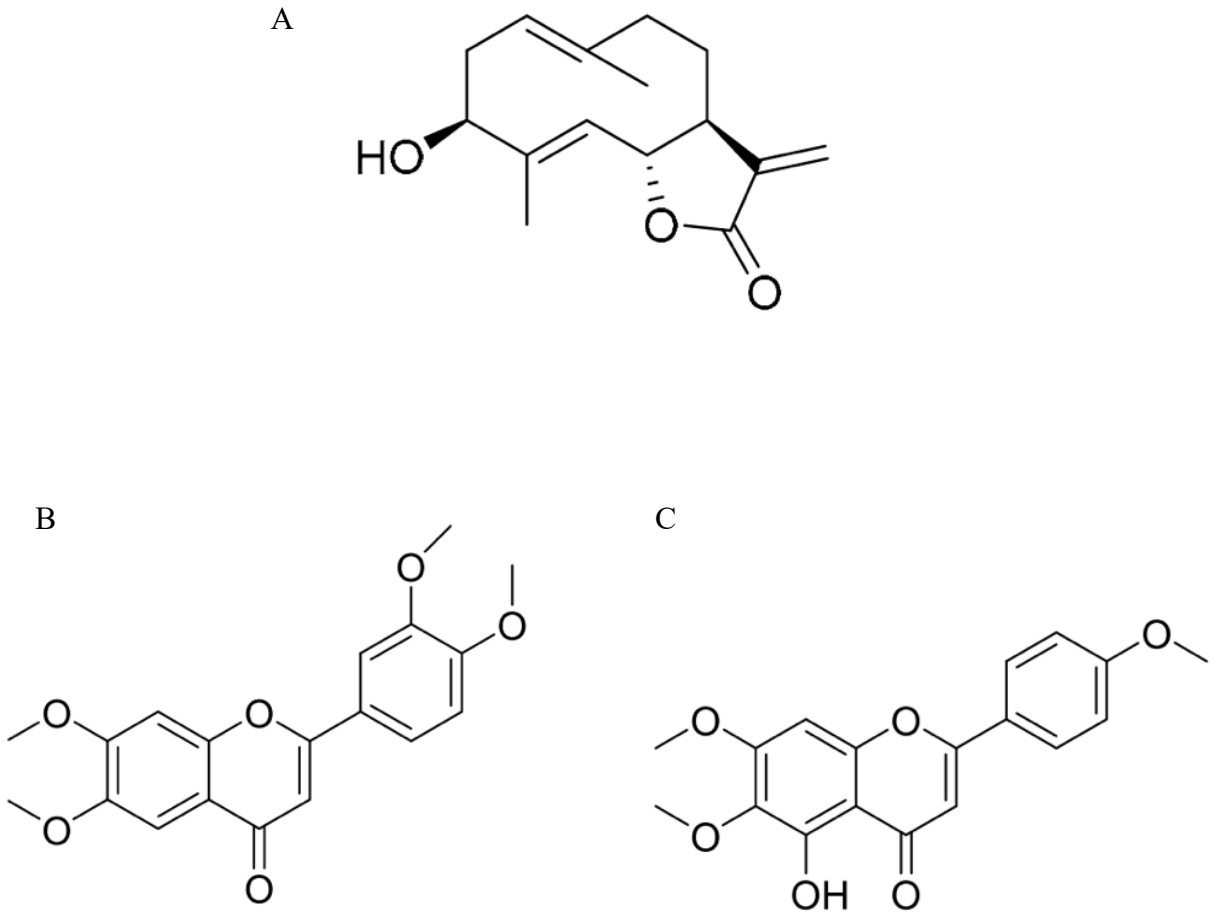


Figure 4.4. Chemical structure of (A) hanphyllin (a sesquiterpenoid constituents of *A. wilhelmsii*), (B) santoflavone and (C) salvigenin (both phenolic constituents of *A. wilhelmsii).* Hanphyllin shows a selective cholesterol-lowering activity (−12.7% at 30 μM); Santoflavone stimulates glucose uptake via the GLUT transporter (+16.2% at 30 μM), while the trimethoxylated flavone salvigenin shows a dual activity in decreasing lipid levels (−22.5% palmitic acid biosynthesis at 30 μM) and stimulating mitochondrial functionality (+15.4% at 30 μM)

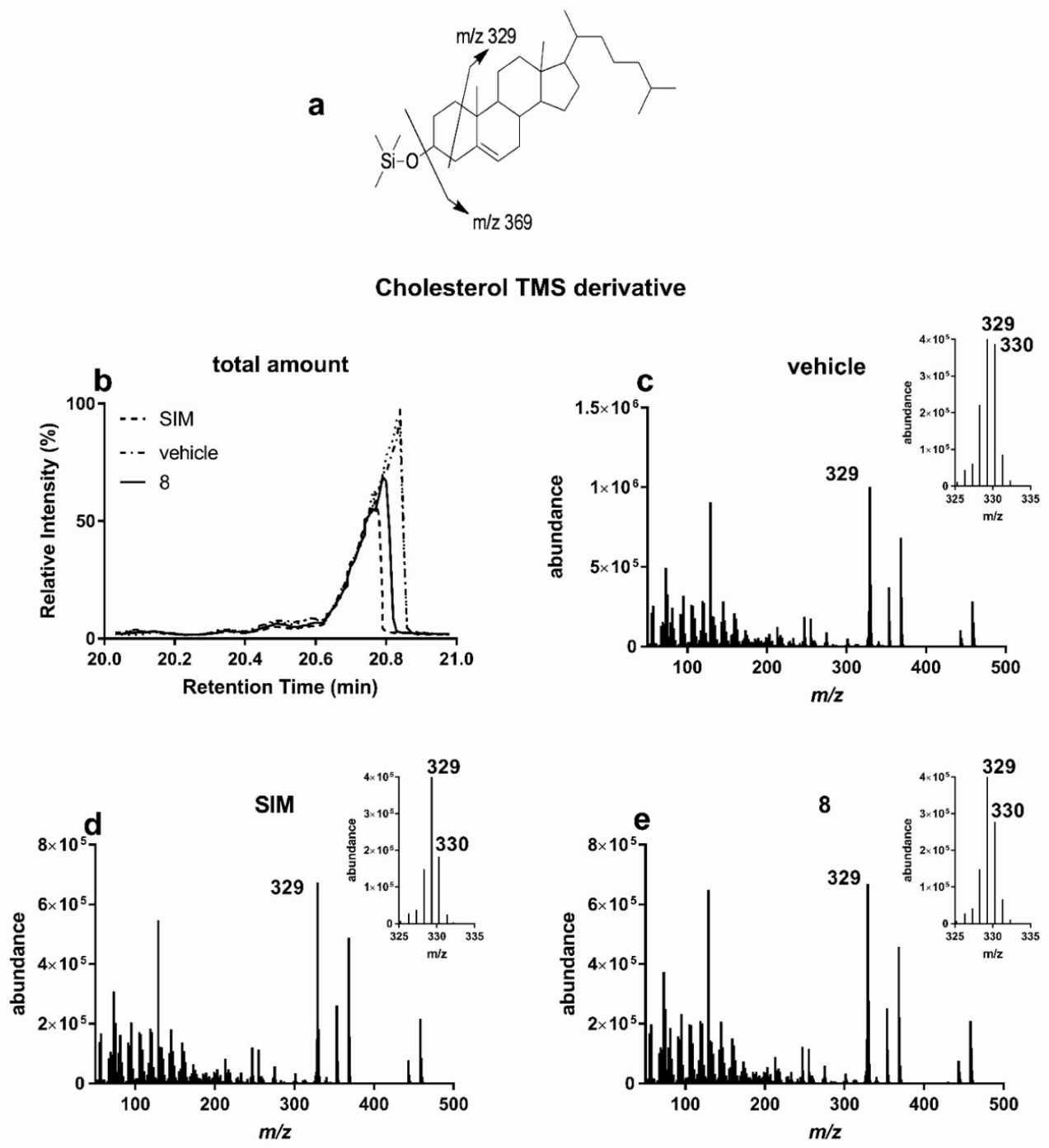


Figure 4.5. Hanphyllin inhibits cholesterogenesis in HuH7 cells. HuH7 cells were grown in the presence of D_2O and treated for 72 h in the presence of simvastatin, Hanphyllin or the corresponding volume of vehicle (DMSO). (a) fragmentation pattern of cholesterol TMS derivative; (b) comparison of gas chromatography-mass spectrometry (GC/MS) spectra (intensity versus retention time) of samples extracted from HuH7 cells treated with hanphyllin, vehicle, or simvastatin. (c–e) MS analysis of GC peaks eluting at 20.8 min and containing undeuterated TMS derivatized cholesterol (*m/z* 458 M–H_20), a cholesterol fragment (*m/z* 329 M-H_20–C_8H_{16}), and their corresponding deuterated forms (see corresponding enlarged insets). (Representative of at least three experiments).

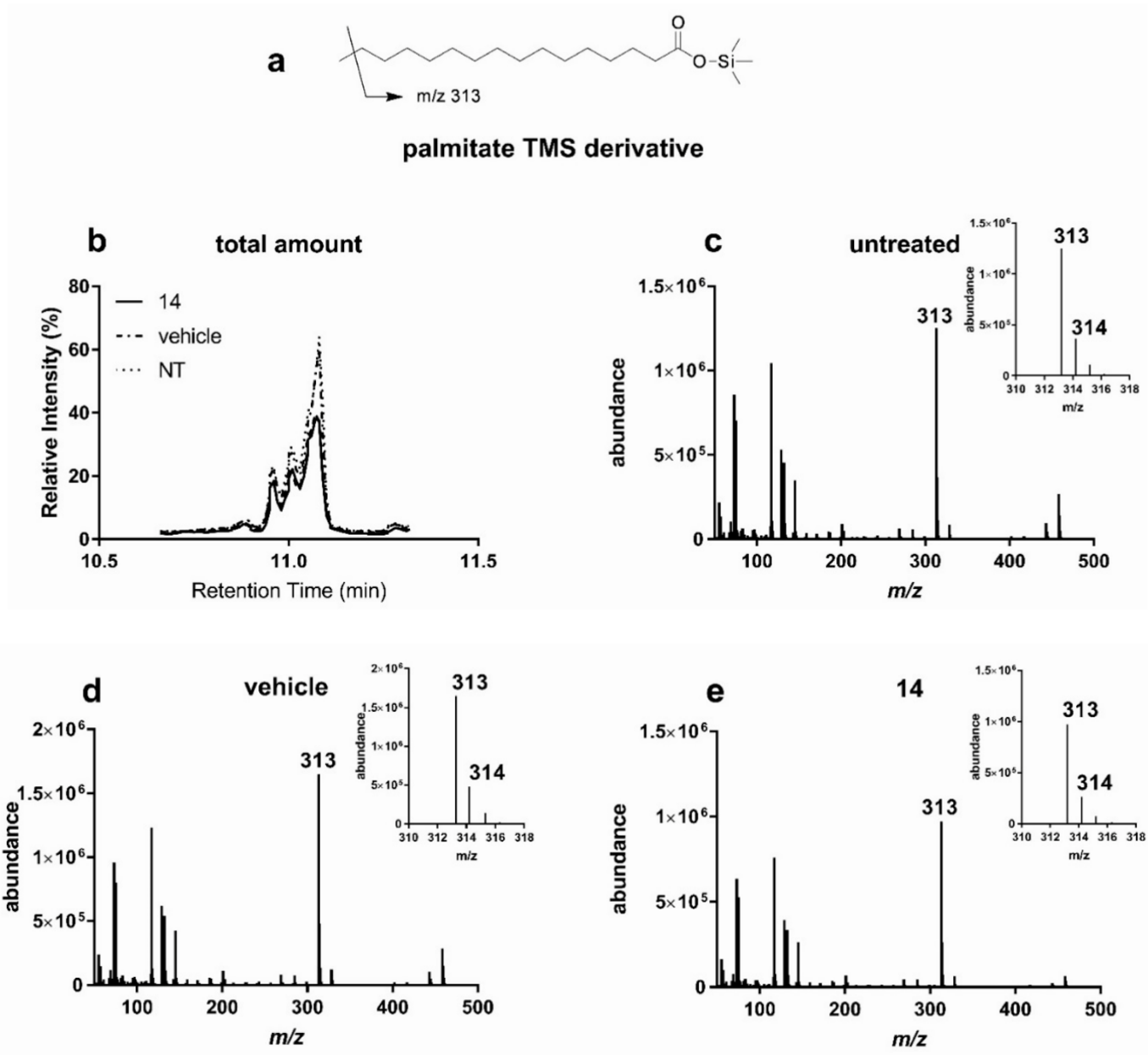


Figure 4.6. Salvigenin inhibits palmitic acid biosynthesis in HuH7 cells. HuH7 cells were grown in the presence of D_2O and treated for 72 h in the presence of salvigenin or the corresponding volume of vehicle (DMSO). (a) Fragmentation pattern of palmitic acid TMS derivative; (b) comparison of gas chromatography-mass spectrometry (GC/MS) spectra (intensity versus retention time) of samples extracted from HuH7 cells treated with salvigenin, vehicle, or left untreated. (c-e) MS analysis of GC peaks eluting at 11.1 min and containing undeuterated TMS derivatized palmitic fragment (*m/z* 313 M-CH_3), and its corresponding deuterated forms (*m/z* 314 and 315 see corresponding enlarged insets). (Representative of at least three experiments)

4.4 Development of an experimental platform to identify lipid-lowering nutraceuticals

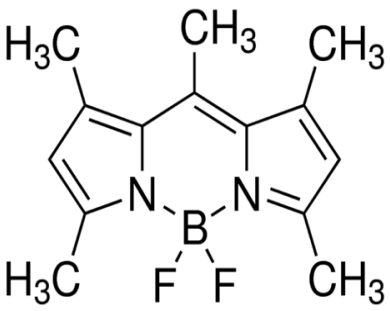
Cardiovascular diseases (CVD), particularly atherosclerotic CVD, are responsible for almost half of all non-communicable disease deaths over the past decade. A major risk factor of cardiovascular disease seems to be hyperlipidemia. Lipid-lowering therapy is one main hallmark treatment used to modulate atherosclerosis.

To identify nutraceuticals endowed with lipid-lowering potential. I used a strategy based on fluorescence staining of biological membranes with fluorescent dyes that have chemical-physical properties similar to the components of biological membranes i.e. lipophilicity and amphipathicity. Natural or synthetic lipophilic and amphipathic fluorescent molecules will spontaneously insert themselves within the biological membranes, mixing with their components and making them fluorescent.
I decided to used BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene, Thermo Fisher Scientific) which is a latest generation fluorophore and can be used as a stain for neutral lipids and as a tracer for oil and other nonpolar lipids, with its nonpolar structure and long-wavelength absorption and fluorescence (**Figure 4.7A, B**).

Briefly, cells were fixed in 3.7% formaldehyde diluted in PBS for 30 min, to be then permeabilized with 0.1% Tryton X100, stained with 2 μM BODIPY 493/503 and visualized under a fluorescence microscope. When indicated, tested compounds (30 μM), Simvastatin (12 μM) or vehicle (DMSO) were added to the cultures for 72 h.

The inhibitory effect on lipogenesis of salvigenin was also visible using immunofluorescence as well as light microscopy. HuH7 cells present intracytoplasmic lipidic droplets (containing mostly triglycerides) that appear electron-dense (**Figure 4.8A, B**) and are stained by BODIPY 493/503, a fluorescent dye staining neutral lipids such as triglycerides (**Figure 4.8C, D**). HuH7 cells treated with compound salvigenin showed a drastic reduction in the number of droplets present intracellularly, confirming the inhibitory activity of the compound toward lipogenesis.

A



B

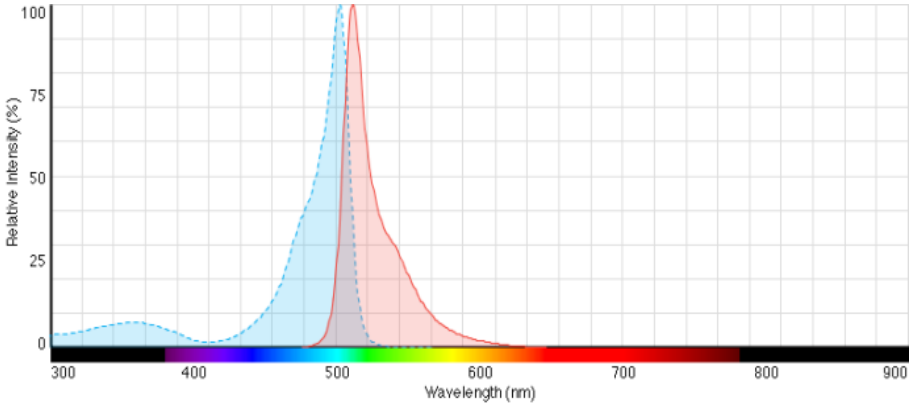


Figure 4.7. (A) Chemical structure of BODIPY 493/503. (B) Excitation and emission spectra of the probe

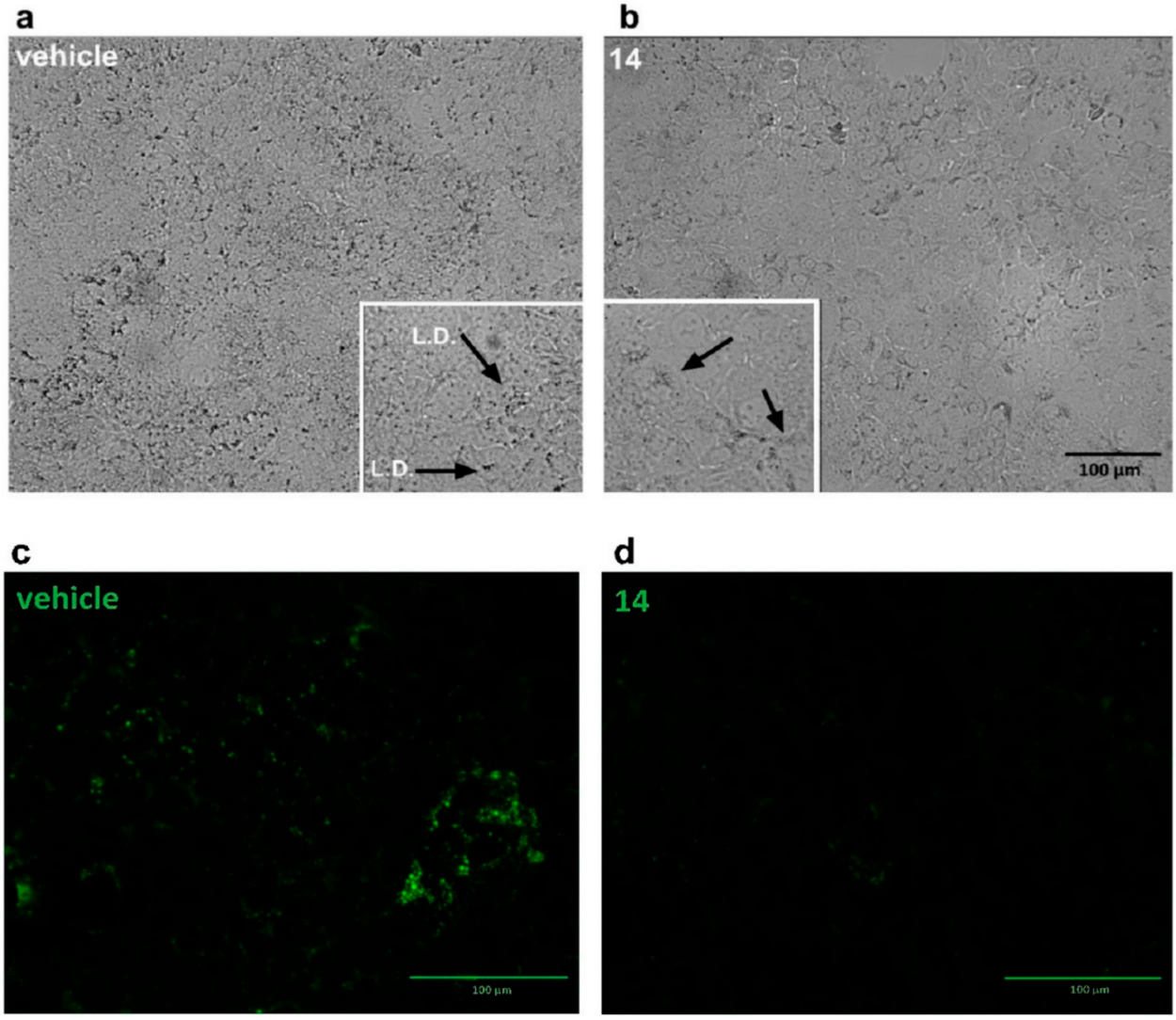


Figure 4.8. Salvigenin (14, in the images) reduces lipid droplets in HuH7 cells. HuH7 cells were grown in the presence of salvigenin (30 µM) or the corresponding volume of vehicle (DMSO). Lipid droplets, visible as electron-dense material in the cytoplasm of HuH7 cells (a,b) or upon staining with the lipid tracer BODIPY 493/503 (c,d), are reduced upon treatment with compound (see enlarged insets and cytoplasmic structures pointed by the arrows and stained by the dye). (Representative of at least three experiments). Scalebar = 100 µM

4.4.1 Direct Infusion Fourier transform-ion cyclotron resonance mass spectrometry (DI-FT-ICR-MS)

Metabolomic approaches are extremely useful tools for probing any change in metabolism accompanying drug treatments and provide invaluable insights in the mechanism of action of complex mixtures and phytocomplexes. Metabolic profiling was performed by Direct Infusion FT-ICR mass spectrometry (DI-FT-ICR-MS), which is characterized by unmatched ultra-high mass accuracy and resolution, that make it highly suitable in metabolite profiling.

