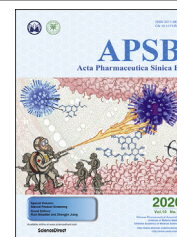




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REVIEW

Nature-derived compounds modulating Wnt/ β -catenin pathway: a preventive and therapeutic opportunity in neoplastic diseases



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Abstract The Wnt/ β -catenin signaling is a conserved pathway that has a crucial role in embryonic and adult life. Dysregulation of the Wnt/ β -catenin pathway has been associated with diseases including cancer, and components of the signaling have been proposed as innovative therapeutic targets, mainly for cancer therapy. The attention of the worldwide researchers paid to this issue is increasing, also in view of the therapeutic potential of these agents in diseases, such as Parkinson's disease (PD), for which no cure is existing today. Much evidence indicates that abnormal Wnt/ β -catenin signaling is involved in tumor immunology and the targeting of Wnt/ β -catenin pathway has been also proposed as an attractive strategy to potentiate cancer immunotherapy. During the last decade, several products, including naturally occurring dietary agents as well as a wide variety of products from plant sources, including curcumin, quercetin, berberin, and ginsenosides, have been identified as potent modulators of the Wnt/ β -catenin signaling and have gained interest as promising candidates for the development of chemopreventive or therapeutic drugs for cancer. In this review we make an overview of the nature-derived compounds reported to have antitumor activity by modulating the Wnt/ β -catenin signaling, also focusing on extraction methods, chemical features, and bio-activity assays used for the screening of these compounds.

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1. Introduction

The Wnt/ β -catenin signaling cascade is an evolutionarily conserved pathway. It has a crucial role in normal embryonic development, by orchestrating a wide range of process including limb, heart, or neural development, axis specification and gastrulation^{1–5}. Moreover, Wnt pathway is one of the main players in the maintenance of adult tissue homeostasis by regulating cell proliferation, migration, differentiation, survival and adhesion, as well as renewal of stem cells^{6–10}. Due to its pleiotropic and essential functions in controlling a great number of process during embryonic and adult life, dysregulation of the Wnt/ β -catenin signaling is associated with many types of diseases, including cancer and neurodegenerative disorders^{5,11–14}, fibrosis^{15,16}, endocrine diseases¹⁷, and metabolic syndrome¹⁸. In view of this crucial role in the pathogenesis of such different kinds of diseases, in the last two decades, most of molecular components of the signaling have been proposed as innovative therapeutic targets^{5,12,14,19–21}. Crucial molecules participating to the signaling seem also to possess a diagnostic/prognostic value in neoplastic diseases^{22–24}, and this further increases the interest of the scientific world on this pathway. Not by chance, many research groups worldwide are engaged in expanding the knowledge on this pathway and its role in the onset and progression of various diseases. Moreover, several pharmaceutical and biotech companies invested, and are currently investing, considerable funds for developing innovative drugs targeting critical steps of this signaling, or for confirming the diagnostic value of molecules participating to the Wnt/ β -catenin cascade. In the last decade, a great number of Wnt pathway targeting compounds, including small molecules and biologics, have been tested as novel therapeutic agents in both preclinical and clinical studies. Most of the studies analyzed the efficacy of these compounds in anticancer therapy^{14,25}, since cancer has been the first disease in which a role of Wnt signaling has been demonstrated²⁶ and, therefore, the knowledge in this field is greater than for other pathological conditions.

Herbal preparations have been used since ancient times as the main source of therapeutic principles for world populations. In the history of medicine, there are many remarkable examples of how the discovery of natural products deeply affected advances in biology and stimulated drug discovery and therapy. Nevertheless, the interest of pharmaceutical companies toward natural compounds, as potential candidates in the drug discovery process, showed a decline during the 1990s and early 2000s, due to the advent of high-throughput screening (HTS) and combinatorial chemistry²⁷. In the last years, with advances of technologies that allow to screen natural products in HTS assays, the interest in plant-derived drugs has progressively increased and a “New Golden Age” for the drug discovery of nature-derived products is emerging^{28,29}. The discovery of nature-derived compounds with strong anti-cancer activity contained in many foods leads also to design chemotherapy regimen combining these compounds with conventional chemotherapeutic agents. During the last decade, several products, including naturally occurring dietary agents as well as a wide variety of products from plant sources, have been identified as potent modulators of the Wnt/ β -catenin signaling and have gained interest among the researchers as promising candidates for the development of chemopreventive or therapeutic drugs for cancer^{30–33}.

In this review, we make an overview of the nature-derived compounds that are reported to have antitumor activity by modulating the Wnt/ β -catenin signaling, also focusing on extraction methods, chemical features, and bio-activity assays

used for the screening of these compounds. In addition, we briefly describe some of the preclinical studies that demonstrated, in *in vitro* and *in vivo* models of cancer, the effect of these nature-derived compounds on the signaling and some clinical trials just completed or that are ongoing, aiming to demonstrate the anti-tumor efficacy of natural agents targeting Wnt signaling components.

2. The Wnt/ β -catenin signaling cascade: the current regulatory model

Wnts are secreted, cysteine-rich glycoproteins that act as ligands to promote receptor-mediated signal transduction pathways in both vertebrates and invertebrates^{2,5,12,34–38}. During synthesis, Wnt proteins are modified by the attachment of an acyl group (palmitoleic acid) and this modification, brought about by the palmitoyl transferase Porcupine (membrane-bound *O*-acyl-transferase family, MBOAT in Fig. 1), is crucial for Wnt secretion and for the binding to the Wnt receptor Frizzled (Fzd)¹² (Fig. 1). Wnt signals are transduced in the canonical, or β -catenin-dependent, pathway and in other two non-canonical, or β -catenin-independent, pathways (Wnt/ Ca^{2+} and the planar cell polarity signaling)^{35–37}. The receptor Fzd is crucial for all Wnt signaling cascades, with the N-terminal Fzd cysteine rich domain (CRD) that acts as the Wnt binding domain³⁵. In addition to the Fzd, the Wnt/ β -catenin pathway needs the low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6) as co-receptors³⁹. The formation of a Wnt–Fzd–LRP6 complex is the trigger for the Wnt/ β -catenin signaling cascade.

β -Catenin was originally recognized as a cadherin-associated protein participating to the cell–cell junctions^{40,41}, but in the last two decades has got increasing interest as one of the most important mediators of the canonical Wnt pathway^{5,6,42}, mainly due to the role of this signaling in tumorigenesis. A huge number of papers on this issue are available in literature, and here we summarize the key aspects of the Wnt/ β -catenin signaling, and refer to other reviews and papers for more details on the molecular mechanism^{6,12,14,25,43–45}. As illustrated in Fig. 1, following the current model, in the “Wnt-OFF” state, β -catenin exists in a cadherin-bound form that regulates cell–cell junctions; the excess of β -catenin that is not segregated on the cell membrane by cadherins, is rapidly phosphorylated by glycogen synthetase kinase-3 β (GSK-3 β) in the adenomatous polyposis coli (APC)/axin/GSK-3 β destruction complex and is then degraded by the ubiquitin–proteasome pathway (Fig. 1). Conversely, in the “Wnt-ON” state, the binding of Wnt to the Fzd receptors and to the LRP5/LRP6 co-receptors inactivates the APC/axin/GSK-3 β destruction complex and the results in β -catenin accumulation in the cytosol and its translocation into the nucleus. Nuclear β -catenin functions as a co-activator for TCF/LEF-mediated transcription and regulates the expression of Wnt target genes that regulates cell proliferation and survival, apoptosis, cell differentiation, cell motility and invasion, and resistance to chemotherapy. Indeed, in non-pathological conditions, β -catenin dynamically has multiple subcellular localizations, that include adherens junctions, where it participates to cell–cell contacts, the cytoplasm, where its levels are tightly controlled by the degradation at the destruction complex, and the nucleus, where it is engaged in transcriptional regulation and chromatin interactions⁴⁶. This β -catenin dynamics is finely regulated by different factors, including growth factors, prostaglandins, and E-cadherin levels, that play in concert

