Review

Development of Orally Administrable Phytochemicals by Nano-Suspension and Nano-Emulsion Techniques

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Abstract: The awareness of the existence in plants of bioactive compounds namely phytochemicals (PHYs), having health properties is progressively expanding. Therefore, their massive introduction in the normal diet, in food supplements, and their use as natural therapeutics to treat several diseases are increasingly emphasized by several sectors. Particularly, most PHYs possessing antifungal, antiviral, anti-inflammatory, antibacterial, antiulcer, anti-cholesterol, hypoglycemic, immunomodulatory, and antioxidant properties have been isolated from plants. Additionally, their secondary modification with new functionalities, to further improve their intrinsic beneficial effects, is extensively investigated. Unfortunately, although the idea of exploiting PHYs as therapeutics is amazing, its realization is far from simple, and the possibility of exploiting them as effective orally administrable drugs is almost utopic. Most PHYs are insoluble in water and, when introduced orally, they scarcely reach the site of action in therapeutic concentrations. Degradation by enzymatic and microbial digestion, occurring in the mouth, stomach, and intestine, as well as fast metabolism and rapid excretion via the kidney, biliary, or lung, strongly limit their in vivo activity. To overcome these drawbacks, several nanotechnological approaches have been used and many PHYs-loaded delivery systems with dimensions of nanometers have been developed. This paper, also by reporting various recent case studies, reviews the foremost nano-suspension and nano-emulsion-based techniques developed for formulating the most relevant PHYs in more bioavailable nanoparticles (NPs), suitable or promising for clinical application. Also, the acute and chronic toxic effects due to the exposure to NPs reported so far, the possible nanotoxicity which could derive by their massive employment, as well as the ongoing actions to improve the knowledge in the field were discussed. The state of the art concerning the actual clinical application of both PHYs and the nanotechnologically engineered PHYs was also reviewed.

Keywords: Nanotechnology application; nano-suspension techniques; nano-emulsion techniques; bioactive constituents of plant; phytochemicals (PHYs); poor water solubility; drug delivery systems (DDSs); toxicological risks of NPs; clinical application of NPs

1. Introduction

Epidemiological studies have evidenced that the ingestion of some foods, including edible plants, is associated to the onset of healthy effects. As an example, the consumption of red wine is related to a reduction in mortality by cardiovascular events triggered by atherothrombosis, due to its capability of decreasing the progression of atherosclerotic lesions [1]. Additionally, green tea has proved to have protective effects on cardiovascular diseases [1]. The bioactive chemical compounds responsible of these benefits are known as phytochemicals (PHYs). Precisely, PHYs are defined as bioactive chemical compounds findable in plants, such as fruits, vegetables, grains, and other plant-derived foods that may supply health benefits, beyond basic nutrition and could help to reduce the risk of major chronic diseases [2]. PHYs are generally produced by plants to help themselves resist fungi, bacteria, and plant virus infections, and also to hamper their consumption by insects and other

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animals [3]. In the years, humans have used PHYs both as poisons and as traditional medicine [3]. Nowadays, the recognition that plants, can be a source of compounds, having health properties, is increasingly expanding worldwide, and both the food market and the sector of natural compounds are involved [1]. Often PHYs are regarded only as research compounds, rather than molecules which could have actual clinical application as possible therapeutics, because proofs of their possible health effects have not been proven yet [4]. However, experts in the field incessantly emphasize their extensive introduction in the normal diet, in food supplements, as well as their use as natural therapeutics to treat several diseases. As of the beginning of January 2022, a total of 130 thousand PHYs have been found [5]., and others will be discovered in the next years. Several PHYs owing many beneficial effects, including antifungal, antiviral, anti-inflammatory, antibacterial, antiulcer, anti-cholesterol, hypoglycaemic, immunomodulatory, and antioxidant activities have been isolated from plants, such as vegetables, herbs, fruits, legumes, oils, spices, nuts, and whole grains [6-11]. Anyway, in Western societies, the industry dealing with natural products and plants progressively searches for the discovery of new PHYs, to be used as possible health promoters safer than synthetic compounds [1]. Parallelly, experts in the field, including scientists, engineers, and technologists, incessantly investigate the possibilities of chemical modification of known PHYs, by the introduction of new functionalities, such as antioxidant, anti-free radicals, and anticancer moieties, to boost up their intrinsic activities. Unfortunately, although the idea of exploiting the strong potential of PHYs for health purposes is brilliant, its realization is far from simple [12]. Additionally, thinking of exploiting extracted PHYs as effective orally administrable health promoters is almost utopic. Solubility in an aqueous medium, permeability across the membrane of epithelial cells, and molecular interactions in the fluids of the gastrointestinal tract (GIT) are key factors that greatly affect the possible beneficial effects of PHYs, by influencing their route to the bloodstream and their final distribution to the targets. In this regard, most PHYs are insoluble in water and, in whatever form they are introduced orally, they hardly will reach the target in therapeutic concentrations. Moreover, early degradation by chemical, enzymatic and microbial digestion, occurring in the mouth, stomach, small and large intestine [12], as well as fast metabolism and rapid excretion via the kidney, biliary, or lung further limit their activity in vivo [13]. Collectively, PHYs generally achieve only nano/picomolar concentrations in cells and tissues, which are doses insignificant for producing a health promotion response. Figure 1 shows the main possible drawbacks related to most PHYs and the events that limit their in vivo beneficial effects after oral administration.

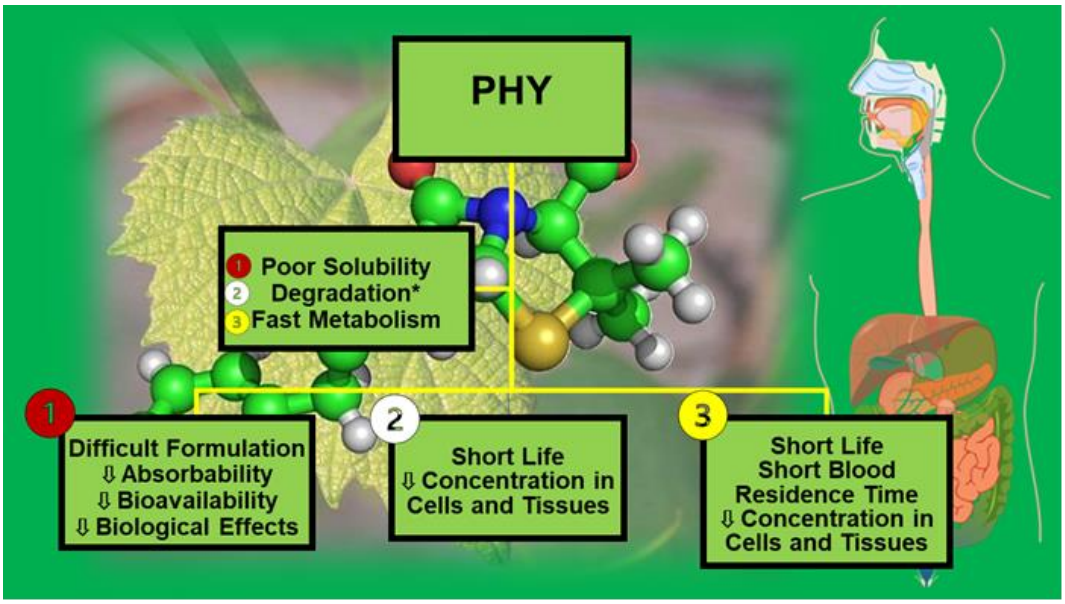

Figure 1. Main possible drawbacks related to most PHYs and events that limit their beneficial effects after oral administration. * Chemical, enzymatic or microbial.

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Additionally, concerning PHYs recognized for having demonstrated beneficial properties in vitro, we must bear in mind that, since when ingested they can be altered by microbial fermentation, also their biological properties may be distorted, and paradoxically converted in toxic effects. In this regard, important methods are necessary to judge safety and efficacy of these products, including potential risks and gaps. Anyway, to overcome the afore-mentioned problems and to allow the exploitation of PHYs as health enhancers, researchers increasingly resort to nanotechnology and nanostructures with dimensions of nanometers (nm). In the years, several PHYs-enriched nanomaterials have been engineered to overcome the poor solubility, permeability, and negative pharmacokinetics of PHYs, and different nanosized delivery systems (DSs)to transport therapeutic concentrations of PHYs to the targets have been developed [12,14].

The following Figure 2 provides an idea of the growing scientific interest in PHYs for healthy purposes and of the consequent application of nanotechnologies for solving their physicochemical issues, during the last 15 years. After having carried out a survey both in Pubmed and in Scopus datasets, we have reported the results obtained in Scopus because they included a greater number of papers, not considered in Pubmed. Particularly, the bar graph in Figure 2 was obtained by carrying out a survey about the number of works published year by year from 2008 so far (excluding the current year), making a search by "nanomaterials OR nanoparticles OR nano-formulation AND phytochemicals" keywords.

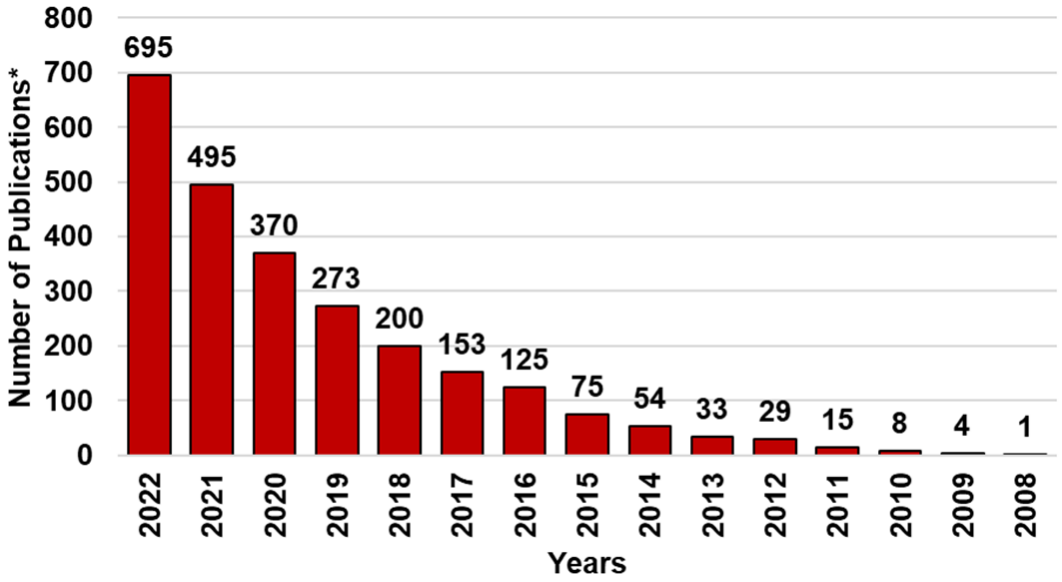

Figure 2. Number of publications per year over the last 15 years according to Scopus. * The research was carried out on 05 April 2023 by the following keywords: nanomaterials OR nanoparticles OR nano-formulation AND phytochemicals.

While the interest in the topic was low up to the year 2015 (less than 100 papers), in the following years, it has grown exponentially, as established by the several papers which have been published. This review, also by reporting several recent case studies, provides an up-to-date overview of the nanotechnological approaches based on nano-suspension (NS) and nano-emulsion (NE) techniques developed so far for formulating the most relevant PHYs in more bioavailable NPs promising for clinical applications by oral administration. We also paid attention to the pending issue relating to the possible toxic effects of NPs on humans, animals, and the environment in a scenario of limited knowledges, and we briefly reported the ongoing actions to improve the expertise in the field and/or to promote the development of increasingly safe nanomaterials.

The last section of the present paper gives a glance to the actual clinical applicability of PHYs and of nanotechnologically engineered PHYs, by reviewing those which are already clinically approved or are currently in advanced clinical trial. Originally, most information provided here has been also graphically presented and statistically analyzed.

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2. Methodology for Literature Search and Study Selection

The literature search was performed using PubMed, and Scopus database considering the period January 2008 up now using first the same keywords used for detecting the publications taken into account in Figure 2 (nanomaterials OR nanoparticles OR nano-formulation AND phytochemicals). Secondly, using nano-suspensions or nano-emulsions in place of nanomaterials, nanoparticles, nano-formulation, we have limited our investigation only to publications concerning these two techniques. Among results, we selected studies, which contained information specifically suitable for the sections which we have decided to report in the present review, Studies published in English language only were included in this review. Conference abstracts, patents and unpublished results were excluded.

3. Phytochemicals (PHYs)

3.1. Phytochemicals and Nutraceuticals: Not Quite the Same

Both PHYs, also known as phytonutrients, and nutraceuticals are bioactive compounds which could be found in edible products, possessing beneficial properties capable of enhancing humans' health, and which could be commonly ingested with the diet. Anyway, while nutraceuticals are essential nutrients for human life necessarily present in edible products, which can derive from animals, plants and fungi, PHYs are non-nutrients, which exclusively originate form plants [15]. Differently from the major nutrients like vitamins, whose reduced intake leads to the onset of serious deficiencies, PHYs are powerful health-boosting nutrient-like substances, whose lower intake does not cause defects [16]. However, these compounds can be invaluable to humans' health, and a diet rich in PHYs is strongly connected with better health [16]. They could improve the ability to detox, as well as could boost the immune system. They may also help protect against age-related diseases, such as diabetes, heart disease, and osteoporosis [16]. PHYs contained in food, when ingested and metabolized, provide benefits. In vitro studies have demonstrated that these plants constituents are multifunctional compounds, with healthy properties like those of conventional drugs and can be considered "pharmaceutical-grade compounds". Although, as above-mentioned, also nutraceuticals could be plants-derived bioactive compounds, we focused our work on PHYs.

3.2. Phytochemicals: An Overview

It has been reported that scientists have already identified over 5,000 different classes of PHYs. Anyway, many more exist undiscovered, as well as much more has to be learned about their potential benefits [17]. Although PHYs can derive from both edible and non-edible plants [18], all plant-based foods including fruits, vegetables, nuts, and herbs contain them. Since processing methods may lower the PHYs content of some foods [19], fresh, whole foods have to be preferred to get the most benefit from PHYs. Additionally, although generally PHYs confer to the plant-derived food containing them a particular bright color, also foods without these characteristics can contain these healthy promoters as well (Table 1). So, if green, purple, red, blue, or yellow vegetables and fruits are containers of colored PHYs [17,20,21], also not brightly colored potatoes, cauliflower and nuts such as almonds, cashews, and hazelnuts contain several PHYs (Table 1). Both tea and dark chocolate, even if not brightly colored, yet they are packed with these health-boosting plant compounds [17,22-24]. Moreover, essential oils (EOs) including pine needles, cedar, and lavender are used as health-promoters, due to their PHYs content (Table 1) [25-27].

Table 1. Sources of PHYs and their main benefits.

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Edible Plants		Essential Oils	Benefits
Brightly Colored	**Not Brightly Colored**		
Berries Cranberries Blackberries Strawberries Cherries Currants Grapes Plums Purple potatoes Red Cabbage Cabbage Kohlrabi Broccoli sprouts Apples Bananas Peaches Antiparasitic herbs Egg yolks Orange peppers Oranges Pumpkins Yellow corn Kale Parsley Romain lettuce Spinach Olive oil Melons	Potatoes Almonds Pecans Pistachios Cauliflower Walnuts Cashews Hazelnuts Tea Dark chocolate Cacao beans Barley Beans Lentils Rice Coffee Mung beans Soybeans Cloves Cinnamon Cumin Nutmeg	Pine needles Cedar Lavender	Boost the immune system [28] Combat OS and FR [29] ⇓ Blood sugar levels [29,30] ⇓ Blood pressure [28] ⇓ Diabetes risk [29,31] ⇓ Serious health issues [29,31] Prevent chronic disease [28,29] Protect from pathogens [32-34] Protect brain and liver [28] ⇓ Cholesterol [28] ⇓ Inflammation [29,35,36] Support detoxification [29,37] Ward off osteoporosis [28]

OS = Oxidative stress; FR = free radicals; ⇓ reduces, lowers.

Note that, we have not reported the beneficial properties of each plant, but a series of health promoting activities attributed to all plants, because each plant, being a source of multiple phytochemicals, each having multiple pharmacological activities, rationally, can possess all the reported properties. Anyway, we have examined the point more in depth in Table 2, where the different beneficial activities were reported for each class of phytocompounds or even for the single phytocompound.

3.2.1. Specific Sources and Benefits of Main Types of PHYs

The most common PHYs comprise polyphenols, including four principal classes of compounds: phenolic acids (caffeic acid, ellagic acid, gallic acid, tannic acid), stilbenes, lignans and flavonoids, such as catechins. Flavonoids in turn include, among others, the subgroups of iso-flavonoids, proanthocyanidins, comprising procyanidins, anthocyanidins, including cyanidins, and anthocyanins, which are anthocyanidins with sugar groups. Also, PHYs encompass carotenoids, coumarins, indoles, organosulfur compounds, isothiocyanates, saponins, tannins, phenylpropanoids, anthraquinones, ginsenosides, terpenoids etc. [38]. In the following Table 2 we have reported the most relevant PHYs, their source and the associated healthy effects [3,39].

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Table 2. Most relevant types of PHYs, their own source, and the associated healthy effects.