Briefly, upon treatment with each nutraceutical, 2×10^6 HuH7 cells were rinsed three times in PBS to be then homogenized in 1 mL of pre-chilled methanol/water 1:1 solution containing 10 nmol of internal standard and centrifuged at 10,000× g for 10 min at 4 ◦C [33]. The resulting supernatants were collected and transferred into new Eppendorf tubes and stored at –80 ◦C. Analyses were performed in direct infusion following a previous protocol [34] employing a Hamilton syringe (250 µL) at a flow rate of 2 µL/min. Data were acquired on a SolariX XR 7T (Bruker Daltonics, Bremen, Germany). The instrument was tuned with a standard solution of sodium trifluoracetate. Mass Spectra were recorded in broadband mode in the range 100–1500 m/z, with an ion accumulation of 20 ms, with 32 scans using 2 million data points (2M). Nebulizing (N_2) and drying gases (air) were set at 1 and 4 mL/min, respectively, with a drying temperature of 200 ◦C. Both positive and negative ESI ionizations were employed. Five replicates of each injection were carried out. The instrument was controlled by Bruker FTMS Control, MS spectra were elaborated with Compass Data Analysis version 4.2 (Bruker), identification of compounds based on accurate MS measurements was performed by Compound Crawler version 3.0 and Metaboscape 3.0 (Bruker). Metabolites signals were normalized using internal standards. Comparisons and differences were analyzed for statistical significance by two-way Anova test and Bonferroni post tests analysis. All graphs, bars or lines indicate mean and error bars indicate standard error of the mean (SEM).

For the first nutraceutical tested, an Annurca Apple Extract (AAE), our metabolite profiling (**Table 1, Figure 4.9**) revealed that it significantly changed the levels of at least 38 key intracellular metabolites in HuH7 cells. The significant elevation of (i) free FAs, (ii) alpha-GPC, (iii) glucose, (iv) the increase in the intracellular level of the PPP intermediates together with (v) the reduction of the intracellular level of glutamine and GSH, all suggest that AAE stimulates glycolysis, lipolysis of membrane lipids, and their β-oxidation. Short chain acyl-carnitines produced by peroxisomal and mitochondrial β-oxidation enter into the Krebs cycle in the form of succinate and further increase mitochondrial respiration, ultimately reducing pyruvate conversion into lactate. By diverting acetyl-

CoA and citrate to the Krebs cycle, AAE inhibits anabolic reactions necessary for FAs synthesis and cholesterogenesis. This metabolite profiling of HuH7 cells allowed us to take a snapshot of some of the metabolic pathways modulated by AAE (**Figure 4.10**), a nutraceutical enriched in procyanidin B2.

Table 1. Fold induction for the indicated metabolites measured upon treatment of HuH7 with AAE.

Metabolic Pathway	Metabolite	Fold change [1]	Metabolic Pathway	Metabolite	Fold Change [1]
Glycolysis			PPP		
	Glucose	1.02 ± 0.08		Ribose 5-P	2.04 ± 0.26
	Glucose 6-P	1.81 ± 0.2		Sedoheptulose	1.24 ± 0.12
	Lactate	0.22 ± 0.10		Sedoheptulose-7P	1.93 ± 0.20
Glycogenolysis			Nucleotides		
	Maltose	1.56 ± 0.13		Xanthine	0.84 ± 0.01
Amino acids				Adenosine	0,96 ± 0.07
	Proline	1.54 ± 0.04		Cytidine	1.33 ± 0.07
	Threonine	1.71 ± 0.07		Guanosine	1.21 ± 0.09
	Glutamine	0.62 ± 0.04		Inosine	1.34 ± 0.07
	Lysine	0.97 ± 0.12		Deoxy-inosine	0.65 ± 0.04
	Histidine	1.08 ± 0.11		GSH	0.57 ± 0.02
	Cysteine	1.52 ± 0.11	β-oxidation		
	Tryptophan	1.19 ± 0.07		Propionyl-carn	0.54 ± 0.05
	Taurine	1.52 ± 0.16		Butyryl-carn	0.56 ± 0.02
	Creatine	1.60 ± 0.09		Valeryl-carn	0.50 ± 0.03
	Glutamic Acid	1.91 ± 0.05			
	Leucine	1.54 ± 0.08	Krebs cycle		
	Tyrosine	1.24 ± 0.12		Citrate	1.86 ± 0.06
	Phenylalanine	1.41 ± 0.02		Fumarate	1.32 ± 0.12
	Aspartic Acid	1.08 ± 0.06		Malate	1.06 ± 0.05
Lipids			Bile acids		
	Cholesterol	0.48 ± 0.08		CDCA	0.50 ± 0.05
	Palmitic Acid	1.78 ± 0.22			
	Palmitoleic Acid	2.45 ± 0.20			
	Stearic Acid	1.17 ± 0.22			
	Oleic Acid	2.34 ± 0.14			
	Myristic acid	1.73± 0.14			
	α - GPC	1.44 ± 0.04			

[1] (n = 5. Shown is mean ± SEM). Annurca polyphenolic extract (AAE). PPP: pentose phosphate pathway; GSH: glutathione; CDCA: chenodesoxycholic acid.

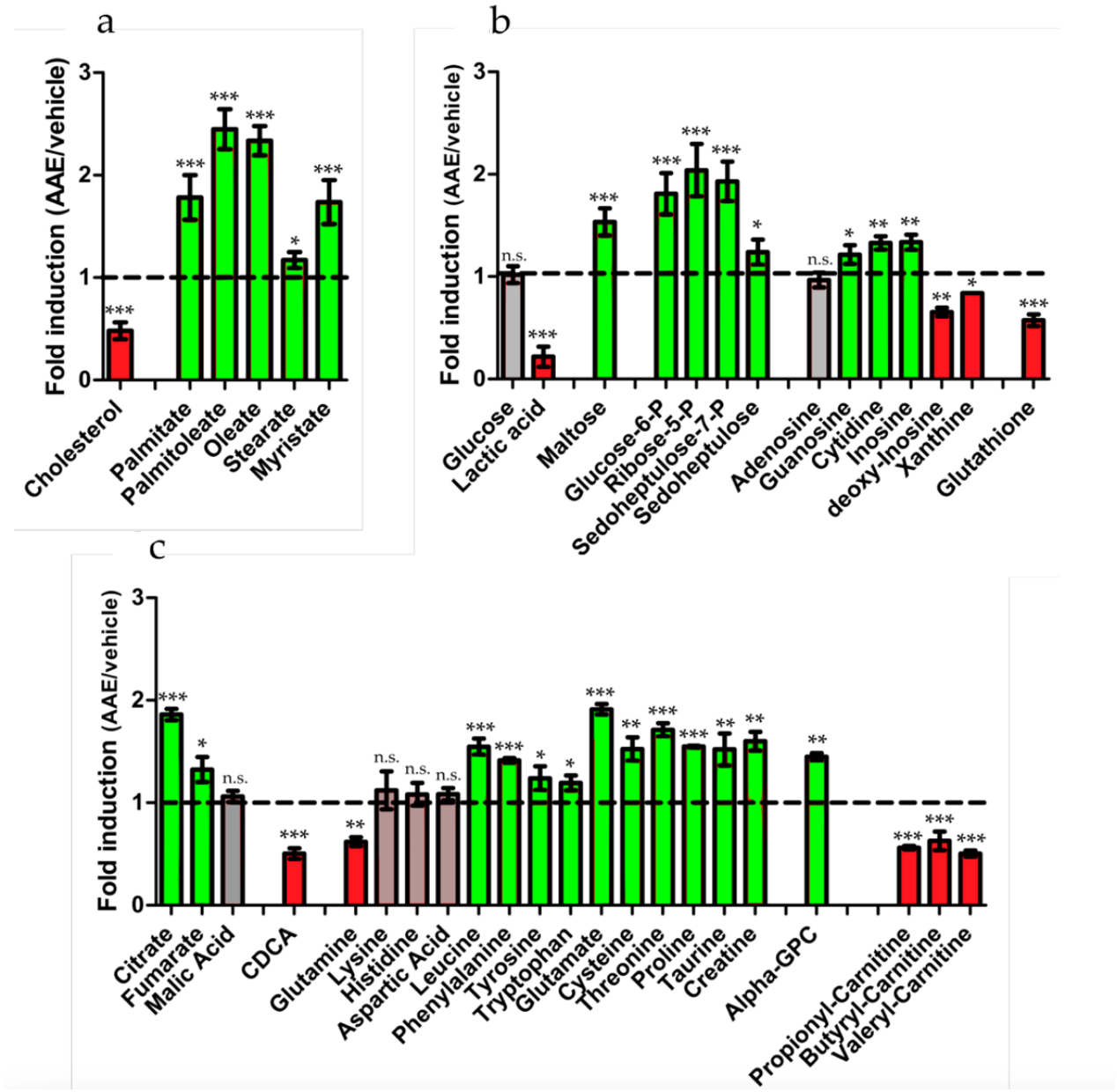


Figure 4.9. AAE diverts HuH7 metabolism from lactic fermentation toward mitochondrial respiration. Metabolomic profiling of HuH7 cells grown for 72 h in the presence of AAE (400 mg/L). Each bar represents the fold change (AAE versus vehicle) in the intracellular concentration of the indicated metabolites (n = 5 measurements, shown is mean ± SEM.Two way ANOVA and Bonferroni post test analysis were performed; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. non statistically different). Colors are used to highlight increased (green), reduced (red) and unaltered (gray) intracellular metabolites.

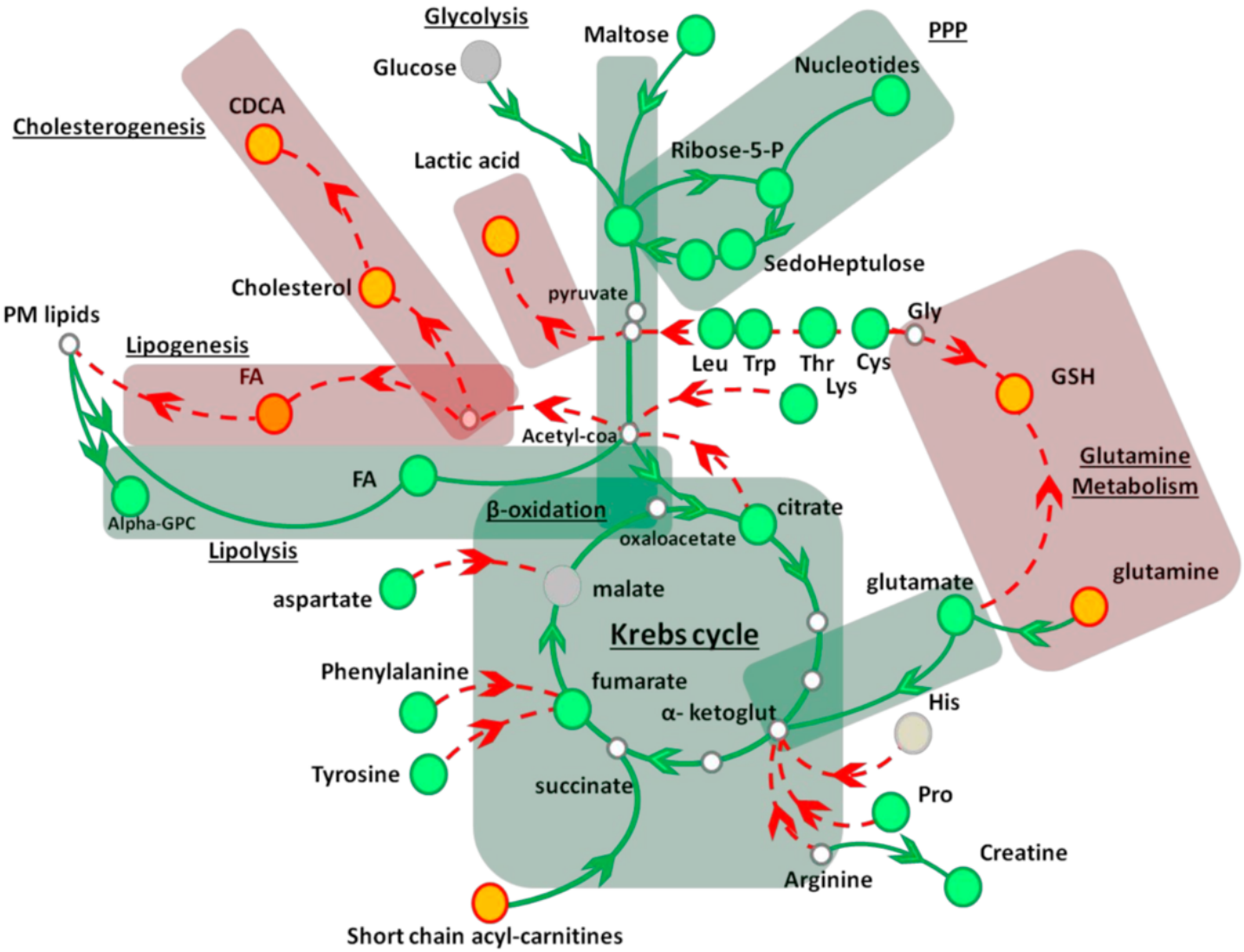


Figure 4.10. AAE influence on HuH7 metabolome. Schematic cartoon depicting some of the metabolic reactions positively (green boxes) or negatively (red boxes) affected by AAE in HuH7 cells. Red and green arrowheads indicate reactions halted or stimulated by AAE, respectively. Orange and green dots indicate metabolites whose intracellular levels resulted to be decreased or increased by treatment with AAE, respectively.

4.5 Development of an experimental platform to identify mitochondria booster

Mitochondrial activity was assessed using the mitochondrial selective probe MitoTracker CMXRos, as pipeline evaluating Mitochondrial Stimulatory Activity of nutraceuticals. This probe accumulates in the intermembrane space of mitochondria and emits fluorescence with an intensity that positively correlates with the difference in potential existing between the mitochondrial matrix and the mitochondrial intermembrane space.

Mitochondria staining of cells and animal tissues was achieved by incubation with MitoTracker® Red CMXRos (Thermo Fisher Scientific); a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. A dye working solution was prepared by diluting a stock solution (10 μM in DMSO) in DMEM to yield a final concentration of 100 nM [37]. For staining of *in vitro* samples, cells were rinsed twice in PBS before adding the dye. For staining of *ex-vivo* tissues, mice livers (soon after animal sacrifice) were washed in PBS by flushing PBS in the portal vein. Cells and tissues were incubated in the presence of the probe for 45 min in a cell incubator at 37 ◦C and 5% CO_2. At the end of the incubation, cells and tissues were rinsed three times in DMEM and once in PBS, fixed in 4% formaldehyde for 30 min to be then permeabilized in 0.1% Triton X-100 in PBS and stained with the nuclear dye DAPI.

Mitochondrial fluorescence was measured in a Perkin Elmer Envision 2105 Multiplate reader (Perkin Elmer), using the inbuilt monochromator and the following parameters: λ excitation 579 nm, λ emission 599 nm for MitoTracker, λ excitation 351 nm, and λ emission 450 nm for DAPI correlated with the total number of cells in each well and was used for normalization; or when indicate under confocal fluorescent microscope Zeiss LSM800 (Zeiss, Jena, Germany) equipped with an electronically switchable illumination and detection (**Figure 4.11**).

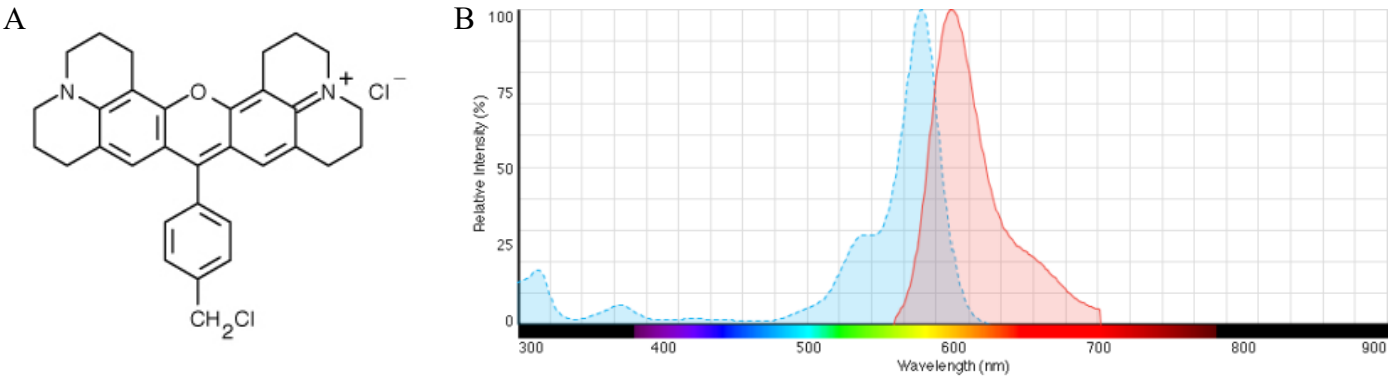


Figure 4.11. (A) Chemical structure of oxidized MitoTracker®Red CMXRos. (B) Excitation and emission spectra of the probe

I applied this strategy to evaluate mitochondrial boosting activity of a nutraceutical formulation produced by grape pomace and enriched in Resveratrol and Polyphenols [35], known as Taurisolo, both *in vitro* and *in vivo*. In particular, I analyzed its effect on *in vitro* cultured hepatic HuH7 cells and of C57BL-6J mice fed a High Fat Diet and treated with the nutraceutical. Appling on this platform Metabolomic approach, I demonstrated the nutraceutical acts as a mitochondrial booster and stimulates catabolic reactions of oxidative phosphorylation (OXPHOS) and mitochondrial ATP production [36].

To demonstrate the treatment with this nutraceutical was increasing mitochondrial respiration activity, we used the mitochondrial probe Mito Tracker CMX-ROS. The dye accumulates into cell organelles depending on their membrane potential. Since the membrane potential existing between the matrix and the mitochondrial intermembrane space is an indication of mitochondrial activity, Mito Tracker CMX-ROS fluorescence intensity correlates with Oxidative Phosphorylation (OXPHOS). When analyzed by fluorimetry, HuH7 cells treated with Taurisolo showed an increased mitochondrial activity compared to cells treated with vehicle (1.20 ± 0.05, **Figure 4.12**) confirming that Taurisolo stimulates mitochondrial activity. This is further supported by the reduction in the intracellular levels of creatine-phosphate (0.38 ± 0.02) indicating that the cells are not recycling ATP produced by glycolysis using the creatine cycle, due to the augmented biochemical activity of mitochondria.

To confirm the hepatoprotective effect *in vivo*, Taurisolo [123 mg/kg/die, a dose equivalent to 800 mg/die (10 mg/kg/die in adults), the one suggested for human] or an equal amount of placebo, were administered to C57BL/6J mice. Upon treatment, mice were sacrificed, livers were excised, and blood was replenished with culture medium supplemented with the mitochondrial probe. The dye faintly stained the hepatocytes of Placebo mice. In contrast, in the livers of Taurisolo mice, the fluorescence emitted by the probe resulted much more intense, confirming an increase in mitochondrial activity (**Figure 4.13**).