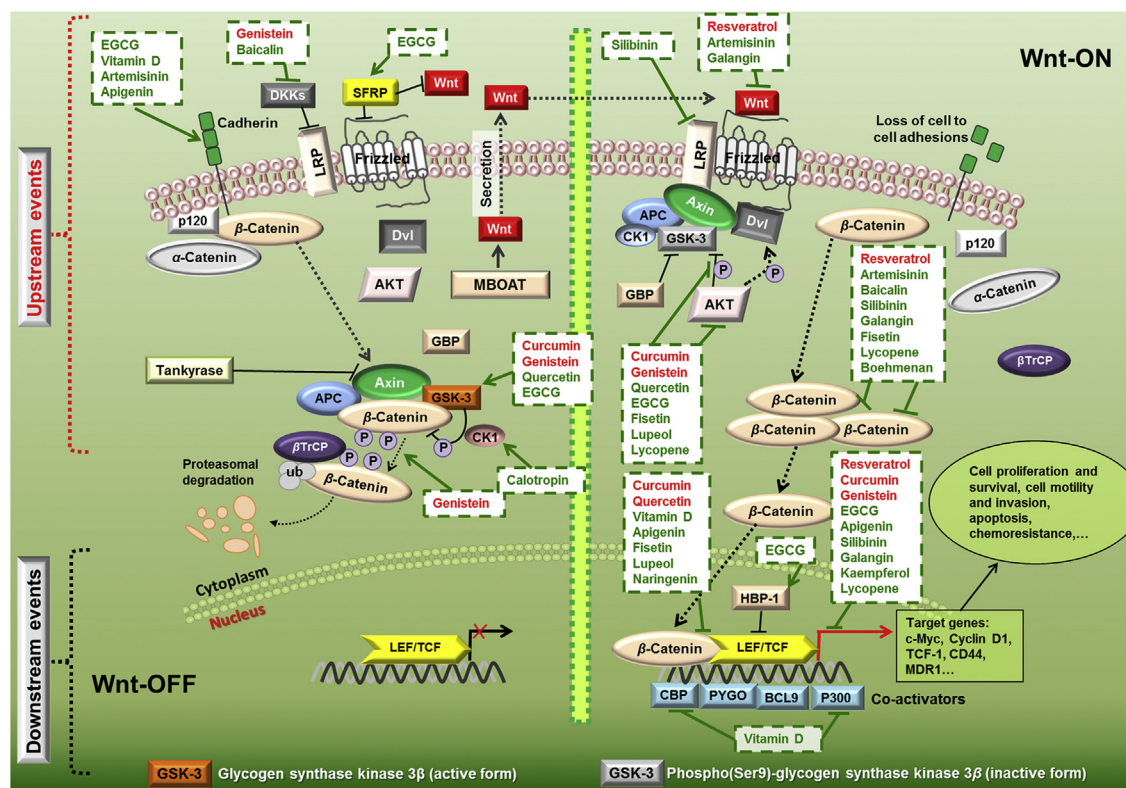


Figure 1 Schematic representation of the current regulatory model of Wnt/ β -catenin signaling, and the Wnt-targeting natural compounds which are currently under investigation. AKT, serine/threonine-protein kinase; APC, adenomatous polyposis coli; BCL9, B cell lymphoma 9 co-activator; β -TrCP, E3 ubiquitin ligase; CBP, CREB-binding protein; CK, casein kinase; Dvl, dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LEF, lymphoid enhancer factor; LRP, LDL receptor-related protein; MBOAT, membrane-bound *O*-acyltransferase; P, phosphorylation; p300, E1A associated protein; PYGO, Pygopus co-activator; sFRP, secreted Frizzled-related protein; TCF, T-cell factor; ub, ubiquitination. The compounds that are currently explored in clinical studies are marked in red colour.

to maintain a fine balancing between Wnt-OFF and Wnt-ON states. Due to the critical role of Wnt/ β -catenin signaling in regulating opposite cell fates (survival vs. cell death, differentiation vs. proliferation, etc.), the preservation of this balance in the different tissues and organs is essential, since aberrant regulation of the signaling cascade and/or the inability of the organism of restoring the right equilibrium might lead to the onset and progression of a wide range of diseases.

3. The Wnt/ β -catenin pathway and cancer: therapeutic perspectives

In the two last decades, numerous studies demonstrated the key role of deregulation or constitutive activation of the Wnt/ β -catenin pathway in tumor initiation, growth, metastasis and dormancy^{6,12,19,21,47}. This signaling is also involved in tumor immunity and cancer stem cell maintenance in different forms of human cancer, mainly solid tumors, including colorectal, gastric, esophageal, prostate, breast, thyroid, lung, ovarian cancer, and hepatocellular carcinoma^{6,12,19,21,47–50}. Therefore, many of the molecular components of the Wnt/ β -catenin signaling have been proposed as innovative targets for cancer therapy^{5,14,19–21,25,46,50}. Furthermore, since the Wnt/ β -catenin signaling is also involved in some chronic diseases associated with an increased risk of developing cancer, such as the inflammatory bowel diseases (Chron's disease and ulcerative rectocolitis), and liver fibrosis,

drug targeting this signaling has also been proposed for cancer prevention^{22,51–53}. The main therapeutic approaches that are currently explored (see for review^{14,25}) involve the use of drugs targeted to molecules that participate to upstream events, such as Wnt ligands, Fzd receptors, LRP5/LRP6 co-receptors, Dishevelled and others, or drugs targeting downstream events such as β -catenin/TCF or β -catenin/CBP interactions.

Several modulators of the signaling have been developed for treatment of cancer and other β -catenin-related diseases, but, up today, none of them has been yet approved or incorporated into clinical practice. The main issue for Wnt antagonists, apart the common safety aspects that could halt the development of new drugs, is the target strategies. Some drugs developed to date display a mechanism of action that involves the targeting of upstream molecules of the pathway such as tankyrase or porcupine. However, these experimental drugs failed to demonstrate a clinical efficacy in neoplastic diseases in which mutations in APC and β -catenin genes have been found, such as colorectal cancer. Even if the direct targeting of β -catenin appears the most relevant pharmacological strategy for overcoming these mutations, β -catenin seems to be an undruggable target, due the absence of ligand binding pockets usually present in enzymes and receptors⁵⁴. These critical issues could be resolved based on the new scientific evidence about the crucial role of this pathway in the immunological response, since the Wnt/ β -catenin signaling in immune cells is activated by extrinsic factors rather than intrinsic mutations⁵⁴. This emerging role of the pathway is

particularly noteworthy if seen in the context of the new golden age of immunotherapy in oncology⁵⁵. The advent of immune checkpoint inhibitors (ICI) is changing the clinical paradigm and the life expectancy, with significant results for progression-free survival and overall survival for patients affected by several cancer diseases such as melanoma and non-small cell lung cancer. Despite the clinical success of these therapies, only certain types of tumors respond to ICI and others such as breast, prostate, and colon are less sensitive^{54,55}. Therefore, there is a scientific and regulatory consensus that suggests the development of new research programs aiming to the optimization of the use of existing ICI in order to improve the clinical management of patients treated with these innovative medicines. Thus, the recent evidence, indicating that Wnt/ β -catenin signaling plays an important role in the regulation of different tumors and their immune sensitivity, opens up new perspective in this field. Specifically, the dysregulation of this pathway seems to be deeply related to the biological function of several immune cells involved in antitumor immunity, immune evasion and exclusion mechanisms that are known to have a central role in non-responders or resistant patients treated with ICI⁵⁴. These findings suggest that the efficacy of cancer immunotherapy by ICI treatment could be significantly improved through a combined strategy with molecules targeting Wnt/ β -catenin pathway. This assumption is sustained by data from *in vitro* and *in vivo* experiments^{56–60}, and from clinical studies that explored the efficacy of combined treatment with Wnt/ β -catenin modulators and checkpoint inhibitors have been recently started^{61,62}.

In this context, natural compounds could represent an alternative and important source useful for increase the availability of molecules with different pharmacological properties able to potentially overcome some critical issues that characterize synthetic and biological molecules that have been developed to date. In particular, the large number of natural compounds known for acting on the Wnt/ β -catenin pathway could be precious not only for developing more efficient and less toxic preventive/therapeutic drugs but also for potentiating the efficacy of the ICI treatment, in a combined therapy. To this purpose, a phase II clinical trial (trial number: NCT03192059) is recruiting patients with advanced and/refractory cervical cancer, endometrial carcinoma or uterine sarcoma in order to study the combination of vitamin D (as co-drug) and curcumin (as supplement) with an immunomodulatory cocktail that also includes the anti PD-L1 pembrolizumab (<https://clinicaltrials.gov/ct2/show/NCT03192059>).

4. Natural molecules targeting Wnt/ β -catenin pathway

The key role Wnt/ β -catenin signaling in the mechanism of cancer development and progression has led to a significant increase of researches aimed to discover new molecule able to modulate several pharmacological target of the pathway. In this context, a large variety of natural compounds has been demonstrated to inhibit or modulate molecules involved in upstream and downstream events of the Wnt/ β -catenin pathway⁶³. The majority of these molecules are polyphenols, mainly flavonoids, but some terpenes and terpenoids are also included (Fig. 2). Almost all of these nature-derived compounds share the feature of having antioxidant and anti-inflammatory properties, and this could in part explain their effects on the Wnt/ β -catenin signaling that is often dysregulated in condition of oxidative stress or

inflammation. Conversely, up today, it has not been available to find common specificities in the chemical structures of these compounds that could be directly related to the effects on the pathway or whose direct interaction with specific targets participating to the Wnt/ β -catenin signaling could be supposed. As reported in Table 1^{3,25,30–33,46,63–98, 99–105}, and illustrated in Fig. 1, all the mentioned compounds have multiple targets involved in both upstream and downstream events of the signaling. Indeed, all these nature-derived molecules are “modulators” of the pathway rather than specific activators or inhibitors, as reported for other synthetic Wnt-targeting drugs¹⁴. This means that they can act in one or in the other ways depending on the cell type and on the state of dysregulation that the signaling has in that specific disease. In other words, as modulators of the pathway, these nature-derived compounds are hypothetically able to preserve (when used in preventive strategies) or to restore (if used as therapeutics or adjuvants) the right equilibrium between Wnt-ON and Wnt-OFF, thus reinstating the signaling aberration that leads to the onset and progression of the diseases. Specifically, in the neoplastic disease, in which the Wnt/ β -catenin pathway is generally, but not always, constitutively activated, these natural modulators act almost always as inhibitors. Conversely, in other diseases such as neurodegenerative disorders, in which the activation of the pathway has neuroprotective ability^{13,14}, they have beneficial effects by functioning as Wnt pathway activators^{106,107}.

As it concerns the use of these nature-derived compounds as a preventive and therapeutic strategy in neoplastic diseases, most of them are still in preclinical development and only three are already under clinical investigations. Therefore, in the following section we focus on the most significant natural compounds that demonstrated significant pharmacological properties in relevant model of neoplastic diseases (Table 1).

Methods used for the pharmacological screening of natural compounds described in this review include bioassay traditionally employed for the evaluation of anticancer potential of every kind of experimental drugs. Specifically, the most used screening systems are cell viability and proliferation assay, cell cycle analysis, wound healing and transwell migration assay. These methods are used to evaluate the potential cytotoxicity and antimetastatic activity. Relating to the scope of this review, the evaluation of compounds that modulate the Wnt/ β -catenin pathway was conducted through the following main techniques: Top/Fop Flash, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot analyses. Top flash is a cell-based luciferase assay system that involves the use of cell lines that are transfected with TCF/LEF reporter for monitoring the activity of Wnt/ β -catenin signaling pathway through the employment of known inhibitor and experimental compound(s). Instead, Fop flash uses mutant TCF-binding site which does not respond to the Wnt signal³². These bioassays were used generally to monitor Wnt signaling pathway activity and to screen agonists or inhibitors.