PHYs	Sources	Health benefits
Carotenoids [40,41]	Carrots, tomatoes, parsley Orange and green leafy vegetables Chenopods, fenugreek Spinach, cabbage, radish, turnips	Antioxidant Protect against uterine, prostate, colorectal, lung, and digestive tract cancers
Phytosterols [42,41]	Vegetables, nuts, fruits, seeds	Suppress the growth of diverse tumour cell lines
Limonoids [41]	Citrus fruits	Inhibit phase I enzymes and induce phase II detoxification enzymes in liver Provide protection to lung tissue, detoxify enzymes
Curcuminoids [41]	Turmeric, curry powder, mango, ginger	Analgesic, anti-inflammatory, anti-cancer, antioxidative, anti-depressive Against hay fever, depression, ⇓ cholesterol and itching risk
Indole compounds [41] (Indole-3-carbinol)	Cabbage, cauliflower, broccoli, kale Brussels sprouts	Strong antioxidant, DNA protector, chemo-preventive, anti-cancer, ⇑ heart health
Alkaloids [43]	Plants (also animals and bacteria)	Antimalarial, antiasthma, anticancer, cholinomimetics, vasodilatory, antiarrhythmic Analgesic, antibacterial, antihyperglycemic, psychotropic, stimulant
Phytoprostanes [44] Phytofurans [44]	Almonds, vegetal oils, olives, algae Passion fruit, nut kernels, rice	Immunomodulators, anti-inflammatory, anti-tumours
Polyphenols [41]	Fruits, vegetables, cereals, beverages, legumes Chocolates, oilseeds	Action against free radicals, anti-inflammatory, anti-allergenic Inhibition of platelet aggregation, against hepatotoxins
Flavonoids * [41]	Fruits, vegetables, cereals, beverages, legumes Chocolates, oilseeds	Action against free radicals, anti-inflammatory, anti-allergenic Inhibition of platelet aggregation, against hepatotoxins
Iso-flavonoids ** [41]	Fruits, vegetables, cereals, beverages, legumes Chocolates, oil seeds	Action against free radicals, anti-inflammatory, anti-allergenic Inhibition of platelet aggregation, against hepatotoxins
Anthocyanidins ** [41] Anthocyanins ** [41]	Fruits, vegetables, cereals, beverages, legumes Chocolates, oilseeds	Action against free radicals, anti-inflammatory, anti-allergenic Inhibition of platelet aggregation, against hepatotoxins
Glucosinolates [41]	Cruciferous vegetables	Protection against cancer of colon, rectum, stomach
Phytoestrogens [41]	Legumes, berries, whole grains, cereals	Protection against bone loss, heart disease, cardiovascular diseases

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PHYs	Sources	Health benefits
	Red wine, peanuts, red grapes	Protection against breast and uterine cancers
Terpenoids [41] Isoprenoids [41]	Mosses, liverworts, algae, lichens, mushrooms	Antimicrobial, antiparasitic, antiviral, antiallergic, anti-inflammatory, chemotherapeutic Antihyperglycemic, antispasmodic
Fibers [45]	Fruits and vegetables (green leafy), oats	⇓ Blood cholesterol, ⇓ cardiovascular disease
Polysaccharides [41]	Fruits and vegetables	Antimicrobial, antiparasitic, antiviral, antiallergic, anti-inflammatory ⇓ Serum, ⇑ defence mechanisms
Saponins [41]	Oats, leaves, flowers, green fruits of tomato	Protection against pathogens, antimicrobial, anti-inflammatory, antiulcer agent
Tannins [41]	Cranberries, currants, blackberries, apples Grapes, peaches, strawberries, almonds Hazelnuts, pecans, pistachios, walnuts, barley Beans, lentils, rice, tea, cacao beans Dark chocolate, antiparasitic herbs	Antioxidant, fight pathogens, ⇓ blood pressure, ⇓ inflammation, ⇓ serious health risks Regulate the immune system
Lutein [41] Zeaxanthin [41]	Egg yolks, orange peppers, oranges, pumpkins Yellow corn, kale, parsley, romaine lettuce Spinach, pistachios, olive oil	Protect retina from damage, ⇑ eye function, ⇑ memory and brain function Promote the body's use of insulin, ⇑ skin health, ⇓ blood pressure, ⇓ inflammation Support heart health
Eugenol [46]	Cloves, cinnamon, cumin, nutmeg, coffee Mung beans, soybeans, bananas, melons Strawberries, tomatoes	Anti-inflammatory, antioxidant, eliminate parasites, fights fungi Inhibits serious health concerns, protects the brain and the liver, ⇓ bacterial biofilm Supports heart and stomach health

⇑ Improved, higher; ⇓ reduced, lower; * are a subgroup of polyphenols; ** are a subgroup of flavonoids.

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As above-mentioned, the known PHYs are thousands. Following, some examples of the most common [1,15].

Polyphenols

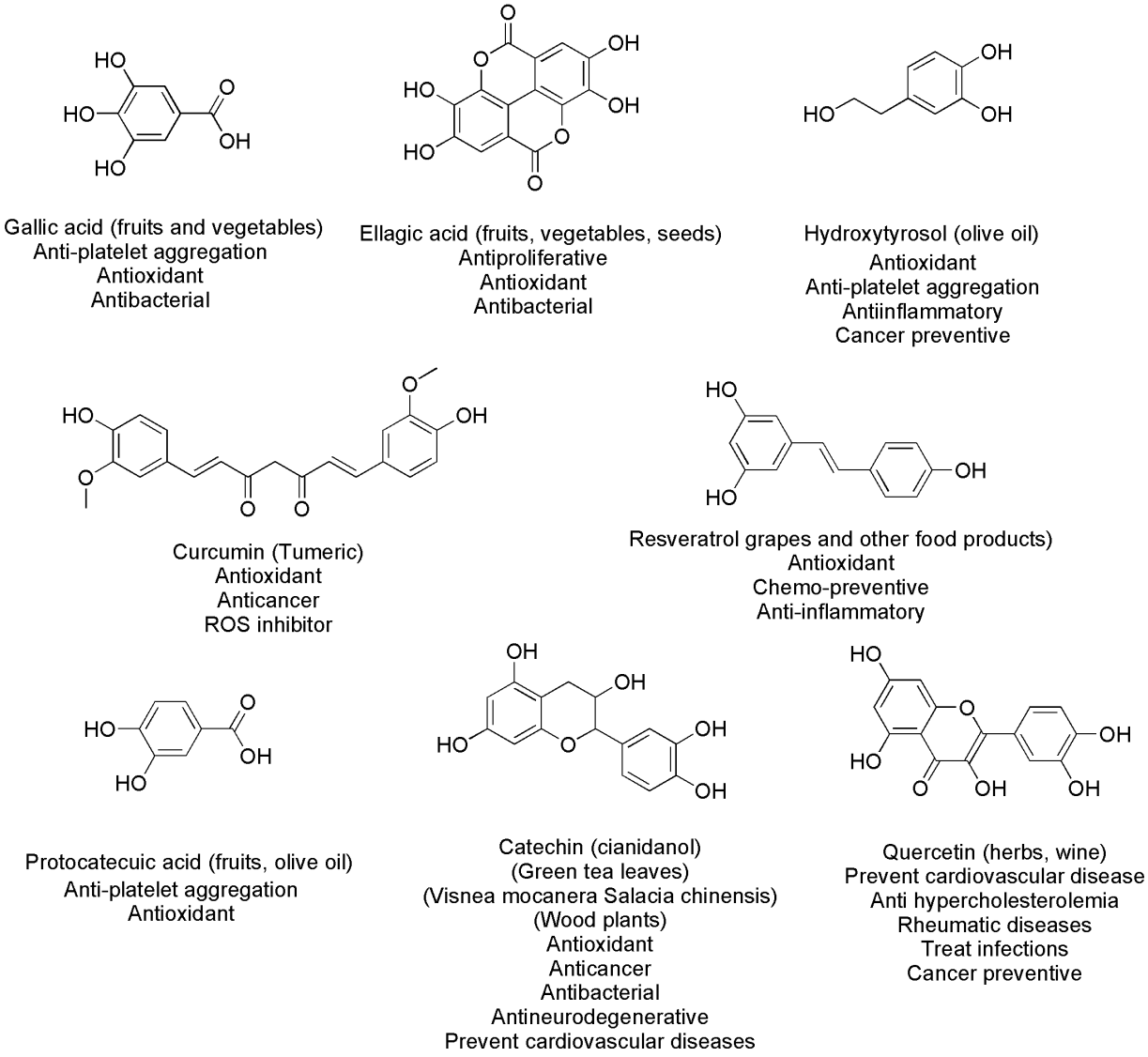

Figure 3. Structures of some plants derived polyphenols. ROS = reactive oxygen species.

Among polyphenols, gallic acid (GA), ellagic acid (EA), and other natural products such as propolis extracts, which are composed of phenolic acids, flavonoids, terpenes, and essential oils, can treat infections sustained by bacteria of several species, including those of sporogenic type. Particularly, GA showed antibacterial activity against *Paenibacillus larvae, Staphylococcus aureus* [3], *Escherichia coli, Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas* spp. P304, *Plesiomonas schigelloides*, and *Schigella flexneri B* [3]. Both in in vitro studies and in animal models, GA and EA have shown activities against several degenerative diseases, such as cardiovascular, cancer, and central-nervous-system-disabling disorders, including Parkinson's disease, Alzheimer, multiple sclerosis, and amyotrophic lateral sclerosis [47-52]. Furthermore, hydroxytyrosol, a polyphenol extracted from extra-virgin olive oil has demonstrated antioxidant, anti-inflammatory and cancer preventive activities. Curcumin from *Curcuma longa* L. rhizome, GA, protocatechuic acid, quercetin, and resveratrol (RES), which are contained in many fruits and vegetables, are capable of inhibiting platelet aggregation and reactive oxygen species (ROS) activity induced by thrombin, collagen, or other agonists [3,15]. Particularly, RES is a phytoalexin derived from grapes and other food products has antioxidant

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and potential chemo-preventive activities and anti-inflammatory effects. Quercetin is found in many foods and herbs, including wine successful in the treatment or in the prevention of diverse conditions including cardiovascular disease, hypercholesterolemia, rheumatic diseases, infections and cancer. Quercetin as a nutritional supplement is well tolerated and has not been linked to serum enzyme elevations or to episodes of clinically apparent liver injury. Also, curcumin (from Turmeric), catechins (from tea leaves, Visnea mocanera Salacia chinensis, wood plants), also known as cianidanol, and RES (from grapes) possess antioxidant effects and can help in preventing and treating cancer, cardiovascular illness, neuronal degenerative diseases, diabetes, and infections. Most of them are inhibitors of platelet aggregation and prevent OS triggered by ROS [15]. As above reported, polyphenols encompass flavonoids which in turn comprise anthocyanins, whose name comes from the Greek words for 'flower' and 'blue.' Anthocyanins give fruits and vegetables their vivid red, blue, and purple colors. The main sources of anthocyanins are red, blue, and purple vegetables, including berries, cherries, currants, grapes, plums, purple potatoes and red cabbage [21]. Scientists have discovered over 700 different types of anthocyanins [16]. In vitro and in vivo studies on healthy properties of anthocyanins established they can decrease serious health risks, eliminate FR, help in controlling weight, in preventing heart disease, and increase insulin sensitivity. Additionally, they reduce inflammation, diabetic complications, protect DNA and the brain. Anthocyanins also boost other PHYs and the activity of other phytonutrients [53-55].

Tannins

Tannins confer to edible plants astringent flavors and bitter tasting, but also a variety of health benefits, such as antioxidant effects [56]. Additionally, they fight parasites, microbes, and other pathogens, lower blood pressure, inflammation, serious health risks, and regulate the immune system [57]. Many different foods could be the sources of tannins, such as tart fruits like cranberries, currants, blackberries, sweeter fruits like apples, grapes, peaches, strawberries, and nuts, such as almonds, hazelnuts, pecans, pistachios, and walnuts. Additional sources of tannins could be barley, beans, lentils, and rice.

Tea is a common way by which people consume tannins, along with cacao beans and dark chocolate [57]. Lastly, antiparasitic herbs, including black walnut, sagebrush, garlic, oregano, *Tribulus terrestris* L., *Mimosa pudica*, neem (*Azadirachta indica*), grapefruit seed, vidanga (*Embelia ribes Burm* F.), carnations etc. contain tannins. Among tannins, ellagitannins (ETs) have shown activities against several degenerative diseases, such as cardiovascular, cancer, and central-nervous-system-disabling disorders, including Parkinson's disease, Alzheimer, multiple sclerosis, and amyotrophic lateral sclerosis. Moreover, ETs are source of GA and EA that, as above introduced, have shown similar activities, both in vitro studies and animal models [47-52].

Lutein and Zeaxanthin

Lutein and zeaxanthin can give foods a rich orange or yellow color, are potent antioxidants, and are mainly known for their role in eye health. These compounds absorb 40-90% of blue light and reduce OS, thus helping to protect retina from damage [58]. The eye functions resulted ameliorated, thus allowing to see with more clarity and to be less bothered by glaring lights [58]. Additionally, lutein and zeaxanthin may also enhance memory and brain function [59], as well as improve the body's use of insulin, and skin health [60], while they lower blood pressure, reduce inflammation, and support heart health [16]. According to their orange and yellow colors, the possible sources are egg yolks, orange peppers, oranges, pumpkins, and yellow corn [60]. However, whopping dose of lutein and zeaxanthin is contained also in green foods. Kale, parsley, romaine lettuce, and spinach are excellent sources of lutein and zeaxanthin, as well as pistachios and olive oil [60].

Sulforaphane

Sulforaphane is a potent antioxidant and is highly researched for its potential health benefits. In vivo and in vitro studies suggest that sulforaphane acts as an anti-inflammatory agent and reduces

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serious health issues. Additionally, this compound could potentially trigger unhealthy cells to die [16]. Also, sulforaphane may boost the ability to fight pathogens, increases detoxification and liver function, lowers autism symptoms, protects eyes, and reduces depression and anxiety [61]. Cruciferous vegetables including cabbage, kale, broccoli sprouts, and kohlrabi are great sources of sulforaphane [61], which need activation by mustard.

Eugenol

Eugenol may act as a strong anti-inflammatory and antioxidant compound and helps eliminate parasites [62,63]. Animal, test-tube, and human cell studies established that eugenol is effective against fungi, inhibits serious health concerns, protects the brain, the liver, reduces bacterial biofilm, as well as supports heart and stomach health [64]. The best source of eugenol is clove. Also, eugenol can be found in multiple herbs and spices such as cinnamon, cumin, nutmeg, and is present in coffee, mung beans, and soybeans, as well as in bananas, melons, strawberries, and tomatoes [46].

Terpenoids

Terpenoids represent one of the largest groups of natural products, mainly extracted from plants, which account for more than 40,000 compounds, but new terpenoid compounds are discovered incessantly every year. They possess anticancer effects against several tumors, including breast, mammary, skin, lung, forestomach, colon, pancreas, and prostate carcinomas. Additionally, most triterpenoids suppress cancer cells without exerting toxicity towards normal cells [3]. Terpenoids, which are found mainly in spices and di-and tri-terpenoids extracted from different typologies of Salvia, such as ursolic and oleanolic acids have shown antibacterial, hypoglycemic, and anticancer properties [10,11].

3.2.2. Let's Eat in Colors!

Since PHYs confer food particular colors, colored foods surely contain PHYs, and possess the same benefits of PHYs having the same color. So, by eating foods from all the different color groups, a wide range of different PHYs can be assumed with a consequent broad spectrum of benefits [65]. The following Table 3 reports the general subdivision of plants-related foods in the different color groups.

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Table 3. General classification of the different color groups *.

Color Group	Foods	PHYs	Properties	Ref
Green	Asparagus, avocados, Celery Cucumbers, Green beans, green peppers, kale, kiwi Spinach, zucchini	EGCG, glucosinolates, indolesisoflavones Isothiocyanates, lutein and zeaxanthin Sulforaphane	Promote wound healing and healthy gums Support arteries, blood cells, eyes, liver, and lungs	[16,65]
Purple	Black beans, blackberries Eggplants, elderberries, plums Purple cabbage, purple grapes, raisins	Anthocyanins, flavonoids, phenols, tannins, RES	Protect against serious health issues Support arteries, bones, brain, cognition, healthy aging, and heart	[16,65]
Red	Cherries, cranberries, kidney beans Red beans. strawberries, tomatoes watermelon	Anthocyanins, ellagic acid, eugenol, hesperidin Lycopene, tannins, quercetin	Protect against heart disease and other serious health issues Support prostate, urinary tract, and DNA health	[16,65]
Yellow	Apricots, cantaloupe, carrots, grapefruit, Yellow pears, yellow peppers Yellow winter squash	α-Carotene, β-carotene, β-cryptoxanthin, lutein Zeaxanthin, hesperidin	Boost the immune system, support heart and vision health	[16,65]
White	Apples, cauliflower Great northern beans Mushrooms, onions	Allicin, ECGC, glucosinolates, indoles, tannins Quercetin	Protect against heart disease and other serious health issues Support arteries, bones, and circulation	[16]

* The present Table has been constructed by authors exploiting information found in literature [16].

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3.2.3. Phytochemicals: A Plethora of Benefits in Vitro Against Poor Findings in Vivo

The clinical development of PHYs as orally administrable therapeutics, is strongly restrained by several physicochemical and pharmacokinetic limitations, mainly including low water solubility, poor bioavailability and deficient targeting [66]. The dissolution in the intestinal fluids of a poor water-soluble compound is very slow, as well as its GIT permeation, and its systemic concentration will hardly be enough for having a significant therapeutic response [1]. Poor water solubility means a low absorption rate at gut level, low bioavailability, and insufficient blood and tissue concentration. Consequently, while tested in vitro, PHYs show a plethora of beneficial effects and high activity, they are often several times less effective when assayed in vivo. Figure 4 reports the main factors that can influence the water solubility of a bioactive compound.

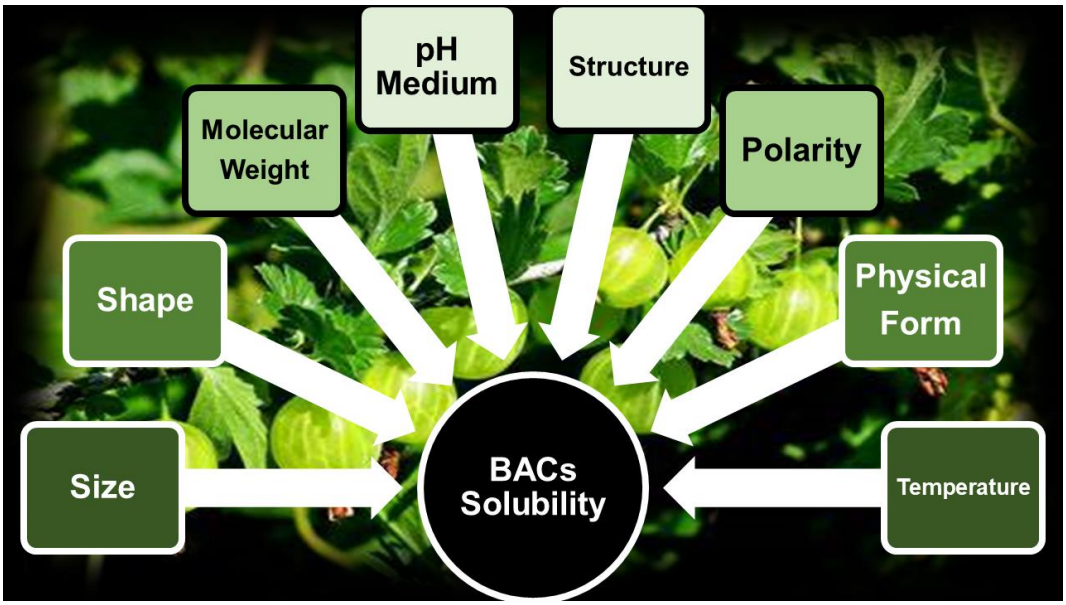

Figure 4. Main factors that can influence the water solubility of bioactive compounds (BACs).

Particularly, unsymmetrical small-size particles with high surface area dissolve better and more quickly. Higher temperatures promote dissolution, while high molecular weight (MW) lowers compounds' solubility. Since it is known that branched polymers are more soluble than linear ones with equal MW, major amount of branching in carbon chains favors solubility. In fact, branched-chain molecules have minor volume/dimension ratio in solution and higher dissolution rate. Molecules arranged in amorphous forms own higher aqueous solubility than the crystalline ones, as well as different polymorphs have different solubility. Additionally, since ionized forms have higher solubility in water and weak acids or weak bases ionize in solution on the base of the pH of medium, pH of solutions can strongly affect the compounds' solubility. An approach to improve the water dispersibility of bioactive compounds could consist of formulating them in colloidal suspensions or emulsions, but when their solubility is excessively low, very high concentrations of surfactants, stabilizers, polymers, osmotic agents, organic solvents, complexing agent, would be required, which may trigger unpleasant side effects, including GIT irritation, in a future oral administration [67,68]. In this context, efficient and low-cost solubilizing methods, which minimize or even avoid the use of harmful excipients, organic solvents, co-solvents, emulsifiers, or other additives are necessary. Currently, nanotechnology is extensively exploited to improve PHYs solubility, to help their formulation and to enhance their absorption and bioavailability, with the final goal to solve the gap between the exciting and very promising results obtained in vitro and the unsatisfying results obtained in in vivo studies [69-72].