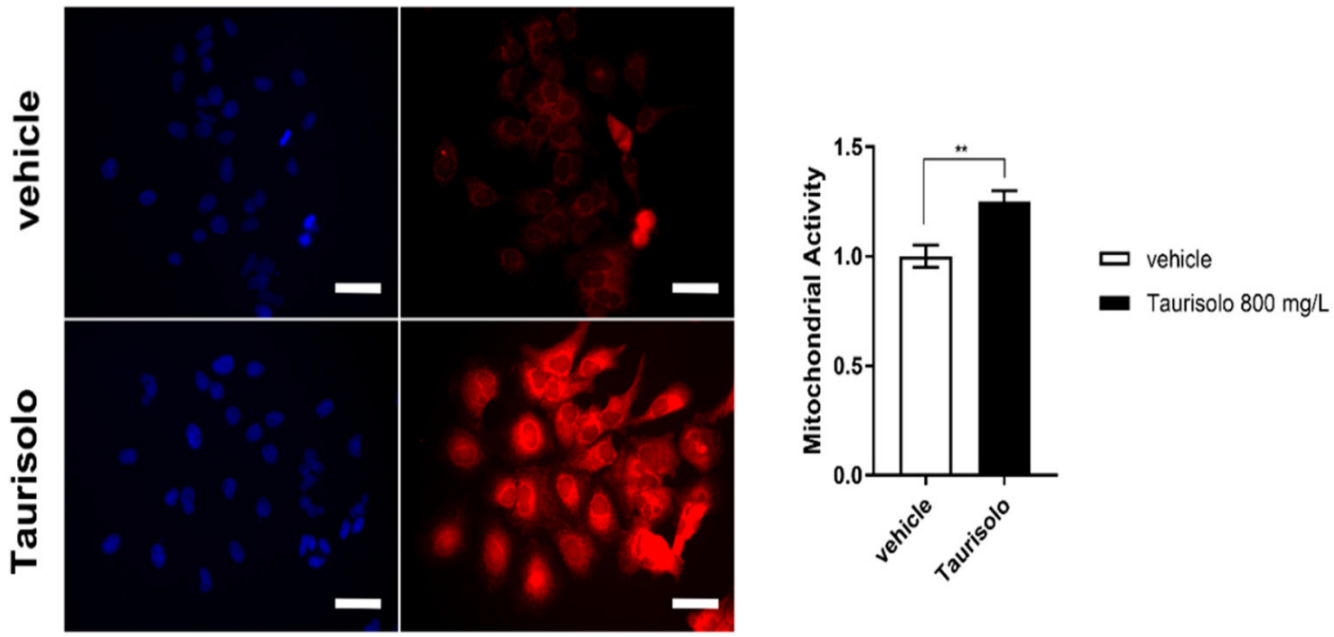


Figure 4.12. Taurisolo shifts HuH7 metabolism toward mitochondrial respiration. Fluorescent emission of MitoTracker CMX-ROS (red channel) visualized by fluorescence microscopy or by spectrofluorimetric measurement in HuH7 cells growing for 72 h in the presence of Taurisolo (800 mg/L) or of vehicle. Cells nuclei were counterstained with DAPI (blue channel). MitoTracker emission was normalized with DAPI to account for the cell number. Each bar represents normalized intensity of Mitotracker in HuH7 treated with 800 mg/L (black bars) Taurisolo, or with vehicle (white bars). Scale bars correspond to 14 μm.

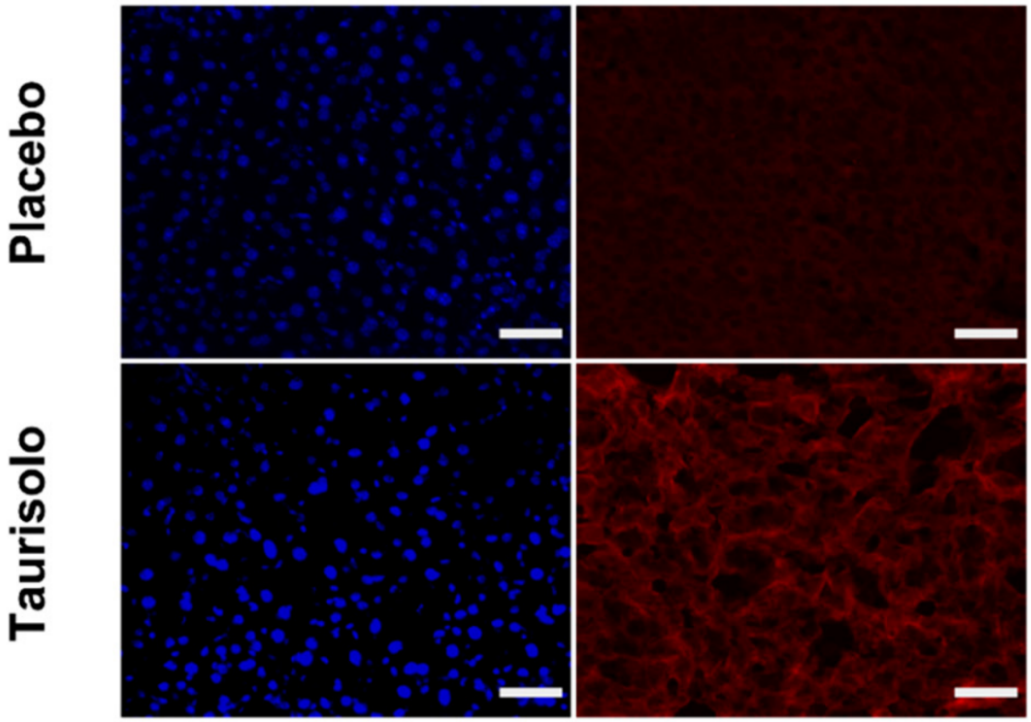


Figure 4.13. The fluorescent emission of Mitotracker CMX-ROS (red channel) was here used to show the increase in mitochondrial membrane potential induced by Taurisolo in murine livers. Liver biopsies collected from mice treated with Taurisolo or placebo were incubated *ex-vivo* with Mitotracker CMX-ROS. UPPER PANEL: A faint fluorescence emission of the probe (red channel) is detectable in the hepatocytes of the placebo group. LOWER PANEL: Increased fluorescence emission of Mitotracker CMX-ROS in hepatocytes of the Taurisolo group. DAPI (blue channel) stains cell nuclei. In G, scale bars correspond to 50 μm.

4.6 Development of an experimental platform to identify insulin-like nutraceuticals

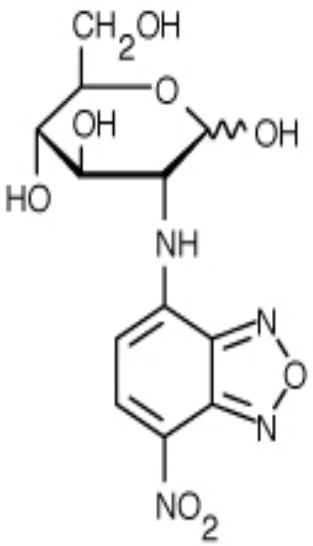
The third pipeline assessed the ability of nutraceuticals to modulate glucose uptake via glucose transporter (GLUT). Handling of circulating glucose levels is compromised in dysmetabolic syndromes, that are often characterized by insulin resistance and hyperglycemia. GLUTs are a family of transporters playing a pivotal role in the uptake of glucose from circulation. Most of them are regulated by insulin receptors via PI3K signaling and respond to insulin stimulation. Insulin stimulation can either increase the number of GLUT receptors (mainly GLUT4) localized on the plasma membrane of the cells as well as promote glycolysis and ultimately increase the activity of passive transporters (GLUT1). GLUT transporter activity is compromised in condition of Insulin resistance and their pharmacological activation improves glucose tolerance and ameliorates hyperglycemia.

Glucose uptake was assessed by measuring the uptake of NBDG, a fluorescent analogue of deoxy-glucose covalently linked to the fluorescent molecule nitro blue tetrazolium (NBT). The uptake of NBDG increases the fluorescence of living cells indicating the activity of the GLUT transporters.

2-NBDG is a fluorescent glucose analog that has been used to monitor glucose uptake in live cells, as an indicator of cell viability. The modification consisting in positioning the NBD in the C-2 position of the glucose, to generate 2-NBDG, allows the phosphorylation of the probe by the hexokinase and the subsequent intracellular metabolization. 2-NBDG is phosphorylated after absorption, to produce the fluorescent derivative (2-NBDG 6-phosphate), from the glycolytic enzyme, hexokinase. The probe cannot proceed along the glycolytic pathway. The reaction enzyme of phosphoglucose isomerase in fact requires Carbon 2 position to be free. The probe therefore accumulates intracellularly without proceeding along the glycolytic pathway. The 2-NBDG can instead be accumulate as glycogen, because the enzyme glycogen synthase uses Carbon 1 and Carbon 4 to be able to convert UDP glucose into glycogen.

Although sensitive to its environment NBD fluorescence typically displays excitation/emission maxima of ~465/540 nm and can be visualized using optical filters designed for fluorescein (**Figure 4.14**).

A



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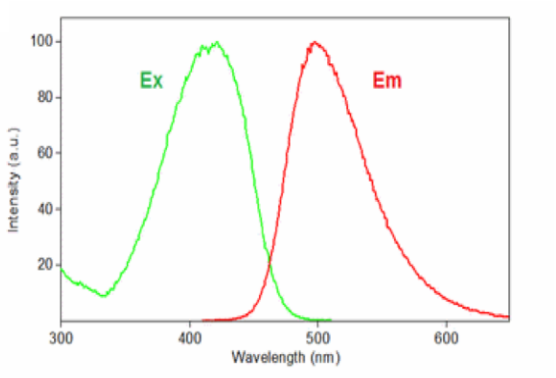

Figure 4.14. (A) Chemical structure of 2-NBDG. (B) excitation and emission spectra of the probe

4.6.1 2-NBDG glucose uptake assay

In these studies [31][35][38], I performed NBDG Glucose Uptake Assay on hepatoma cell line HuH7. It is well known that glucose uptake by liver cells is insulin-independent, physiologically. The cell line, I chose as biological platform, displays altered PI3K-AKT signaling for this reason they promptly respond to an insulin short stimulation by augmenting glucose uptake.

In particular, HuH7 cells were plated (5×10^3/well) in a black, clear bottom, 96-well microtiter plate (Perkin Elmer, Waltham, USA) in a final volume of 100 μL/well of culture medium. Once cells had reached 80–90% of confluence, the culture medium was carefully removed and replaced with 100 μL of HBSS containing 100 μM 2-DG, 0.4 g/L BSA, and 1.3 mM $CaCl_2$ (in the absence of any growth factors or FBS) and when indicated the tested compounds. Plates were incubated at 37 ◦C for 1 h. Cell medium was replaced with the same HBSS supplemented with 6 μM 2-NBDG. Plates were incubated with the fluorescent probe for 45 min to be then washed twice in PBS. Uptake of 2-NDBG was measured in a Perkin Elmer Envision 2105 Multiplate reader (Perkin Elmer), using the inbuilt monochromator and the following parameters: λ excitation 471 nm, λ emission 529 nm, and monochromator cut off 360 nm. After the measurement of 2-NDBG, cells were fixed in 3.7% paraformaldehyde for 30 min to be then permeabilized in 0.1% Triton X-100 in PBS and stained with the nuclear dye DAPI (30 μM). This second fluorescence measurement correlates with the total number of cells in each well and was used for normalization. DAPI fluorescence was measured using the following parameters: λ excitation 351 nm and λ emission 450 nm. Data analysis for glucose uptake is reported as the ratio between intracellular 2-NDBG fluorescence and DAPI fluorescence ± s.d.

In particular, **Figure 4.16** showed results dealing with Taurisolo stimulation glucose uptake on liver cells. The nutraceutical used is a grape pomace extract from the grape cultivar “Aglianico”, enriched in Resveratrol, Catechins, and Procyanidins. Administered to humans, Taurisolo acts as an antioxidant and reduces oxidized-LDL serum levels, as a circulating oxidative stress biomarker, and of Trimethylamine N-oxide (TMAO) a known cardiovascular risk factor marker. In murine models, both acute and chronic consumption of Taurisolo (i) protects the blood–brain barrier and reduces brain damages in rat undergoing ischemic injuries, (ii) reduces Radical Oxygen Species (ROS) produced by endothelial cells during oxidative stress, (iii) reduces Thromboxane TxB2 biosynthesis and (iv) promotes Nitric Oxide production. Our results show that Taurisolo promotes glucose uptake,

hepatic glycolysis, fatty acid beta-oxidation leading to an overall reduction of hepatic triglycerides and cholesterol levels and a better response to insulin.

Precisely, Huh7 cells were incubated at 37 ◦C for 1 h. HBSS was then supplemented with insulin (1 nM, 10 nM or 100 nM), Taurisolo (400 mg/L or 800 mg/L) in the presence or in the absence of the PI3K inhibitor Ly294002 (10 μM) (**Figure 4.15**). As shown in **Figure 4.16**, 24 h treatment with either 400 mg/L or 800 mg/L of Taurisolo, promotes glucose uptake in HuH7 (2.0 ± 0.5 and 2.2 ± 0.5 over vehicle, respectively). Surprisingly, the amount of glucose uptake upon Taurisolo treatment is comparable to that promoted by 10 nM and 100 nM insulin, (1.3 ± 0.3 and 2.3 ± 0.5, respectively) suggesting that Taurisolo might stimulate GLUT transporters using an insulin-like mechanism (i.e., involving the AKT and PI3K kinases). To verify the PI3K-dependent Taurisolo effect on glucose uptake, the nutraceutical was administered to cells in the presence of the PI3K inhibitor Ly294002. As a consequence of the signaling cascade connecting insulin receptors to PI3K, glucose uptake following insulin stimulation was reduced by Ly294002. In contrast, Taurisolo activity was unchanged by Ly294002, suggesting a mechanism for the nutraceutical different from that used by insulin. Moreover, Taurisolo does not affect insulin activity, as the latter did not change in the presence of the nutraceutical.

The same strategy was also used to demonstrate the antidiabetic activity of extracts obtained from *Cissus rotundifolia*, a Saudi medicinal and edible plant. All the compounds obtained from purification of the bioactive fractions (indicated from 1 to 7) of extract were evaluated for their activity in the promotion of glucose uptake in hepatic cells. As shown in **Figure 4.18**, 1 h treatment with fraction 7 (800 mg/ L) promoted glucose uptake in HuH7 (2.0 ± 0.2 over vehicle). On the contrary, the other tested fraction 1−6 failed in stimulating glucose uptake. The amount of glucose uptake upon treatment with 7 was comparable to that promoted by 10 and 100 nM insulin (1.9 ± 0.1 and 2.2 ± 0.1, respectively). The main constituent of fraction 7, the coumaroyl glucoside (**Figure 4.17**) might stimulate GLUT transporters using an insulin-like mechanism. Dose−response experiments revealed that the compound promotes glucose uptake with an EC value of 7.7 ± 0.1 μM.

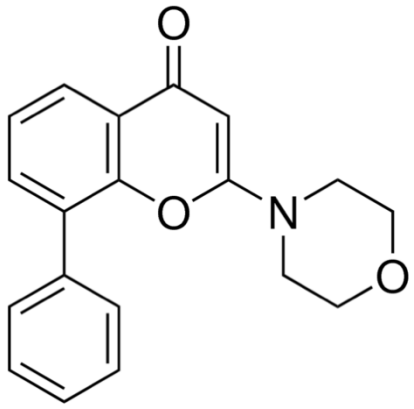

Figure 4.15. Chemical structure of Ly294002, a reversible inhibitor of phosphatidylinositol 3-kinase (PI3K) that acts on the ATP binding site of the enzyme.

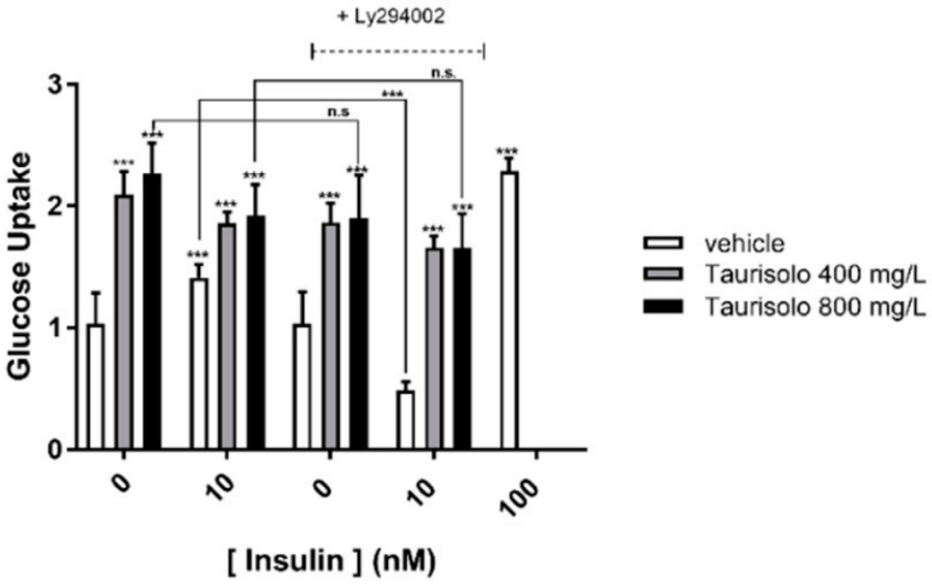

Figure 4.16. Glucose uptake of HuH7 cells grown for 72 h in the presence of 400 mg/L (gray bars), 800 mg/L (black bars) Taurisolo, or of vehicle (white bars). When indicated 10 nM insulin and/or 10 μM LY294002 was added to the cell.

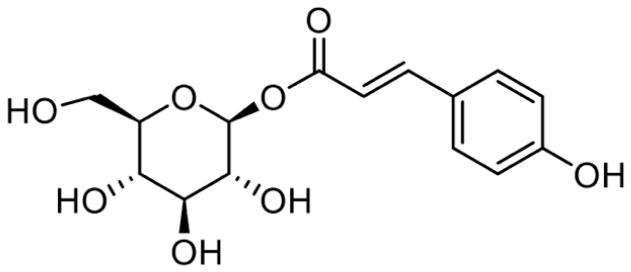

Figure 4.17. Chemical structure of *Cissus rotundifolia* fraction 7

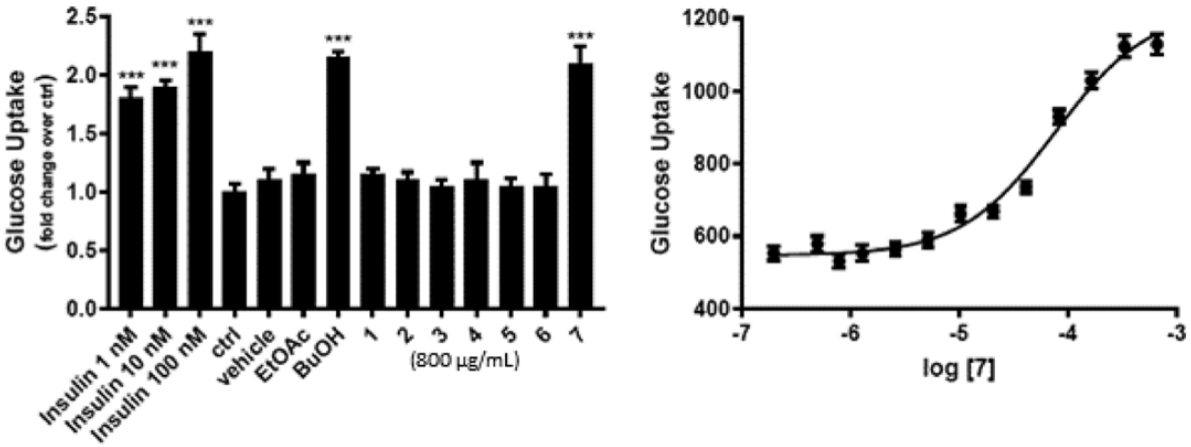

Figure 4.18. (Left) Glucose uptake of HuH7 cells treated for 1 h with the indicated amount of insulin (control), fractions *Cissus rotundifolia* extract (2.0 mg/mL) and compounds 1−7 (800 µg/mL), or vehicle (DMSO) (data are representative of n = 3 measurements, shown as mean ± SD; ***p < 0.001). (Right) Dose−response curve for fraction 7 promoting glucose uptake in HuH7 cells. The graph is representative of three independent experiments (mean of three replicates ± SD)

4.7 Conclusions

The metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance, obesity, atherogenic dyslipidemia, and hypertension. The syndrome is posing substantial concern since it has reached epidemic proportions worldwide. It is widely recognized that botanicals may serve as effective agents for the prevention or treatment of metabolic syndrome since they contain biologically active secondary metabolites that, by exerting multiple mechanisms of action, may potentiate each other's activity, or have a synergistic effect.

Phytocomplexes were proved endowed with nutraceutical potential in many human conditions. The hundreds of different metabolites contained in nutraceutical formulation act in synergism and allow this extract to be effective in a plethora of different biological contexts: as antioxidant, as modulator of lipid and cholesterol anabolism, as mitochondria booster.
The pipelines applied allow to confirm *in vitro* the cholesterol lowering activity of nutraceuticals and enlighten the molecular mechanism behind them.

The metabolite profiling of cells allowed us to take a snapshot of some of the metabolic pathways modulated by nutraceuticals of interest. The picture obtained is very useful to explain some metabolic effects exerted by nutraceuticals, especially when these consist of complex mixtures of components, each acting on different intracellular targets.

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CHAPTER 5

Setting-up new approaches to identify nutraceuticals supporting antineoplastic therapies

5. Experimental pipelines to identify nutraceuticals supporting anti-cancer treatments

Cancer is defined as the abnormal tissue growth due to an uncontrolled cell division. It arises as consequence of a plethora of consecutive genetic alterations, developed in the cell and triggered by environmental factors, among which toxicants, radiations, inadequate and unbalanced diet [1]. Recently, several studies have shown an association between diet and cancer and a positive "onco-protective" effects exerted by nutraceuticals on human cells [2].

Regulatory guidelines do not approve nutraceuticals as chemotherapeutic agents. However, these can instead be used as supplements to support chemotherapy treatments. If nutraceuticals have positive or negative effects during chemotherapy, it is still unclear. During cancer treatment, patients take nutraceuticals to alleviate drug toxicity and improve long-term results. Some nutraceuticals may work in synergism with cytotoxic chemotherapy by inducing cell growth arrest, cell differentiation, unbalancing the redox state of cells; in other cases, high levels of them may interfere with the chemotherapeutic treatment [1].