4.1. Flavonoids

4.1.1. Genistein

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) is the main isoflavone in soy (*Glycine max*) and was firstly extracted from *Genista tinctoria* L., a flowering plant of the family Fabaceae. It is considered a dietary phytoestrogen and the main

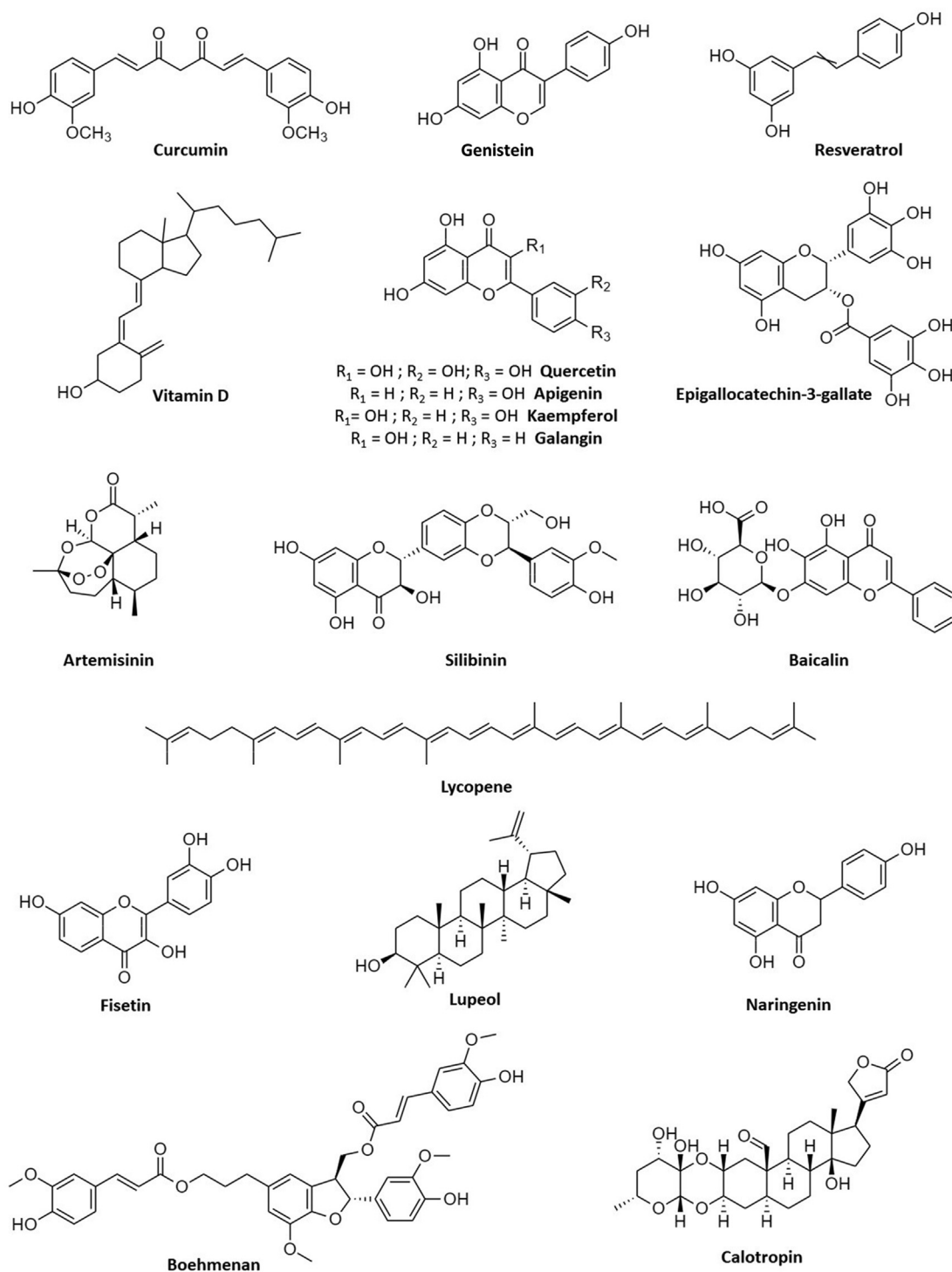


Figure 2 Molecular structures of nature-derived compounds that modulate the Wnt/ β -catenin signaling.

sources of this isoflavone are soybean and soybean products^{108,109}. Many different extraction techniques were reported in literature as effective for the recovery of isoflavones from natural sources and ultrasound assisted extraction and Quick Easy Cheap Effective Rugged and Safe (QuEChERS) methodology were recently reported as good strategies with high recovery yield¹¹⁰. Genistein is one of the main secondary metabolites in soy-derived

food and together with other isoflavones entities and dietary phytoestrogen, it is consumed in significant quantities worldwide¹¹¹. Moreover, genistein has different biological interesting effects, such as anti-inflammatory and pro-apoptotic effects, modulation of steroidal hormone receptors and metabolic pathways. According to literature, genistein is also able to change the expression of variety of Wnt target genes leading to the induction

Table 1 Wnt pathway targeting natural-derived compounds that are under investigation on neoplastic diseases.

Compound and chemical class	Effect on Wnt/ β -catenin pathway	Stage of development	Ref.
Curcumin polyphenols: curcuminoids - diaryl eptanoides	Inhibits p-GSK3 β , β -catenin, E-cadherin and N-cadherin, cyclin D1 and the nuclear expression of dishevelled proteins. Prevents β -catenin/TCF DNA binding and transactivation.	Phase I in metastatic colon cancer patients in combination with 5-fluorouracil (NCT02724202) Phase I in advanced colorectal cancer in association with irinotecan (NCT0185985) Phase II in locally advanced rectal cancer in combination with capecitabine and radiation therapy before surgery (NCT00745134) Phase III in locally advanced or metastatic adenocarcinoma of the colon in combination with celecoxib (NCT00295035)	46,63–65
Genistein polyphenols: flavonoids - isoflavones	Inhibits of AKT phosphorylation and suppression of GSK-3 β dephosphorylation. Promotes β -catenin phosphorylation Suppresses β -catenin/TCF-driven transcription. Induces the overexpression of E-cadherin, reduces the activity of WNT-1 and its targets such as c-Myc and cyclin D1. Reduces the gene expression of <i>Wnt-7a</i> . Inhibits the effect of DKK1. Downregulates of TCF reporter activity. Reduces the expression of <i>Wnt5a</i> , <i>Sfrp1</i> , <i>Sfrp2</i> and <i>Sfrp5</i> .	Phase I/II in combination with FOLFOX or FOLFOX-Avastin for the treatment of metastatic colorectal cancer (NCT01985763)	3,31,46,63,66-71
Resveratrol Polyphenols: stilbenoids	Decreases the expression of β -catenin, c-Myc, MMP7 and Survivin. Downregulates WNT-2 and upregulate AXIN2 in Colo16 cells and Myc-tagged TCF4 protein. Upregulates of WNT-1	Phase I in patients with suspected or documented colon cancer (NCT00256334)	30,46,63,72–74
Vitamin D secosteroids	Decreases the binding of β -catenin to TCF. Increases E-cadherin and DKK1	Observational clinical study in normal subjects and colorectal cancer patients (NCT00399607)	25,46,75,76
Quercetin polyphenols: flavonoids - flavonols	Inhibits the binding between β -catenin and TCF. Inhibits GSK-3 β phosphorylation. Increases the expression of E-cadherin.	Preclinical: <i>in vitro</i> and <i>in vivo</i>	33,46,68,77–79
EGCG (epi-gallocatechin-3-gallate) polyphenols: catekines	Downregulates the expression of p-GSK3 β . Increases the expression of GSK3 β and Reduces the level of β -catenin and its target genes. Inhibits the expression of β -catenin/TCF-4 receptor activity. Upregulates HBP1. Increases secreted frizzled-related protein expressions and E-cadherin	Preclinical: <i>in vitro</i> and <i>in vivo</i>	46,80–82
Artemisinin sesquiterpenes	Decreases the expressions of <i>Wnt5a/b</i> resulting in the suppression of β -catenin and in an increase of E-cadherin	Preclinical: <i>in vitro</i> and <i>in vivo</i>	83
Apigenin polyphenols: flavonoids - flavones	Inhibits β -catenin/TCF/LEF interaction. Increases of E-cadherin and decreases nuclear β -catenin, c-Myc, and cyclin D1	Preclinical: <i>in vitro</i> and <i>in vivo</i>	84–88
Baicalin polyphenols: flavonoids	Reduces β -catenin mRNA and protein. Induces of DKK1 expression, reduces the expression of c-Myc Inhibits of microRNA-217 (negative regulator of DKK1)	Preclinical: <i>in vitro</i> and <i>in vivo</i>	89,90
Silibinin polyphenols: flavonoids	Suppresses Wnt co-receptor LRP6 expression. Reduces of β -catenin, cyclin D1 and c-Myc	Preclinical: <i>in vitro</i> and <i>in vivo</i>	91,92
Galangin polyphenols: flavonoids - flavonols	Reduces the expression at protein and mRNA levels of <i>Wnt3a</i> and β -catenin. Downregulates β -catenin intracellular levels and the expression of β -catenin/TCF-dependent genes, such as cyclin D1 and c-Myc.	Preclinical: <i>in vitro</i> and <i>in vivo</i>	32,93,94

(continued on next page)

Table 1 (continued)