3.2.4. Improving Solubility of Bioactive Compounds

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Bioactive compounds' solubility can be improved by two modalities, which are both based on the reduction of particles size to micrometer or nanometer dimensions.

Particle Engineering Techniques (PETs)

Particle engineering techniques consist of old and novel methods to improve the solubility and bioavailability of a compound. By using PETs, the physicochemical, micromeritic and biopharmaceutical properties of a compound are changed mainly reducing its particles size [73]. In addition to wet-milling, dry-milling, high-pressure homogenization (HPH), ultra-high-pressure homogenization (UHPH), the novel PETs comprise the supercritical fluid technologies and the cryogenic technologies [73-75]. The techniques belonging to these PETs are reported in Figure 5.

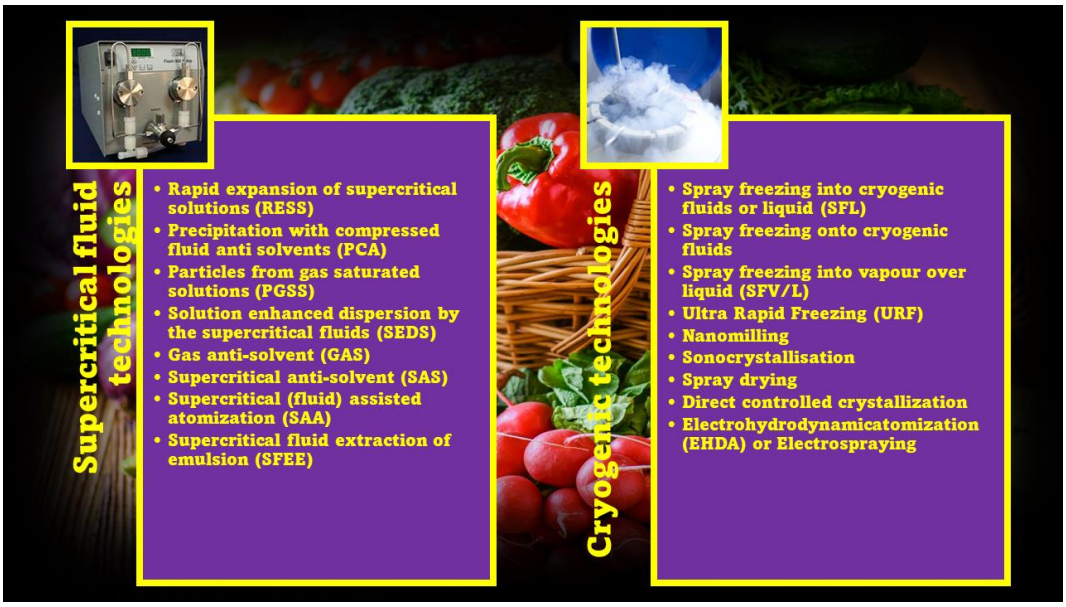

Figure 5. Supercritical fluid technologies and cryogenic technologies.

Formulation Approaches (FAs)

FAs aim to obtain solid, lipid or amorphous nano-formulations from colloidal dispersions, in turn prepared using mixtures of water/oil phases, stabilizers, solvents/co-solvents, by using PETs, such as spray-drying (SD), milling, and other techniques reported in Figure 5. Table 4 collects the most commonly used techniques with the related advantages and disadvantages.

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Table 4. Advantages and disadvantages of the most used techniques and approaches for enhancing the solubility of bioactive compounds. *.

Methods	Advantages	Disadvantages	Ref.
Emulsion solvent extraction Double-emulsion solvent extraction	Low-cost	⇑Residual solvent ⇓Encapsulation efficiency (EE) Thermal degradation Multi-steps processes Micronization step needed	[15]
SD	Improved bioavailability Taste masking Modified release achievable Aseptic manufacturing Fine powders Improved stability	High overhead Possible thermal degradation Not for every compound Not widely available Multi-steps processes Micronization step needed	[15]
Liquid anti-solvent technique	Low-cost	⇑Residual solvent ⇓ EE Thermal degradation Multi-steps processes Micronization step needed	[15]
Spray freeze drying (SFD)*	Higher rate of freezing Independent control over particle size	⇓ Biological activity Possible protein denaturation Excipients required	[15]
Spray freezing into liquid (SFL)*	Higher level of biological activity High degree of atomization Ultra-rapid freezing (URF) Formation of amorphous highly porous NPs	Possible ⇑ viscosity of the feed liquid Limited applications ⇑ Cost equipment Time and energy-intensive	[15]
Thin film freezing (TFF) **	High-yield products Flexibility on processable drugs Large-scale production Simple, efficient, robust process ⇑ stability of the protein product	⇑-Cost equipment	[15]
Supercritical fluid extraction (SFE) method	Single-step process Controllable particle size Controllable morphology Controllable crystallinity Monitorable residual solvent	⇑-Cost equipment	[15]
Solvent evaporation technique	Low-cost	⇑Residual solvent ⇓EE Thermal degradation Multi-steps processes Micronization step needed	[15]

* The present Table has been created by authors exploiting information found in literature [15]; ** Cryogenic particle engineering methods [76]; ⇑ improved, higher; ⇓ reduced, lower.

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Except for SD, SFD, SFL and TFF, all the techniques in Table 4 have the disadvantages of retaining a lot of residual solvent and of allowing low percentages of EE%. Additionally, except for cryogenic methods, they could cause reduction in the biological activity of bioactive compounds by thermal degradations or other undesirable events. All are multi-stage processes, frequently requiring an additional micronization step by air jet milling to obtain the needed particle size and size distribution, which could cause occasional crystallographic defects in the products [15]. On the contrary, although high costly, the SFE method is a single-step process requiring shorter operation time. In the SFE method, the residual solvent content can be monitored, and micronized dry powders with controllable particle size, morphology, and crystallinity can be achieved [15].

4. Nanotechnology

Currently, nanotechnology is the most promising science, engineering, and technology conducted at the nanoscale (1-100 nm), used to improve the bioavailability of bioactive compounds.

4.1. Advantages of Nanotechnology Application

By using nanotechnology and NPs, in addition to improve the solubility of bioactive compounds, their delivery, and cell uptake, it is possible to protect them from early degradation and fast metabolism. A typical PHY engineered by nanotechnology is EA, a polyphenol found in fruits and vegetables, whose several healthy properties are unfortunately associated with very poor solubility and numerous pharmacokinetic drawbacks. Several studies exist on the adoption of appropriate nanomaterial-based devices to enhance EA solubility, its hydrophilic-lipophilic balance (HLB), and GIT absorbability, as well as to protect it from early metabolism [77-83]. In particular, an EA high-water solubility was achieved using cyclodextrins [81,82], pectin [83], and polyester-based dendrimers [83].

Furthermore, by formulating PHYs using NPs, it can be realized their controlled and targeted release, which is essential for having an effective administration. A controlled delivery results in a higher concentration at the target, thus allowing to reduce the overall administered dose and consequently the systemic toxicity [1]. Both internal and external factors can control the specific release of bioactive compounds, including pH, temperature, ultrasound or magnetic fields applications, light incidence, type and physicochemical features of NPs, as well as the chemical structure and the physicochemical features of bioactive compounds themselves [1].

Stimuli-sensitive nano-capsules loaded with a bioactive paclitaxel derivative and possessing an oil core were shown to improve the anticancer effects of the encapsulated compound following the oral administration, due to targeted delivery and a controlled long-term release [84]. The improved effects allowed to decrease the dosage and the administration frequency, thus improving the patient compliance [84]. The layer-by-layer self-assembly of pH-sensitive building blocks proved to be a promising approach to obtain biomaterials with customized properties, which were successfully applied as stimuli-responsive nanocarriers [15]. Starting from biocompatible pH-dependent polyelectrolytes, nontoxic nanocarriers with high permeability were designed [84].

In addition, the encapsulation of bioactive compounds in properly functionalized NPs can allow an increased cellular uptake and a slower drug release, thus improving the drug bioactivity, and contributing to a sustained therapy [15]. The effects of phospholipid composition on the pharmacokinetics and biodistribution of epirubicin-loaded liposomes were investigated, demonstrating a significantly prolonged circulating time, reduced clearance and reduced heart toxicity [85]. Furthermore, carrying bioactive compounds in NPs can favor their distribution in specific brain areas, thus providing more valuable benefits in neuro-regenerative treatments, while minimizing their accumulation in the systemic circulation and related toxic side effects [86]. Collectively, nanotechnology provides nano-formulation techniques, which, by using NS and NE approaches, and/or different types of nanomaterials enhance the solubility of bioactive compounds, PHYs included (Figure 6).

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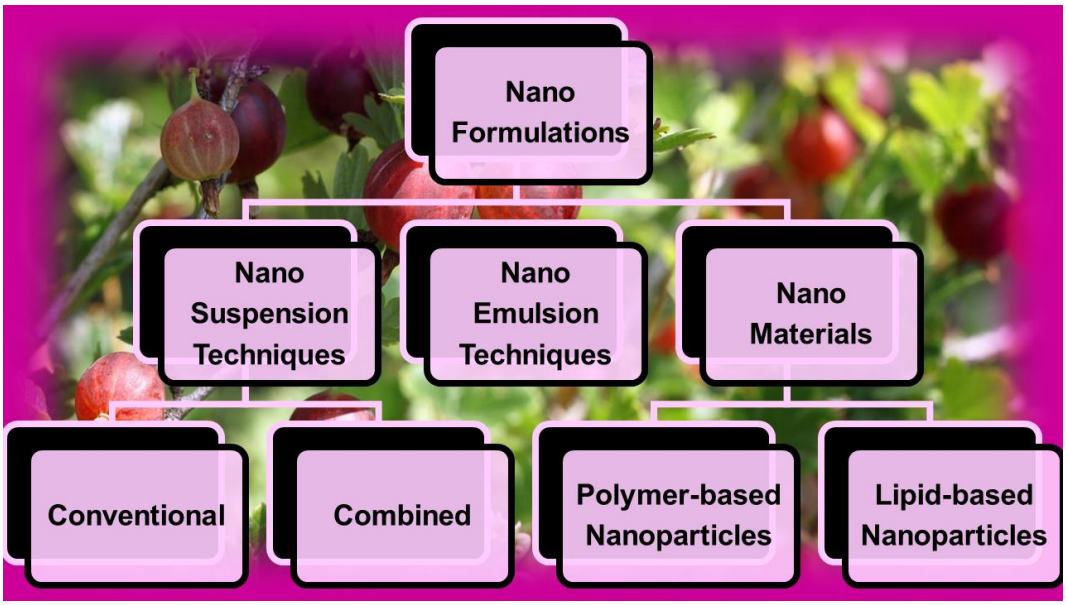

Figure 6. Main nano-formulation techniques.

Due to the incessantly increasing interest in nanotechnology for manipulating poorly soluble bioactive compounds including PHYs and engineering them in more soluble and bioavailable dosage forms potentially suitable for clinical applications, the numbers of studies in the field are enormously improved in the last decade and especially in the past five years (Figure 2). In this regard, we though that a single paper cannot be sufficient to review all type of nano-formulation approaches recently developed to formulate PHYs. On the other hand, according to a recent research paper, up to year 2019, while liposomes were the most studied NPs for nano formulating PHYs, NEs were the nanotechnological approach less considered, and NSs were even not reported [87]. Additionally, since for their production, GRAS ingredients are usually employed, in addition to liposomes, NSs and NEs can be considered the less toxic and the most suitable tactics for clinical applications purposes. Accordingly, with the aim of emphasizing the potential of these promising nano-formulation techniques and stimulating scientists to further study and use them, we decided to focus the present review specifically on NSs- and NEs-based techniques.

4.2. Nanosuspension and Nanoemulsion Approaches

4.2.1. Nanosuspension Techniques

These techniques are suitable to improve solubility and bioavailability of both hydrophilic and lipophilic bioactive compounds. A nanosuspension consists of an aqueous colloidal dispersion of NPs, stabilized by surfactants, co-surfactants, and polymers [15].

Drug-loaded NPs achieved by this technique usually possess high dispersibility and solubility, and can allow a sustained, controlled, and targeted delivery of the loaded drug, as well as are endowed with improved stability and therapeutic effects [88]. NS technique encompasses both conventional and combined approaches. The conventional approaches to prepare NSs consist of the bottom-up (B-U) and top-down (T-D) methods as reported in Figure 7 and Table 5.

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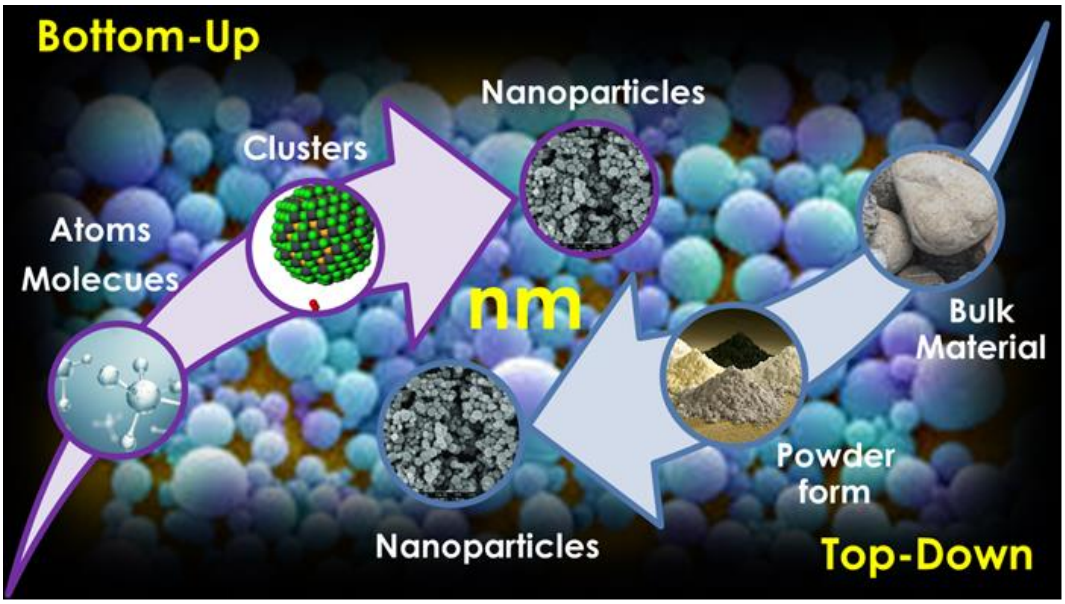

Figure 7. Bottom-up (B-U) and top-down (T-D) methods.

Table 5. Suspension-based nano-formulation techniques.

Nano-suspension techniques		
Conventional techniques		**Combined techniques (CTNI)**
Bottom-up (B-U)	Top-down (T-D)	Nanoedge™ Technique (Baxter Healthcare) H 69 Technology (SmartCrystal® technology group) H 42 Technology (SmartCrystal® technology) H 96 Technology (SmartCrystal®, Abbott/Soliqs, Ludwigshafen, Germany) Combination Technology (CTNO)

Particularly, the T-D techniques are referred to as physical methods, which start from particles with large dimension and reduce their size to nanoscale dimensions by media milling technique, HPH, UHPH, or supercritical fluid processes. On the contrary, the B-U methods are referred to as chemical and physical methods, which start subjecting the atomic bioactive compound to precipitation, melt emulsification, coacervation, inclusion complexation, supercritical fluid extraction, or liquid antisolvent precipitation, causing self-association and self-organization, forming nanosized materials [15,88]. Differently, the combined approaches mix both a B-U phase, such as precipitation with a subsequent T-D approach, such as HPH. They consist of Nanoedge™ Technique (Baxter Healthcare), H 69 Technology (SmartCrystal® technology group), H 42 Technology (SmartCrystal® technology), H 96 Technology (SmartCrystal®, Abbott/Soliqs, Ludwigshafen, Germany) and Combination Technology (CTNO) as reported in Table 5.

Particularly, Nanoedge™ Technique combines the microprecipitation of the PHY in water and the homogenization techniques giving a better particle size distribution and better stability. Usually, the precipitation is performed using water-miscible solvents including methanol, ethanol, and isopropanol and leads to the obtainment of an amorphous precipitate [15]. In the Nanoedge™ technology, an evaporation step is included to yield solvent-free starting material, which is further processed by HPH, using piston-gap homogenizers, or by sonication. The homogenization phase allows to achieve in short time nanosized particles (80–700 nm), endowed with great stability and impedes the further crystal growth [15]. H 69 Technology makes part of the SmartCrystal® technology group and is like the Nanoedge™ approach, except for an immediate treatment of the micro-precipitate with cavitation, particle collision, and shear forces. Highly stable drug nanocrystals in the range of 20–900 nm can be obtained [15].

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H 42 Technology belongs to the platform of SmartCrystal® technology as well. In this case, the B-U step which consists of the PHYs precipitation by SD carried out in aqueous media containing surfactants, is followed by the usual HPH phase (T-D phase), for further particle size reduction. Solvent-free dry intermediates, and small drug nanocrystals, are obtainable after a reduced number of HPH cycles (170–600 nm) and short processing times. Unfortunately, since high temperatures are necessary during SD, this technique is unsuitable for processing thermolabile compounds [15].

Differently, H 96 Technology (SmartCrystal®, Abbott/Soliqs, Ludwigshafen, Germany) involves a B-U pre-treatment step by FD, followed by the usual T-D step for particle size reduction through HPH. In this case, H 96 technology is suitable to process thermolabile or expensive drugs, due to the low temperatures and the high yields of the FD [15]. Finally, combination technology (CTNO), without using organic solvents, combines two T-D approaches. Particularly, a low-energy pearl milling phase of an aqueous macro-suspensions that provide particles of 600–1500 nm, is associated with the usual HPH phase, thus obtaining particles with a size of 250–600 nm. This approach permits a limited risk of crystal growth during storage, providing NPs with enhanced physical stability. In addition, to allow reducing processing times and costs, CTNO is suitable for scaling up [89].

Nano-suspensions-based Phytochemicals Delivery Systems

Table 6 reports some examples of PHYs nano-formulated by both conventional and combined nanosuspension techniques.

Table 6. Examples of plant derived bioactive compounds nano-formulated by nanosuspension conventional and combined techniques.