There are many nutraceuticals which are used against various forms of cancer due to their anticancer activities including tea polyphenols, resveratrol, flavonoids and curcumin [3][4][5]. The mechanism of action of these nutraceuticals has not been fully elucidated: they have been shown (i) to up- or down-regulate DNA methylation and alter histone acetylation; (ii) to activate specific miRNAs; (iii) to block inflammatory pathways by inhibiting NF-κB and subsequently down-regulate b-cell lymphoma-2 (Bcl-2), cyclin D1, metallo-proteinases-9 (MMP-9), angiogenic factors and IL-6 pathways; (iv) to inhibit proliferation pathway such as Wnt/beta catenin (**Figure 5.1**).

In this scenario, it would be auspicial to develop biological pipeline to test antineoplastic activity of nutraceuticals and identify the mechanism underpinning their activity.

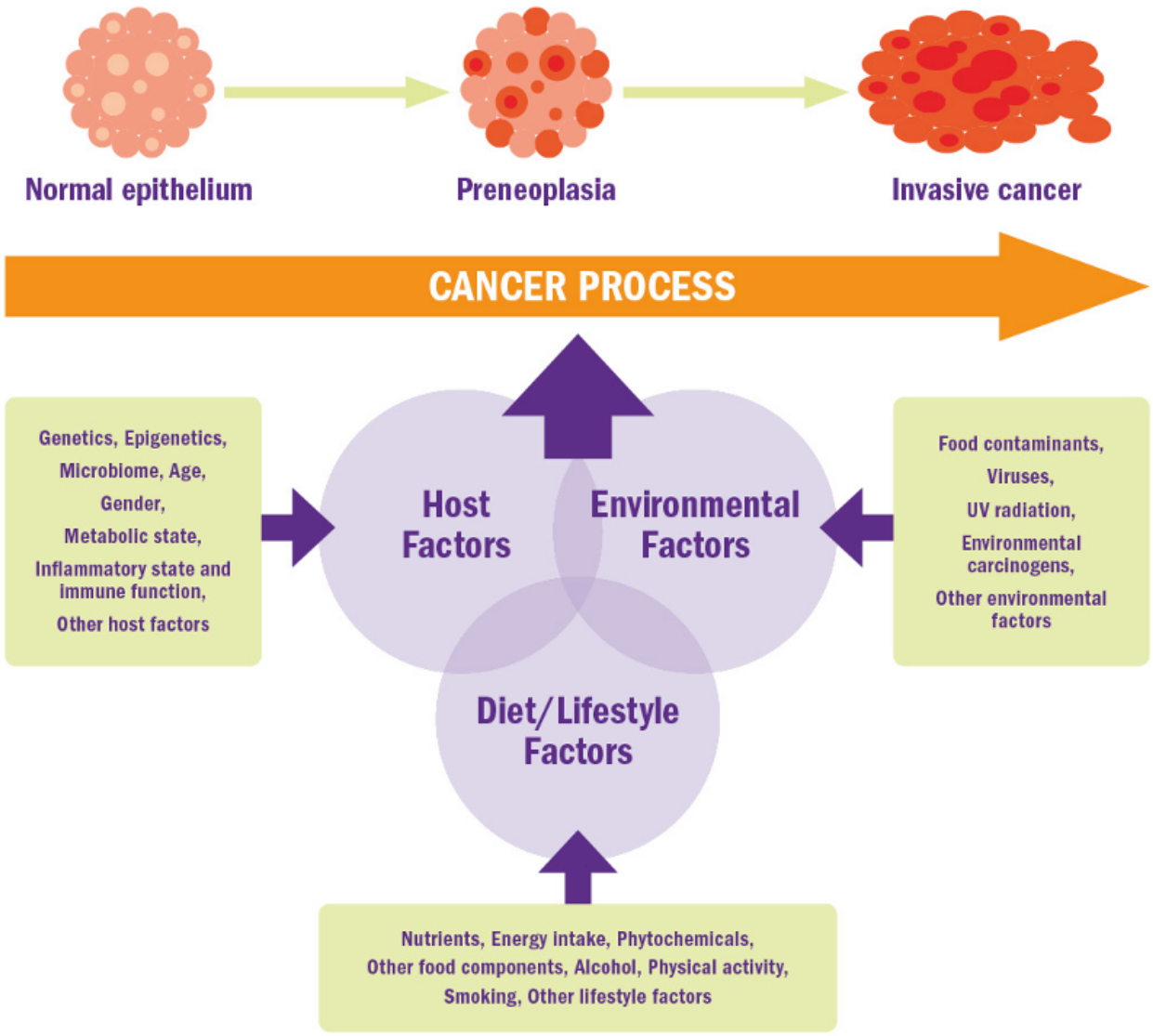


Figure 5.1. Diet, nutrition and physical activity, other environmental exposures and host factors interact to affect cancer progression

5.1 Cancer stem cells and traditional anti-cancer treatments

Currently anti-cancer treatments include surgery, radiotherapy, chemotherapy and adjuvant chemotherapy. Despite advances in this field, the high degree of tumour heterogeneity contributes to treatment failure [6]. Several factors contribute to resistance, progression of tumour and relapse after therapy with cancer stem cells (CSCs) being recently identified as one of these factors [7][8].

Conventional therapies affect tumours by killing preferentially cells with high proliferative potential. Traditional chemotherapeutic agents are usually cytotoxic drugs able to interfere with mitosis and induce cell damage and death: methotrexate, gemcitabine and mercaptopurine are able to prevent DNA and RNA synthesis; anti-microtubule agents, such as vinca alkaloids and taxanes (vincrastine, nocodazole, paclitaxel, docetaxel) which block cell division by preventing microtubule functions; topoisomerase inhibitors, such as irinotecan, that affect the activity of topoisomerase I and topoisomerase II inhibiting both replication and transcription.

Stem cells are essential for adult organism. This pool of undifferentiated cells perpetuates by self-renewal and possesses the ability, at any stage of the adult life, to generate by differentiation a multitude of lineages of mature cells. However, stem cells can undergo a process of transformation producing tumours with peculiar biological properties [9]. CSCs are transformed stem cells. In virtue of their capacity to self-renew and to produce the differentiated progeny, they sustain a tumour by forming, replenishing and increasing the tumour mass. They have been detected in many forms of cancer (haematological malignancies as well as solid tumours like brain, breast, prostate and colon cancer) but differently from their differentiated counterpart they resist to chemotherapeutic agents, ultimately causing relapse after treatment (**Figure 5.2**).
However, CSCs are able to enter in a quasi-quiescent state thus they are less proliferative and less sensitive to traditional anti-cancer drugs. Upon treatment, they remain viable and can eventually re-establish the tumour [10] .

In CSCs, drug effluxing pumps, such as ATP-binding cassettes drug transporters, are hyperactive; anti-apoptotic proteins like Bcl-2 are upregulated [11] and epigenetic mechanisms, including DNA methylation or histone modification, confer them resistance to cell death [12]. Moreover, activation of DNA damage checkpoint responses, increased DNA repair capacity [13][14] and a particular niche of surrounding cells helps and protects CSCs.

Most of the abovementioned activities rely on an aberrant signalling activity of the Wnt/β-catenin pathway. In the effort to kill CSCs, Wnt pathway is nowadays considered as the golden target to address [12][15]. However, it is essential to understand the molecular mechanisms behind its regulation to ultimately antagonize it and block CSCs.

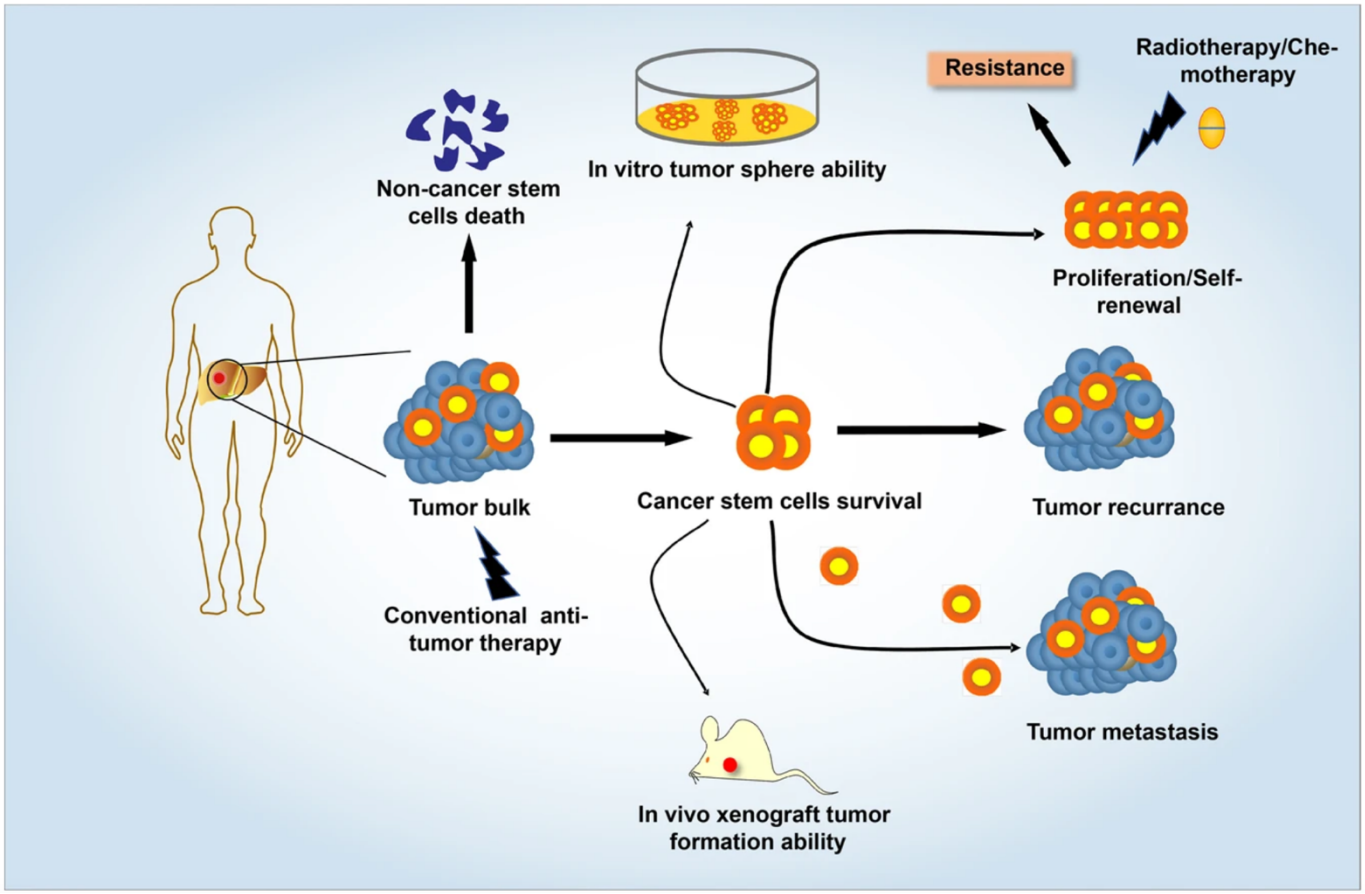


Figure 5.2. The characteristics of cancer stem cells. CSCs resist to conventional treatment, for this reason upon treatment, they remain viable and can eventually re-establish the tumour. In vitro, a single CSC possesses the capability to form tumour spheroids, which represent the self-renew and proliferation ability of CSCs. In vivo, a small number of cancer stem-like cells can trigger tumour forming in mice. In addition, CSCs have inherent drug resistance and dormancy characteristics, as well as the ability to trigger distant metastasis of cancer [16]

5.2 Wnt signaling pathway

The Wingless/Int1 (Wnt) signaling pathway regulates several cell processes such as cell-fate determination, migration, polarity, neural patterning, organogenesis [17] and plays a key role during embryonic development [18].
Wnt pathway acts via interconnected branches, historically divided into canonical (or β-catenin-dependent) and non-canonical (or β-catenin-independent) networks. The activation of this pathway is triggered by Wnt proteins and class F GPCRs family members called Frizzled (FZD) receptors (**Figure 5.3**)[19].

Briefly, in the β-catenin-dependent pathway, Wnt binding to FZD receptors induces the recruitment of the scaffold protein Dishevelled (Dsh/Dvl). The latter promotes β-catenin stabilization via inhibition of Adenomatosis Polyposis Coli (APC) β-catenin-destruction-complex and its translocation into nucleus, where β-catenin regulates TCF/LEF-dependent transcription. However, most of the signal events involved are not understood at a molecular level. Generally this pathway is considered to be heterotrimeric G-proteins-independent although new evidences suggest the opposite [20].

In contrast, G-proteins are known to be crucial in β-catenin-independent pathways which include an increasing number of complex intracellular networks: FZD/planar cell polarity, Wnt/Ca^{2+}, Wnt/cAMP, Wnt/Ror, Wnt/Rac and Wnt/Rho pathways. Their downstream effects include cell migration, cytoskeleton reorganization and planar cell polarity [20] .

Wnt signalling defects have dramatic consequences on embryo development and they cause several human pathologies, including breast, colon and skin cancer, glioblastoma, skeletal defects and human birth defect disorders [21][22].

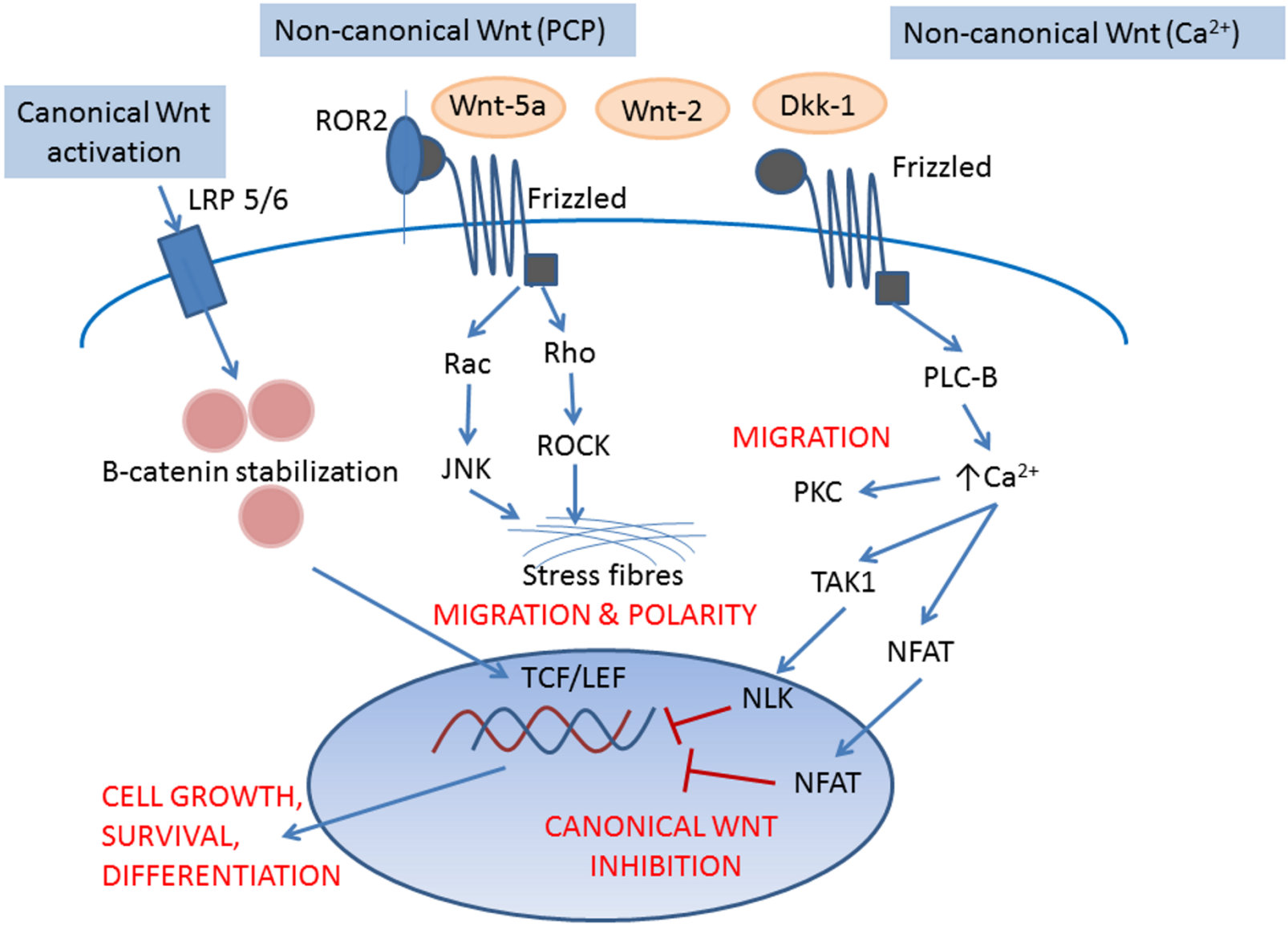

Non-canonical Wnt (PCP)
Non-canonical Wnt (Ca2+)
Canonical Wnt activation
ROR2
Wnt-5a
Wnt-2
Dkk-1
Frizzled
LRP 5/6
Frizzled
Rac
Rho
PLC-B
MIGRATION
B-catenin stabilization
JNK
ROCK
PKC
↑Ca2+
Stress fibres
TAK1
MIGRATION & POLARITY
NFAT
TCF/LEF
NLK
NFAT
CELL GROWTH, SURVIVAL, DIFFERENTIATION
CANONICAL WNT INHIBITION

Figure 5.3. Overview of canonical and non-canonical Wnt signaling. In canonical Wnt signaling, absence of Wnt ligands (Wnt signaling inactive state, left) leads to phosphorylation of β-catenin by the destruction complex, which contains the scaffold protein Axin, APC and the kinases GSK3β and casein kinase (CK1α). In this state, β-catenin is phosphorylated by GSK3β, ubiquitinated and targeted for proteasomal degradation. The canonical pathway is activated upon binding of secreted Wnt ligands to Fzd receptors and LRP co-receptors (Wnt signaling active, right). LRP receptors are then phosphorylated by CK1α and GSK3β, which recruits Dishevelled (Dvl) proteins to the plasma membrane where they polymerize and are activated. This results in stabilization and accumulation of β-catenin which then translocates into the nucleus. There, β-catenin forms an active complex with LEF (lymphoid enhancer factor) and TCF (T-cell factor) proteins by displacing TLE/Groucho complexes and recruitment of histone modifying co-activators. This transcriptional switch leads to a change of multiple cellular processes. Non-canonical Wnt signaling is defined by β-catenin-independent mechanisms of signal transduction. During Wnt/PCP signaling, Wnt ligands bind to the ROR-Frizzled receptor complex to recruit and activate Dvl. Dvl binds to the small GTPase Rho by de-inhibition of the cytoplasmic protein DAAM1. This leads to rearrangements of the cytoskeleton and/or transcriptional responses. Wnt/Ca2+ signaling is initiated by G-protein triggered phospholipase C activity leading to intracellular calcium fluxes and downstream calcium dependent cytoskeletal and/or transcriptional responses [19]

5.3 The role of nutraceuticals in the regulation of the Wnt pathway

Emerging studies suggest the chemopreventive role of nutraceuticals such as curcumin, resveratrol, polyphenols in cancer [23][24]. In particular, they have shown to prevent or delay tumor progression by targeting the Wnt signaling pathway.
During the last decade, many products have been identified as potent modulators of the Wnt/*β*-catenin signaling and have gained interest among the researchers as promising candidates to develop new therapeutic drugs for cancer [25].

Several Wnt modulators have been discovered to target this crucial pathway but, up today, they failed to demonstrate a clinical efficacy in cancer characterized by mutations in APC and β-catenin genes. Even if targeting directly β-catenin appears the most relevant pharmacological strategy, β-catenin seems to be an undruggable target. In this context, natural compounds could represent an alternative useful source to potentially overcome some critical issues that characterize synthetic and biological molecules till now developed.

The bulk of these natural compounds are polyphenols, mainly flavonoids, but also some terpenes and terpenoids are included. All the mentioned compounds seem to have multiple targets, even if the mechanism of action is still obscure.

The aim of this thesis chapter is to present three studies in which I set up new approaches to test Wnt inhibitory activity of well known nutraceuticals to then investigate their mechanism of action.

5.4 Development of an experimental platform to identify cell growth regulators

Herein [26], I performed a biological pipeline to test the Wnt inhibitory activity of two nutraceuticals extracted from two common southern Italian apples, *Malus pumila Miller* cv. 'Annurca' (AAE) and *Malus domestica* cv 'Limoncella' (LAE), respectively. I here showed that both Annurca and Limoncella apple extracts act as Wnt inhibitors, mostly thanks to their polyphenolic contents.

In these studies [27][28] I performed an unprecedented metabolite analysis using Direct Infusion Fourier Transform-ion cyclotron resonance mass spectrometry (DI-FT-ICR-MS) strategy to investigate molecular mechanism underpinning AAE activity.

5.4.1 Wnt pathway activity measurement in cells using the TCF-GFP constructs

Activation of the Wnt canonical pathway induces β-catenin stabilization and nuclear translocation. Stabilized nuclear β-catenin forms a complex with members of the T-cell factor family of DNA binding proteins (TCF/LEF), promoting the transcription of Wnt-responsive genes. TCF proteins bind to the conserved DNA binding sequence 5'-(A/T)(A/T)CAA(A/T)G- 3' located on the minor groove of the DNA[29] , by a conserved HMG domain [30].