Compound and chemical class	Effect on Wnt/ β -catenin pathway	Stage of development	Ref.
Fisetin polyphenols: flavonoids	Downregulates β -catenin and TCF4 and its target genes such as cyclin D1 and MMP7. Reduces phosphorylation of GSK3- β and decreases β -catenin stabilization. Modulates the interaction between β -catenin and LEF/TCF-2 leading to the downregulation of c-Myc, Brn-2 and Mitf and its downstream targets	Preclinical: <i>in vitro</i> and <i>in vivo</i>	95–97
Lupeol triterpenoids	Reduces the nuclear expression of β -catenin and the formation of β -catenin/TCF4 complexes. Downregulates the expression of GSK-3 β , leading to the suppression of AKT1, PI3K, β -catenin, c-Myc and cyclin D1 mRNA expression	Preclinical: <i>in vitro</i>	98–100
Kaempferol polyphenols: flavonoids - flavonols	Inhibits β -catenin/TCF transcriptional activity	Preclinical: <i>in vitro</i>	88,101
Lycopene carotenoids	Inhibits β -catenin, c-Myc and cyclin E protein levels. Reduces activation AKT and GSK-3 β phospho-inhibition. Induces a reduction in TCF/LEF reporter activity, β -catenin and cyclin D1.	Preclinical: <i>in vitro</i>	63,102
Naringenin polyphenols: flavonoids - flavonones	Suppresses β -catenin/TCF transcriptional activity	Preclinical: <i>in vitro</i>	32,103
Boehmenan polyphenols: lignans	Decreases cytosolic and nuclear β -catenin and c-Myc	Preclinical: <i>in vitro</i>	104
Calotropin carotenoids	Decreases cytosolic and nuclear β -catenin by increasing its phosphorylation mediated by CK1 α	Preclinical: <i>in vitro</i>	105

of cell-cycle arrest, apoptosis and/or inhibition of epithelial–mesenchymal transition (EMT) and metastasis¹¹². Indeed, genistein is a multitarget drug with several pharmacological activities including chemopreventive and anticancer efficacy in various type of tumors. It affects cell cycle, angiogenesis and inhibits metastasis¹¹³. Differently from curcumin, genistein bioavailability after oral administration does not represent a critical issue; the molecule is readily available as reported in preclinical and human trials¹¹⁴.

Several *in vitro* and *in vivo* studies analyzed the effect of genistein on Wnt/ β -catenin pathway and demonstrated an inhibitory effect through the modulation of different key molecules. Genistein regulates the upstream components of the β -catenin/TCF pathway by inhibiting AKT phosphorylation and suppressing GSK-3 β dephosphorylation, thus facilitating the ubiquitylation and degradation of β -catenin⁶⁶. Sarker et al.⁶⁷ showed that genistein increases the expression of GSK-3 β , induces β -catenin attachment to GSK-3 β and promotes β -catenin phosphorylation levels, leading to the suppression of prostate cancer growth. Other studies have also demonstrated that genistein potently suppresses β -catenin/TCF-driven transcription in AGS gastric cancer cells^{33,68}. Su et al. demonstrated that genistein induces the overexpression of E-cadherin and reduces the activity of WNT-1 and its targets such as c-Myc and cyclin D1 in mouse mammary epithelial cell line HC11^{115,116}. Furthermore, an *in vivo* study showed that genistein was able to inhibit Wnt pathway reducing cyclin D1 in mammary ductal epithelium of animals³¹. Genistein was also demonstrated to reduce the gene expression of Wnt-7a in endometrial adenocarcinoma cells^{31,69}.

Recent *in vitro* study on several renal cancer (RCC) cell lines and *ex vivo* analyses performed on tissues from 43 patients affected by clear renal cancer demonstrated that genistein induces apoptosis, inhibits proliferation and invasion through the downregulation of TCF reporter activity and miR-1260b, that has known to be overexpressed in renal cancer tissues, affecting cancer aggressiveness⁷⁰. Its biological function was also strictly correlated with the β -catenin-dependent pathway in RCC cell lines. The study of Hirata et al.⁷⁰ demonstrated that genistein inhibited Wnt-signaling by regulating miR-1260b expression in renal cancer cells.

In another *in vivo* study on colon cancer murine model, Zhang et al.⁷¹ demonstrated that genistein inhibited Wnt target genes cyclin D1 and c-Myc and its signaling, reducing the expression of *Wnt5a*, *Sfrp1*, *Sfrp2* and *Sfrp5*. Results obtained from this study suggested that genistein possesses protective role in the development of early colon neoplasia.

As previous reported, genistein has been extensively studied as drug for cancer prevention and treatment. Over the years, 29 clinical trials have been conducted (on ClinicalTrials.gov), and 1 of 29 aimed to correlate the effect of genistein with the inhibition of the Wnt/ β -catenin pathway (NCT01985763). This study is a phase I/II clinical trial that was conducted hypothesizing that the combination of genistein with the standard of care might reduce chemotherapy resistance and improve response rates in patients. The study was designed in order to administrate genistein with FOLFOX or FOLFOX-Avastin in 13 patients with stage IV colon or rectal neoplasms. The trial was completed in 2018 but no results have been published to date.

Relating to the safety profile, conflicting results have been described in scientific literature. It has been reported the potential toxic effects of genistein on fertility and fetus development. Some studies demonstrated that genistein has a negative effect on ovarian differentiation, estrous cyclicity, and fertility in a rodent model. However, data from clinical trials for the determination of these potential critical issues about the use of genistein are still lacking¹¹⁴.

4.1.2. Quercetin

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a polyphenol compound widely diffused and a common phytochemical in human diet and can be found in nuts, tea, onions, apple and in plant sources. Quercetin can also be found in dietary supplements¹¹⁷. Quercetin exhibits many beneficial activities for human health and in particular an abundant antioxidant activity due to free radicals scavenging leading to the protection of human DNA from oxidative damages¹¹⁸. Moreover, epidemiological studies highlighted that quercetin shows anti-inflammatory, antitumor, antiviral, antiallergic, and anti-edematous activities, and may provide prevention from cardiovascular pathologies¹¹⁹. Due to these multiple activities attributed to quercetin, in recent years its extraction and determination have acquired a growing interest. At date, the extraction of quercetin from natural sources can be achieved by some innovative and green techniques: molecular imprinted polymers (MIPs) were used for the recovery of this polyphenol from red wine¹²⁰ and deep eutectic solvents (DESs)-based polymeric monolithic cartridges were applied for the extraction of quercetin from *Ginkgo biloba*, a China native large tree¹²¹. Quercetin has also been detected in some traditional remedies such as in *Saururus chinensis* leaves used in Korean traditional medicine for the treatment of pain and other affections¹²².

Quercetin has been considered a potential antitumor compound, based on the reported action on Wnt pathway, as described by *in vitro* and *in vivo* studies^{66,123}. Specifically, *in vitro* studies demonstrated that quercetin acts by inhibiting the binding between β -catenin and TCF in SW480 colorectal cancer cell line and in HEK293 cells transfected with constitutively active mutant β -catenin gene^{77,124}. The anticancer efficacy of quercetin was also confirmed in other cancer cell lines including leukemia and lymphoma cells¹²⁵, colon cancer¹²⁶, melanoma¹²⁷ and hepatocarcinoma¹²⁸.

Recent studies also suggested a promising effect of quercetin in triple negative breast cancer (TNBC) both in *in vitro* and *in vivo* models. Specifically, Srinivasan et al.⁷⁸ have shown that the treatment of MDA-MB-231 and MDA-MB-468 cells with quercetin increases the expression of E-cadherin and downregulates vimentin levels in TNBC, both markers of mesenchymal-to-epithelial transition. This effect was mediated by the modulation of β -catenin and its target genes such as cyclin D1 and c-Myc. Similar effects have been reported by Sultan et al.⁷⁹ in MDA-MB-231 and MDA-MB-157 TNBC cells and in a TNBC xenograft mouse model. The authors showed that quercetin induces apoptosis in TNBC cells by reducing the expression of different molecular markers including β -catenin. Moreover, the evaluation of anticancer activity of *in vivo* quercetin treatment in the TNBC model demonstrated the inhibition of tumor xenograft growth by 41.7% than the control group. These data support the potential use of this molecule for the treatment of TNBC⁷⁹. Despite quercetin is being evaluated in oncologic trials as anticancer drug, none of

these clinical studies evaluated its effect on the Wnt/ β -catenin signaling.

4.1.3. Apigenin

Apigenin (4',5,7-trihydroxyflavone) is a secondary metabolite from common plants, belonging to the flavone class of flavonoids. Apigenin can be found in many plants and food sources (parsley, celery, celeriac, cilantro and oregano) and even in Chinese traditional remedies (*i.e.*, *Scutellaria barbata* D. Don) and it is particularly abundant in chamomile flowers (Asteraceae)^{129,130}. Throughout literature, several methods have been used for the extraction of apigenin including conventional techniques (*i.e.*, maceration and heat reflux extraction) and more advanced ones (microwave assisted extraction and ultrasound assisted extraction). Among the more innovative techniques, supercritical carbon dioxide approach gained an increasing interest due to high effectiveness and less environmental impact compared to the other methods. Most recently, a green and highly efficient technique for the recovery of apigenin from a Chinese traditional plant was developed by Yang and Mei¹²⁹ obtained by coupling ultrasound assisted extraction technology with supercritical fluid extraction. Apigenin is known as a flavonoid with many interesting beneficial biological actions including antioxidant, antimutagenic, anticarcinogenic, antiinflammatory, and antiproliferative¹³¹. Due to its antitumor activity and low toxicity, apigenin has been widely researched and observed to suppress different human cancer both *in vitro* and *in vivo* studies¹³⁰ and many molecular target have been described for apigenin in human cancer¹³¹.

Apigenin was the first molecule belonging to the flavonoid class known to have an effect on Wnt pathway³³. This natural compound possesses anticancer capacity both *in vitro* and *in vivo*, by suppressing cell migration and invasion, and inducing apoptosis and cell cycle arrest^{84,132}. Its antitumor ability has been ascribed to the inhibition of β -catenin/TCF/LEF interaction, that suppresses the β -catenin nuclear entry and the expression of Wnt target genes in SW480 and HCT15 human colorectal cancer cells^{85,86} and in U2OS and MG63 osteosarcoma cells¹³³. *In vivo* evidence suggested that apigenin significantly reduces tumor volumes and completely inhibits lymphonodal, liver and lung metastases in a transgenic mouse model of prostatic adenocarcinoma and these effects have been related to an increase of E-cadherin and a decrease of nuclear β -catenin, c-Myc, and cyclin D1⁸⁷.