PHY	NPs/Mechanical Method (MM)/ Combined techniques (CTNI)	Particle size	Characteristics
β-carotene [90] (B-U)	*n*-Octenyl succinate-modified starch (NPs)	300-600-nm-sized particles	⇑ Dispersibility ⇑Coloring strength, ⇑ Bioavailability
Quercetin [91] (B-U)	Maltodextrin (NPs)	753-nm-sized particles	Water-re-dispersible ⇑ RSA ⇑ ORAC
Quercetin [92] (T-D)	HPH (MM) + spray-drying (MM)	400-nm-sized particles	⇑ Antioxidant ⇑ Anti-inflammatory ⇑ Anticancer properties ⇑ Water solubility ⇑ Oral bioavailability
α-Tocopherol [93] (B-U)	Supercritical assisted process	150-nm-sized particles	⇑ Solubility ⇑ Bioavailability
All-trans retinoic acid (ATRA) [94]	Nanoedge™ Technique	155-nm-sized particles	Orally administrable 30′ Operation time
Resveratrol (RES) [95]	H 69 Technology	150 nm-sized NPs	Orally administrable 10 cycles HPH/1200 bar
RES [15]	H 42 Technology	200 nm-sized NPs	Orally administrable 1 HPH cycle at 1500 bar
Hesperidin [15] *	CTNO	599 nm-sized NPs	⇑ Solubility Long-term stability Orally administrable Topically applicable Five homogenization cycles (1000 bar)

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Rutin [15] **	CTNO	600 nm-sized NPs	⇑ Solubility Orally administrable Topically applicable 1 Cycle of HPH at 100 bar
Apigenin [15]	CTNO	275 nm-sized NPs	1 Cycle HPH at 300 bar
Isoliquiritigenin [96] T-D	Wet media milling (MM)	238 nm-sized NPs (HPC-SSL) 354 nm-sized NPs (PVP-K30)	⇑ Solubility ⇑ Cytotoxicity ⇑ Cellular up-take ⇑ Apoptosis induction ⇓ Toxicity
Celastrol [97] B-U	Antisolvent precipitation method	148 nm-sized NPs	Stable in plasma Orally administrable ⇑ EE% ⇑ DL% ⇑ Solubility (vitro) ⇑ Cytotoxicity (vivo) ⇑ Cumulative release (48h)
Curcuma Longa L. Extracts [98] B-U	Supercritical fluid expansion	47 nm-sized NPs	⇑ Solubility
Naringenin (NRG) [99] B-U	Antisolvent sonoprecipitation	117 nm-sized NPs	⇑ Absorption in GIT ⇑ Dissolution ⇑ Oral bioavailability
NRG [100] B-U	Precipitation-ultrasonication	118 nm-sized NPs	⇑ Drug dissolution ⇑ Pharmacokinetic profile ⇑ Stability
NRG [101] B-U	HPH	81 nm-sized NPs	⇑ Intracellular ROS level ⇑ Mitochondrial membrane potential ⇑ Caspase-3 activity ⇑ Lipid peroxidation status ⇓ GSH levels ⇑ Antitumor activity on DLA cells ⇑ Life span ⇓ Cancer cell number ⇓ Tumor weight
Glaucocalyxin A [102] B-U	Precipitation-ultrasonication	143 nm-sized NPs	⇑In vitro antitumor activity ⇑ In vivo anticancer efficacy
Oleanolic acid (OA) [103] B-U	Organic solvent evaporation	100 nm-sized NPs	⇑ Stability ⇑ Saturation solubility ⇑ Dissolution rate ⇑Cytotoxicity ⇑ Bio-efficacy ⇑ Bioavailability

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P. guajava L. extracts [104] B-U	Nanoprecipitation	241-327 nm-sized NPs	⇑ Antihyperglycemic activity ⇑ Physical parameters ⇑ Hepatic parameters ⇑ Renal parameters ⇑ Absorption ⇓ Metabolism ⇑ Stability
Nigella sativa L. [105] B-U	Nanoprecipitation	N.R.	⇑ Total phenolic content ⇑ Total flavonoid contents ⇑ Antioxidant activity ⇑ Antidiabetic activity ⇑ Antibiofilm activity ⇑ Bioavailability

RSA = radical scavenging activity; ORAC = oxygen radical absorbance capacity; * Hesperidin nanocrystals can be found in the Platinum Rare cosmetic product (La Prairie, Volketswil, Switzerland); ** Rutin nanocrystals are in a cosmetic product launched by Juvena, St. Margrethen, Switzerland [15]; DL = drug loading content; GSH = glutathione reductase; DLA = Dalton lymphoma ascites; ⇑ improved, higher, high; ⇓ decreased, lower.

Considering the most recent case studies reported in Table 6, the poorly water-soluble flavonoid extracted from licorice root, namely isoliquiritigenin, effective against several forms of cancer, was nano-formulated by Qiao and colleagues using a T-D approach [96]. Hydroxypropyl cellulose-SSL (HPC-SSL) and polyinylpyrrolidone-K30 (PVP-K30) were used as stabilizers and particles with mean sizes of 238 nm and 354 nm respectively, were achieved. Both NSs showed a lamelliform or ellipse shape, higher dissolution rate of isoliquiritigenin, improved cytotoxicity and enhanced cellular uptake [96]. Additionally, while the developed NSs caused an apoptosis rate 7.5-10-fold higher than that caused by the not formulated isoliquiritigenin, toxicity on normal cells (HELF) was lower [96].

Also, celastrol (CSL), that is one of the main components of *Tripterygium wilfordii* Hook f., having significant antitumor activity, but poor solubility, low oral bioavailability and systemic toxicity, was nano-formulated by Huang et al. by way of a B-U technique [97]. Particularly, through an antisolvent precipitation method with poloxamer 188 (P-188) as stabilizer, CSL nanosuspensions (CSL-NSs) were prepared having nanosized spherical-shaped particles, with high EE (98%) and DL (87%). Upon its nano-formulation, CSL dissolution in vitro was greatly enhanced, and its cumulative drug release reached approximately 69.20% within 48 h [97]. Additionally, in in vivo experiments, CSL-NSs (3 mg/kg, i.g.) displayed a significantly enhanced tumor inhibition rate (TIR) in comparison with that of CSL suspension when administered orally [97].

SC-CO_2 extracts obtained from the de-oiled *C. longa Linn* (turmeric) rhizome was converted to NPs, by a B-U nano-suspension technique, performing a supercritical fluid expansion method using SC-CO_2 [98]. The production of particles was based on the expansion of the supercritical solution and provided nanosized almost spherical particles with significantly improved dissolubility [98].

The poor aqueous solubility and low oral bioavailability of narigenin (NRG) was addressed by preparing NRG nanosuspensions (NRG-NS) using polyvinylpyrrolidone (PVP K-90) and Tween 80 as stabilizers via an antisolvent sono-precipitation method [99]. Optimized conditions provided NRG-NSs with smallest particle size of 117 nm and zeta potential of −15 mV into an amorphous form possessing higher absorption in GIT, as well as improved dissolution rate and oral bioavailability [99].

More recently, NRG was nano-suspended by a precipitation-ultrasonication method using different surfactants and polymers such as sodium cholate (SC), sodium lauryl sulphate (SLS), polyethylene glycol 4000 (PEG), polysorbate 80 (Tween® 80), poloxomer-188 and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS or Vitamin E-TPGS) [100].

The best nano-formulation showed small particles of 118 nm, increased drug dissolution rate in simulated gastric fluid pH 1.2 (SGF) and phosphate buffer pH 6.8 (PB), an improved pharmacokinetic profile compared to pure NRG and was stable over a period of six months [100].

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As a continuation of their previous research, Rajamani et al. prepared NRG-loaded NSs using TPGS to evaluate the ability of the TPGS-coated NRG-NS to reverse the drug-resistance in human breast adenocarcinoma MCF-7 cells and animal models [101]. The treatment with NRG-NS significantly increased intracellular ROS level, mitochondrial membrane potential, caspase-3 activity, lipid peroxidation status (TBARS) and decreased GSH levels when compared to free NRG treatment in MCF-7 cells, while exhibited dose-dependent in vitro antitumor activity on DLA cells [101]. Additionally, a significant increase in the life span, associated to a decrease in the cancer cell number and tumor weight were noted in mice [101].

By a precipitation-combined ultrasonication method, glaucocalyxin A (GLA), which is a PHY component with multiple pharmacological activities affected by poor solubility, has been formulated in NSs [102]. The GLA-NSs were obtained as spherical particles with a smooth surface, small size (143 nm), and DL% of about 9%. In contrast to the free drug solution, GLA NSs showed higher in vitro antitumor activity against HepG2 cells (IC50 value of 1.793 vs. 2.884 μg/mL at 24 h, $p < 0.01$), and better anticancer efficacy on H22 bearing mice (54.11% vs. 36.02% tumor inhibition rate) [102].

Also, sucrose ester (SE)-stabilized oleanolic acid (OA) NSs (SEOA NSs) for enhanced delivery were prepared via organic solvent evaporation methods, achieving spherical SEOA NS particles (~100 nm in diameter) which were stable over a month at 4°C. The best performant SEOA 4121 NS showed a great increase in saturation solubility (1.89 mg/mL vs. 3.43 μg/mL), dissolution rate, cytotoxicity, bio-efficacy and bioavailability [103].

NSs-based formulations of the flavonoid-rich fraction of *P. guajava* L. extracts with enhanced antihyperglycemic activity and best physical parameters, were prepared using PVA, through the nanoprecipitation method and tested in vivo on type 2 diabetes in high-fat diet-fed, streptozotocin-induced diabetic animals [104]. Upon oral administration, the developed NSs restored the normal level of blood glucose in the first hour and showed beneficial effects on various hepatic and renal parameters [104]. Additionally, NSs enhanced the absorption, decreased the metabolism, and improved the stability of flavonoids [104].

Nigella sativa L.-based NSs were prepared and their composition, as well as their bioactivities in terms of antioxidant, antidiabetic, antibacterial, and hemolytic activities were investigated and compared with those of the not formulated ethanolic extract [105]. The results revealed that the NSs of *N. sativa* seeds showed a total phenolic and a total flavonoid content higher than those of the ethanolic seed extract. NSs showed antioxidant and antidiabetic activity, as well as biofilm inhibition activity against *Escherichia coli* higher than those of both the extract and ciprofloxacin. Additionally, the study showed that NSs had enhanced the bioavailability of bioactive plant compounds as compared to the ethanolic extract [105].

4.2.2. Emulsion-Based Techniques

As suspension methods, emulsions techniques can be used to reduce particles size of both hydrophilic and hydrophobic bioactive compounds, thus improving their solubility and bioavailability, and obtaining orally administrable formulations suitable for pharmaceutical applications [1,106]. Emulsions technology involves the encapsulation of bioactive compounds in small droplets mixing an aqueous phase (w) with an oil one (o) and obtaining either water in oil (w/o), oil in water (o/w), or bi-continuous colloidal dispersions, which are stabilized using specific additives, such as generally-regarded-as-safe (GRAS) pharmaceutical surfactants, co-surfactants, and emulsifiers (Table 7) [1,107].

Emulsions encompass micro-emulsions (micro-sized droplets, not considered in this paper), NEs (100-500 nm droplets), self-emulsifying drug delivery systems (SEDDSs), which in turn include self-nanoemulsifying drug delivery systems (SNEDDSs) and self-micro-emulsifying drug delivery systems (SMEDDSs), so classified based on the dimensions of their NPs. Additionally, self-double-emulsifying drug delivery systems (SDEDDSs) represent a further evolution of conventional SEDDSs (Table 7) [1].

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Table 7. Main types of NEs and common oily phases.

Type of NEs		Oily Phase	Advantages	Drawbacks	Ref.
NEs o/w or w/o (100-500 nm)		Captex 355 Captex 8000 Witepsol Myritol 318 Isopropyl myristate Capryol 90 Sefsol-218 Triacetin Isopropyl myristate Castor oil Olive oil	PHYs protection Controlled release Sustained release ⇑ DL%	No high-melting PHYs 5-10% additives* required	[1]
SEDDSs o/w **			⇑ Oral bioavailability Possibility of easy scale-up ⇑ DL% Allow delivering peptides/lipids without the risk of digestion	5-10% additives* required For low therapeutic dose PHYs Many parameters The physico-chemical properties of PHYs can influence the efficiency of oral absorption and the performances of SEDDSs.	[1]
SNEDDSs ** (<50 nm)	SMEDDSs ** (100–200 nm)				
SDEDDSs ** (w/o/w) or (o/w/oil)					

SNEDDSs = self-nanoemulsifying drug delivery systems; SMEDDSs = self-micro-emulsifying drug delivery systems; SDEDDSs = self-double-emulsifying drug delivery systems; * surfactants, co-surfactants, and stabilizers; ⇑ high, higher; ** anhydrous systems.

By NE techniques, it is possible to obtain PHYs-based formulations characterized by particles of 100–500 nm, endowed with improved solubility, stability, bioavailability, and extended half-life. Upon the use of suitable additives (5-10%), isotropic, transparent, and kinetically stable suspension are achievable [108,109]. NEs are generally prepared using either low energy techniques (LET), not involving mechanical devices, or high energy techniques (HET), needing the use of mechanical devices and strong agitation [107].

Among NEs, SEDDSs are anhydrous nano-dispersions obtained by spray drying or freeze drying a mixture of an oil phase, surfactants, co-surfactants/co-solvents, and a lipophilic bioactive compound. SEDDSs are particularly suitable for orally delivering lipophilic bioactive compounds because they spontaneously arrange in colloidal emulsions when mixed with water or with fluids in GIT, after ingestion of capsules filled with the SEDDSs [1,110]. SEDDSs include SNEDDSs (droplets size < 50 nm), SMEDDSs (droplets size of 100–200 nm), and SDEDDSs. The latter can form water-in-oil-in-water (w/o/w) or oil-water-in-oil double emulsions in GIT fluids, thus representing novel self-emulsifying formulations, expressive of a further evolution of conventional SEDDSs [111].

High Energy and Low Energy Methods

The high energy methods involve the use of a mechanical device such as high-pressure valve homogenizers, microfluidizers and ultra-sonicators, while the low-energy methods, use the energy input deriving from the chemical potential of the components used to from NEs. In the latter case, the NE forms at the oil and water phase interface, by gentle mixing of the components, and its formation depends on factors such as temperature, composition, and compounds solubility. Table 8 reports the main adopted high energy and low energy methods and their recent applications in nano formulating PHYs.

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Table 8. Main adopted high and low energy methods and their recent applications in nano-formulating PHYs.

Technique	Method	PHYs	Activity	Particle size (nm)	Ref.
HET	High pressure homogenization (HPH)	Jackfruit pulp extract* rich in carotenoids	Antioxidant	166	[112]
		Lycopene	Radicals scavenging activity	92.6	[113]
		Lentil	⇓ Blood pressure ⇓ Bad cholesterol (LDL) ⇑ Good cholesterol (HDL) ⇓ Heart disease risk	149	[114]
	Microfluidization (MF)	β-carotene	Antioxidant	140-160	[115,116]
	Ultrasonication (US)	Essential oils (EOs)(*Zataria multiflora*)	Antibacterial	91-211	[117]
		RES	⇓ Diseases by oxidative stress	20.4 **; 24.5 ***	[118]
LET	Phase Inversion Temperature (PIT)	Cinnamon EO	Antioxidant Antimicrobial	101	[119]
	Phase Inversion Composition (PIC)	Rosmarinic acid (RA)	Antiviral Anti-inflammatory Antioxidant	70-100	[120]
	Spontaneous Emulsification (SE)	Key lime (*Citrus aurantifolia*) EO Kaffir lime (*Citrus hystrix*) EO Calamansi lime (*Citrofortunella microcarpa*) EO	Antibacterial	21 28 60	[121]

* Rich in carotenoids; ** NE; *** NE-cyclodextrin inclusion complex.

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Novel Nano-emulsion Preparation Techniques

New emulsification approaches are being increasingly developed to increase the range of materials which can be formulated, the available operating conditions, and to simultaneously lower the production costs. Water-in-oil NEs can be prepared by condensing water vapor on subcooled oil-surfactant solution. The NEs formed using the condensation approach have dimensions of about 100 nm. Particularly, an oil bath is placed in a humid environment with appropriate concentration of surfactant, and on decreasing the temperature below the dew point, water condensation is induced on the oil surface resulting in NE formation. The process is simple, rapid, scalable, and energy efficient with potential application in processed foods [122]. Additionally, pickering emulsions (PNEs) are NEs stabilized by solid particles (for example colloidal silica) which adsorb onto the interface between the water and oil phases [123]. Typically, the emulsions are either water-in-oil or oil-in-water emulsions, but other more complex systems such as water-in-water, oil-in-oil, water-in-oil-in-water, and oil-in-water-in-oil also exist. The use of PNEs overcomes the problems associated with surfactant desorption and Ostwald ripening. Low energy approach cannot be used to produce PNEs, while the high energy approach prevents adsorption of the particles on the droplets. The vapor condensation method used for PNEs preparation is a single step process and has several advantages over the conventional techniques such as the use of low concentration of NPs [124].

NE-based Phytochemicals Delivery Systems

NEs-based DSs have been exploited for formulating herbal drugs, whole plant extracts, a single PHY or mixtures, which are poorly insoluble, unstable in highly acidic pH and undergo liver metabolism if orally administered as free. Interestingly, NE-based drug delivery systems (DDSs) allow to minimize the side effects due to the possible drug accumulation in the non-targeted areas; so that their oral administration is authorized also in pediatric and geriatric individuals [125]. As examples, NE techniques were used to nano-formulate turmeric, curcumin (diferuloylmethane), and di-benzoyl-methane (a structural analog of curcumin). Tannins, stilbenes, and flavonoids, which demonstrated in vitro antioxidant effects, have been encapsulated in nano drops by NE methods [1]. Also, bioactive lipids and carotenoids were formulated as NEs by observing, respectively, more stability against autoxidation and increased bio-accessibility [1].

In this regard, curcumin-loaded lipid NEs (CmLN) functionalized with a nona-arginine peptide (R9-CmLN) have been prepared by Simion and colleagues, using triacylglycerol as the oil phase and Tween-20 as emulsifier [125]. When used in therapeutically relevant concentration, R9-CmLN demonstrated low hemolytic activity, low cytotoxicity, and anti-inflammatory effects. Additionally, in vivo biodistribution studies in mice revealed high accumulation of R9-CmLN in the liver and the lungs, suggesting their potential therapeutic applications in different inflammatory pathologies localized in such organs [125].

The limited efficacy of curcumin due to its low oral bioavailability was overcome by developing SNEDDS by the group of Nazari-Vanani [126]. An optimal formula for a SNEDDS comprised ethyl oleate/Tween 80/PEG 600 (50/40/10 w/w) which formed 11.2-nm uniform droplets by mild agitation. In in vivo experiments in rats orally administered with the SNEDDS, the curcumin C max was increased of 3.95 times, while its bioavailability was enhanced by 194.2%, compared to the curcumin suspension in water [126]. In another study, to enhance the bioavailability of curcumin and its impact on the levels of docosahexaenoic acid (DHA), which is an important long chain omega-3 polyunsaturated fatty acid (PUFA), Sugasini and Lokesh developed a curcumin-loaded NE using a phospholipid core material (Lipoid™) [127]. Particularly, curcumin was dissolved in coconut oil, sunflower oil, or linseed oil, and the NEs were achieved after mixing with Lipoid™ using HPH. Experiments in rats demonstrated high levels of curcumin in serum liver, heart and brain, and a significant increase in DHA levels of serum and lipid tissue [127].

Moreover, curcumin-loaded NPs were prepared by a particular emulsion technique referred to as emulsion–diffusion–evaporation method [128]. Briefly, curcumin was dissolved in acetone and

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ultrasonicated, stirred for 1 h at 55 °C, and finally heated in an oven up to the complete evaporation of the organic solvent, achieving 32 nm-sized NPs [128].

Experiments carried out on diabetic rats evidenced a significant reduction of the blood glucose level, while an increasing of that of insulin, in the group treated with the developed curcumin-enriched NPs [128].