To measure Wnt pathway activity in cells, I generated a TCF/LEF reporter construct presenting the cDNA of GFP under the control of the TCF/LEF promoter.

The construct, together with the mutated version of it (named mut construct), was cloned in the vector pcDNA 3.1-cGFP between the restriction sites Nru1 and Hind3. The mut construct presents a double mutations AT > GC in each of the octamer forming the promoter's consensus sequence (**Figure 5.4**). These octamers are essential for the promoter activity and, thus, the substitution of these peculiar nucleotides abrogate the binding of TCF/LEF to its DNA binding site [29].
In order to test our TCF/LEF reporter assay functionality, HEK293 cells lines were used. HEK293 cells do not express endogenous FZD4, thus we transiently transfected them with (HA)-tagged wt FZD4 (HA-FZD4-wt). As source of Wnt ligands U87MG glioblastoma cells were used. The medium of these cells is particularly enriched in Wnt ligands. For this reason, we used it as conditioned medium in our assay.

HEK293 cells were transfected with either TCF/LEF wt-GFP or TCF/LEF mut-GFP reporter under several conditions.
When HEK293 cells were transfected with TCF/LEF wt-GFP alone, no TCF/LEF signal could be measured confirming that in these cells the Wnt pathway is inactive. Conditioned medium was unable to activate the reporter, confirming that HEK293 cells do not express FZD receptor. Similarly, when the TCF/LEF construct was co-transfected with HA-FZD4-wt we could not measure any TCF/LEF signal.

When HEK293 cells expressing TCF/LEF wt-GFP and HA-FZD4-wt were cultured in the presence of U87MG conditioned medium, I measured expression of GFP. This result confirmed that TCF/LEF activation was dependent on the presence of both FZD4 and Wnt (**Figure 5.5A**).
On the contrary, HEK293 cells transfected with TCF/LEF mut-GFP and HA-FZ4-wt did not express GFP neither in the presence nor in the absence of U87MG medium, confirming the specificity of the readout. (**Figure 5.5B**)

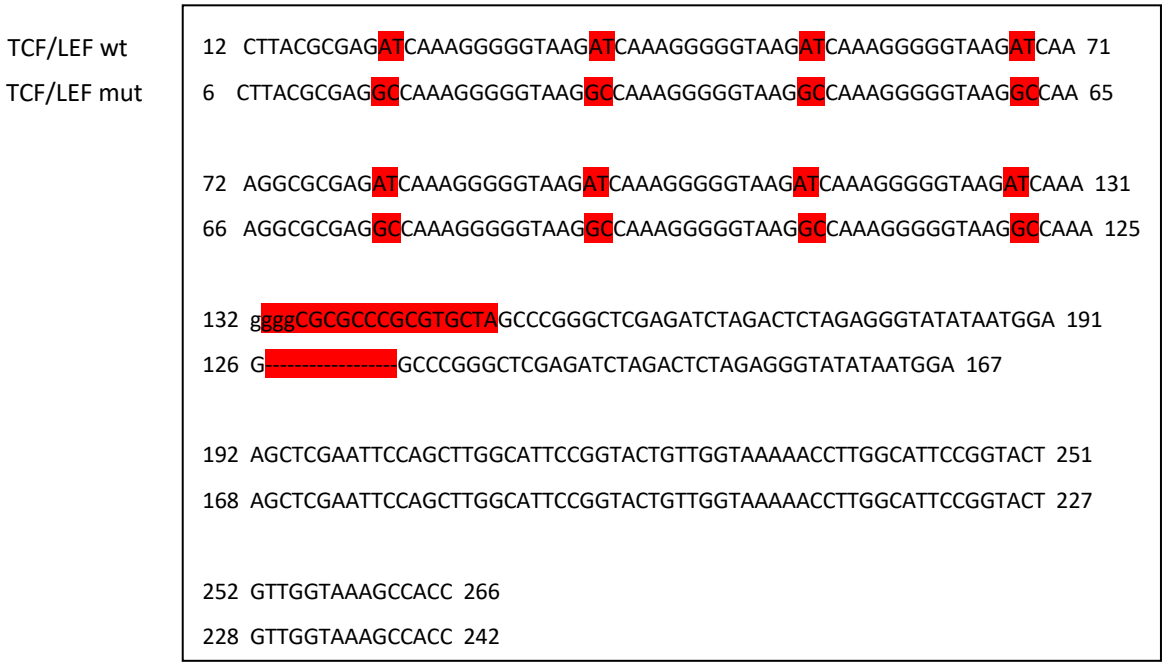


Figure 5.4. TCF/LEF wt and TCF/LEF mut sequence alignment shows the position of the double mutations. They occur in each of the octamer forming the consensus sequence for the TCF/LEF promoter

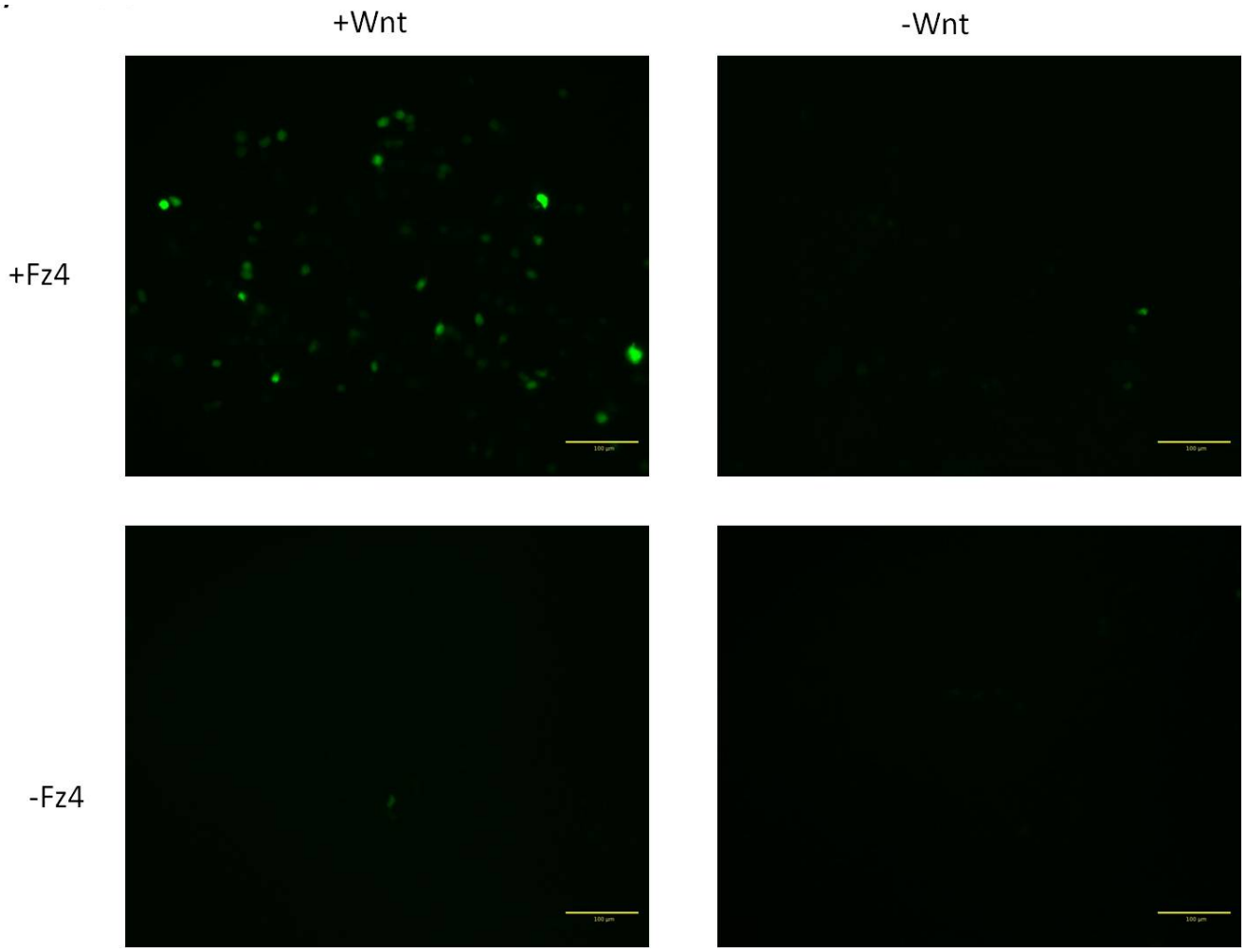


Figure 55.A. Representative images of TCF/LEF reporter assay. HEK293 cells expressing TCF/LEF wt-GFP were co-transfected or not with HA-FZD4-wt (+/- Fz4) and cultured with or without U87MG medium (+/- Wnt). GFP expression is dependent on FZD4 expression and Wnt availability. Scalebar: 100µm.

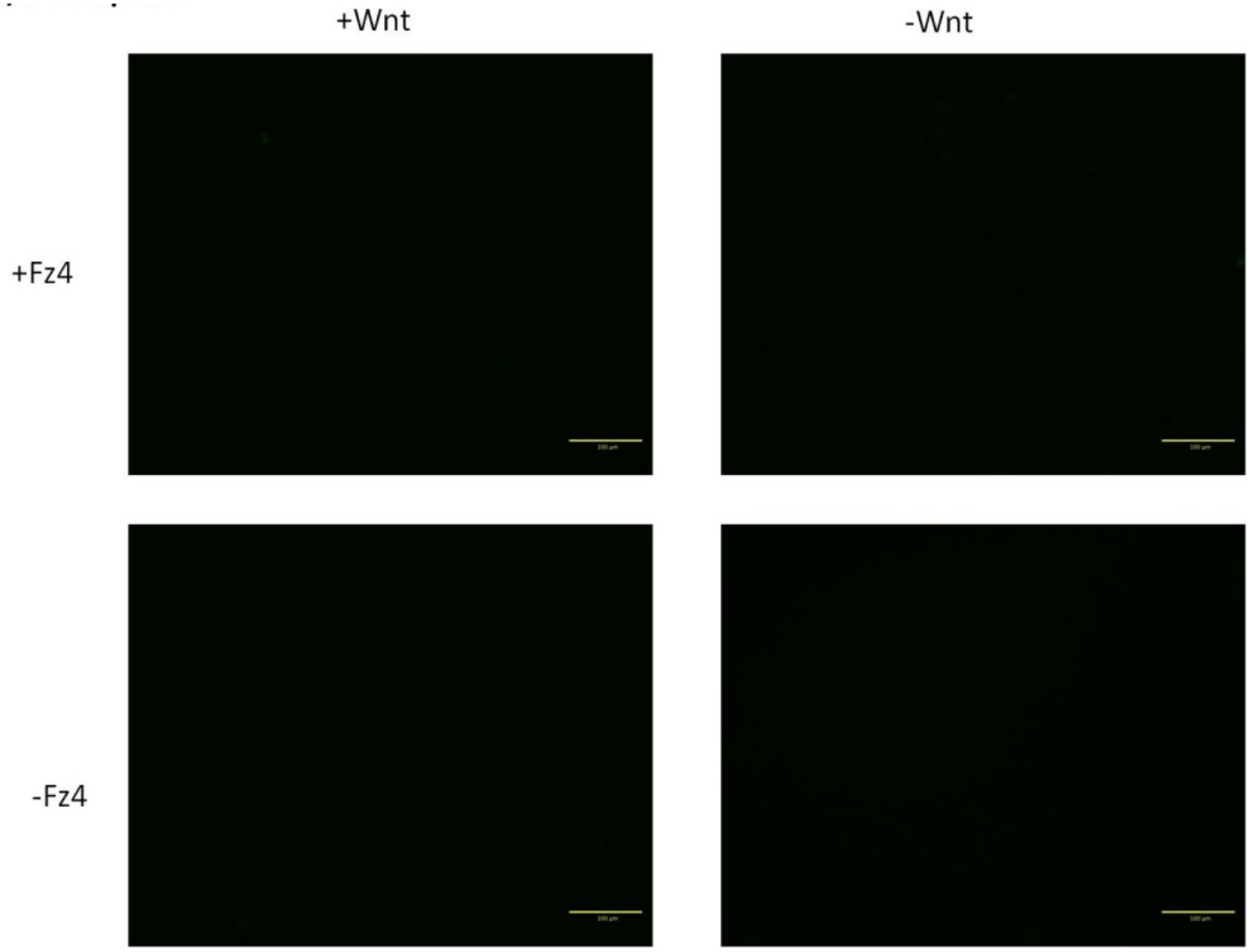


Figure 5.5B. Representative images of TCF/LEF reporter assay. HEK293 cells expressing TCF/LEF mut-GFP were cotransfected or not with HA-FZD4-wt (+/- Fz4) and cultured with or without U87MG medium (+/- Wnt). No GFP expression could be detected. Scalebar: 100µm.

5.4.1.1 Wnt inhibitory activity of *Malus Pumila miller* cv Annurca and *Malus domestica* cv Limoncella Apple Extracts on both *in vitro* and *ex vivo* cultures

In this study [26], based on the above mentioned biological pipeline, I tested on *in vitro* cultures of cells carrying Familial Adenomatous Polyposis (FAP) mutations and on *ex vivo* biopsies of FAP patients, the WNT inhibitory activity of two apple cultivars, native to Southern Italy, namely "Annurca" (AAE) and "Limoncella" (LAE) to identify the mechanism underpinning their activity.
In FAP syndrome, a mutation in the protein APC, leads to hyperactivation of the Wnt/β-catenin signaling pathway and, in turn, to uncontrolled intestinal cell proliferation and a high risk for the formation of adenocarcinomas.

I. Materials and methods

I.1 Nutraceuticals Preparation

Annurca (*Malus Pumila Miller* cv Annurca) apple fruits and Limoncella (*Malus Domestica* cv Limoncella) were collected from Valle di Maddaloni (Caserta, Italy), in October 2016, when fruits had just been harvested. Annurca fruits were reddened for about 30 days, and then analyzed. Lyophilised peels and flesh (10 g) of Limoncella and Annurca apple samples were treated with 60 mL of 80% methanol (0.5% formic acid), homogenized for 5 min by ultraturrax (T25-digital, IKA, Staufen im Breisgau, Berlin, Germany) and shaken on an orbital shaker (Sko-DXL, Argolab, Carpy, Italy) at 300 rpm for 15 min. Then, the samples were placed in an ultrasonic bath for another 10 min, before being centrifuged at 6000 rpm for 10 min. The supernatants were collected and stored in darkness, at 4 ◦C. The pellets obtained were re-extracted, as described above and with another 40 mL of the same mixture of solvents. Finally, the extracts obtained were filtered under vacuum, the methanol fraction was eliminated by evaporation, and the water fraction was lyophilized. To obtain polyphenol-enriched fractions of Annurca apple extract (AAE) and Limoncella apple extract (LAE) (in the text referred to as PEF(AAE) and PEF(LAE), respectively) the dry extracts were dissolved in distilled water and slowly filtered through an Amberlite XAD-2 column, packed as follows: Resin (10 g; pore size 9 nm with particle sizes of 0.3–1.2 mm; Supelco, Bellefonte, PA, USA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 × 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionized water for removal of sugars and other polar compounds. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen. The extracts were stored at −80 ◦C until HPLC analysis. Before performing the biological tests, each extract was

dissolved in DMSO at a final concentration of 300 mg/mL. Food grade Limoncella apple extracts (IndLAE) were produced at MB-Med (Turin, Italy) starting from fresh Limoncella Apples. Upon lyophilization of peels and flesh of Limoncella apples, samples were treated with ethanol/water (70:30 v/v) for 24 h at 40 ◦C to extract phenolic compounds and generate food grade Limoncella Apple Extracts (in the text referred to as IndLAE).

I.2 Cell cultures

HEK293, CaCo-2, and U87MG cells were grown in DMEM (#41965-039, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (#10270, GIBCO), Glutamax (#35050-061, GIBCO) and Pen/Strep (#15070-063, GIBCO). HEK293 transfection was performed using Polyethylenimine (Sigma Chemical Co., St. Louis, MO, USA). CaCo-2 transfection was performed using Lipofectamine (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Both cell cultures and human biopsies were analyzed for viability using Trypan blue, Propidium Iodide (PI) and Acridine-Orange (AO) staining. A pre-mixed AO/PI solution was directly added to cell samples for viability analysis, using a fluorescent cell counter.

I.3 Human Biopsies from FAP Patients Biopsies

Biopsies from Familial Adenomatosis Polyposis patients were kindly provided by Prof. G.B. Rossi. The study was approved by the Ethics Committee of the University Federico II of Naples. For all the patients enrolled in this study, polypectomy was part of their clinical treatment plan and it was scheduled and performed independently from this research. Immediately after excision, biopsies were rinsed in physiological saline. Samples were then digested with Trypsin for 10 min at room temperature (RT) and centrifuged at 400 rpm for 10 min at RT. Isolated cells were then counted and cultured at the confluency of 100,000 cell per mL in DMEM, supplemented with 10% FBS, Glutamax and Pen/Strep. When indicated, apple extracts were added at a concentration of 400 mg/L. After 24 and 48 h of incubation, cell viability was measured with using Trypan blue, Propidium Iodide (PI) and Acridine-Orange (AO) staining.

II. Results and discussion

II.1 Wnt Inhibitory Activity of AAE and LAE

I used, as a biological platform, human embryonic HEK293 cells, transiently expressing both the Wnt receptor Frizzled 4 (FZD4) and a Wnt pathway reporter DNA construct to evaluate the activity of nutraceuticals on Wnt/β-catenin signaling. Three different Wnt reporter constructs were used. The first, hereinafter referred to as TCF-wt GFP, presents the coding sequence of GFP under the control of an optimized Wnt pathway responsive promoter. In the second, hereinafter referred to as TCF-mut GFP, the WNT responsive promoter was mutagenized to become unresponsive to Wnt. Finally, a third reporter construct (cmv GFP) presents the coding sequence of GFP under the control of a constitutive cmv promoter. The TCF-mut GFP and cmv GFP constructs were here considered as negative controls.

The FZD agonist, Wnt5A, induced GFP expression in HEK293 cells, transiently transfected with TCF-wt GFP. On the contrary, Wnt5A did not affect GFP expression in cells transfected either with TCF-mut GFP or with cmv GFP, confirming the specificity of the platform. At the endogenous level, HEK293 cells express several other FZD receptors. However, differently from FZD4, these did not respond to WNT5A stimulation. As shown in Figure S1, in the absence of FZD4, Wnt5A stimulation does not influence GFP expression in HEK293. Wnt5A has been shown to activate both the Wnt/β-catenin pathway as well as one of the "non-canonical" branches of Wnt signaling, the Wnt/Ca2+ pathway. In our biological system, Wnt5A increased GFP expression, mainly as a consequence of Wnt/β-catenin pathway activation. Inhibitors of Nuclear Factor of Activated T-cells (NFAT) and Protein Kinase C (PKC) (both key elements of the WNT/Ca2+ pathway) did not affect GFP expression induced by Wnt5A.

The combination of Wnt5A and a recombinantly expressed FZD4 allowed us a clear interpretation of the effect of apple extracts on the Wnt/β-catenin signaling pathway.

AAE and LAE both worked efficiently as Wnt inhibitors and reduced Wnt activity elicited by Wnt5A. The EC_{50} of WNT pathway inhibition were 140 ± 16 mg/L and 330 ± 23 mg/L for AAE and LAE, respectively (**Figure 5.6A, B**). AAE and LAE did not affect GFP expression in cells transfected either with TCF-mut GFP or with cmv GFP, confirming that the two extracts affect the Wnt/β-catenin pathway.

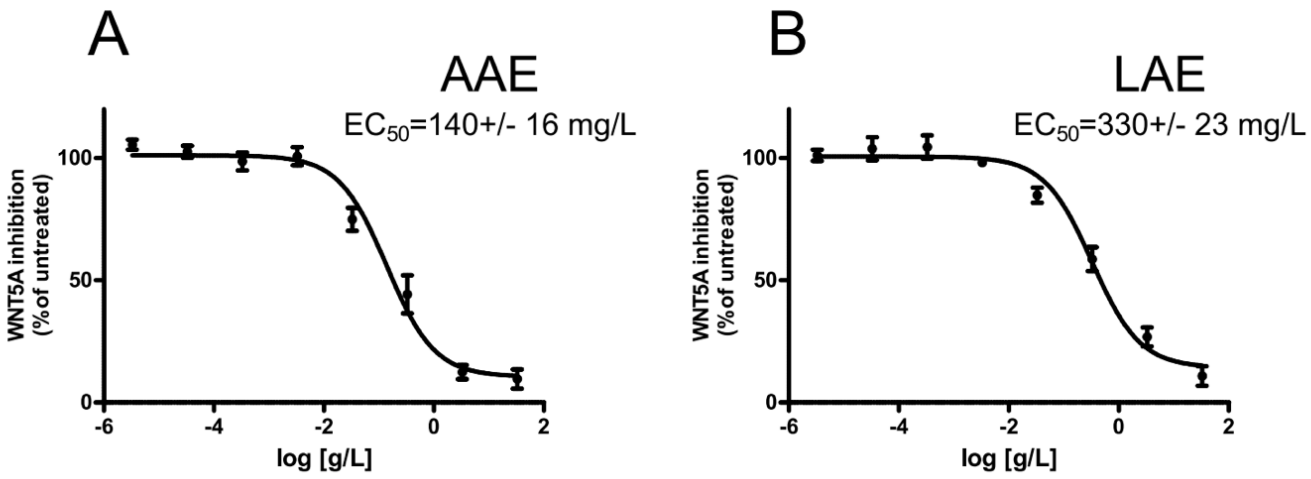


Figure 6. AAE and LAE act as Wnt pathway inhibitors. (A, B) Dose-response curves represent AAE (A) and LAE (B) modulation of Wnt/β-catenin pathway in HEK293 cells co-expressing FZD4 and the Wnt reporter construct (TCF-wt GFP). Values indicate changes in GFP expression (expressed as mean percentage change over untreated samples). Values are reported as mean ± SD (n = 3 replicates). EC50 values for each sample are shown on the corresponding graph and are reported as mean ± SEM (n = 5 independent experiments)

II.2 Mechanism Underpinning AAE and LAE Wnt Inhibitory Activity

We thus moved to identifying the Wnt pathway branches inhibited by AAE and LAE.