4.1.4. Baicalin

Baicalin is a flavonoid compound found in roots of different species of the genus *Scutellaria* (*S. laterifolia* and *S. galericulata*), and in particular it is the active metabolite responsible for the biological action of *Scutellaria baicalensis* Georgi, a Chinese medicinal plant used to treat psoriasis¹³⁴. The more common techniques applied for the extraction and isolation of baicalin are heat reflux extraction (HRE), ultrasound-assisted extraction (UAE) supercritical fluid extraction (SFE)¹³⁵. Moreover, other more sophisticated techniques were developed for the isolation and purification of baicalin, such as deep eutectic solvents, ultrahigh pressure technology¹³⁶ and molecular imprinted polymers¹³⁷. Baicalin exhibits an anticancer activity toward many kinds of cancers, including ovarian, prostate, breast and pancreatic cancers, esophageal squamous cell carcinoma, as well as Burkitt lymphoma¹³⁸. The exact mechanism of action is not completely understood but it seems that it acts through multiple mechanisms involving the induction of apoptosis and the modulation of

different pathways also including the Wnt/ β -catenin signaling^{89,139}. Jia et al.⁸⁹ demonstrated that the treatment with baicalin of colon cancer DLD1 and HCT-116 cells inhibits their proliferation and induces apoptosis. This effect was mediated by the induction of DKK1 expression, a relevant antagonist of Wnt signaling pathway, thus reducing the expression of β -catenin and c-Myc. The authors also demonstrated that the mechanism of action of baicalin on these cells also involves the inhibition of microRNA-217, negative regulator of DKK1. Therefore, it was shown that baicalin induces apoptosis through the miR-217/DKK1-mediated inhibition of Wnt signaling pathway⁸⁹. Baicalin was tested also on triple negative breast cancer cell line MDA-MB-231 and mouse mammary cancer cell line 4T1⁹⁰. The molecule did not affect cell viability but demonstrated a potential effect on migration and invasion in a dose dependently manner, and reverted epithelial-to-mesenchymal transition process by targeting β -catenin signaling. The same authors in an *in vivo* xenograft metastasis tumor model of 4T1 breast cancer cells also confirmed these results. Specifically, the compound reduced the number of liver and lung metastases through the downregulation of epithelial-to-mesenchymal transition markers and the inhibition of β -catenin in tumor tissues⁹⁰.

4.1.5. Silibinin

Silibinin is a polyphenol belonging to the flavolignan class. It is the major active compound in the extract of *Silybum marianum* (commonly named milk thistle plant) seeds, commonly used as a medicinal plant in CTM, is recently gaining interest in western societies for the treatment of liver diseases and diabetes. In nature, Silibinin exists in a mixture of two diastereomers, silibinin A and silibinin B in a ratio of 1:1. Typically, the extraction of this polyphenol, as also described in *European Pharmacopoeia*, is achieved by two solvent steps using *n*-hexane and methanol, respectively. This procedure is achieved by means of traditional Soxhlet apparatus and requires long time of processing. In order to reduce time of extraction and to improve the recovery of silibinin, many improved methodologies have been studied, such as the pressurized liquid extraction technique developed in a recent work¹⁴⁰. Silibinin has been traditionally used as nutritional supplement for hepatoprotection, but it has also demonstrated to exert anticancer activity in different *in vitro* and *in vivo* models of solid tumors such as skin, breast, lung, colon, bladder, prostate and kidney carcinomas. It has been reported that this effect are correlated with the modulation of Wnt/ β -catenin pathway. Specifically, *in vitro* test showed that silibinin inhibited migration and invasiveness of PC3 prostate cancer cells through different mechanisms also including the increment of E-cadherin at cell membrane and the reduction of nuclear β -catenin¹⁴¹. Silibinin-induced suppression of cell growth correlated to the inhibition of the Wnt/ β -catenin signaling was also demonstrated in the human colorectal carcinoma cell line SW480 and in the xenograft model, where the compound inhibited tumor growth by decreasing the expression of β -catenin, cyclin D1, c-Myc¹⁴². Another study demonstrated that silibinin acts as suppressor of the Wnt co-receptor LRP6 and that the antitumor activity is mediated by the inhibitory effect on Wnt/LRP6 signaling in prostate and breast cancer cells⁹¹. The antitumor activity of silibinin was also confirmed *in vivo* in *Apc^{Min/+}* transgenic mouse model of intestinal carcinogenesis. The natural compound prevented the polyp formation in small intestine and colon and this chemopreventive effect was mediated by the decrease of β -catenin levels and its

transcriptional activity⁹². Similar results were also obtained in other *in vivo* models of colon carcinogenesis^{143,144}.

4.1.6. Galangin

Galangin (4*H*-1-benzopyran-4-one,3,5,7-trihydroxy-2-phenyl or 3,5,7-trihydroxyflavone) is a polyphenol compound naturally occurring in plants of the genus *Alpinia* of the Zingiberaceae family (*Alpinia officinarum*, *A. galangal*). These herbs have been widely used as traditional remedies in different regions of the world like Asian and African countries, for the treatment of many diseases. Moreover, *A. officinarum* is reported as a safe food spice for non-medical usages¹⁴⁵. Extraction of bioactive compounds and in particular galangin from this genus has been purchased by the application of several techniques (*i.e.*, Soxhlet extraction, maceration, ultrasonication, and soaking) and different solvents such as petroleum ether, ethyl acetate and water^{146,147}. Galangin has been suggested to have antimutagenic, antioxidant, and muscle contraction inhibitor properties¹⁴⁸. In recent years, scientific studies focused on the evaluation of its beneficial effects on animal models in terms of anticancer activity^{93,149} and vascular disorders treating¹⁵⁰. Galangin belongs to the class of flavonol and possesses anti-oxidant, anti-inflammatory and antitumor activities as demonstrated in a variety of *in vitro* and *in vivo* models⁹³. *In vitro* studies on the human oesophageal carcinoma cell lines Eca9706, TE-1, and EC109 showed that treatment with galangin resulted in cell growth inhibition, induction of apoptosis and cell cycle arrest. The molecular analysis revealed that galangin reduced the expression of *Wnt3a* and β -catenin at protein and mRNA levels. The antitumor efficacy was also confirmed in nude mice with xenograft tumors, where the treatment increased TUNEL positivity and the level of the onco-suppressor p53 and reduced the expression of *Wnt3a* and of the proliferation marker Ki-67⁹³. Other *in vitro* studies indicated that galangin is able to suppress the proliferation of colorectal and hepatocarcinoma cell lines due to the downregulation of β -catenin intracellular levels and the transcription of β -catenin/TCF-dependent genes, such as cyclin D1 and c-Myc⁹⁴.

4.1.7. Fisetin

Fisetin (7,3',4'-flavon-3-ol) is a bioflavonoid occurring in plants. It is found in dietary sources like strawberries, apple, persimmons and onions and in many species of plants like Eudicotyledons and Fabaceae family (genus *Acacia*), among the others¹⁵¹. As other polyphenols, fisetin shows many beneficial biological actions, for instance the ability to scavenge free radicals leading to a high antioxidant activity. Moreover, many different studies were performed to evaluate its anti-inflammatory and antiallergic activity and recently, this polyphenolic compound gained an increased interest because of its action as memory-enhancing agent in rodents¹⁵². Like other flavonoids, fisetin possesses antiproliferative activity against different cancers and many reviews have been published focusing on this chemopreventive potential^{151,153,154}. The extraction of Fisetin from natural sources has been performed by applying acidic conditions (typically by HCl)¹⁵⁵ and in some cases, an ultrasonic-assisted extraction was described¹⁵⁵. Scientific literature indicated that fisetin is a potent anticancer agent against different solid tumors such as lung, breast, colon and pancreatic cancer. It has been demonstrated that fisetin regulates Wnt signaling in colon cancer cell line HT-29 through the downregulation of β -catenin and TCF4 and its target genes such as cyclin D1 and matrix metalloproteinase^{795,96}. The modulation of Wnt pathway by fisetin was also demonstrated in *in vitro* and

in vivo melanoma models⁹⁷. Specifically, the *in vitro* study on 451Lu melanoma cells showed that fisetin induces a reduction of cell viability and G1 cell cycle arrest. This effect was correlated to the suppression of Wnt signaling and specifically to the decreased phosphorylation of GSK3- β associated with decreased β -catenin stabilization⁹⁷. Fisetin also modulates the interaction between β -catenin and LEF/TCF-2 that resulted in the downregulation of the TCF target genes *c-Myc*, *Brn-2* and microphthalmia-associated transcription factor (*Mitf*). In the *in vivo* study, carried out on 451Lu-xenografted nude mice, fisetin induced the inhibition of tumor growth that is mediated by the inhibition of the β -catenin/*Mitf* signaling⁹⁷.

4.1.8. Kaempferol

Kaempferol (3,4',5,7-tetrahydroxyflavone), is a flavonol, a kind of flavonoids, contained in many dietary and plant sources (in Angiosperm family in particular), such as grapes, tomatoes, broccoli, tea, cabbage in *Ginkgo biloba* leaves and *G. max* among the others. Typically, the extraction of this flavonoid was achieved by using solvents (mainly polar solvents like methanol, ethanol and water) and different approaches were applied (from conventional solvent extraction to ultrasound and microwave assisted extraction). One very convenient extraction recently described for kaempferol was achieved by supercritical fluid technology as reported by Ortega and co-workers in 2018¹⁵⁶. Being a flavonoid, kaempferol exhibits an abundant antioxidative activity by reducing superoxide ions and lowering the formation of reactive oxygen species. Kaempferol is also a promising anticancer agent by promoting inhibition of angiogenesis and apoptosis. Kaempferol additionally exerts many others health perspectives in terms of antidiabetic, anti-inflammatory, antiaging and antiallergic activity¹⁵⁷. It was demonstrated that this compound inhibits Wnt pathway in colorectal cancer cell lines SW480 and HEK293, through the suppression of the β -catenin/TCF transcriptional activity⁸⁸. Qin et al.¹⁰¹ have shown that kaempferol has anti-proliferative and anti-invasive properties in retinoblastoma SO-RB50 cells. The effect was explained on the basis of the interaction with estrogen-related receptor α that induced the suppression of Wnt/ β -catenin signaling¹⁰¹.