Aiming at developing an effective anticancer agent against oral squamous cell carcinoma (OSCC), curcumin was formulated as curcumin-loaded lipid NEs (CUR-NEs), obtaining 100 nm-sized particles. In in vitro investigations on OSCC HSC-3 cells, CUR-NEs exhibited significant cytotoxic effects on OSCC cells in a dose-dependent manner, compared with the control [129].

Hu and co-authors manufactured a SDEDDS loaded with both epigallocatechin-3-gallate (EGCG) and α-lipoic acid (ALA) (EA-SDEDDS), having improved photo-stability respect to free EGCG and equal antioxidant activity respect to a solution of EGCG and ALA [130]. Particularly, a modified two-step method was used and optimized to prepare EA-SDEDDS. In the first step, a primary emulsification was achieved by adding the aqueous phase containing EGCG to the oily one consisting of macadamia oil, cetostearyl alcohol, ALA (6g/L) and polyglycerol polyricinoleate (PGPR) as a hydrophobic emulsifier, using an overhead stirrer. In the second step, the primary emulsion was furtherly mixed with different types of hydrophilic emulsifiers (S721, P10, L23. and S40) [130].

In a further study, the in vivo poor antioxidant activity of EGCG was significantly increased when it was formulated as NE by Koutelidakis et al. [131]. Particularly, in a typical experiment w/o, o/w and double emulsions were prepared and administered to mice by gavage. After 2-hour administrations the total antioxidant capacity (TAC) was measured with Ferric-Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) assays in plasma and some tissues (especially colon, jejunum, heart, spleen). While no toxic effects were observed, the EGCG emulsion II (o/w), which contained 10% olive oil and 0.23 mg/mL esterified EGCG in fatty phase, exerted an antioxidant effect in mice plasma remarkably higher than that of the aqueous solution of EGCG. Additionally, in several tissues of mice administered with emulsion II were observed values of TAC higher than those observed in animals treated with emulsions I and III [131].

Table 9 reports some examples of plants-derived bioactive compounds nano-formulated by NE techniques obtaining emulsion-based DSs.

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Table 9. Emulsion-based formulation for delivering PHYs.

PHY	Emulsion type/Method	Additives	Results	Ref.
Curcumin	NE	Tween-20	⇓ Toxicity ⇑ Bioavailability ⇑ Bioactivity ⇑ Anti-inflammatory	[125]
	o/w SNEDDS Mild agitation	Tween 80 PEG 600	⇑ Oral bioavailability ⇑ C max	[126]
	NE/HPH	PEG (3%)	⇑ Oral bioavailability ⇓ DHA levels	[127]
	Emulsion–diffusion evaporation	N.R.	⇓ Blood glucose levels, ⇑ Insulin	[128]
	Interfacial prepolymer deposition and SE	Lipoid 100	Inhibition of OSCC cell ⇓ PI3K/Akt/mTOR ⇑ miR-199a	[129]
EGCG (E) + ALA (A)	SDEDDS	PGPR S721, P10, L23. and S40	⇑Photo-stability Antioxidant ⇑EE	[130]
EGCG	NE (o/w)	BC WPI	No toxicity ⇑ Antioxidant	[131]
Carotenoids (*Paprika Oleoresin*)	SMEDDS	Tween 80	⇑ Solubility	[132]
Lutein	SMEDDS	Tween 80 Labrasol TranscutolHP/Lutro-E400 [1]	⇑ Solubility ⇑ Bioavailability	[133,134]
Polymethoxyfla-vones (PMFs)	NE	Tween 20 Tween 85	⇑ Dissolution rate	[135]
β-Carotene	o/w NE	Tween 20	⇑ Emulsion stability ⇑ Solubility ⇑ Bioaccessibility	[136]
Lycopene	Microemulsion (ME)	ESE 3GIO SML	⇑ Solubility	[137]
Quercetin	SNEDDS	Tween 80 PEG 400	⇑ Solubility	[138]
Naringenin Hesperetin	NE	Glycerin	⇑ Solubility ⇑ In vitro stability No cytotoxic (vitro) No hemolytic (vivo) Anti-inflammatory	[139]
Baicalein	NE/HPH	PEGM Sodium oleate Hoechst 33258 3,3-DODOXAP	⇑ Oral bioavailability ⇑GIT permeability ⇑Transcellular transport ability ⇓ Cytotoxicity	[140]
Imperatorin	NE/HSS NE/HPH	Polaxamer 188	⇑ Bioavailability Antiploriferative (MDA-MB-231)	[141]

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Pandanus conoideus Lamk (red fruit)	SNEDDS	PPG Tween 20	⇑ Cytotoxic activity	[142]
Pandanus conoideus Lamk (red fruit)	NE/high-speed mixer	PPG SEPIGEL 305™	Antioxidant	[143]
Plantago lanceolata L.	SNEDDS	Labrasol or Kolliphor RH 40 Transcutol HP	⇑ Solubility ⇑ Permeability ⇑ Bioavailability ⇑ Pharmacological effects	[144]
Bay Leaves extracts (*Eugenia polyantha* Wight)		Tween 80 PEG 400		[145]
Myricitrin		Capryol 90 Cremophor RH 40 PEG 400 Cremophor EL Transcutol HP		[146]
Myricitrin		Cremophor EL35 Dimethyl carbinol		[147]
Quercetin		PEG 200 Tween 40 Tween 60 Tween 80 PEG 400 Transcutol HP		[148]
Baicalin		Peceol® (14.3%, w/w) Kolliphor® EL (57.1%, w/w) Transcutol® P (28.6%, w/w)		[149]
AITC	Emultion solvent evaporation	Polyvinyl alcohol (PVA) (3%)	⇓ Degradation ⇓ Volatility ⇑ Shelf life Sustained release ⇑ Toxicity on tumor	[150,151]
			⇑⇑ Anti-cancer activity ⇓ toxicity	[152]
BITC	US	Tween 80 Decyl-β-d-glucopyra-noside	⇑ EE%	[153]
BITC	US	Tween 80	⇑ EE% Good DL% MDA-MB-231 cells inhibi-tion	[154]
	Heating stirring-soni-cation		Good long-term stability ⇑⇑⇑ EE Sustained release ⇑ Cytotoxicity (MDA-MB-231)	[155]
SEO	o/w NEs	Tween 20 Tween 80	⇑ Antibacterial effects	[156]

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	o/w NEs Votexed/sonication	Tween 80	⇑ Antibacterial effects ⇓ Biofilm formation	[157]
RES	SNEDDS	Capryol 90 Cremophor EL Tween 20	⇑ Oral bioavailability ⇑ Anti-fatigue effect	[158]
	SMEDDS	Labrafil Labrasol RH40	⇑ Oral bioavailability	[159]
Astaxanthin + α-tocopherol	κ-carrageenan o/w NE/SE	Span® 80 (1%) PEG (1%) Tween® 20 (1%)	⇓ Toxicity ⇓ Hyperglycemia ⇓ Diabetes Complications ⇑ Wound healing	[160]
	κ-carrageenan o/w NE Ultrasonication	None		
Cloves	SMEDDS	Tween 20 Tween 80	⇑ Anticancer effects ⇑ Antibacterial effects	[161]
Cloves	US	Tween 20 Tween 80 PEG	Cytoprotective effects ⇑ Anticancer effects ⇓ Toxicity	[162]
Kaempferol	HPH	Egg-lecithin Tween 80	⇑ Permeation Safe Antioxidant capability ⇑ Drug into rat's brain ⇓ C6 cell viability	[163,164]
β-Carotene	o/w NE/US and MF	Casein	⇑ Water dispersibility ⇑ Chemical stability	[165]
	o/w NE/HPH	Porcine gelatin	⇑ Water dispersibility ⇑ Stability ⇑ Dispersibility in foods	[166]
Astaxanthin	o/w NE/SE	Lecithin	⇑ Stability ⇓Photodegradation	[167]
Curcumin	o/w NE/HPH	SDS	⇑ Bioavailability ⇑ Antioxidant effects ⇑ Lipids digestion	[168]
	o/w NE/MF	Lecithin Tween 20 SMP	⇑ Stability ⇑ Antioxidant effects	[169]
	o/w NE/SE	Tween 80	⇑ Antimicrobial effects	[170]
Ginger EO	o/w NE/US	Tween 80	⇑ Antimicrobial effects ⇑ Antioxidant effects	[171]
Capsaicin	o/w NE/HPH+US	Tween 80	⇑ Antimicrobial effects ⇑ Physical properties	[172]

[1] co-surfactant; OSCC = oral squamous cell carcinoma; EGCG = epigallocatechin-3-gallate; ALA = α-lipoic acid; EE = entrapment efficiency; 3GIO = tri-glycerol monooleate; SML = sucrose monooleate; PEG = polyethylene glycol; PGPR = polyglycerol polyricinoleate; BC = bacterial cellulose; WPI = whey protein isolate; ESE = ethoxylated sorbitan esters; PEGM = poly(ethylene glycol) monooleate; 3,3-DODOXAP = 3,3-dioctadecyloxacarbocyanine perchlorate; DHA = docosahexaenoic acid; HSS = high-speed shearing; SEO = *Satureja Montana* essential oil; PPG = polypropylene glycol; MDA-MB-231 = cell model of late-stage breast cancer; RES = resveratrol; SDS = sodium dodecyl sulphate; SMP = sucrose palmitate; ⇑ = increasing, improvement, higher, up-regulated; ⇓ reduced, lower.

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Naringenin and hesperetin are citrus flavonoids possessing well-documented protective effects on cardiovascular system. Unfortunately, their poor water solubility, affecting their bioavailability, strongly restricts their therapeutic use. To address these issues, they were recently encapsulated into lipid NEs (LNEs) achieving flavonoids loaded LNEs which showed nanosized particles of 190-200 nm (naringenin) and 193-218 nm (hesperetin), negative zeta potential, an EE over 80%, good in vitro stability and steady release of the cargo. Additionally, while the LNEs did not exhibit in vitro cytotoxicity, and did not provoke lysis of mouse erythrocytes, they exerted significant anti-inflammatory effects [139].

The potential of NE techniques as enhancers of drugs solubility, oral bioavailability and stability, was demonstrated by Yin et al when they prepared NEs-based baicalein DSs [140]. Particularly, baicalein (BCL) possessing important pharmacological activities but poor solubility and low stability in the GIT, was formulated using a NE technique, in which a HPH process was exploited to minimize the quantity of surfactants [140]. BCL-loaded NEs were achieved which demonstrated ~90 nm-sized particles, EE > 99%, and oral bioavailability of BCL 525% higher than that of BCL suspensions. Additionally, BCL-loaded NEs exhibited excellent intestinal permeability and transcellular transport ability, while cytotoxicity was acceptably low for oral purposes [140].

Liang and co-workers used NE techniques to formulate imperatorin, having antitumor, antibacterial, anti-inflammatory, anticoagulant activities, and myocardial hypertrophy inhibitory effects [141]. An optimized preparation required 1.39 g of egg lecithin, 0.21 g of poloxamer 188, and 10.6% soybean oil, as stabilizers and oily phase respectively, thus providing imperatorin-loaded spheres, showing round globules of relatively uniform shape and sizes within 200 nm. The imperatorin-loaded lipid NPs allowed a significantly enhanced bioavailability of imperatorin and inhibited MDA-MB-231 cell proliferation, thus resulting promising for the treatment of late-stage breast cancer [141].

The PHY constituents of *Pandanus conoideus* Lamk (red fruit) are endowed with significant antitumor activity against breast cancer but are poorly adsorbable in GIT. To address this issue, Satria and co-workers prepared SNEDDS-type formulations (particles size 193 nm) of the *Pandanus conoideus* Lamk (red fruit)'s red oil extract [142]. Once tested in vitro against MCF-7 breast cancer cell lines, the red oil-loaded SNEDDS formulations showed good cytotoxic activity, higher than that demonstrated by the not formulated *P. conoideus* extract at the equivalent dose [142].

More recently the red oil (*P. conoideus*) previously reported was formulated by the NE technique in the form of a conventional NE, as a cream NE and as a NE gel, intended for skin application. The NEs were prepared employing sucrose palmitate as emulsifying agent and a brute force method, using Ultra-Turrax homogenizer as a high-speed mixer [143].

The red fruit oil-based conventional NE showed pseudoplastic flow properties, spherical shape, and an average particle size of 103 nm. The cream NE demonstrated plastic flow properties, and an average particle size of 392 nm, while that in the form of gel revealed plastic flow properties, and an average particle size of 144 nm. In antioxidant experiments using 2.2-diphenyl-1-picrylhydrazyl (DPPH) assay, the cream and gel nano-suspensions showed IC50 values of 6.14 and 48.85, respectively [143].

Likewise, herbal drugs such as *Plantago lanceolate* [144], ethyl acetate extracts of bay leaves (*Eugenia polyantha* Wight) [145], myricetin [146,147], quercetin [148], and baicalin [149], were also developed in SNEDDS formulations (Table 9) to increase their solubility, permeability, bioavailability, and pharmacological effects.

Particularly, the low oral bioavailability of myricitrin was improved preparing myricitrin (M)-loaded SMEDDS consisting of an oil phase (ethyl oleate), a surfactant (Cremophor EL35) and a co-surfactant (dimethyl carbinol) [147]. The prepared M-SMEDDS exhibited stable physicochemical properties, small droplets (22 nm), negative zeta potential (−23 mV) and high EE (92.7%) [147]. The in vitro release study showed that the release of myricitrin from M-SMEDDS was significantly higher than that from a free myricitrin solution, while the oral bioavailability of M-SMEDDS was 2.47-fold higher than that of the free drug [147].

Some natural isothiocyanates (ITCs), including sulforaphane (SFN) isolated from broccoli in 1992, allyl isothiocyanate (AITC) abundant in mustard, horseradish and wasabi, benzyl isothiocyanate (BITC) from garden cress, and phenethyl isothiocyanate (PEITC) from watercress demonstrated

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a plethora of pharmacological effects [150]. Currently, some of them are under Phase I and II clinical trials to assess their safety, tolerance, pharmacokinetics, and therapeutic benefit in the context of different types of cancer, diabetes, kidney disease, skin disorder, blood/vascular disease, asthma and autism [150].

Unfortunately, the results of these clinical trials only partially confirmed the promising beneficial potential demonstrated during in vitro studies, due to their instability, low bioavailability, and, concerning cancer, because of the influence of the complex tumor microenvironment [150].

In the last five years, Encinas-Basurto et al., encapsulated AITC into poly (lactic-glycolic acid) nanoparticles (PLGA NPs) to extend its shelf life and enhance its antiproliferative properties, using an emulsion-solvent evaporation method [151]. The obtained AITC PLGA NPs had a particle size of about 200 nm, polydispersity >2%, and negative zeta-potentials (−8.0mV). These NPs demonstrated reduced degradation, volatility and an extended shelf life when compared with free AITC [151]. In vitro experiments on cancerous HeLa and MDA-MB-231 cells showed that the sustained release of AITC from polymeric NPs resulted in a significant toxicity towards tumor cells. Subsequently, the same group modified the surface of AITC-loaded PLGA NPs using a specific antibody to target the Epidermal Growth Factor (EGF) receptor overexpressed on the epithelial squamous carcinoma cells [152]. AITC-loaded PLGA NPs showed more effective anti-cancer properties when compared with free AITC. The attachment of the anti-EGFR antibody on the NPs' surfaces further enhanced their cytotoxicity towards the tumor cells, and reduced toxicity against normal cells [152].

Kumar et al. encapsulated BITC in a NE through ultrasonication using Tween 80 or decyl-β-d-glucopyranoside as stabilizers [153]. The average size of NE particles was about 32 nm and EE was 99%. The nano-DD showed good stability at pH 5, 7 and 9, while in highly acidic or basic conditions (pH 2 and 12) aggregation occurred, probably due to ineffective electrostatic repulsion and hydrolysis [150].

In a recent research, Uppal et al. developed cerium oxide NPs (CONPs)- based DDSs using the ultrasonic nano-emulsification method [154]. The synthesized NPs (size ≤ 5 nm) were then loaded with BITC. The average particle size of BITC-loaded CONPs was about 5 nm, while zeta-potential value was about −15 mV. The formulation achieved showed high EE and good DL, while demonstrated to significantly inhibit the viability in MDA-MB-231 cells. The same group has also produced a new BITC-based NE system employing the heating stirring-sonication method and rhamnolipid as biosurfactant [155]. The optimized NE exhibited good long-term stability, very high EE, sustained release of BITC and increased cytotoxicity against MDA-MB-231 cells as compared to BITC alone [155]. Collectively, from data in literature, it results that NE techniques are the main method used for the nano-formulation of BITC providing BITC-loaded NPs with very high EE%, enhanced absorption and bioavailability, prolonged shelf-life, sustained release of BITC, as well as improved anti-cancer activity against several cancer cell lines [155].

Oil in water (o/w) NEs composed by *Satureja Montana* essential oil (SEO), owing high content of active PHYs and several biological effects, were prepared using Tween 20 or Tween 80 as emulsifiers [156]. The achieved SEO-NEs were analyzed in terms of hydrodynamic diameter, zeta-potential and polydispersity index, which confirmed the formation of homogeneous in size stable NEs. Microbiologic experiments carried out on Gram-positive and Gram-negative clinical isolates established that the NE-based formulation preserved and improved the antimicrobial activity of pristine SEO [156].

More recently, Rinaldi and colleagues formulated SEO, preparing and optimizing o/w NEs composed of SEO and Tween-80 and achieving 112 nm-sized NPs [157].

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) evaluated by the microdilution method, showed that the SEO-based NEs exhibited higher inhibitory effects against planktonic *E. coli* than SEO alone. Additionally, SEO formulations enabled an efficient reduction of the biofilm produced by the strong producer strains at sub-MIC concentrations. On these results, SEO-based NEs could be promising to ensure food safety quality, and to counteract the antibiotic resistance of poultry associated *E. coli*, if applied/aerosolized in poultry farms [157].

A SNEDDS for RES capable to exert anti-fatigue activity was developed by Yen et al. to improve RES bioavailability and was evaluated for its anti-fatigue activity in rats. The optimized SNEDDS was composed of Capryol 90, Cremophor EL, and Tween 20 and showed nanosized particle of

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approximately 41 nm. Such RES-SNEDDS not only enhanced the oral bioavailability of RES upon administration in rats, but also exerted improved anti-fatigue pharmacological effect [158].

More recently, RES-loaded SMEDDS with particles in the range 22-26 nm have been developed with (SMEDDS-1) and without inhibitory excipients (SMEDDS-2) to increase RES oral bioavailability, by inhibiting intestinal metabolism [159]. Results demonstrated that while similar physicochemical properties between inhibitory SMEDDS-1 and non-inhibitory SMEDDS-2 were observed, bioavailability of RES was increased up to 76.1% in SMEDDS-1 [159].

Astaxanthin and α-tocopherol, which showed activity in wound healing in diabetics, were recently formulated with κ-carrageenan to obtain bicomponent NEs (AS-TP@KCNEs) [160]. In vitro and in vivo experiments on diabetic mice demonstrated that AS-TP@KCNEs were biocompatible and possessed healing properties that accelerated wound closure and exhibited better control of hyperglycemia, thus reversing the diabetes mellitus complications [160].

Spices have been known to exert numerous functions useful against different diseases along with strong anti-cancer potential. Particularly, clove and turmeric are spices with strong anti-cancer potential.