This is very important, especially when searching for therapeutic agents to use for FAP patients. Since APC is a midstream component of the Wnt pathway, its mutations make the manipulation of most of the upstream signaling components therapeutically ineffective. To be active in FAP patients, Wnt inhibitors should act either downstream to APC or on "non-canonical" Wnt pathway branches. One of the most active non-canonical branches positively contributing to Wnt signaling is the one involving the EGF Receptor (EGFR). Once activated, the EGFR pathway bypasses APC and leads, via AKT, to β-catenin activation. We thus challenged AAE and LAE to compete with LiCl and EGF, two inducers of the Wnt pathway, both acting downstream to APC. LiCl binds directly to GSK-3β and inhibits the β-catenin destruction complex. In contrast, EGF activates the EGFR pathway, that, via AKT, promotes β-catenin detachment from the Plasma Membrane and its nuclear translocation. In our biological system, both LiCl and EGF induced, in a dose-response manner, GFP expression in HEK293 transfected with TCF-wt GFP (**Figure 5.7A, C**). On the contrary, they both did not have an effect on cells transfected either with TCF-mut GFP or with cmv GFP (**Figure 5.7A, C**). AAE and LAE failed to inhibit activation of the WNT pathway induced by 15 and 30 mM LiCl.

However, they both reduced Wnt pathway activity induced by 5 and 10 mM LiCl (results for LAE are depicted in **Figure 5.7B**). Moreover, at all the tested concentrations, the extract abolished the WNT stimulatory activity of EGF (**Figure 5.7D**). These results prove that the apple extracts inhibit WNT pathway activation induced by LiCl and EGF and are thus suitable Wnt inhibitors for FAP cells carrying APC mutations.

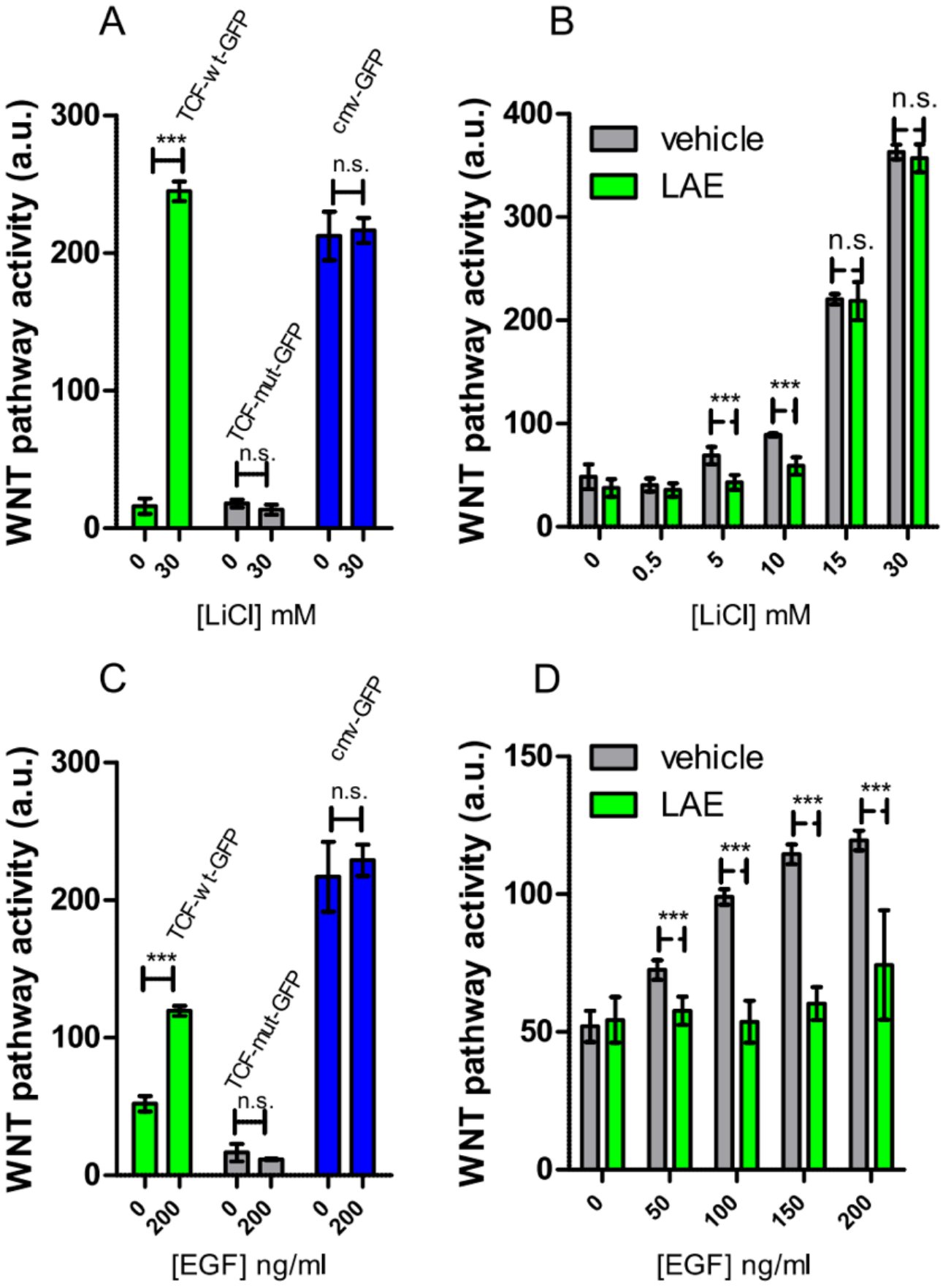

A
WNT pathway activity (a.u.)
300
200
100
0
TCF-wt-GFP
TCF-mut-GFP
cmv-GFP
***
n.s.
n.s.
0
30
0
30
0
30
[LiCl] mM
B
WNT pathway activity (a.u.)
400
300
200
100
0
vehicle
LAE
***
***
n.s.
n.s.
0
0.5
5
10
15
30
[LiCl] mM
C
WNT pathway activity (a.u.)
300
200
100
0
TCF-wt-GFP
TCF-mut-GFP
cmv-GFP
***
n.s.
n.s.
0
200
0
200
0
200
[EGF] ng/ml
D
WNT pathway activity (a.u.)
150
100
50
0
vehicle
LAE
***
***
***
***
0
50
100
150
200
[EGF] ng/ml

Figure 7. LAE inhibits the Wnt pathway, acting downstream to APC. (A) The histogram shows the WNT pathway activity induced by LiCl (30 mM) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (B) Wnt pathway activity of cells treated with the indicated concentration of LiCl in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L); (C) Wnt pathway activity induced by EGF (200 ng/mL) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (D) WNT pathway activity of cells treated with the indicated concentration of EGF in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L). Values are reported as mean ± SEM (n = 5). *** $p < 0.05$, n.s. indicates

II.3 Wnt Inhibitory Activity of LAE and AAE on CaCo-2 Cells

The suitability of LAE and AAE for APC treatment was further proved *in vitro*, by testing the Wnt inhibitory activity of AAE and LAE on CaCo-2 cells. This colon cancer cell line presents a mutation in the APC gene and is commonly used as in vitro cell culture prototype for FAP cells. CaCo-2 cells were transiently transfected with TCF-wt GFP. Thanks to the GFP reporter construct, the small percentage of Wnt active cells can be easily followed (**Figure 5.8A**). Upon treatment with AAE or LAE (400 mg/L, 48 h), GFP expression decreased in CaCo-2 cells (**Figure 5.8A, B**), indicating that the extracts efficiently inhibited the Wnt pathway in these cells.

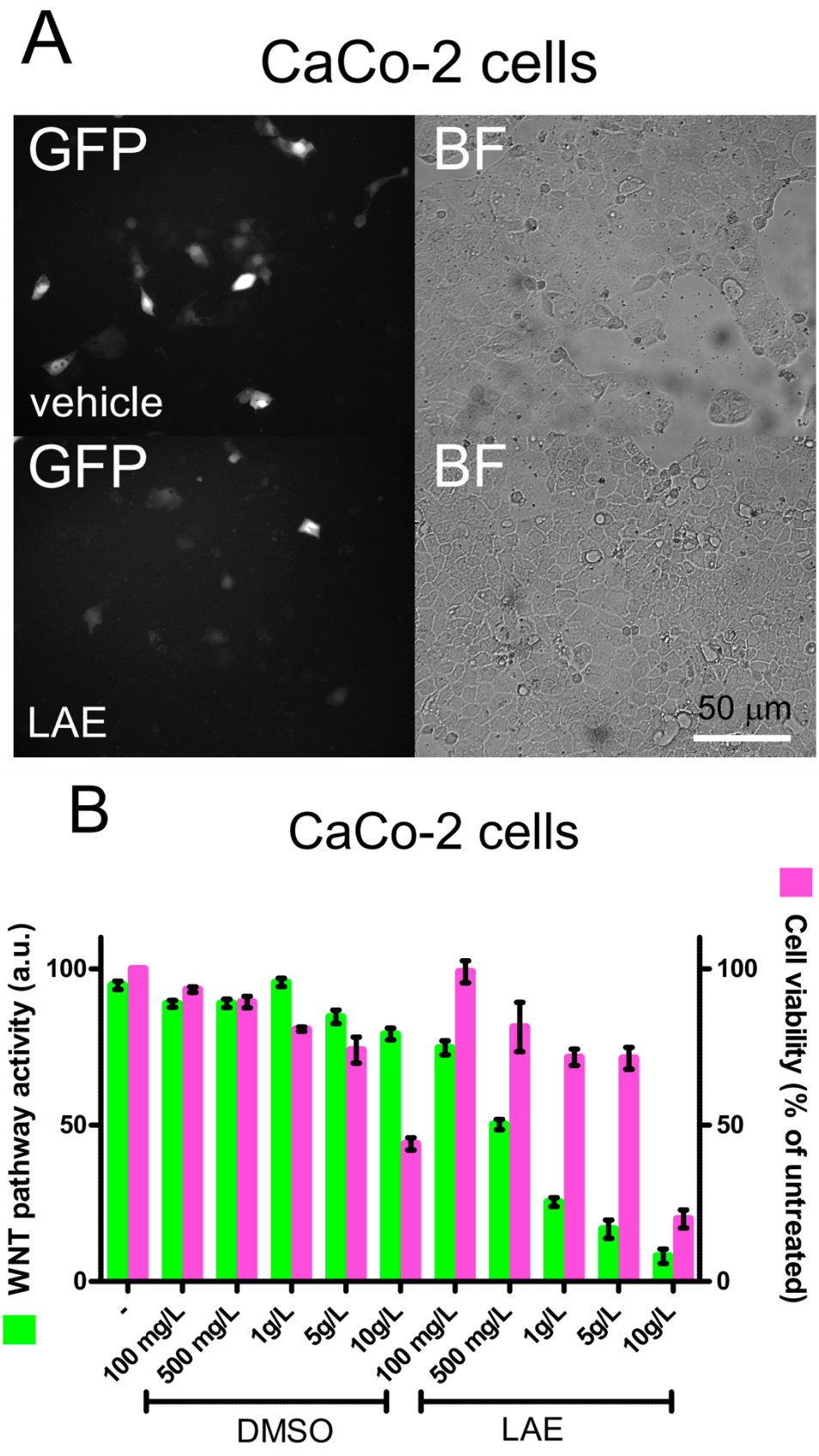

A
CaCo-2 cells
GFP
BF
vehicle
GFP
BF
LAE
50 µm
B
CaCo-2 cells
WNT pathway activity (a.u.)
Cell viability (% of untreated)
100
50
0
100
50
0
-
100 mg/L
500 mg/L
1g/L
5g/L
10g/L
100 mg/L
500 mg/L
1g/L
5g/L
10g/L
DMSO
LAE

Figure 8. AAE and LAE act as Wnt inhibitors in CaCo-2 cells. (A) Activity of the Wnt reporter construct TCF-wt GFP in CaCo-2 cells cultivated for 48 h in the presence or in the absence of LAE (400 mg/L) (representative of five experiments) (BF = Bright Field; scale bar is shown); (B) WNT pathway activity (green bars) and cell viability (magenta bars) of CaCo-2 cells transfected with TCF-wt GFP and cultivated in the absence (−) or in the presence of the indicated concentration of LAE (or of the corresponding dilution of DMSO). Values on the left axes indicate changes in GFP expression (a.u.). Values on the right axes indicate changes in cell viability expressed as percentage of untreated cells (−)

II.4 Wnt Inhibitory Activity of LAE and AAE on Human Biopsies

AAE and LAE were tested in an *ex vivo* system of FAP cells. Human colon biopsies were cultured *in vitro* soon after their resection from FAP patients (**Figure 5.9**). In unsupplemented media, these *ex vivo s*amples survived and duplicated for up to two days (**Figure 5.9A**), and then underwent growth arrest. Treatment with AAE or LAE resulted in a decreased proliferation rate and survival of the *ex vivo* cultures (**Figure 5.9A, C**).

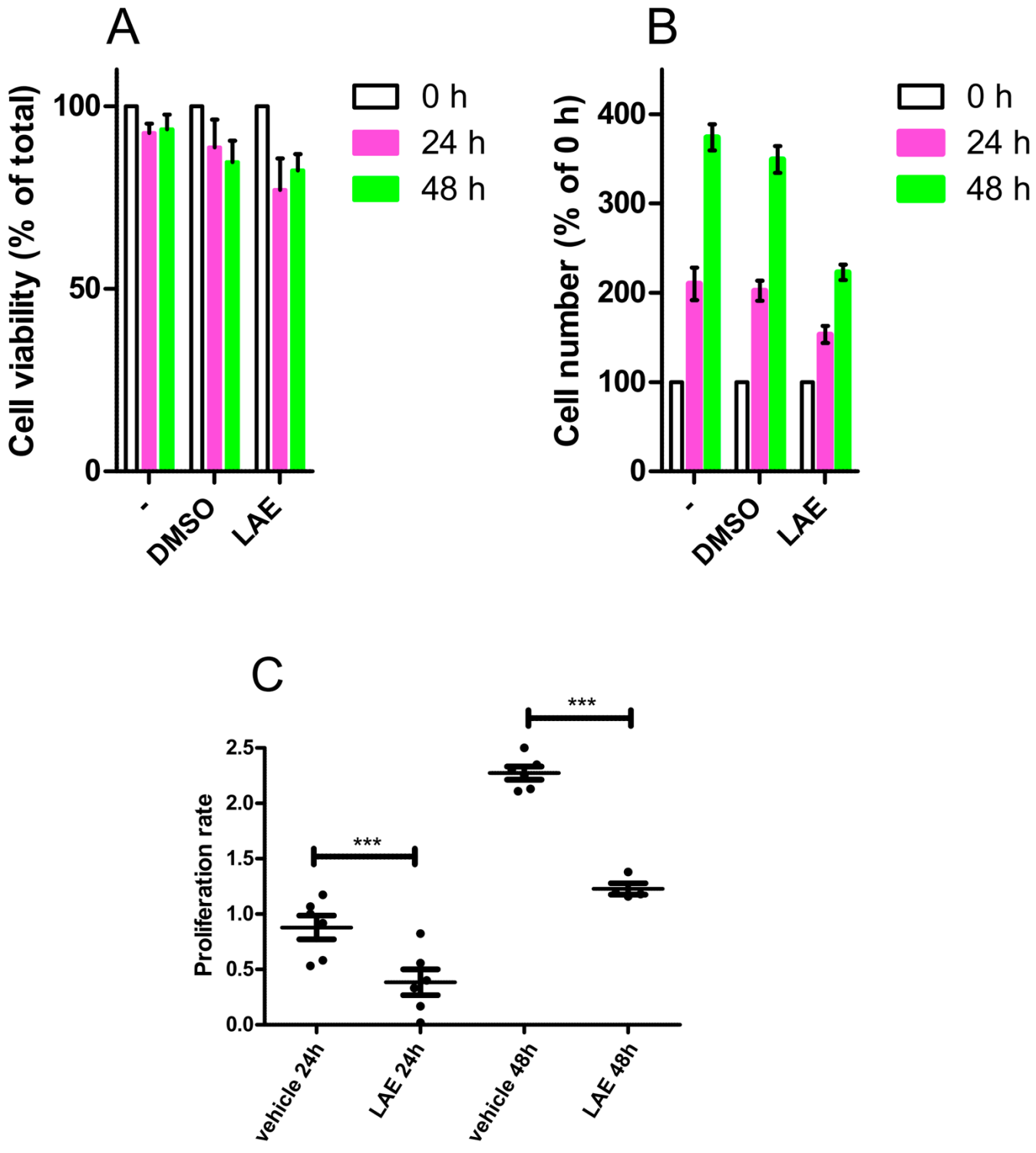


Figure 5.9. AAE and LAE affect *ex vivo* cultures of cells carrying FAP mutations. Cell viability, cell number and proliferation rate of human colonic biopsies cultured for 24 h and 48 h in a culture medium supplemented with LAE (400 mg/L) or with vehicle (DMSO). Values are expressed as mean ± SEM (n = 9). *** $p < 0.05$.

5.4.2 Growth promotional effects exerted by Annurca Apple Polyphenols on Hair Follicles (HFs)

A human healthy hair follicle (HF) undergoes cyclical rounds of growth, active fiber production (anagen), degeneration (catagen) and rest (telogen). Upon hair loss, HFs undergo a premature miniaturization and shorten their permanence in anagen. Several pharmaceutical therapies are approved by FDA such as Finasteride and Minoxidil, but they produce adverse effects on patients. Recently, dietary supplements, popular over-the-counter products, have indeed been shown to increase anagen rate in Hair Loss patients.
Despite the increased usage of nutraceuticals for hair growth, studies concerning their mechanism of action are far from being complete.

Herein, I used Annurca Apple polyphenolic extract of (AAE) to stimulate proliferation of keratinocytes and induces production of keratins. I here investigated the metabolomic profile of murine HFs treated with AAE. C57BL/6 mice received a topical treatment with a cosmetic foam containing AAE. Upon 4 weeks of treatment, murine HFs were extracted from the skin and their metabolome were analyzed by Direct Infusion Fourier Transform-ion cyclotron resonance mass spectrometry (DI-FT-ICR-MS), a technique well explained in **Chapter 4**.

I. Materials and methods

I.1 Nutraceutical Composition
Composition of AAE cosmetic foam: AnnurtriComplex (industrial procyanidinic extract of Annurca apple PGI (Protected Geographical Indication) (*Malus pumila Miller* cv. Annurca) produced by MB-Med (Turin, Italy)) 6% (w/v), water, glycerin, decylglucoside, polysorbate, maltodextrin, potassium sorbate, sodium benzoate, silica. The Placebo foam was formulated identically but did not contain AAE.

I.2 Animals
Wild-type C57BL/6 mice (7 weeks old, postnatal day 49) were used in all experiments to test the effect of cosmetic foam containing AAE. All animals received human care and were maintained in separate cages at 22–24 ◦C and fed a general rodent diet. Differently from other published protocols, here animals were left unshaved and received a topical treatment with 2 cm3 of the indicated cosmetic foam (this would correspond to 2–4 mL of foam for an adult human scalp) for 4 weeks, twice a week.

Only male animals were used in this study. All animal experiments were performed in compliance with ethical guidelines and approved by the University of Naples Federico II.

I.3 Histology

After 4 weeks of treatments with AAE, mice were sacrificed and their dorsal skin immediately excised and immersed in Phosphate Buffer Saline (PBS). 1 cm^2 of skin biopsies were fixed overnight in 4% formalin, washed in PBS, dissected and embedded in paraffin. 5–10 µm sections were deparaffinised and stained with haematoxylin and eosin using standard procedures. For ex-vivo staining of mitochondria, mice skin biopsies, immediately after excision, were rinsed in PBS and located in six multi-well plates. The staining solution was prepared by diluting MitoTracker CMX-Ros in Dulbecco Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) (without fetal bovine serum) to yield a final concentration of 200 nM. Tissues were incubated for 40 min in a Cell Culture incubator at 37 ◦C, supplemented with 5% CO2. At the end of the incubation, tissues were rinsed three times in DMEM, fixed in 4% formaldehyde (diluted in PBS pH 7.4), embedded in Paraffin or in OCT and sectioned with a Leica 3200 Cryostat (Ramsey, MN, USA). 10–20 µm sections were washed in 96%, 90% and finally in 80% EtOH solution (5 min wash) to be then covered with a glycerol/PBS solution 1:1 (to avoid drying) and visualized under a fluorescent microscope.