4.1.9. Naringenin

Naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one) is a flavonoid belonging to the flavonone subclass. Naringenin is known as one of the most ingested flavonone by people because of its vast distribution in *Citrus* genus. Indeed, it is the most abundant flavone in grapes, tangelo, blood orange, lemons, pomelo, and tangerines, and it is found in cherries grapefruit and cocoa¹⁵⁸. Naringenin possesses board biological beneficial effects for human health including antioxidant, anti-inflammatory, antidiabetic, antiproliferative and anticancer activity¹⁵⁹. The extraction of naringenin has been performed by the techniques typically applied for other flavonoids and recently, a green and efficient extraction methodology based on ultrasound-assisted deep eutectic solvents extraction was developed¹⁶⁰. As mentioned above, naringenin has demonstrated to have several pharmacological activities such as antioxidant, antitumor, anti-inflammatory, antiadipogenic and cardioprotective effects. These have been demonstrated in preclinical and in clinical trials. Scientific evidence on the activity that naringenin exerts on Wnt- β -catenin pathway has been also described. The natural compound demonstrated to suppress Wnt signaling in AGS human gastric

cancer cells. Naringenin acts suppressing the TCF transcriptional activity in a concentration-dependent manner¹⁰³.

4.2. Other polyphenols

4.2.1. Curcumin

Curcumin [(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a diarylheptanoid (curcuminoid) extracted from the rhizome of *Curcuma longa* L., a flowering plant of the ginger family (Zingiberaceae)¹⁶¹, cultivated in tropical and subtropical regions, such as Asia and Central America. *C. longa* L., also known with the common name of turmeric, is widely used in traditional medicines such as in Ayurvedica and Chinese traditional medicine (CTM). The extraction of curcuminoids, and in particular curcumin, from the rhizome of *C. longa*, firstly achieved in 1818 by Vogel and Pelletier¹⁶², has been vastly described in literature and many different approaches were applied^{163–165}. Indeed, throughout literature many conventional techniques were reported as organic solvents extraction, steam distillation, hot and cold percolation, use of hydrotrope, and alkaline solution. In addition, several advanced techniques have been studied like supercritical fluid extraction, microwave-assisted extraction, ultrasound-assisted extraction, and enzyme assisted extraction. Most recently, Patil and co-workers¹⁶⁶ described an advanced three-phase partitioning and batch extraction as an innovative technique suitable for the extraction of curcuminoids from natural sources. *C. longa* is utilized also in culinary uses as yellow/orange spice, therefore, curcumin can be assumed for medical purposes if utilized in traditional herbal remedies or as dietary ingredient. Curcumin expresses interesting *in vitro* and *in vivo* biological activities such as anti-inflammatory¹⁶⁷, antioxidant¹⁶⁸ and neuroprotective¹⁶⁹ among the others. In addition, anticancer activity of curcumin was widely investigated⁶⁴. The biological effect is mediated by the modulation of different pathways known to have a crucial role in pathological conditions, including the Wnt/ β -catenin pathway. Curcumin acts on different critical molecular components of the signaling cascade, whose overexpression is involved in cancer initiation, progression and resistance. For example, in breast cancer cells curcumin induces cell proliferation arrest and apoptosis, also inhibiting their migration and invasion of through the inhibition GSK-3 β , β -catenin and its nuclear localization, E-cadherin, and cyclin D1^{65,170}. Other studies demonstrated that curcumin inhibits β -catenin, p-GSK3 β protein expression, cyclin D1 and c-Myc in non-small-cell lung cancer (NSCLC) and medulloblastoma cell lines and dramatically decreases proliferation and the epithelial–mesenchymal transition in SW620 human colon cancer cells, through different mechanisms including also the downregulation of the Wnt pathway^{171–173}. Curcumin has also a promising antitumor activity when used to target cancer stem cells in colorectal, breast and lung cancer through a specific action on Wnt/ β -catenin pathway and its target genes^{174–178}.

A recent *in vivo* study in a mouse model of colon cancer has demonstrated a new mechanism of regulation of Wnt/ β -catenin pathway by curcumin, leading to an antitumor effect through the downregulation of β -catenin and TCF4. The authors demonstrated that the action of curcumin on the pathway was mediated by the downregulation of the microRNA (miR)-130a and miR-21. Specifically, the miR-130 was revealed as negative regulator of curcumin anticancer activity. In fact, the overexpression of miR-130a in SW480 cells abolished the curcumin-induced inhibition of cell proliferation as well as the downregulation of β -catenin, and this

suggests that curcumin regulates Wnt/ β -catenin pathway by inhibiting miR-130a¹⁷⁷. Another recent study conducted by Marjaneh et al.¹⁷⁹ explored the efficacy of a novel formulation of phytosomal curcumin in combination with 5-FU in a mouse model of colitis-associated colon cancer by evaluating the expression of cyclin D1, beclin, E-cadherin, and p-GSK3 α / β . Results showed that the association strongly reduces inflammation and tumor volume and number, through modulation of the Wnt pathway and E-cadherin.

The potent antiproliferative effect observed in *in vitro* and *in vivo* model of solid tumors of lung, breast, gastrointestinal tract and central nervous system has led to explore the pharmacological potential of curcumin also in clinical trials. In the last 10 years, 33 clinical studies have been conducted in order to evaluate the antitumor potential of curcumin in cancer patients¹⁸⁰. Specifically, four of them have been designed in order to use curcumin for targeting the Wnt/ β -catenin pathway in CRC. They are evaluating the safety and efficacy of curcumin in association with 5-FU and with irinotecan in two phase I studies as preoperative neoadjuvant standard radiation therapy and chemotherapy in a phase II study, and in combination with celecoxib in a phase III clinical trial. These studies are in ongoing and no results have been published to date⁴⁶. Although a potential anticancer effect has been also demonstrated in patients, further studies are warranted to validate the use of curcumin as preventive and therapeutic treatment as monotherapy or in combination with standards of care. Curcumin and its derivatives have been considered safe as shown by the clinical trials conducted until now. The main side effects reported are mild and mainly affecting the gastrointestinal system¹⁸¹. As known, the bioavailability of curcumin is a critical issue that affect antitumor efficacy in patients. New clinical development strategy aiming to define a better patient population through specific biomarkers is needed in order to optimize and potentially validate the use of curcumin as anticancer medicines.

4.2.2. Resveratrol

Resveratrol (3,5,4'-trihydroxy-trans-stilbene, **2**) is a stilbene contained in a wide range of food sources such as grape, blueberries and peanuts. In particular, grape peel contains high quantities of resveratrol and its glycosides¹⁸². Resveratrol represents one of the most interesting natural bioactive compounds because of its high beneficial potential in health applications. Indeed, several *in vitro* and *in vivo* studies have highlighted the antioxidant, anti-inflammatory, cardioprotective, neuroprotective and antitumor actions¹⁸³. Many efforts have been faced in the extraction of resveratrol and the recovery from grape peel remain challenging because of the poor solubility of this stilbene in water and in other common solvents typically used for solvent-based extraction. Many approaches have been applied and the optimum quantitative extraction of resveratrol from grape peel (after infection with powdery mildew) has been gained by using an aqueous solution of ethanol at 60 °C¹⁸⁴ and other methods focused on the conversion of piceid (a resveratrol glucoside derivative) to resveratrol to improve the extraction yield by acid hydrolysis, heat application or enzyme treatment^{185–187}. Recently, in order to achieve a more rapid and economic procedure, Averilla and co-workers¹⁸², described an innovative method combining enzymes and heat to improve the extraction recovery.

As mentioned, resveratrol is a potent antioxidant and anti-inflammatory agent that has been extensively studied during the last years. It has been demonstrated that resveratrol possesses antiproliferative and antimetastatic activity through the

modulation of different mechanisms^{30,33,188}. In particular, it has been demonstrated that resveratrol decreases the expression of β -catenin in the nucleus of colon cancer cells and this seems to be correlated to the downregulation of molecular regulators of β -catenin localization^{30,189}. Very recently, Mineda et al.¹⁹⁰ reported that resveratrol induces apoptosis and arrests cell proliferation in human uterine sarcoma cell line MES-SA through a dose-dependent downregulation of β -catenin and c-Myc. Geng et al.¹⁹¹ have also demonstrated that resveratrol was able to inhibit proliferation, migration and invasion of multiple myeloma cells (U266 and LP1) through a reduced expression of nuclear β -catenin, c-Myc, MMP7 and survivin. Other authors showed that resveratrol inhibits gastric cancer cell line MGC-803 through downregulation of the Wnt/ β -catenin signaling pathway¹⁹². Specifically, the antitumor effect was mediated by the reduction of the expression of β -catenin, c-Myc, and cyclin D1¹⁹².

Cilibrasi et al.⁷² demonstrated that resveratrol inhibits proliferation and motility in glioma stem cells lines isolated from patients affected by glioblastoma multiforme. The efficacy of resveratrol in these models was strongly correlated with the upregulation of WNT-1 and MYC^{72,193}.

Resveratrol also inhibits proliferation of human osteosarcoma cells lines MG-63 and U2-OD by downregulating the expression of β -catenin^{194,195}. After treatment with resveratrol, a downregulation of Wnt/ β -catenin pathway target genes, such as β -catenin, c-Myc, cyclin D1, MMP-2 and MMP-9 was shown, while E-cadherin level increased¹⁹⁵.

Resveratrol has also a promising effect on breast cancer stem-like (BSCS) cells isolated from MCF-7 and SUM159, and exhibited antitumor potential in a xenograft model of SUM159 cells in NOD SCID mice. Resveratrol inhibits the growth of tumors through different mechanisms including the reduction of the expression of β -catenin and cyclin D1 in both *in vitro* and *in vivo* models¹⁹⁶.