In this context, Nirmala et al. developed an oil-based NE of cloves (*Syzygium aromaticum*) buds and tested its anti-cancer efficacy against thyroid cancer cells (HTh-7). The cloves loaded NE showed anti-proliferative effects against thyroid cancer cells, with apoptosis seen as the mode of cell death [161].

More recently, to improve the medicinal properties of *Syzygium Aromaticum* L, the *S. Aromaticum L.* bud EO was nano-formulated using the ultra-sonication NE technique obtaining a nanosized DSs (SABE-NE) with 131 nm-sized particles [162]. While the produced SABE-NE induced apoptosis response and significant cellular death in HT-29 cancer cells, HFF normal cells indicated confined cytotoxic impacts. Moreover, in vivo tests on mice livers demonstrated the cytoprotective properties of SABE-NE [162].

Since kaempferol (KPF) has been reported to induce glioma cell death, Colombo et al. prepared NEs containing KPF with and without chitosan to investigate their potential for KPF brain delivery following intranasal administration, and to evaluate their antitumor activity against glioma cells [163]. KPF-loaded NE (KPF-NE) and KPF-loaded mucoadhesive NE (KPF-MNE) were prepared by HPH technique which demonstrated significantly higher permeation capability across the mucosa in ex vivo diffusion studies [163]. Both types of KPF-NE were safe for the nasal mucosa and able to preserve KPF antioxidant capability. Additionally, KPF-MNE enhanced significantly the amount of drug into rat's brain following intranasal administration, and reduced C6 glioma cell viability through induction of apoptosis to a greater extent than either free KPF or KPF-NE [163].

Other case studies have been reported in literature and Table 9 on the formulation of β-carotene, astaxanthin, curcumin, ginger EO and capsaicin by NE technology, using both HPH, SE, US or MF methods and obtaining nanosized droplets with significantly improved physicochemical and biological properties [164-172].

NSs- and NEs-based Phytochemicals Formulations: The State of The Art in Graphs

In this following section we have provided some graphical interpretation of information reported in the previous Sections 3.2.1. and 3.2.2. Particularly, Figure 8 gives us a scenario concerning the main NSs- and NEs-based PHYs-loaded formulations developed in the last recent years grouped according to their PHY content.

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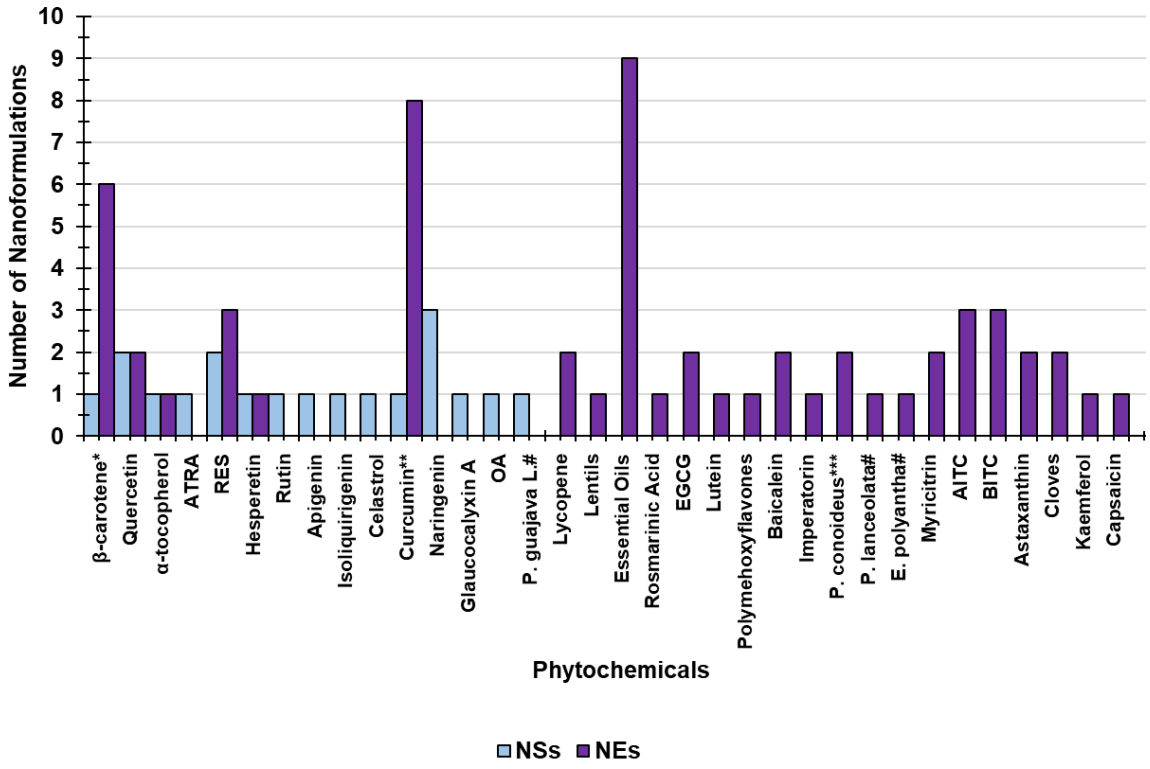

Figure 8. Main NSs and NEs-based PHY formulations described in the last recent years. * Including also other carotenoids; ATRA = all *trans* retinoic acids; RES = resveratrol; ** including also *Curcuma Longa* extracts; OA = oleanolic acid; EGCG = epigallocatechin-3-gallate; *** red fruits; # plants extract; AITC = isothiocyanate; BITC = benzyl isothiocyanate.

The results evidence that the NE-based technologies are the most adopted and studied (75.6%), respect to the NS-based ones (24.4%). Secondly, concerning the type of plants-derived bioactive compounds used to prepare NE-based formulations finalized to treat human diseases, EOs and curcumin are the most chosen (11.5 and 10.3% respectively), followed by carotenoids (7.7%). Differently, concerning NS-based formulations here considered, naringenin (3.8%), quercetin (2.6%) and resveratrol (2.6%) are the PHYs most used, while no NS-based formulation of EOs has been recently developed.

Figure 9 shows that the B-U methods (63.2%) are the preferred ones to develop NSs containing PHYs, followed by the CONI (2.6%) and by the T-D ones (only 1.1%).

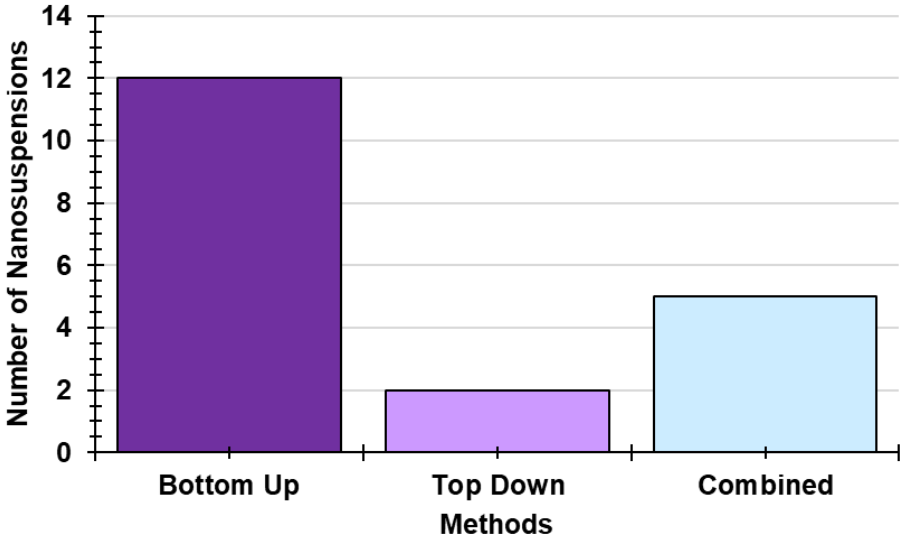

Figure 9. Main NSs methods.

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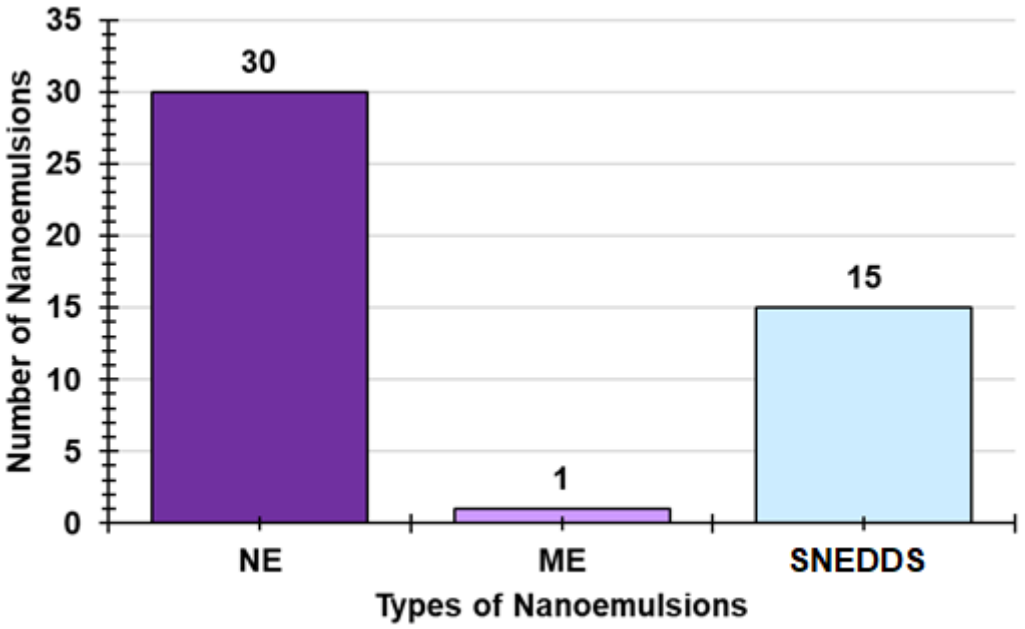

Figure 10. Main types of NEs.

Within the three main types of NEs reported, the most developed in literature are the conventional ones (65.2%), SNEDDSs (including SMEDDSs, SNEDDSs and SDEDDSs) are the 32.6%, while ME are only 2.2% (Figure 10). Figure 11 shows the frequency with which HET are employed to develop NEs vs that of LET, thus evidencing that the first are preferred to the second ones (64.3 vs. 35.7%).

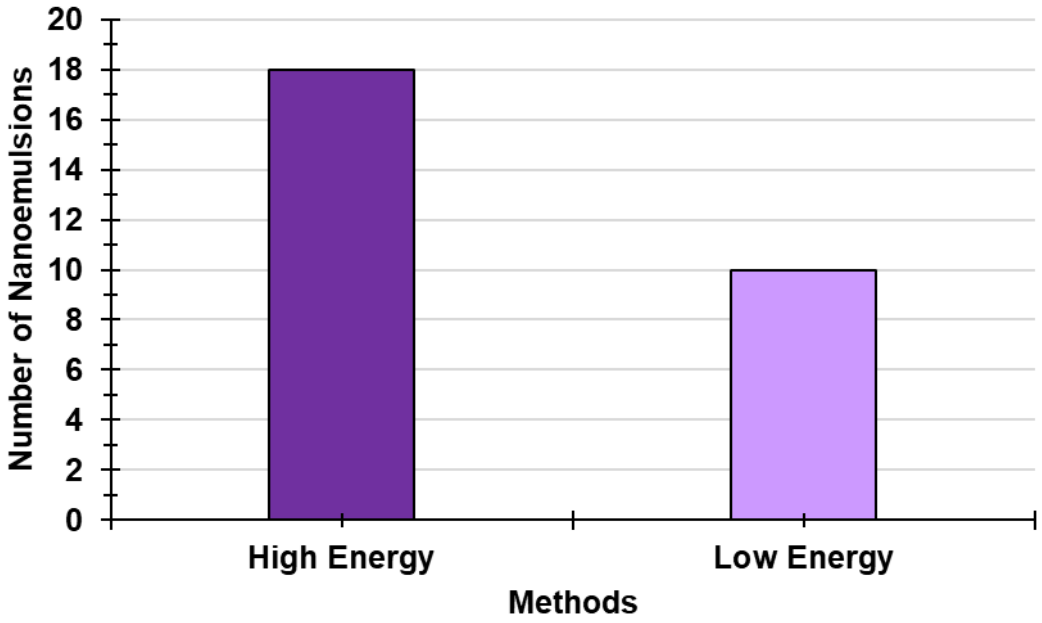

Figure 11. Main types of methods to prepare NEs.

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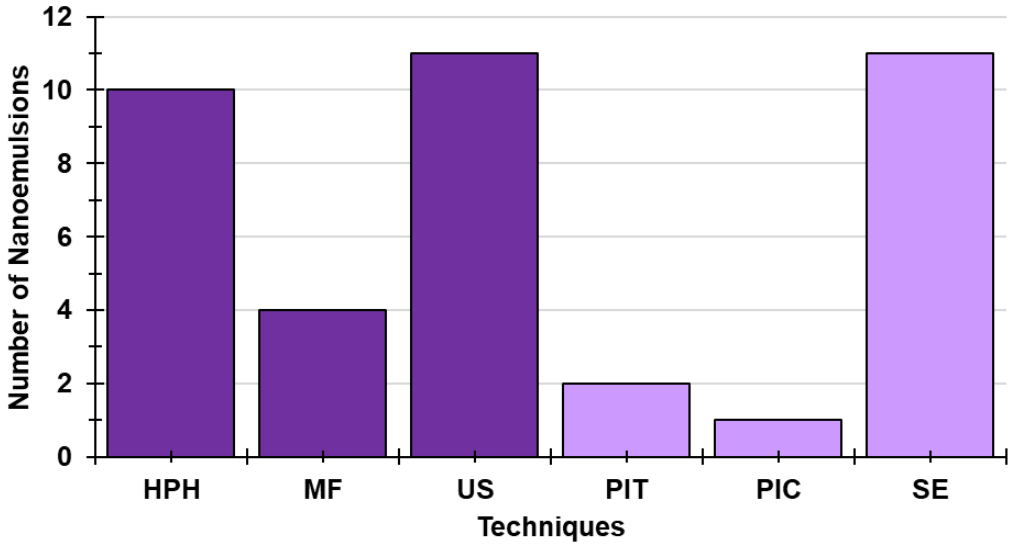

Figure 12. Main techniques of HET (purple) and of LET (light purple).

Finally, within the HET, US and SE are the most applied (28.2%), followed by HPH (25.6%), while the frequency of utilization of MF (10.3%), PIT (5.1%) and PIC (2.6%) was remarkably lower (Figure 12).

5. Nanomaterials and Nanoparticles: What We Know and What We Should Know

The use of nanotechnology in products regulated by the FDA, such as foods, cosmetics, medical devices and drugs has been enduring for several decades. According to the FDA's Center for Drug Evaluation and Research (CDER), drug products containing nanomaterials are very different from conventional ones and should have a particular attention. Since the early 1970s, there has been an incessant increase in the number of approved drug products containing nanomaterials, and more than 60 applications have been approved so far, but interest continues to rise [173].

Together with liposomes and nanocrystals, NEs are among the most common types of drug products containing nanomaterials being approved, due to the use of GRAS material for their development. Drug products containing nanomaterials are unique and possess nonpareil chemical, physical, or biological properties different from those owned by traditional drugs [173]. Importantly, the presence of nanomaterials in a drug formulation may positively or negatively impact the quality, safety, or efficacy of the product, mainly because drug products containing nanomaterials may follow a different pathway in the body compared to that of a not nano-sized drug [173] (Table 10). After a drug product formulated as NPs enters the bloodstream, it could interact with specialized immune cells called macrophages, which can engulf and transport it to the target site, such as that where bacteria, fungi, viruses or tumor cell reside. Differently, these areas are typically difficult to reach for a not nano-formulated drug. Also, a drug formulated as NPs often has a special coating that can prevent it from the immune cells attack, thus having the possibility to circulate in the bloodstream for prolonged time, and to reach untouched the tumor tissues or infected areas. The ability to target areas of the body and to bypass others possessed by nano-formulated drugs can significantly reduce the risk of side effects, such as toxicity to nontarget organs, and potentially increase the effectiveness of the treatment. For these reasons, nanomaterials are most frequently used to formulate drugs intended for the treatment of cancer or infections [173].

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Table 10. Post-administration events and related advantages concerning nanomaterials-based and not nanomaterials-based drug formulations (NDF NNDF).

Post-administration event	NNDF	NDF	Result	Advantages	Ref.
Interaction with specialized immune cells	NO	SI	Easier and faster macrophage-mediated targeting	⇓ Risk of side effects	[173]
Possible presence of a special coating	NO	SI	Capability to by-pass immune cells attack	⇓ Toxicity to non-target organs ⇑ Effectiveness	
		SI	Prolonged residence time in bloodstream		

5.1. Ongoing Actions to Address Challenges Related to Nanotechnology

With the aim to inform agency guidance and regulatory review, the Office of Testing and Research (OTR) in CDER's Office of Pharmaceutical Quality has been conducting research to better understand the manufacturing and quality issues associated with drug products containing nanomaterials. Particularly, OTR is establishing clear standards to pave the way for approval of future generics containing nanomaterials. Currently, studies are focused on identifying the critical processes and the material properties that can impact the quality of the drug products containing nanomaterials. Figure13 puts in evidence some of factors which could impact the quality of nanomaterials-based drug formulation within the context of efficacy and safety. Obviously, manufacturers should select and implement the right quality control measures so that any variability can be captured and accounted for.

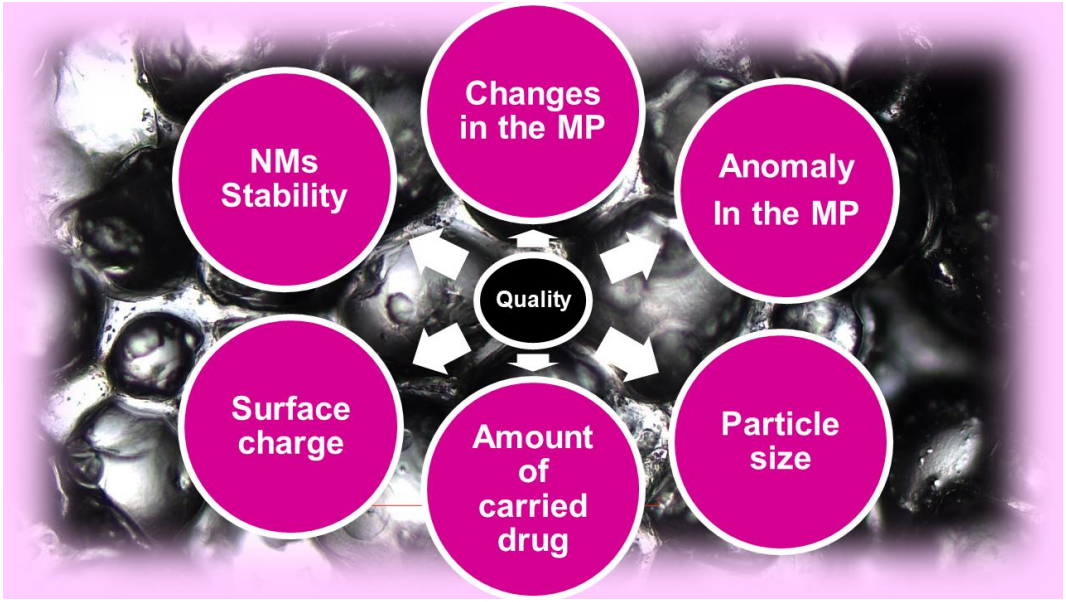

Figure 13. Main factors that could impact the quality of nanomaterials-based drug formulation within the context of efficacy and safety. NMs = nanomaterials; MP = manufacturing process.