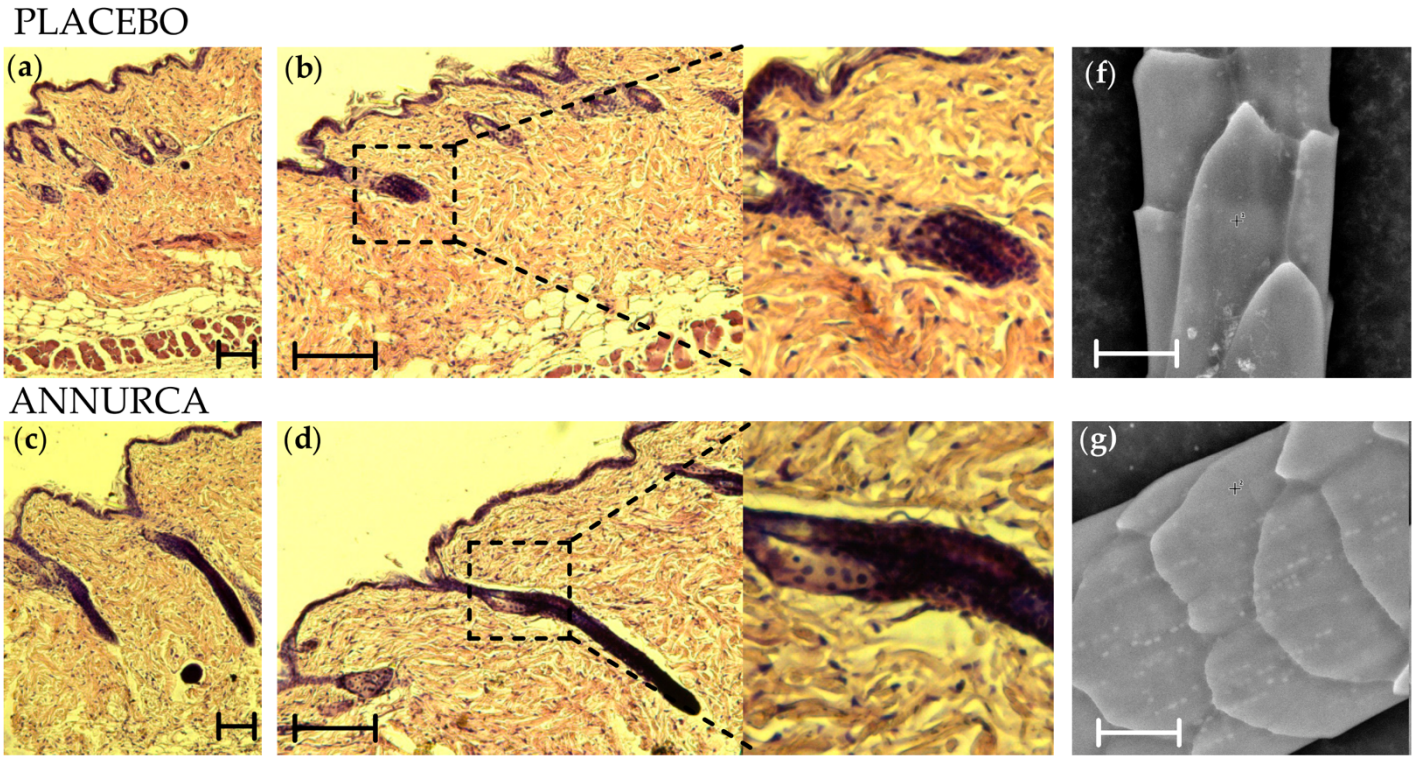
I.4 Mass Spectrometry-Based Metabolomic, Statistics and Analysis Analyses

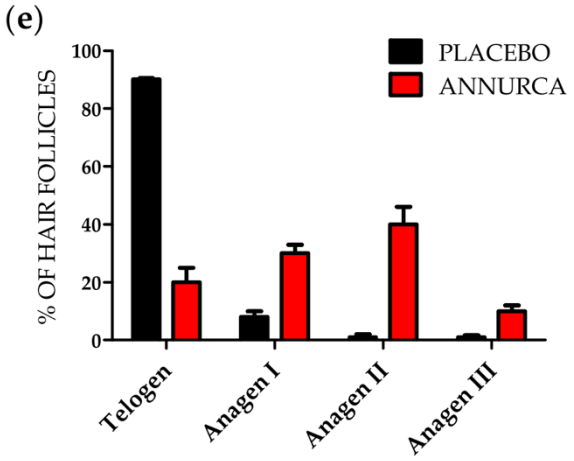
After excision, tissues were immersed in Phosphate Buffer Saline (PBS). Hair shafts were plucked out with a sterile tweezers and immediately covered with a solution of PBS at RT (Room Temperature). To allow detachment of HF cells, plunked HFs were incubated for 15 min in PBS supplemented with 5 mM EDTA. Hair shafts were removed with a cell strainer and HF cells were centrifuged for 5 min at 500 rpm. The cell pellets were washed twice in PBS and split in two aliquots. The first pool of cells was solubilized in 1 mL of Trizol (Invitrogen, Carlsbad, CA, USA) and stored at −80 ◦C for qPCR analysis. The second pool was homogenized in 1 mL of pre-chilled methanol/water 1:1 solution containing 10nmoL of internal standard and finally centrifuged at 10,000× g for 10 min at 4 ◦C [31]. The resulting supernatants were collected and transferred into new Eppendorf tubes and stored at −80 ◦C.

II. Results and discussion

II.1 Topical treatment with AAE increases Keratin Content in Hair Shafts

After 4 weeks of treatment, C57BL/6 mice (11 weeks old) were sacrificed and their dorsal skin excised. Skin biopsies were embedded in paraffin and prepared for histology. As expected, HFs of mice treated with placebo were mostly in Telogen/Anagen I phase (**Figure 5.10A, B**). Differently, HFs of mice treated with AAE appeared increased in length (**Figure 5.10C, D**). To confirm HFs of mice treated with AAE being in a production phase of the hair cycle, percentage of Sulfur were evaluated by SEM-EDX. Compared to placebo, AAE-treated hairs showed an increased content of Sulfur and thus of keratins (**Figure 5.10F, H**). Since keratins are only produced by anagen HFs, our SEM data confirm histochemical analysis indicating that AAE topical treatment accelerated exit of murine HFs from telogen phase.


PLACEBO
(a)
(b)
(f)
ANNURCA
(c)
(d)
(g)


(e)
% OF HAIR FOLLICLES
100
80
60
40
20
0
PLACEBO
ANNURCA
Telogen
Anagen I
Anagen II
Anagen III

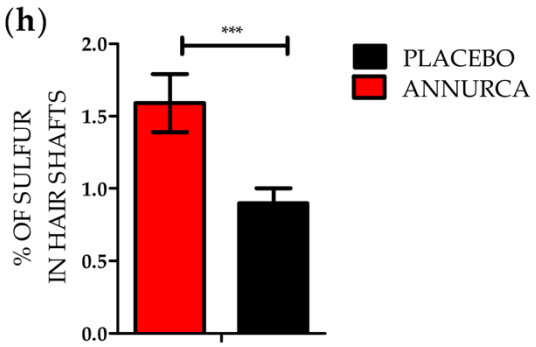

(h)
% OF SULFUR IN HAIR SHAFTS
2.0
1.5
1.0
0.5
0.0
***
PLACEBO
ANNURCA

Figure 5.10. AAE induces early exit from telogen and increases keratin content of hair shafts in murine HFs. C57BL/6 mice were treated topically with a foam supplemented either with AAE or with a placebo. Foams were applied on the dorsal skin of 7-week-old mice. After 4 weeks of treatment, mice were sacrificed and HFs classified following morphological criteria. Haematoxylin-eosin staining of HFs of mice treated with placebo (a–b) showing HFs mostly in Telogen/AnagenI phase. HFs of mice treated with AAE appeared in a later stage of Anagen, mostly Anagen II (c–e). (f–h) SEM (SEM-EDX) analysis of hairs extracted from mice treated with placebo (f) or with AAE (g). SEM quantitative analysis indicates (h) an increase in Sulfur concentration (cystine, methionine, cysteine and cysteic acid all abundant amino acids of hair keratins) in hairs of mice treated with AAE. Values in € and (h) are reported as mean ± SEM (n = 8, *** $p < 0.001$). Scale bars in a-d correspond to 200 μm. Scale bars in (f) and (g) correspond to 10 μm

II.2 Treatment with AAE Alters the Intracellular Levels of HF Key Metabolites

The metabolic content of HF cells plucked out of mice treated topically with AAE were analyzed by DI-FT-ICR mass spectrometry.

Complex profiles were obtained in both positive and negative ionization. The high mass accuracy isotopic distribution and comparison with available standards, ensured confident identification. By screening for intracellular metabolites with similar alteration tendency in all the AAE-treated mice, glutaminolysis, pentose phosphate pathway (PPP), amino acid oxidation, mitochondrial β-oxidation and arginine metabolism became our focus.

Our metabolite profiling revealed that the topical treatment for 4 weeks with a cosmetic foam containing AAE significantly changed the levels of at least 18 key intracellular metabolites. The (i) significant elevation of glucose, (ii) glutamine, (iii) glycine, (iv) the increase in the intracellular level of the PPP intermediate ribulose 5P together with (v) the reduction of the intracellular level nucleotides and deoxy-nucleotides all together suggest that AAE causes a reduction in the utilization of glucose and glutamine for PPP, a metabolic pathway that in HFs, supports nucleotide biosynthesis (**Figure 5.11, Table 5.1**). Interestingly, all the reaction involved seems to share NADPH as cofactor. This electron carrier is involved in many anabolic reactions including synthesis of ribose 5P, of nitrogen-containing bases and of nucleotides. AAE probably affects the overall redox environment of the cell, affecting NADPH production and redox potential. The reduction of the intracellular level of citrulline, the only catabolite of arginine requiring NADPH for its production and the reduced intracellular level of GSH seems to confirm and support our hypothesis that NADPH dependent reaction are halted in AAE-treated HFs (**Figure 5.12**).

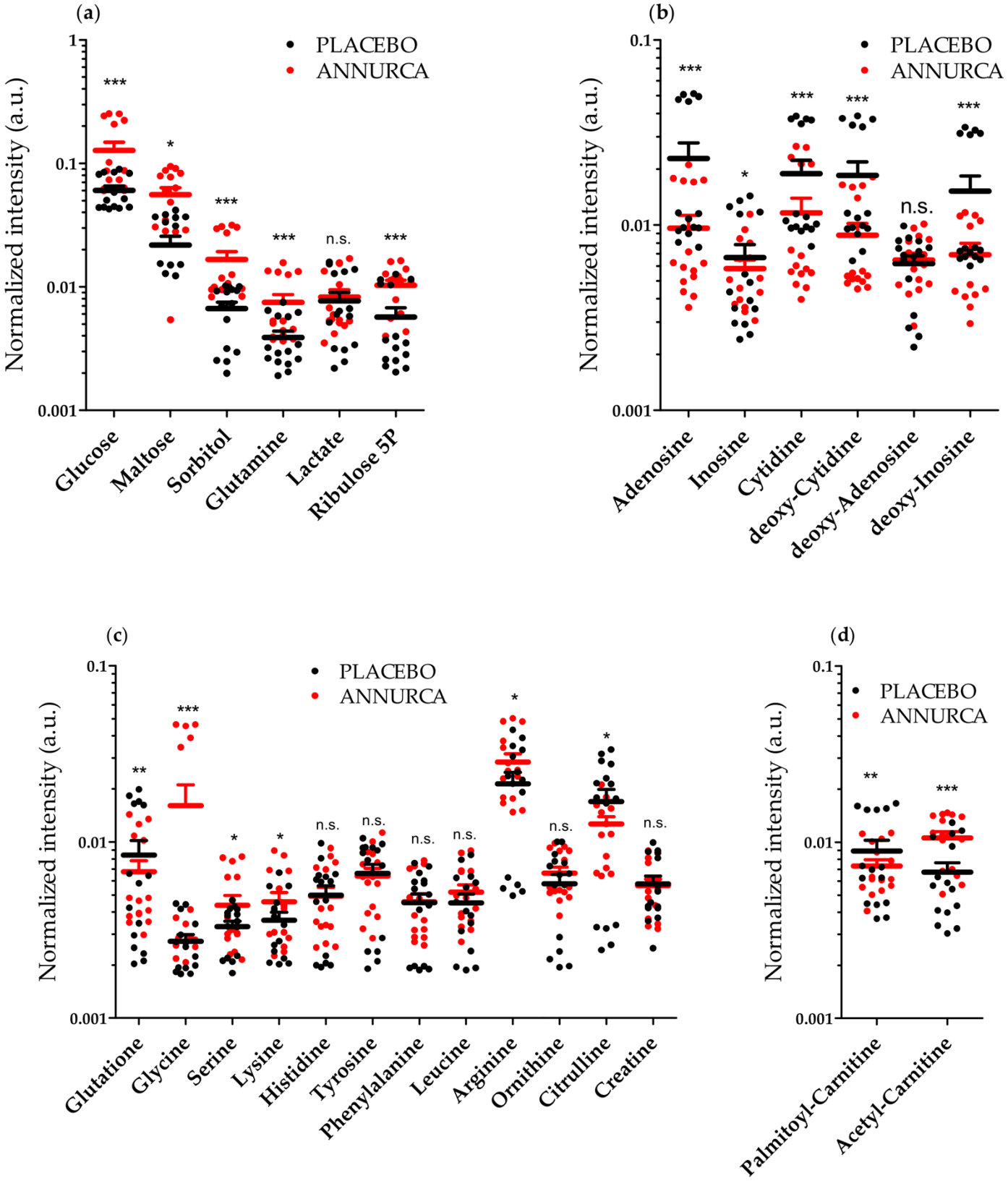

(a)
PLACEBO
ANNURCA
Normalized intensity (a.u.)
1
0.1
0.01
0.001
***
*
***
***
n.s.
***
Glucose
Maltose
Sorbitol
Glutamine
Lactate
Ribulose 5P
(b)
PLACEBO
ANNURCA
Normalized intensity (a.u.)
0.1
0.01
0.001
***
*
***
***
n.s.
***
Adenosine
Inosine
Cytidine
deoxy-Cytidine
deoxy-Adenosine
deoxy-Inosine
(c)
PLACEBO
ANNURCA
Normalized intensity (a.u.)
0.1
0.01
0.001
**
***
*
*
n.s.
n.s.
n.s.
n.s.
*
n.s.
*
n.s.
Glutatione
Glycine
Serine
Lysine
Histidine
Tyrosine
Phenylalanine
Leucine
Arginine
Ornithine
Citrulline
Creatine
(d)
PLACEBO
ANNURCA
Normalized intensity (a.u.)
0.1
0.01
0.001
**
***
Palmitoyl-Carnitine
Acetyl-Carnitine

Figure 5.11. AAE diverts HF metabolic pathways from PPP and amino acid oxidation. Metabolomic analysis (Carbohydrates (a); nucleotides (b); amino acids (c); carnitine derivatives (d)) of HF cells extracted from C57BL/6 mice treated topically for 4 weeks with a foam containing AAE (red dots, ANNURCA) or a Placebo (black dots PLACEBO). Each point represents the normalized intensity of the metabolite (n = 15 measurements, mean ± SEM. Two way ANOVA and Bonferroni post-test analysis were performed; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s.: non statically different)

Metabolic Pathway	Metabolite	Fold Change [1]	Metabolic Pathway	Metabolite	Fold Change [1]
Glycolysis			PPP		
	Glucose *	2.4 ± 0.2		Ribulose 5P *	3.2 ± 0.1
	Lactic acid	1.1 ± 0.2			
Glycogenolysis			Nucleotides	Adenosine *	0.4 ± 0.1
	Maltose	2.2 ± 0.1		Cytidine *	0.6 ± 0.1
	Sorbitol	3.1 ± 0.2		Deoxy-cytidine *	0.5 ± 0.1
Amino acids				Deoxy-inosine *	0.5 ± 0.1
	Glutamine *	2.4 ± 0.2			
	Glycine	6.2 ± 0.2	β-oxidation	Palmitoyl-carnitine	0.8 ± 0.1
	Serine	1.3 ± 0.2		Acetyl-carnitine	1.6 ± 0.2
	Lysine	1.3 ± 0.2			
	GSH *	0.9 ± 0.1			
	Arginine	1.3 ± 0.2			
	Citrulline *	0.7 ± 0.2			

[1] (n = 15. Shown is mean ± SEM); * indicates metabolites requiring NADPH for their anabolism/catabolism.

Table 5.1. Fold induction for the indicated metabolites measured upon *in vivo* treatment with AAE

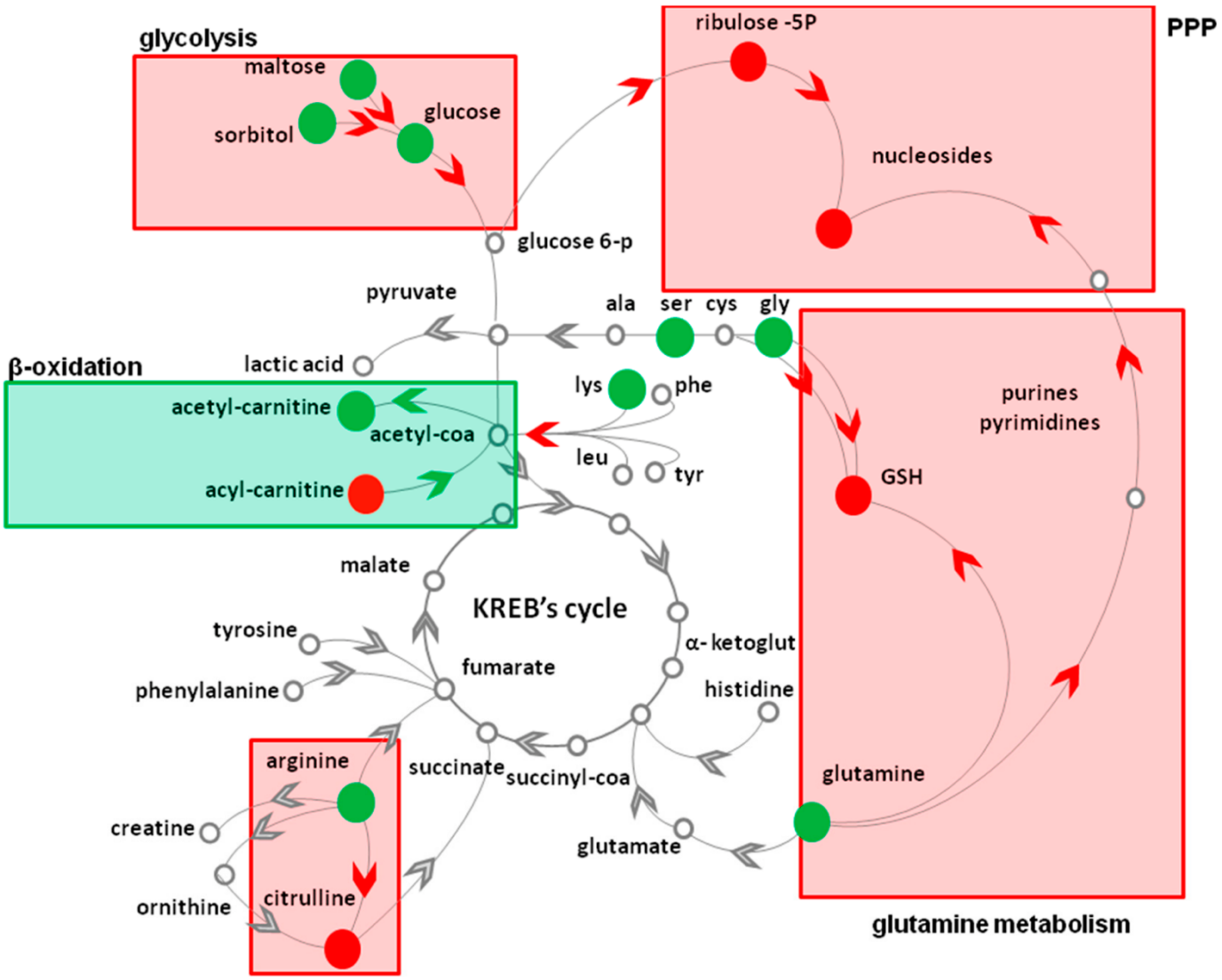


Figure 5.12. Schematic cartoon depicting some of the metabolic reactions positively (green box) or negatively (red boxes) affected by AAE in murine HFs. Red and green arrowheads indicate reactions halted or stimulated by AAE, respectively. Red and green dots indicate metabolites whose intracellular concentration resulted decreased or increased by treatment with AAE, respectively.