It has been also demonstrated that resveratrol improves the efficacy of temozolomide by increasing the sensitization of glioma resistant to the drug, both *in vitro* and *in vivo*, through the inhibition of WNT-2 and β -catenin expressions¹⁹⁷.

The antitumor effect of resveratrol has been also investigated in clinical trials. To date, 20 clinical studies were published on *ClinicalTrials.gov*. Two of 20 studies support the use of resveratrol as anticancer due to the effect of resveratrol on the Wnt/ β -catenin pathway. A phase I clinical study (NCT00256334) was conducted on 11 patients with suspected or documented colon cancer. The primary outcome of the study was based on the investigation of the potential effect of resveratrol on Wnt signaling both in colon cancer and normal colonic mucosa. Results obtained from the study demonstrated that resveratrol was not able to inhibit Wnt pathway in colon cancer but showed a statistically significant inhibition of Wnt target gene, cyclin D1 and axin II, in normal colonic mucosa, suggesting a potential use of resveratrol as a preventive treatment for colon cancer⁷³.

As it concerns the pharmacokinetic profile, recent clinical studies reported that resveratrol is rapidly absorbed after oral administration and is well tolerated with no adverse reactions reported^{198,199}. However, resveratrol has low bioavailability due to rapid and extensive metabolism in the intestine and liver but new formulations effective for improving its absorption and bioavailability have been already developed⁷⁴.

In any case, although numerous *in vitro* and *in vivo* studies and various clinical trials have been conducted up today, further clinical investigations are warranted in order to characterize the

potential use of Resveratrol as preventive or treatment strategy in cancer.

4.2.3. Epigallocatechin-3-gallate (EGCG)

Epigallocatechin-3-gallate ([$(2R,3R)$ -5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl]3,4,5-trihydroxybenzoate, EGCG), a type of catechin, is the ester of epigallocatechin and gallic acid. EGCG is the most abundant catechin in tea (*Camellia sinensis* and *C. assamica*) and tea-based ready-to-drink beverage and can also be easily assumed with diet by the consumption of apples (apple skin), onions and hazelnuts and by the administration of dietary supplements²⁰⁰. As all the other polyphenol compounds, EGCG has a strong antioxidant activity leading to an interesting beneficial potential for human health. Indeed, due to the anti-inflammation, anti-mutagenic and anti-viral activities, EGCG has a good potential for the prevention of chronic diseases like diabetes and cancer among the others²⁰¹. The extraction of this polyphenol was purchased by the application of always greener and more efficient techniques, such as ultrasound assisted extraction²⁰⁰ and deep eutectic solvents technology²⁰².

EGCG is a potent antioxidant that has been extensively studied as treatment of several diseases including cancer as preventive or treatment strategies. *In vitro* and *in vivo* studies confirmed that this natural compound exerts its potential antitumoral action through different mechanism also including the modulation of the Wnt/ β -catenin signaling. Studies on colorectal cancer stem cells demonstrated that EGCG suppresses the spheroid formation from the human colon cancer cells DLD-1 and SW480, by inducing apoptosis and inhibition of proliferation⁸⁰. Specifically, EGCG downregulated the expression of p-GSK3 β , increased the expression of GSK3 β and reduced the level of β -catenin and its target genes⁸⁰. The pharmacological activity of EGCG on cancer stem cells (CSCs) was also confirmed on lung cancer A549 and H1299 cells. EGCG reduced lung CSCs activity by inhibiting tumorsphere formation, decreasing lung CSCs markers, suppressing proliferation and inducing apoptosis. These effects were mediated by the downregulation of crucial molecules of the Wnt/ β -catenin signaling including p-GSK3 β ²⁰³. The EGCG antitumor activity mediated by an effect on the Wnt/ β -catenin pathway was also confirmed in other cell lines such as gastric and colon cancer^{204,205}. Other studies showed that EGCG modulates the pathway by decreasing β -catenin levels and inhibiting the β -catenin/TCF-4 reporter activity⁸¹, and/or by inducing HBP-1, one of the specific transcriptional repressor of the signaling²⁰⁶.

The anticancer potential of EGCG was also confirmed by *in vivo* experiments. The combined administration of EGCG with fish oil significantly reduced the number of tumors through the modulation of β -catenin in *Apc*^{Mim/+} transgenic mouse model of intestinal carcinogenesis²⁰⁷. Similar results were obtained in a mouse model of liver carcinogenesis where EGCG was used in combination with theaflavin⁸². Specifically, in this mouse model, the EGCG/theaflavin association was able to reduce the expression of β -catenin and its phosphorylated form, increase secreted frizzled-related protein expressions and E-cadherin, and decrease the transcription of Wnt target genes such as cyclin D1 and cMyc⁸².

4.2.4. Boehmenan

Boehmenan is a lignan from the Chinese medicinal plant *Clematis armandii*, named "Chuan-Mu-Tong" in *Chinese Pharmacopoeia* used against inflammatory conditions²⁰⁸. Lignans are fiber-associated polyphenols ubiquitous in human diet derived by

phenylalanine found in plants and many common foods such as grains, nuts, seeds, legumes, vegetables, tea, coffee or wine^{209,210}. Lignans are associated with many beneficial biological effects in mammals including antioxidant and antitumor actions. Indeed, numerous studies have shown that boehmenan possesses potent cytotoxic effects against many cancer cell lines^{208,211}. In particular, boehmenan is able to reduce viability of colon cancer cells RKO, SW480, HCT116 through the decrease of cytosolic and nuclear β -catenin and c-Myc¹⁰⁴.

4.3. Terpenes and terpenoids

4.3.1. Artemisinin

Artemisin ($(1R,4S,5R,8S,9R,12S,13R)$ -1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0.4,13.0.8,13] hexadecan-10-one) is an active sesquiterpene present in the extract of *Artemisia annua* (commonly called Qinghao), a medicinal plant included in the CTM for the treatment of fever and febrile illness²¹². Artemisinin is widely known as an antimalarial agent and was firstly extracted using a reproducible procedure (a low-temperature procedure, followed by a separation of acid portion from neutral one) by Youyou Tu and collaborators in 1971²¹³. Due to its typical and unique peroxide-containing lactone structure and to the new biological properties ascribed, including antitumor and anti-infective activities, artemisinin has recently received increasing attention²¹⁴. But the extraction of artemisinin is actually very challenging, because of the low concentration contained in the plant sources and, to fulfill the world demand of these valuable compounds, no single method alone is reliable. Therefore, many efforts have been applied in order to improve the production rate of artemisinin by using both conventional and advanced approaches²¹⁵.

Even if artemisinin is used over the years as anti-malarial treatment, in the recent years there is an increasing interest on the anticancer properties of this drug and its semi-synthetic derivatives artesunate and dihydroartemisinin. *In vitro* and *in vivo* studies revealed that these three drugs possess antitumor and anti-metastatic activities even when used at very low concentrations^{216–220}. The exact mechanism that supports the use of artemisinin as neoplastic agent is not completely understood. However, scientific literature hypothesizes different possible mechanisms including the modulation of Wnt/ β -catenin pathway. A recent study conducted by Tong et al.⁸³, which evaluated the effects of artemisinin and its semi-synthetic derivatives in two different lung cancer cell lines, A549 (carcinoma *in situ*) and H1299 (adenocarcinoma from metastasis nodule) and in the A540 xenograft mice model, showed that these three drugs were able to inhibit cell proliferation, also significantly suppressing cell migration and invasion, and consistently reduce tumor volume and weight *in vivo*. The authors provided evidence that these compounds act by inhibiting the Wnt/ β -catenin signaling.

4.3.2. Lupeol

Lupeol is a natural bioactive pentacyclic lupine-like triterpenoid²²¹. It is found in several natural sources such as medicinal plants (*Senegalia visco*, *Abronia villosa*, *Gossampinus malabarica*, *Strobilanthes callosa*, *Ficus cordata*, *Albizia adianthifolia*, *Mimosa invisa*, *Klainedoxa gabonensis* and *Turraeanthus africanus* among the others) and dietary vegetables and fruits (mango pulp and peels)^{222,223}. The extraction of lupeol has been performed by different methodologies, both conventional, such as maceration and Soxhlet, and more advanced techniques

(sonication, microwave and high hydrostatic pressures). By the comparison of the recovery yields, the most suitable approach seems to be sonication-based extractions^{224,225}. Lupeol exhibits many pharmacological activities such as antioxidant, anti-inflammatory, anti-hyperglycemic, anti-dyslipidemic and anti-mutagenic effects and exerts beneficial effects on different diseases, leading to the conclusion that this bioactive secondary metabolite is a multi-target agent with a promising pharmacological potential²²¹. In particular, lupeol has selective antitumor potential on various human cancer cells. The anticancer activity of lupeol involves different mechanisms also including the suppression of Wnt/ β -catenin signaling. A recent *in vitro* study conducted by Wang et al.⁹⁸ explored the effect of lupeol on two colorectal cancer cell lines: SW480 and HCT116. In these cells, lupeol was able to inhibit proliferation and migration and to promote apoptosis, and these effects were correlated with the down-regulation of transcriptional activity and protein expression mediated by the Wnt/ β -catenin signaling. Specifically, lupeol decreased expression of β -catenin and TCF4 protein and reduced mRNA and protein expression of the downstream targets c-Myc and cyclin D1. The authors suggested that it is possible that potential mechanism of action could be correlated with the reduction of nuclear β -catenin expression and β -catenin/TCF4 interaction, with subsequent suppression of the signaling⁹⁸. Zhang et al.⁹⁹ demonstrated that lupeol induced apoptosis in the hepatocellular carcinoma HCCLM3 cells in a time- and dose-dependent manner through different mechanisms including the decrease of GSK-3 β phosphorylation in Ser9, with a concomitant suppression of AKT1, PI3K, β -catenin, c-Myc and cyclin D1 genes transcription⁹⁹. That the anticancer effect of lupeol is mediated by the targeting of the Wnt/ β -catenin signaling was also demonstrated in melanoma, in colorectal cancer and in prostate cancer cell lines^{100,226,227}.