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To reduce variations in product quality, OTR encourages the use of advanced manufacturing techniques. In this context, OTR has been collaborating with scientists at the University of Connecticut (grant numbers HHSF223201310117C, HHSF223201610121C and 1U01FD005773-01) to develop a platform for the continuous manufacturing of nanomaterials, which should allow better control over the manufacturing and quality of the process, and which should potentially lead to higher quality products. In nanomaterial formulations, excipients play a more significant role than in traditional ones, but their characterization inside complex matrices has been only recently applied and their critical attributes are not yet well understood. Regarding this, OTR collaborates with CDER's Office of Generic Drugs to determine if current characterization tools and standards for excipients are sufficient to support generic product development, or if different ones are needed. This research is funded in part by the Generic Drug User Fee Amendments (known as GDUFA II). Currently, very few nanomaterial-containing drug products have generic versions on the market. Additionally, to better evaluate the nanoproduct quality, safety and efficacy, OTR research also focuses on determining by in vivo and in vitro advanced analytical experiments, how the drug is released from the nano carriers, and on establishing the relationship between the in vivo and the in vitro measurements.

5.2. Providing Nanotechnology Guidance and Information

The Nanotechnology Risk Assessment Working Group (NRAWG) is an organization that works to assess the potential impact of nanotechnology on pharmaceuticals. It aims at developing standards for nanomaterials used in drug development and at facilitating the advancement of the nanotechnology. Promisingly, the working group established that in most cases the current evaluation practices are adequate to evaluate medicines that include nanomaterials. On the other hand, the CDER has worked over the past several years to understand which are the properties of nanomaterials when they are used in drug products, to inform and ensure the development of a regulatory framework that appropriately could assesses the impact of the unique physical properties of NPs on the safety and efficacy of nanomedicines. Recently, CDER issued a draft guidance for industry titled "Drug Products, including Biological Products, that Contain Nanomaterials" [174]. CDER projects involved research on the nanomaterial characterization and safety assessment in drug products, aimed at identifying the limitations of current test methods to assess the quality and safety of NPs-based therapeutics, and at evaluating the influence of nanotechnology application on the product characteristics, including stability and content uniformity. In this context, several peer-reviewed research articles which inform the scientific community on findings and advancement have been reported in literature [175-180].

5.2.1. Safety of Nanocarriers

From years, we are assisting to an exponential growth of nanotechnology. In nanomedicine, NPs are used as pharmaceutical drug carriers with applications in both diagnostics and therapy. These NPs, including polymeric NPs, nano-emulsions, liposomes and solid NPs, are suggested to have potential clinical applications [166]. However, their clinical applicability depends on different parameters such as their physical and chemical properties, drug loading efficiency, drug release and most importantly the low or no toxicity of the carrier itself [181]. Nanocarriers have unique properties very different from those of small drug molecules, such as nano-size, high surface to volume ratio, and are capable to infuse efficiently from the intestinal barrier to circulation. Currently, the toxic impact of NPs properties is not totally cleared [182]. The actual safety of nanomaterials-based drug formulations should not be underestimated, and more studies focusing on the risks associated to an extensive use of NPs and nanotechnology are necessary. In fact, despite our increased exposure to NPs, information regarding NPs' safety is limited and the research on safe NPs and/or on safety of NPs lags behind that on the possible application of NPs [183]. As represented in Figure 14, reporting the number of scientific papers published from the year 2000 so far (except the ongoing year 2023) reporting on NPs and those on safe NPs, it is evident that, while the research on NPs is enormous (787,017

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papers), that focused on the development of safe NPs as well as the studies concerning their toxicity are dramatically limited (10942 papers, 72-fold lower).

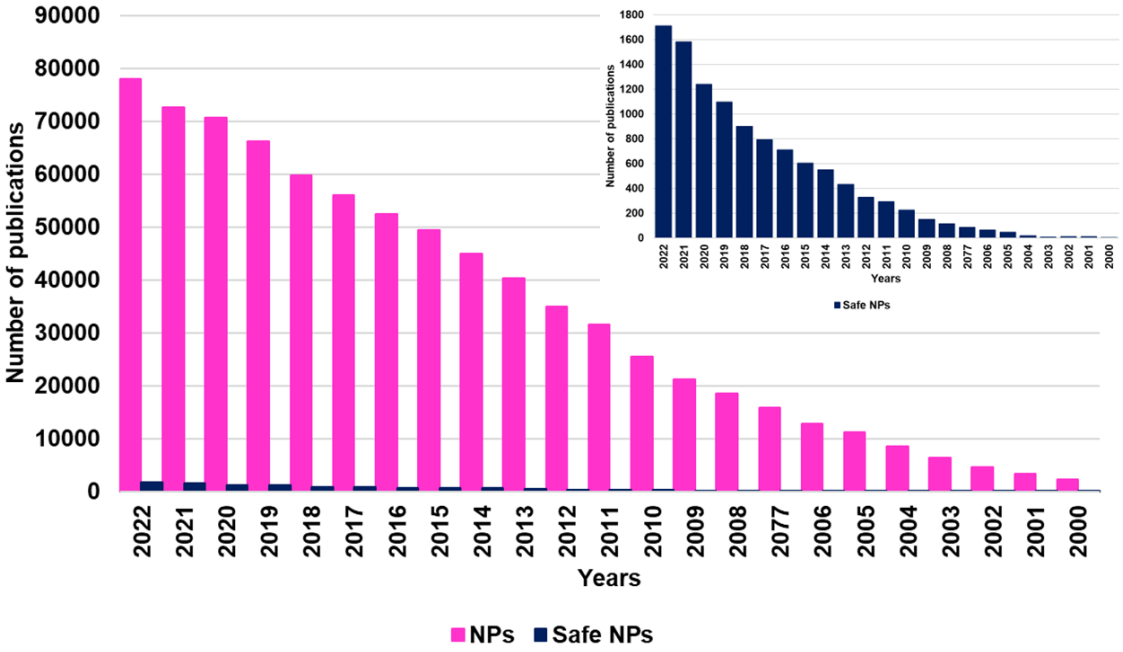

Figure 14. Papers were identified in Scopus database from the year 2000 until 2022 using the key words nanoparticles (fuchsia) and safe nanoparticles (blue).

Despite the unequivocal advantage of using NPs for clinical application, some studies have suggested that NPs can be toxic. NPs could display molecular toxicity, cell toxicity, tissue toxicity or immunological toxicity. Exposure to NPs may be through lungs, injection, ingestion or skin absorption, while the organs distribution includes liver, spleen and kidney. The brain is suggested as a potential target for NP distribution, however direct evidence is still lacking [181]. NPs may enter cell by endocytosis and exert toxic effects, causing mitochondrial defunction, OS, inflammation, and DNA damage, both in animals, and humans [184,185]. Size, surface modification, surface charge, composition, shape, and aggregation state of NPs are key factors in dictating NPs distribution in different organ systems, following their exposure, and in dictating for their possible toxicity [181]. Increasing proofs have demonstrated nanotoxicity by the induction of autophagy [186,187]. Additionally, the possible penetration of NPs into the skin, which could lead to damage to epidermal cells or their possible accumulation in secondary organs following biodistribution are also of great concern [188,189]. These studies have demonstrated the ability of NPs to accumulate in cells and to induce organ-specific toxicity, and due to the ever-increasing human exposure to NPs, the design of progressively safer nanomaterials and the development of strict guidelines for their development with regards to toxicity testing are urgent [181]. On the other hand, there are also growing reports on the safety evaluation of nanocarriers, and there is a scenario of rising findings establishing that certain bioactive compound loaded on nanocarriers are efficient and safe thus being usable in medicine. In this regard, it has been demonstrated that after ingestion of biodegradable polymers chitosan-sodium alginate-oleic acid-based NPs loaded with lutein (LNCs) with dose of 10 mg/kg body weight, no mortality and no morphological and clinical changes in rats were revealed [182]. Table 11 reports some examples of nano-formulations that demonstrated to be safe in in vivo or in vitro experiments.

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Table 11. Some examples of nano-formulations that demonstrated to be safe in in vivo or in vitro experiments.

Formulation	Cargo	Administration route	Animal model Disease	Tests	Results	Ref.
SLN	Irinotecan	Rectal	Mice Cancer	Gel properties Pharmacokinetics Morphology Anticancer activity Immunohistopathology	Easily administered to the anus Rapid and Strong gelling No damage to the rat rectum No body weight loss	[190]
Liposome	SP60015 (JNK inhibitor) Pitavastatin	Intravenous (iliac vein)	Male mice Aneurysm	Binding in vitro Binding in vivo Charging capacity Recharging capacity Drugs release	Good drug transport Targeted drug release Repeatable drug release Safe	[191]
[P(bAsp-co-APIA)-PEG]	Docetaxel	N.A.	N.A. Cancer RA	DL% EE% Drug release	Biodegradable Biocompatible ⇓ Toxicity pH sensitive	[192]
PS-NPs	*Dictyophora indusiata*	Gavage	Male mice IBD Colitis	Disease activity index Hystological analysis Myeloperoxidase activity Goblet cells	Effect against colitis Ameliorated intestinal injury ⇓ oxidative stress ⇓ pro-inflammatory cytokine ⇓ Inflammation ⇑ mucins	[193]

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				Mucous thickness Nitrogen oxide Cytokines Proteins	⇑ Tight junction proteins (TJs) Restored intestinal microbiome ⇓ Harmful bacterial flora ⇑ Beneficial bacterial flora	
[P(Asp-g-Im)-PEG]	Indole-3-acetic acid	Subcutaneous injection	Female nude mice Skin cancer	DL% EE% Morphology Drug release Cells viability Haemolysis Anticancer activity	⇓ Systemic toxicity (physiologic pH) ⇑ Antitumor efficacy ⇑ Accumulation in cancer cells ⇑ Release in cancer cells	[194]
mPEG-PCL micelles	Curcumin	Intravenous injection	Rats */Mice ** Breast cancer	DL% EE% Size Drug release Haemolysis In vivo organ toxicity In vivo anticancer effects	⇓ Systemic toxicity (physiologic pH) ⇑ Antitumor efficacy ⇑ Accumulation in cancer cells ⇑ Circulation time No mortality No organs toxicity No organs degeneration No necrosis No neutrophils No activation of immune response	[195]

SLNs = solid lipid NPs; JNK = c-Jun N-terminal kinase, a proinflammatory signaling molecule; [P(b-Asp-co-APIA)-PEG] = pH sensitive poly{(benzyl-L-aspartate)-co-[N-(3-aminopropyl) imidazole-L-aspartamide]}-poly(ethylene glycol); N.A. = not applicable; IBD = inflammatory bowel disease; RA = rheumatoid arthritis; N.A. = not applicable; PS-NPs = polysaccharide-based nanocarriers; [P(Asp-g-Im)-PEG] = Poly(aspartic acid-graft-imidazole)-poly(ethylene glycol); mPEG-PCL = mono methoxy poly (ethylene glycol)-poly (e-caprolactone) di-block copolymers; * mortality and in vivo toxicity (kidney, liver, heart, spleen); ** antitumor activity.

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However, while a vast array of nanocarrier is under development, many of which are undergoing advanced clinical trials, relatively few have achieved full translation to clinical practice. This slow uptake may be due, in part, to the need for a more rigorous demonstration of safety in these new nanotechnologies. Following, we have provided a Table (Table 12) in which the main studies on acute and chronic toxicity of the most used NPs have been included.

Table 12. Acute and chronic toxicity of NPs.

Acute toxicity			
NPs Type	**Animal/Cells**	**Toxicity**	**Ref.**
Fe_2O_3 NPs** ZnO NPs**	Human mesothelioma cells Rodents' mesothelioma cells	⇓ Overall cell culture activity ⇓⇓⇓ DNA content	[196]
CuO, TiO_2 ZnO $CuZnFe_2O_4$,	A549 cells	Cytotoxicity DNA damage ⇑ OS by ROS Oxidative lesions	[197]
Nano-C60 fullerene aggregate	Human dermal fibroblasts Human liver carcinoma cells (HepG2) Neuronal human astrocytes	⇓ Normal cellular function Lipid peroxidation ⇑ ROS Membrane damage	[198]
TiO_2 NPs	Brain microglia (BV2)	⇑ ROS Neurotoxicity	[199]
Chronic toxicity			
NPs Type	**Animal/Cells**	**Toxicity**	**Ref.**
CNTs	Mice *	Asbestos-like pathogenicity	[200] [201]
MWCNTs	Female mice	Breast cancer metastasis	[202]
Inorganic NPs	Rodents Non rodents	Genotoxicity Carcinogenesis Embryotoxicity	[203]
Al_2O_3 NPs ZnO NPs	Rats#	Hepato-renal toxicities ⇓ Hepatic expression of mtTFA and PGC-1α proteins	[204]
Au NPs	*Daphnia magna*	Mortality ⇓ Reproductive development ⇓ Reproductive fitness ⇓ Total eggs offspring Aborted eggs	[205]
ZnO NPs	*Mytilus galloprovincialis*	⇓ Transcription of key genes involved in DNA damage/repair, antioxidation and apoptosis	[206]
TiO_2 NPs	Rats	Inflammation Lung injury ⇓ Alveolar macrophage function	[207]

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ZnO NPs** $ZnCl_2$ NPs**	*P. subcapitata*	Cytotoxicity	[208]
ZnO NPs	RAW 264.7 cells BEAS-2B cells	Cytotoxicity OS	[209]

** Soluble NPs; CNTs = Carbon nanotubes; * Abdominal cavity; MWCNTs = Multi-walled CNTs; # oral administration.

As mentioned above, the toxic effects of NPs are dependent on their size, shape, their chemical composition, their extent of agglomeration, crystallinity, composition, and particle size distribution [181]. Also, like all drugs, the dissolution rate of NPs can affect their acute toxicity. Unlike polymeric NPs or lipid-based NPs, inorganic NPs such as metal oxide NPs including zinc, iron, and silver oxide NPs, are believed to dissolve after exposure, and it is supposed that the release of free ions associated with these inorganic NPs contributes to their toxicity. In this regard, studies have demonstrated that insoluble NPs of cerium oxide, titanium dioxide and zirconium dioxide exhibited no toxicity when used in human mesothelioma cells at concentrations up to 30 μg/mL [196]. In contrast, soluble NPs of iron oxide and zinc oxide were toxic at similar concentrations after three days of exposure [196]. This study is supported by several others works on chronic toxicity of metal NPs suggesting that the solubility of inorganic NPs is a key property in assessing their toxicity by ROS formation and inactivation of enzymes [208,209]. A study focused on the evaluation of toxicity of different metal oxide particles (CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_3O_4, Fe_2O_3) and that of carbon NPs and multiwalled carbon nanotubes (MWCNT), demonstrated that when tested on the human lung epithelial cell line A549, cytotoxicity, DNA damage and oxidative lesions were determined mainly for CuO NPs [197]. Importantly, acute toxicity assessment of NPs is not sufficient to evaluate their safety, because exposure to NPs is a continuous daily process, such as the exposure of workers in the manufacturing sector, or the exposure through daily applied cosmetics. Further, the dissolution or degradation of NPs may take a significant amount of time, possibly much longer than the elimination of the therapeutic they are carrying, while the products of degradation of NPs may themselves be toxic [181]. Finally, the biodistribution and accumulation of NPs may change over time [182]. Therefore, studies regarding the chronic exposure outcomes of NPs are necessary, which should include studies assessing the chronic use of NPs in humans both in clinical and industry settings, and their bioaccumulation in the environment. Chronic exposure conditions need to be studied differently from acute exposure, since they could involve several steps that cannot be simulated in a single-step acute toxicological exposure [181]. Chronic studies of NP toxicity should try to cover all the toxicology observations (hematology, analysis of organs and tissues, genetic analysis). Further, to enhance the rigor of the data, chronic studies should use proper advanced analysis and an efficient number of animals. In some cases, chronic studies should be performed over the lifetime of the animals (typically 2 years for rodents) [203]. In addition, in in vivo chronic toxicity studies, route of exposure, dose, frequency, duration of exposure, animal age and sex need to be considered, along with understanding the physicochemical properties of the NPs involved, including their composition, size, shape, charge, aggregation status and degradation [181].

Titanium Dioxide: The European Case

Related to the initial work of CDER, which focused on evaluating the role of zinc oxide and titanium dioxide (TiO_2) NPs in sun creams, studies were carried out to examine if TiO_2 NPs could penetrate normal skin [210]. The in vivo study using a pig model demonstrated that the TiO_2 NPs did not penetrate the dermis [210]. Anyway, additional investigations carried out in the subsequent years have shown that TiO_2 NPs can penetrate the protective barriers of the human body and accumulate in the liver, lungs, digestive system with carcinogenic and genotoxic effects [211].

As a consequence, the use of TiO_2 as a food coloring in food, food supplements, and feed was banned starting from 2020 in France and subsequently in all Europe starting from mid-2022, on a proposal from the European Commission approved on 8 October 2021 by the European Food Safety

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Authority (EFSA) committee [212]. Anyway, concerning far pharmaceutic products, TiO_2 is still frequently used in solid oral pharmaceutical forms, such as tablets (coating), soft and hard capsules, pastes, gels for oromucosal and sublingual use, as well as for semi-solid cutaneous and vaginal formulations [213].

On request of the EU Commission, the European Medicine Agency (EMA) recently evaluated the possible impact of the removal of TiO_2 from the list of the authorized food additives on medicinal products. The final feedback from EMA established that no single material has been identified that could provide the same combination properties that are unique to TiO_2 (opacity, enhancing contrast, protection from UV light and finish/smoothness of the resulting product). Possible alternatives could be calcium carbonate, talc, and starch, but disadvantages have been identified with these alternatives [213].

Collectively, the rapid feasibility of replacing TiO_2 was not confirmed, mainly because each medicinal product containing TiO_2 need an individual review and assessment, which will require investigation of alternatives, product reformulation, generation of new data related to manufacture, dissolution and stability etc. and potential new clinical data, which subsequently will have to be assessed by the national authorities and EMA [213].

The direct and indirect impacts on medicines for human and veterinary use are expected to be aggravated in a scenario where only Europe would be the only region globally to ban TiO_2 as excipient in medicines. The pharmaceutical industry should develop new drug formulations potentially for Europe only. Additionally, considering the scale of the use of TiO_2, the time and costs involved in the reformulation, and the volume of products impacted, the replacement of TiO_2 will almost certainly cause significant medicines shortages and discontinuations of medicines from the EU/European Economic Area (EEA) market with major implications for patients and animals. Collectively, it will not be possible to carry out the work for all products simultaneously and prioritization of products reformulation will be necessary. Taking the times estimated by the pharmaceutical industries, the Quality Working Party (QWP) committee concluded that a transition period of 10 years or even longer would be required for the phasing out of TiO_2 in medicines [213].