5.4.3 Annurca Apple Polyphenols Protect Murine Hair Follicles from Taxane Induced Dystrophy

Chemotherapy-induced alopecia (CIA) is a common side effect of conventional chemotherapy and represents a major problem in clinical oncology. As we have described above, nutraceuticals seem to be safe and effective option for CIA as supplements to support chemotherapy treatments.
Moreover, in virtue of its mechanism of action, AAE is herein proven to be compatible with chemotherapy regimens. Herein, we analyze new aspects of the molecular mechanism behind AAE activity by analyzing how the apple extract affects the metabolism of PUFA, a class of lipids and signaling molecules involved in HFs homeostasis. PUFA signaling influences as well response to chemotherapy and is extremely important in terms of immunosuppression, tumor growth and tumor relapse.

I. Materials and methods

I.1 Animals for in vivo experiments
Wild-type C57BL/6 mice (7 weeks old, postnatal day 49) were used in all experiments to test the effect of cosmetic foam containing AAE. All animals received human care and were maintained in separate cages at 22 ◦C–24 ◦C and fed a general rodent diet. Differently from other published protocols, here animals were left unshaved and received a topical treatment with 2 cm^3 of the indicated cosmetic foam for 4 weeks. Only male animals were used in this study.

I.2 Ex vivo culturing of Murine HFs wild-type
Wild-type C57BL/6 mice were used for the ex vivo experiments. Only male animals were used in this study. 12 weeks old mice (postnatal day 84) were sacrificed and their dorsal skin were immediately excised and immersed in Phosphate Buffer Saline (PBS). 1 cm2 of skin biopsies, were rinsed in PBS and located in 6 multiwell plates. Biopsies were cultivated in 1 mL of Dulbecco Modified Eagle Medium (41965-039, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (10270, GIBCO), Glutamine (35050-061, GIBCO), Penicillin and Streptomycin (15070-063, GIBCO). Tissues were incubated for 8 days in a Cell Culture incubator at 37 ◦C, supplemented with 5% CO_2. When indicated, Paclitaxel (700 nM), Docetaxel (700 nM) and/or AAE (400 mg/L) were added to the culture medium. The medium was replenished every 3 days. At the end of the incubation, tissues were rinsed three times in DMEM and fixed in 4% formaldehyde diluted in PBS (pH 7.4). Nuclei were stained with DAPI and visualized under a fluorescent

microscope. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray (EDX) analysis of hair shafts were performed with a bench top Phenom XL (Alfatest, Milan, Italy) following manufacturer instructions and as already described.

I.3 Metabolite extraction from Murine Tissues and Mass Spectrometry-Based Metabolomic

The same described in **5.4.2 paragraph**.

II. Results and discussion

II.1 Treatment with AAE Alters PUFAs Metabolism in Murine HFs

We treated C57BL/6 mice with a cosmetic foam containing AAE (topically and for 4 weeks) to then extract their HFs and analyze their metabolome by Direct Infusion Fourier Transform-ion cyclotron resonance mass spectrometry (FT-ICR-MS), a technique endowed with ultra-high mass accuracy and resolution. We prove that AAE increases the intracellular levels of the growth promoting prostanoid Prostaglandins F2α (PGF2α). On the contrary, AAE reduces the intracellular levels of PUFA epoxides stimulating their conversion into the corresponding inactive diols and promoting their usage as β-oxidation substrates (**Table 5.2, Figure 5.13**).

We finally use an *in vitro* assay and prove for the first time that AAE can inhibit taxane induced dystrophy in *ex vivo* murine HFs. In taxane-containing chemotherapy regimen, AAE could be even more indicated, considering the selectivity of this chemotherapy agent for mitotic cells.

PUFA	Metabolite	Fold Change [1]	PUFA	Metabolite	Fold Change
ARA			DHA		
	ARA 20:4ω-6 *	1.0 ± 0.1		DHA 22:6ω-3 *	0.9 ± 0.2
	Tetranor 12-HETE	3.4 ± 0.2		17-HDoHE	1.0 ± 0.1
	14,15-DiHETrE	3.5 ± 0.1		13-HDoHE	1.1 ± 0.1
	2,3-Dinor-6-keto-PGF1α	1.6 ± 0.2		19,20-DiHDPA	1.3 ± 0.1
	15-Keto-13,14-dihydroPGA2	1.0 ± 0.1			
	PGF2α	1.6 ± 0.1	α-LA		
	13,14-dihydro-PGF2α	2.3 ± 0.2		α-LA 18:3ω-3 *	1.1 ± 0.1
	15-deoxy-Δ12,14-PGJ2	1.1 ± 0.1	LA		
				LA 18:2ω-6 *	0.9 ± 0.1
EPA				9,10-DHOME	specific for AAE
	EPA 20:5ω-3 *	0.9 ± 0.1		13-HpODE	2.3 ± 0.1
	5-HEPE	1.0 ± 0.1		9,12,13-TriHOME	1.3 ± 0.1

Table 5.2. Fold induction for the indicated metabolites measured upon *in vivo* treatment with AAE

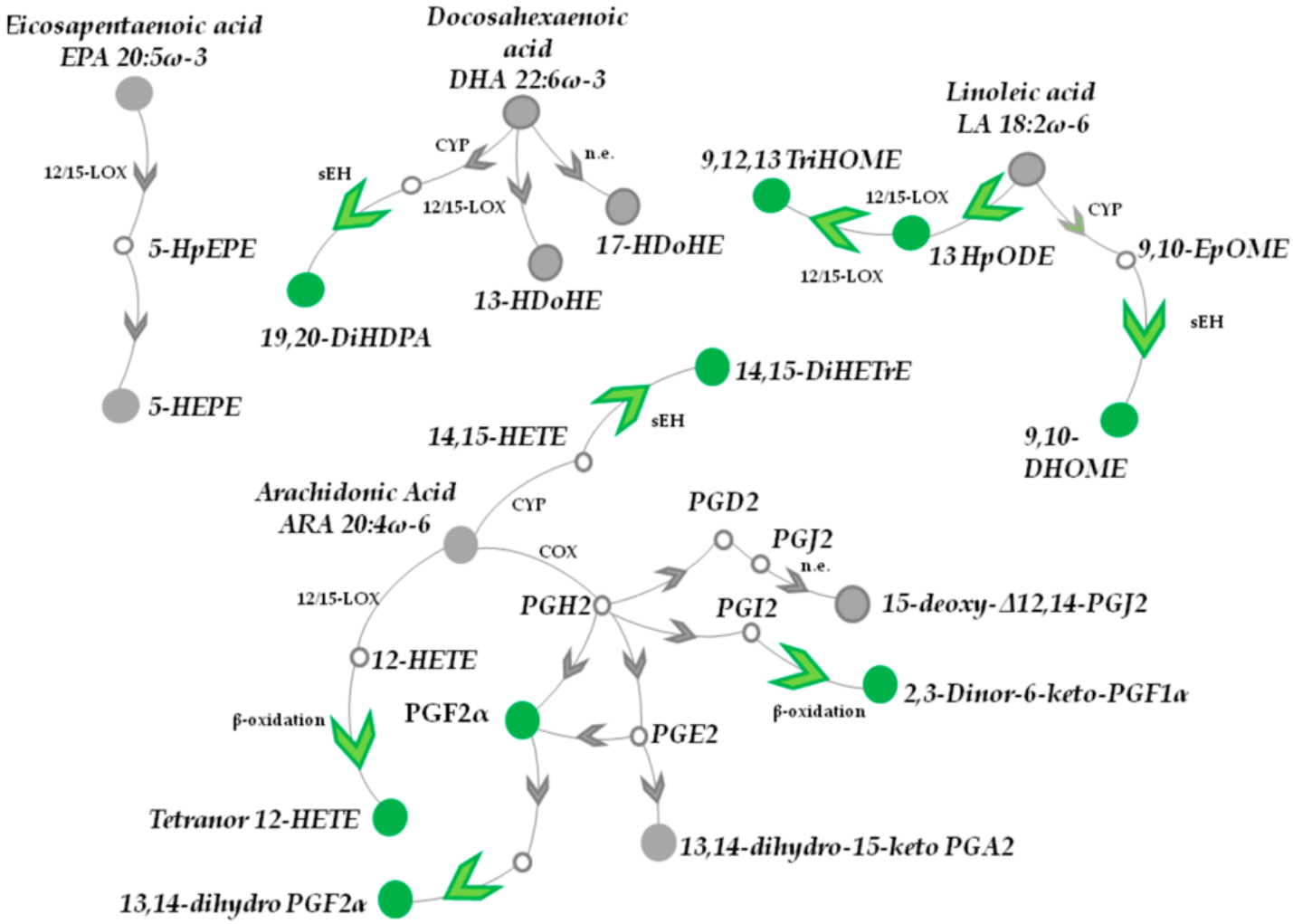


Figure 5.13. Schematic cartoon depicting some of the metabolic reactions positively affected by AAE in murine HFs. Green arrowheads indicate reactions stimulated by AAE. Green dots indicate metabolites, whose intracellular concentration resulted increased by AAE. (COX cyclooxygenase; LOX, lipoxygenase; CYP, cytochrome P450; sEH, soluble epoxide hydroxylase; n.e. non enzymatic.

II.2 AAE Protects Murine HFs from Taxane Induced Follicular Dystrophy

Here we used skin biopsies of 12 week old C57BL/6 mice (anagen phase) to perform an ex-vivo incubation of HFs in the presence of paclitaxel (700 nM) or docetaxel (700 nM) for 7 days. We further tested if 400 mg/L of AAE (AAE active dose for hair promoting effect) or the corresponding amount of vehicle (DMSO), was able to reduce the HF dystrophy induced by the two taxanes. After 7 days of incubation, skin biopsies were processed for histology and classified following morphological criteria. In the absence of any treatment, *ex vivo* cultures of HFs were mostly in Anagen (**Figure 5.14 A**). Treatment with Docetaxel (**Figure 5.14C, D**) massively affected HFs, that showed clear signs of chemotherapy induced damage. When HFs were treated with docetaxel in the presence of AAE, HFs appeared much less damaged by the treatment (**Figure 5.14F**).

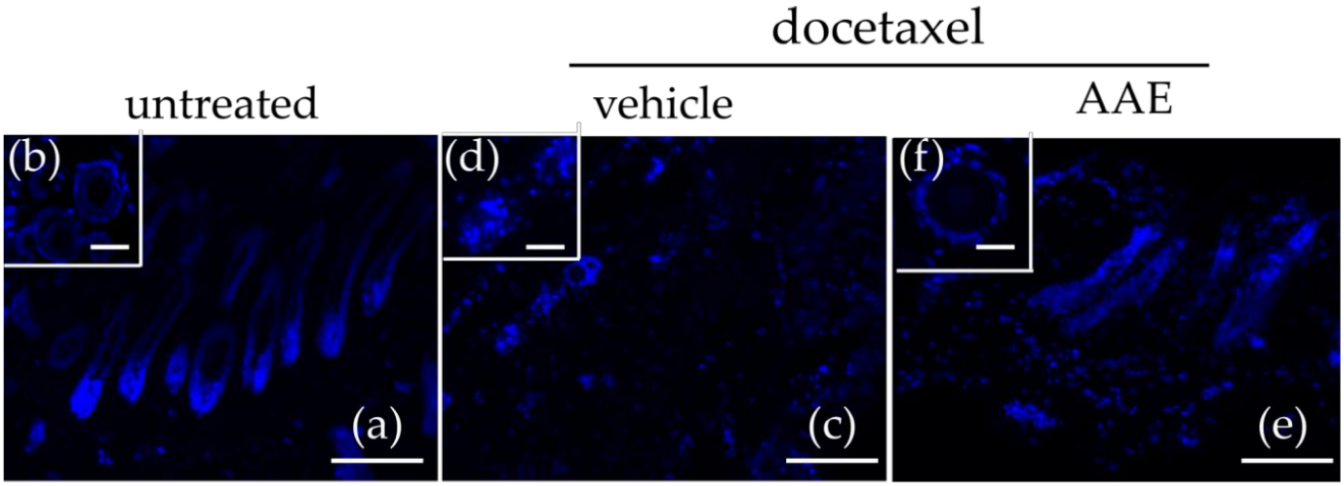


Figure 14. AAE protects murine HFs from taxanes induced dystrophy. 12 week old C57BL/6 mice were sacrificed and their skin biopsies incubated in vitro in the absence or in the presence of Docetaxel (700 nM), AAE (400 mg/L) or vehicle. Upon 7 days of *ex vivo* culturing, biopsies were fixed and processed for histology. Nuclei were stained with DAPI and HFs classified following morphological criteria. (a,b) Untreated HFs in late Anagen phase; (c,d) HFs treated with Docetaxel showing severe signs of follicular dystrophy (e,f) HFs of mice treated with docetaxel in the presence of AAE appearing less damaged by the treatment with the taxane. Scale bars correspond to 50 µm

II.3 AAE Preserves Keratin Production in Murine HFs Treated with Taxanes

To confirm the protective effect exerted by AAE against taxane induced dystrophy, hair shaft microstructure and keratin content of HFs were evaluated by SEM-EDX. To analyze the hair shaft extension occurred only during the period of the in vitro incubation, we evaluated only hair shafts section located in the HFs or closest to them. Differently from untreated HFs (**Figure 5.15A**), hair shafts of HFs treated with Docetaxel (**Figure 5.15B, C, E, G**) appeared frequently damaged and when analyzed by EDX presented a drastic decrease in Sulphur content (**Figure 5.15I**). Since cystine, methionine, cysteine and cysteic acid are abundant amino acids of hair keratins, the percentage of Sulphur in the hair shaft can be considered a measure of keratine amount. We have already shown that AAE is able to increase Sulphur content in hair keratins. Our morphological data and SEM-EDX analysis thus suggest that 400 mg/L AAE were able to protect, at least *in vitro*, murine HFs from taxane induced dystrophy and to preserve in vitro keratin production.

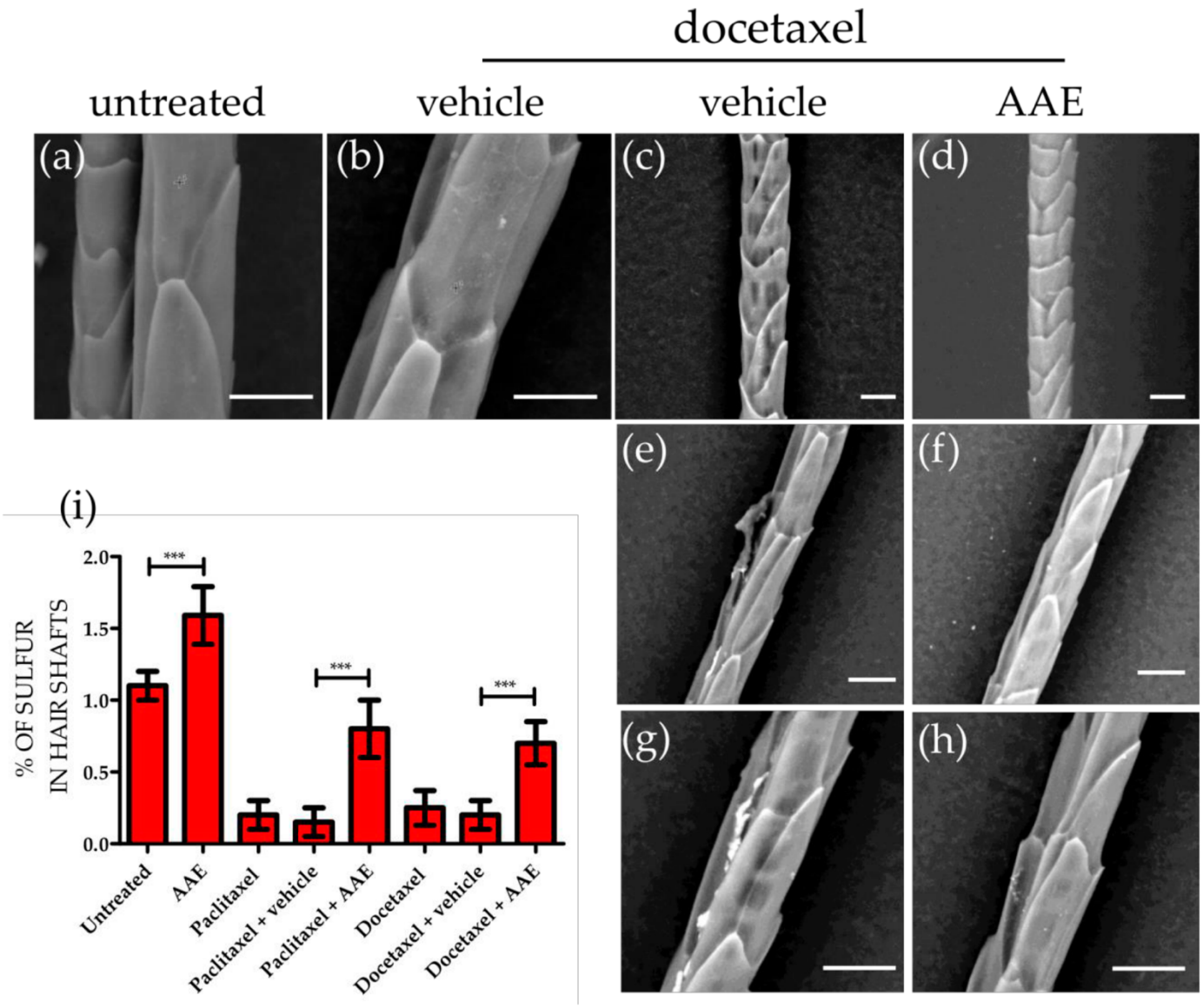


Figure 5.15. AAE preserves keratin content in murine HFs treated with taxanes. Hair shafts plucked out from mice biopsies treated as in Figure 2 were analysed by SEM-EDX. Morphology of hair shafts extracted from untreated skin biopsies (a) or treated with docetaxel (700 nM; b,c,e,g) and showing signs of hair shaft damage. Morphology of hair shafts extracted from HFs treated with Docetaxel in the presence of AAE (d,f,h) showing signs of hair shaft protection exerted by AAE (Scale bars correspond to 10 μm). (i) SEM-EDX quantitative analysis indicates a decrease in Sulphur content in hairs shafts extracted from HFs treated with taxanes. In the presence of AAE the Sulphur content of hair shafts is partially preserved (mean ± s.e.m.; n = 8, *** $p < 0.001$)

5.5 Conclusions

Annurca Apples have been showing their potential as nutraceuticals in many human conditions and pathologies. The plethora of different biological contexts in which AAE is active must be attributed to the hundreds of different metabolites it contains [47]. The resulting molecular synergism allows AAE to act as antioxidant, as modulator of lipid and cholesterol anabolism as well as against stress and aging.

I here showed that apple extracts from both Annurca (AAE) and Limoncella (LAE) are endowed with inhibitory activity toward Wnt/β-catenin signaling, an intracellular pathway involved in many forms of cancer. I showed that both AAE and LAE are able to inhibit Wnt signaling in cells carrying APC mutations. In both *in vitro* cultures of cells as well as in *ex vivo* biopsies of FAP patients, AAE and LAE blocked the proliferation and duplication rates of colon-rectal cancer cells.

AAE and LAE Wnt inhibitory activity can be partially ascribed to their polyphenolic content.
However, none of the major constituents of their polyphenolic fractions, tested as pure molecules, exerted strong Wnt inhibitory activities, suggesting the importance of the whole pool of polyphenols for the activity of the extracts. Our preliminary results suggest that the Wnt inhibitory activity of AAE and LAE can be ascribed either to the antioxidant activity of the entire polyphenolic mixture or to the effects that the latter may exert on its own components, protecting them from detrimental chemical modifications occurring in physiological environments.

Using a murine model and high-resolution mass spectrometry, we have as well disclosed some of the molecular details behind AAE hair promoting effect.
Dealing with the metabolite profiling described above, the use of an *in vivo* system and the metabolite profiles of HFs depicted by high resolution mass spectrometry technique, allowed us to take a snapshot of some of the metabolic pathways activated by Annurca polyphenols in HFs. We are, of course, not sure to have recovered all the different cell lineages present in HFs. However, their specific contribution to the full metabolic profile we, here, present is not possible to determine. Clearly, the picture we obtained is not yet complete, but it is useful to explain some of the hair growth promoting effects of Annurca Polyphenols.

Our analysis is further extended to show the effect exerted by AAE on PUFAs, a class of lipids involved in HF signaling and homeostasis. By monitoring the change in the intracellular levels of PUFA and of their metabolites we could show that AAE hijacks most of them toward β-oxidation.

The metabolic switch induced by AAE in HFs has interesting consequence in terms of compatibility of AAE with chemotherapy regimens. By blocking glutaminolysis, AAE impairs the synthesis of nitrogen containing bases and of deoxy-nucleotides. The absence of metabolites necessary for DNA replication and RNA production explains why AAE does not promote cancer cell growth and mitosis. Moreover, by reducing nucleotide synthesis, AAE makes HFs resistant to chemotherapy agents like taxanes, that selectively inhibit mitotic and highly proliferating cancer cells. This peculiar mechanism, together with the anticancer property we have described for AAE in colon rectal cancer cells, suggests that AAE could represent a safe nutraceutical option.

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