4.3.3. Lycopene

Lycopene is a frequently occurring natural compound of the carotenoids class. It is an highly unsaturated hydrocarbon (11 conjugated and 2 unconjugated double bonds) found in abundant quantity in red fruits and vegetables, including tomato, watermelon, pink grapefruit, apricots, pink guava, and papaya²²⁸, and it is responsible for the red color of these foods or plants. Lycopene exhibits important biological actions and its ability to suppress oxidation, inflammation, angiogenesis and cell migration and proliferation has been evaluated on different cell types²²⁹. Due to these activities, this compound was vastly studied for the treatment of different pathologies such as in the adjuvant therapy of diabetic neuropathy²³⁰ and in the prevention of age-related macular degeneration and proliferative vitreoretinopathy²³¹. Lycopene was extracted firstly in 1910 and it was usually purchased by using organic solvents. At date many extraction techniques have been developed in order to improve the reaction rate^{232,233}. Indeed, Briones-Labarca and co-workers²³⁴ have recently achieved an optimization of extraction yield of lycopene from tomato pulp by high hydrostatic pressure extraction (HHPE) technology.

Lycopene is a potent antioxidant and has antitumor activity in different type of solid cancers due to the modulation of Wnt/ β -catenin signaling and others pathways. These functions are associated with an antiproliferative and pro-apoptotic effect in breast, colon, and prostate cancer cells²³⁵. *In vitro* studies on prostate cancer-derived stromal cells lycopene reduced Wnt/ β -catenin signaling by affecting β -catenin nuclear localization. In normal prostate stromal cells treated with IGF-I, lycopene reduced

the activation of AKT and the GSK3 β phospho-inhibition¹⁰². The inhibition of Wnt/ β -catenin signaling by lycopene was also confirmed in human breast cancer cell lines MCF-7 and MDA-MB-231. In these cellular models, the treatment with lycopene induced a reduction in TCF/LEF reporter activity, β -catenin and cyclin D1²³⁶.

4.3.4. Calotropin

Calotropin is a toxic cardenolide, a steroid with a 5-membered lactone ring found in many plant sources such as Asclepiadoideae family and *Calotropis* genus (*C. procera* and *C. giagantea*). Calotropin, together with other cardenolides, were studied and extracted from the leaves and latex of *C. procera* by Seiber and colleagues²³⁷ using, as extractive solvent, ethanol 95%. As a cardenolide, calotropin is considered a cardioactive compound and possesses a specific inotropic, chronotropic, and dromotropic effects that are found in natural sources²³⁸. Moreover, calotropin exhibits significant cytotoxicity against several cancer cells, indeed, it inhibits the proliferation of colorectal cancer cells both *in vitro* and *in vivo* tests²³⁹. Calotropin seems to have a potent Wnt signaling inhibitory activity. It was demonstrated in several colon cancer cell lines that this natural compound caused a substantial decrease of β -catenin in the cytosol and nucleus through the increase of its CK1-mediated phosphorylation and subsequent degradation¹⁰⁵.

4.4. Secosteroids

4.4.1. Vitamin D

Vitamin D is a lipophilic fat soluble vitamin produced in human body from sterols by the action of UV light on the skin, but it can be also obtained from dietary sources of both vegetal (in form of vitamin D₂ or ergocalciferol) and animal origin (in form of vitamin D₃ or cholecalciferol) and the primary sources include fatty fishes and fortified foods (milk)^{240,241}. The two principal techniques for the extraction of vitamin D are liquid-liquid extraction and solid-phase extraction. On the other hand, other methods have been developed by combining different extractive procedures and adding derivatization steps in order to increase the recovery rate²⁴². Vitamin D is related to many biological pathways and it is involved in bone health by promoting calcium absorption, regulation of gene expression, immunity, cardiovascular health and antioxidant regulation.

1 α ,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active metabolite of Vitamin D₃, possesses antitumor activity as demonstrated by both *in vitro* and *in vivo* studies, as well as a protective ability in different neoplastic diseases, mainly colorectal cancer. It mediates the biological action mainly *via* the binding to the vitamin D receptor (VDR) The crosstalk between the VDR and Wnt/ β -catenin signaling is known and mediates, at least in part and in certain models, the antitumor potential of 1 α ,25-dihydroxyvitamin D₃⁷⁵. The use of 1,25(OH)₂D₃ as anti-cancer medicine is however hampered by its hypercalcemic effects at therapeutic doses, but several analogues that retain the antitumor actions without hypercalcemic effects are in developing⁷⁵.

Several *in vitro* studies demonstrated that 1,25(OH)₂D₃ inhibits the Wnt/ β -catenin pathway in human colon cancer cells by three main mechanisms. First, it induces the interaction between VDR and β -catenin thus decreasing the binding of β -catenin to TCF. Second, it increases E-cadherin expression, leading the relocation of β -catenin from nucleus to plasma membrane^{75,76}. Thirdly, 1,25(OH)₂D₃ augments the expression of Dickkopf (DKK)-1, a

Wnt signaling inhibitor²⁴³. These molecular events lead to the inhibition of several β -catenin/TCF target genes such as *c-MYC*, *TCF1*, *LEF1*, *AXIN2*, *PPAR γ* , and *CD44* in human colon cancer cells^{76,244,245}. The antitumor efficacy of 1,25(OH)₂D₃ in colon cancer, both *in vitro* and *in vivo*, was confirmed by others studies^{246–251}, and similar results were also obtained in *in vitro* and *in vivo* models of breast cancer^{252,253} and in an *ex vivo* model of human uterine leiomyomas²⁵⁴.

The promising results obtained in the preclinical experimentation have led to the evaluation of the potential anticancer activity of vitamin D₃ also in clinical trials. However, scientific evidence obtained to date are not yet completely persuasive⁷⁵. A large number of studies in cancer patients are ongoing in different type of cancers, as recoverable in *ClinicalTrials.gov*, but there is only one clinical trial that specifically evaluated the effect of vitamin D, alone or in combination with calcium, on the Wnt/ β -catenin signaling in cancer patients. It was a randomized double blind chemoprevention trial that assessed these effects in rectal mucosa biopsies from 104 participants at baseline and one-year after (Table 1). The results obtained from this trial showed that vitamin D, alone or in association with calcium, could modify APC, β -catenin, and E-cadherin expression in patients at risk for colorectal neoplasms, supporting the use of vitamin D as potential chemopreventive treatment in colorectal cancers²⁵⁵.

The safety profile of vitamin D and its synthetic derivatives is well known, and hypercalcemia is the main adverse event recognized²⁵⁶. Despite the numerous clinical trials completed and ongoing, further large and well-designed clinical studies are needed in order to evaluate the potential use of vitamin D as anticancer medicines.

5. Conclusive remarks and future perspective

As widely discussed in this review, due to the crucial role recognized to the Wnt/ β -catenin pathway in cancer initiation and progression, the possibility of targeting this signaling cascade is a great opportunity to develop more effective and anticancer drugs. However, some worries are licit when we consider the risks of targeting a pathway critical in tissue homeostasis and stem cell maintenance²⁵. In general, the use of drugs inhibiting signal transduction pathways crucial for embryonic development, such as the Wnt/ β -catenin pathway, could be a double-edged sword, since they can act like “molecular embodiments of Dr. Jekyll and Mr. Hyde”²⁵⁷. Under this point of view, the use of nature-derived molecules that are able to modulate the pathway might limit this risk. As modulators, they could successfully revert aberrant Wnt signaling in pathological situations without interfering with the critical role of this pathway in tissue homeostasis and repair.

The discovery of new promising drugs, specifically targeting upstream and downstream events of the signaling, more and more seems to be an attractive preventive and therapeutic strategy not only for cancer but also for many other types of diseases for whose dysregulation of the Wnt/ β -catenin pathway an association has been demonstrated. In particular, dysregulation of this pathway has been recently proposed as a novel pathomechanism leading to neurodegenerative disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD) and others^{13,14,258–262}, and targeting the Wnt/ β -catenin signaling has been suggested as new therapeutic opportunities for these brain diseases for which no cure is currently available^{14,263–265}. Differently from the Wnt-targeting anticancer therapies, the knowledge about the role of Wnt/ β -

catenin signaling in neurodegenerative diseases is still in its infancy. There are not clinical evidences available, but only data concerning some preclinical therapeutic approaches, performed on cellular and animal models^{14,258,264,265}. Among the bioactive modulators of the Wnt/ β -catenin pathway that are under preclinical investigation as possible therapeutics for neurodegenerative diseases, there are many natural-derived compounds that seem to promote neuronal differentiation and for which a neuroprotective ability has been demonstrated. These include curcumin^{266–268}, resveratrol¹⁰⁷, ginsengoids^{269,270}, salidroside²⁷¹, and others^{272,273}. Until today the therapeutic potential of natural-derived compounds against neurodegenerative disorders has been hampered by their poor bioavailability and consequent scarce delivery to the brain, but innovative delivery systems that could enhance their neuroavailability and therefore their neuroprotective activity are in developing²⁷⁴, and this will reinforce their possible application as preventive and therapeutic strategy against these kind of diseases.

During the last decade, several natural compounds have been identified as modulators of the Wnt/ β -catenin signaling, and most of the study aimed to demonstrate the efficacy of these modulators as tumor preventive and/or therapeutic drugs. The recently discovered involvement of this pathway in the onset of other diseases, and in particular in neurodegenerative disorders, for which a disease-modifying therapy does not exist yet, has increased the attractiveness of these natural compounds, and is stimulating for enhancing their possible use as dietary supplements or drugs in preventive and/or therapeutic new strategies against such different kind of diseases.

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Author contributions

Gloria Brusotti, Enrica Calleri and Annalucia Serafino conceived and designed the manuscript. Gianluca Sferrazza, Marco Corti, and Annalucia Serafino co-worked to the writing of the manuscript and figures. Pasquale Pierimarchi, Caterina Temporini critically reviewed the manuscript.

Conflict of interests

The authors have no conflicts of interest to declare.

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