5.2.2. Strategies to Reduce the Toxicity of Nanoparticles

It has been reported that NSs, NEs and SLNPs are the fewer toxic ones being formulated using food grade ingredients that have been generally recognized as safe (GRAS) by the FDA, such as lipids, proteins, polysaccharides and surfactants [214]. Anyway, efforts are being made to limit the toxicity of nanomaterial. Table 13 collects the main strategies that have been developed to prepare safer NPs.

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Table 13. The main strategies developed to prepare safer NPs.

Strategy	Features	Type of NPs	Results	Ref
Use of next-generation lipids*	⇑ High potency Biodegradability	SLNPs	Rapid elimination from plasma ⇑ Tolerability in preclinical studies ⇑ In vivo potency	[215]
Surface coating strategies**	Biocompatibility ⇑ Colloidal stability ⇓ Degradation Faster excretion ⇓ Accumulation Reversible coating Altered dispersion state	Polymeric SLNPs Inorganic NPs.	⇑ Dispersion state ⇓ Agglomeration ⇓ Cellular up-take ⇓ Pro-fibrogenic effects	[216]
			⇓ Lung toxicity	[217]
		AuC/PF127 NCs	⇑ Stability ⇑ Biocompatibility Photodynamic therapeutic	[218]
Doping	Altered density of surface reactive chemicals ⇓ Binding energy of metal ions to oxygen ⇓ NPs dissolution ⇓ Toxic ions release ⇓ ROS generation	Inorganic NPs.	⇓ Dissolution ⇓ Toxicity	[219]
			⇓ Dissolution ⇓ Toxicity	[220]
Surface chemistry properties modifications	Altered charge density Altered hydrophobicity	CNTs	⇓ Pro-fibrogenic effects ⇓ Uptake in THP-1 and BEAS-2B cells	[221]
		AuNPs	⇓ Toxicity ⇓ Uptake in cells	[222]
		Fe_2O_3 NPs	⇑ Stability ⇓ Toxicity ⇑ Biocompatibility	[223-226]

* The surface of NPs can be covered with various substances such as polymers in single- or multi-layers that can be either complete or incomplete; ⇓ reduced, decreased; ⇑ increased, higher, improved, high; AuC/PF127 NCs = carbynoid-encapsulated gold nanocomposites (NCs) functionalized with pluronic-F127 (PF127).

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The most used materials for coating NPs surface include polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), poly(N–isopropylacrylamide) (PNIPAM), zwitterionic polymers such as poly(carboxybetaine) (PCB), poly(sulfo-betaine) (PSB), phosphorylcholine-based copolymers and polysaccharides such as dextran and chitosan [227-231]. In this context, single-walled carbon nanotubes (SWCNTs) and MWCNTs may induce inflammation, fibrosis and promote cancer progression, due to their surface chemistry, length, and aggregation state [232,233]. By the surface coating strategy, using a nonionic triblock copolymer (PF108), Wang et al. improved their dispersion state, reduced their agglomeration, cellular uptake and pro-fibrogenic effects [216]. Particularly, experiments in vitro on bronchial epithelial BEAS-2B cells and phagocytic THP-1 cells and in vivo using mice lungs, demonstrated a decreased toxicity due to a decrease in pro-inflammatory cytokine (IL-1), to a low deposition in the lung, and to a high protection against pulmonary fibrosis. Also, Mutlu et al. demonstrated that CNTs coated with PF108 protected against lung toxicity and were cleared from the lungs after 90 days compared to non-coated CNTs, which aggregated and induced granulomatous lung inflammation and fibrosis [217]. More recently, Rosso et al., by coating previously synthetized ligand-free carbynoid-encapsulated gold nanocomposites (Au@Carbynoid NCs) with pluronic-F127 copolymer (PF127) achieved a fully biocompatible colloidal solution of Au@Carbynoid/Copolymer nanocomposite (NC), as confirmed by cytotoxicity studies on human skin fibroblasts [218]. Due to the stability of the colloidal dispersions of Au@Carbynoid NCs functionalized with PF127 and their biocompatibility, the green carbynoid based NCs are promising as drug-carrier in biological applications. Doping technique, specifically used for reducing toxicity of inorganic NPs, alters the crystal structure of materials through the addition of impurities to improve chemical and physical properties [234-236]. Possible dopants include aluminum, titanium and iron, while FSP is a well-established technique used in NP doping which uses a rapid combustion method. ZnO NPs have wide applications in cosmetics, such as sun creams and electronics, but ZnO-induced pulmonary inflammations have been reported in humans. George et al. synthesized Fe-doped ZnO NPs by FSP and assessed their cytotoxicity in vitro using RAW 264.7 and BEAS-2B mammalian cells. Results demonstrated a decreased ZnO dissolution, which correlated to a reduced in vitro cytotoxicity [237]. Subsequently, also in vivo studies showed the reduced toxicity of Fe-doped ZnO NPs in zebrafish embryos and rodent lungs [238]. Among surface modification to reduce NPs toxicity, alteration of charge density and hydrophobicity have been reported to improve the efficacy of some NPs in biomedical applications and their targeted drug delivery ability [181]. Alteration of surface chemistry properties of NPs can be achieved by covalent binding of functional groups such as anionic, nonionic and cationic groups onto their surface. In this context, Li et al. synthesized and assessed the toxicity of CNTs functionalized with anionic, nonionic and cationic surface groups in vitro and in vivo. CNTs with the anionic groups (carboxylate and polyethylene glycol) displayed the lowest pro-fibrogenic effects and uptake in THP-1 and BEAS-2B cells [181]. Surfaces of iron oxide NPs, whose toxicity is attributed to the release of hydroxyl radicals were modified by functionalization with organic compounds such as aldehyde, carboxyl and amino groups, thus resulting in their stabilization, in a decreased toxicity, and in an increased biological compatibility [223-226].

6. Phytochemicals-Loaded Nanomedicines: Where Are We and Where Are We Going?

The following Table 14 and Table 15 give us the current scenario of the actual clinical application of PHYs (Table 14) and of nanotechnological PHYs (Table 15) developed so far.

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Table 14. Clinical status and biological activity of PHYs currently applied on humans.

Compound	Source	Activity	Status*
Artemisinin	Artemisia annua	Anticancer	Phase 3
Ursolic acid	Fruits (waxes of apples, pears)	Antioxidant	Phase 2
Thymoquinone	Herbs Spices	Hepatoprotective Antioxidant Anticancer	Phase 2
Sulforaphane	Brassica vegetables	Anticancer Antioxidant Antimicrobial Anti-inflammatory	Phase 2
PEITC	Watercress	Anticancer (lung, oral)	Phase 2
Not specified ITC	Cruciferous vegetables	Bladder cancer	Phase 1
Sinomenine	Roots of *Sinomenium acutum*	Anti-inflammatory Anti-rheumatic	Phase 3
Silibinin	Milk thistle Coffee	Hepatoprotective Anticancer	Phase 4
Catechin	Green tea Beans	Antioxidant	Phase 4
Salvianolic acid B	Red sage	Antioxidant Angiogenetic	Phase 2
RES	Grapes Blueberries Raspberries Mulberries	Antioxidant Anti-inflammatory Cardioprotective Anti-carcinogenic	Phase 4
Quercetin	Fruits Red onions Kale	Anti-inflammatory Anticancer	Phase 3
Paclitaxel	Bark of Pacific yew tree	Mitotic inhibitor in cancer Chemotherapy	Approved (Taxol®) FDA (1998)
Genistein	Plants (lupins, fava beans, soybeans)	Anticancer Anti-inflammatory	Phase 3
Lycopene	Tomato	Antioxidant Anticancer	Phase 4
EGCG	Green tea White tea, Black tea	Antioxidant, Chemo-preventive	Phase 3
Epicatechin	Woody plants	Antioxidant	Phase 2
Caffeic acid	Coffee Eucalyptus	Anticancer Antioxidant Anti-inflammatory	Phase 3
Camptothecin	Stem wood of the Chinese tree	Anticancer	Phase 1

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	Camptotheca acuminate		
Combretastatin	Bark of *Combretum caffrum*	Anticancer	Phase 2
Curcumin	Tumeric	Inhibition of tumor cell proliferation Anti-inflammatory	Phase 4

* Clinical status was obtained by http://clinicaltrials.gov (top clinical status in drugs of intervention category). Accessed on 06 April 2023.

Table 15. PHYs-based nano-formulations currently applied on humans.

Compound	Formulation type	Indication	Status*
Camptothecin	PEG–drug conjugate	Gastric cancer	Phase 2
	Polyglutamic acid–drug conjugate	Colon cancer Ovarian cancer	Phase1/2
	Cyclodextrin NP	Solid tumors, renal cell carcinoma, rectal cancer, non-small-cell lung cancer	Phase1/2
	HPMA–drug conjugate	Solid tumors	Phase 1
	Fleximer–drug conjugate	Gastric cancer Lung cancer	Phase 1
Curcumin	Liposome	Advanced cancer	Phase 1
Irinotecan	Liposome	Metastatic pancreatic cancer	Onivyde® Approved: FDA (2015)
Paclitaxel	NPs albumin-bound	Breast cancer Non-small cell lung cancer Pancreatic cancer	Abraxane® Approved: FDA (2005, 2012, 2013)
	Polymeric micelle	Ovarian cancer	Phase 1
	Polymeric NPs	Peritoneal neoplasms	Phase 1
	Liposome	Ovarian cancer Breast cancer Lung cancer	Phase 1/2
	Liposome	Triple-negative breast cancer	Phase 2
	Liposome	Solid tumors Gastric cancer Metastatic breast cancer	Phase 2

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	PEG-PAA polymeric micelle	Gastric cancer Breast cancer	Phase 2/3
	DHA–drug conjugate	Melanoma Liver cancer Adenocarcinoma Kidney cancer Non-small-cell lung cancer	Phase 2/3
	Polyglutamic acid–drug conjugate	Lung cancer Ovarian cancer	Phase 3
	Polymeric micelle	Advanced breast cancer	Phase 3
	Micelle	Ovarian cancer Primary peritoneal cancer	Apealea® Approved: EMA (2018)
	PEG-PLA polymeric micelle	Breast cancer Lung cancer	Genexol-PM® Approved: marketed in Korea and Europe (2007)
	HPMA–drug conjugate	Solid tumor	Phase 1
Vincristine	Liposome	Acute lymphoid leukemia	Marqibo® Approved: FDA (2012)

* Clinical status was obtained by http://clinicaltrials.gov (top clinical status in drugs of intervention category). Accessed on 06 April 2023.

In total (not formulated PHYs (Table 14) and nano-formulated ones (Table 15) the types of PHYs clinically applied are 23, but only 5 (21.7%) are currently administered to humans in form of NPs (camptothecin, curcumin, irinotecan, paclitaxel and vincristine) (Figure 15).

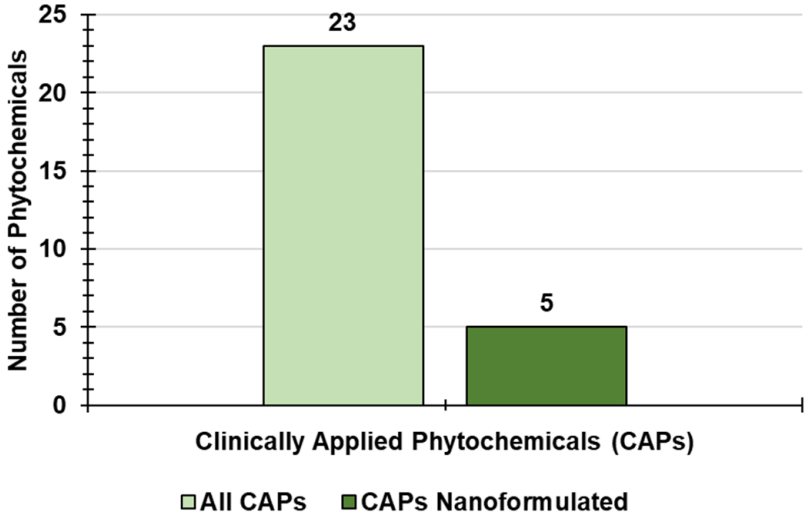

Figure 15. PHYs clinically applied.

All of these five are finalized to counteract tumors, and while vincristine and irinotecan are administered only as NPs (Table 15), the others are dispensed also as not nanotechnological engineered drugs (Table 14 and Table 15). While for vincristine, curcumin and irinotecan only one nano-formulation exists and irinotecan-based NPs and vincristine-based NPs are already approved and

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marketed, for camptothecin and paclitaxel 5 and 13 different nano-formulations exist respectively, and 3 out 13 (23.0%) paclitaxel-based nano-formulations are already marketed (Table 15, Figure 16), while those camptothecin-based are yet in clinical trial.

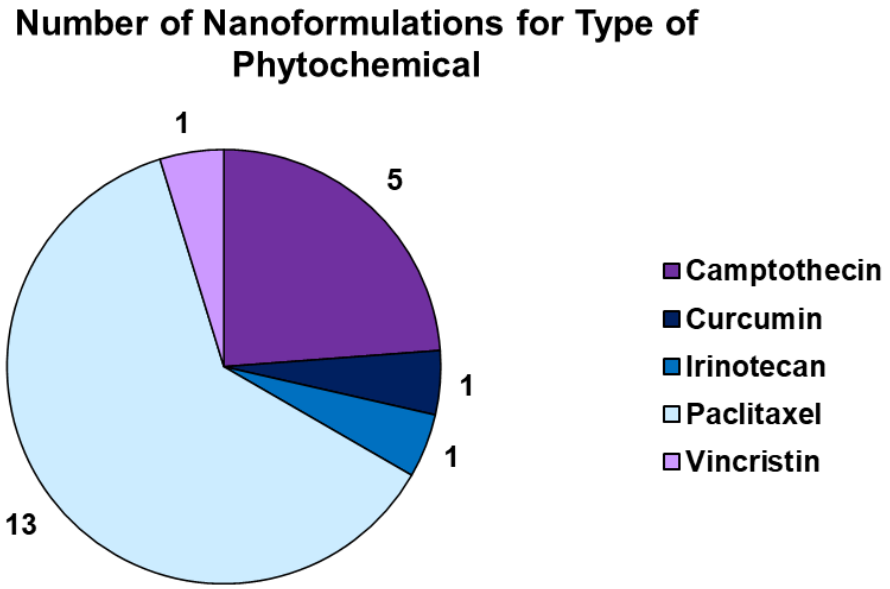

Figure 16. Number of nano-formulations existing for type of PHY clinically applied.

Curiously, among not nano-formulated PHYs only paclitaxel is currently clinically approved (Table 14). Figure 17 shows the clinical status of not formulated PHYs (Figure 17a) and of those nano-formulated (Figure 17b).

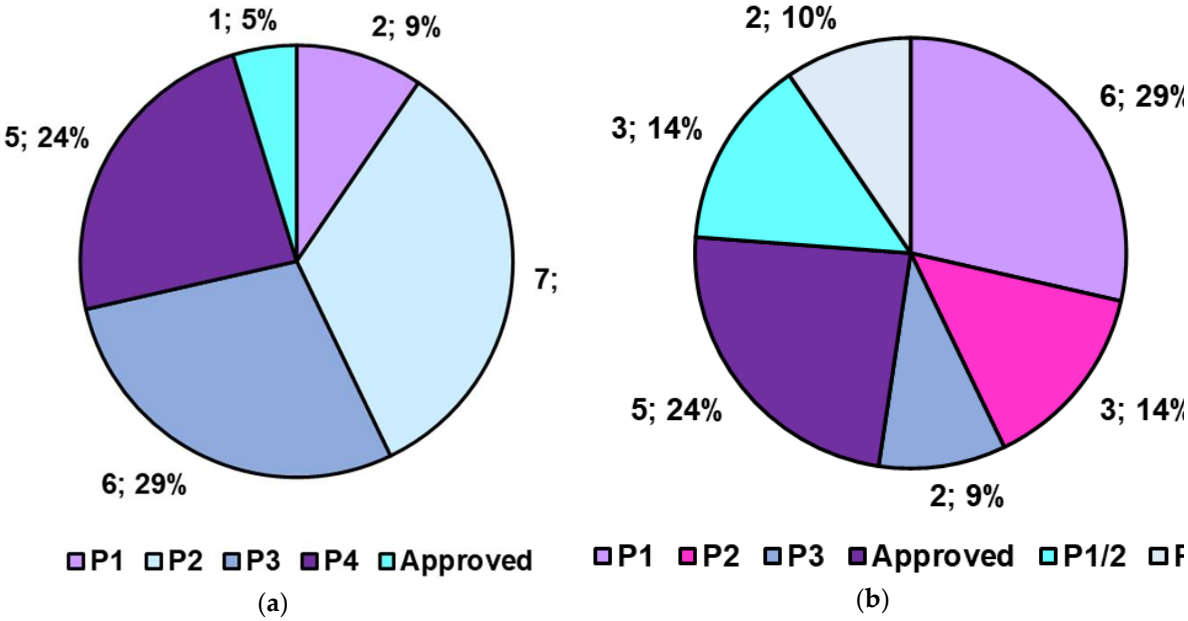

Figure 17. Clinical status of not formulated PHYs (**a**) and of PHY-based nano-formulations (**b**).

Collectively, while only one out of 21 (4.8%) not formulated PHYs is currently approved, the PHY based NPs clinically approved are 5 out of 21 (23.8%).

Phase 1 and phase 2 clinical trials are currently ongoing, to assess the feasibility of combination therapies using associations of curcumin with 5-fluoro uracile (Phase 1) and taxotene (Phase 2) to counteract multi drug resistant metastatic colon cancer and resistant metastatic prostate cancer [239].

7. Conclusions

With this review we have offered to the scientific community a complete scenario concerning the most relevant PHYs, their sources and their pharmacological properties. By reporting several case studies, the main NS and NE techniques, in the past and recently developed to nano-formulate different types of PHYs have been reviewed. Originally, the most information provided in the present paper has been organized in reader-friendly tables, has been graphically presented and also statistically analyzed.We have realized that, the interest in the use of nanotechnology to solve the solubility and bioavailability issues of pharmacologically active molecules (phytocompounds in the present case), is growing so rapidly, that the review of only two of the least exploited nanotechnological approaches among those which have been reported so far, that is NSs and NEs, has led to a very notable and important work. We have decided to deal with these nanoformulation techniques, precisely because, in our opinion, they are underestimated despite their great potential and the limited toxicity, due to the use of GRAS ingredients compared, for example, to the use of inorganic and organic NPs. Anyway, based on the number of case studies found in the literature, we conclude that, between the NSs and NEs techniques, those based on NEs may be the most advantageous, as they are capable of obtaining nanoformulations made of smaller particles than NSs, thus providing nanomaterials with increased solubility, bioavailability and pharmacological efficacy, as well as reduced toxicity. We also think that among NEs techniques reported here, those preparing SNEDDSs (including SMEDDSs, SNEDDSs and SDEDDSs) are remarkably ingenious as they leads to the obtainment of solid nanoformulations, therefore more stable, than only once ingested by exploiting biological fluids and peristalting movements of stomach provides nanoemulsions readily and easily absorbable, avoiding phenomena of early degradation by enzymatic and microbial digestion, occurring in the mouth. We are confident that the present review will inspire the interest of a large audience of experts in the sector of plants-derived products and nanomaterials and will encourage an increasing research work to improve the current results, and to solve the pending issue regarding the possible toxic effect of NPs, thus increasing the number of PHYs-based nanoformulations in clinical trials and then clinically approved.

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